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Immunophenotypic features of metastatic lymph node tumors to predict recurrence in N2 lung squamous cell carcinoma

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Key words

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Patients with mediastinal lymph node metastasis (N2) in squamous cell carcinoma (SqCC) of the lung have poor prognosis after surgical resection of the primary tumor. The aim of this study was to clarify predictive factors of the recurrence of pathological lung SqCC with N2 focusing on the biological characteristics of both cancer cells and cancer-associated fibroblasts (CAFs) in primary and metastatic lymph node tumors. We selected 64 patients with pathological primary lung N2 SqCC who underwent surgical complete resection and investigated the expressions of four epithelial–mesenchymal transition-related markers (caveolin, clusterin, E-cadherin, ZEB2), three cancer stem cell-related markers (ALDH-1, CD44 variant6, podoplanin) of cancer cells, and four markers of CAFs (caveolin, CD90, clusterin, podoplanin) in both primary and matched metastatic lymph node tumors in the N2 area. In the primary tumors, the expressions of all the examined molecules were not related to recurrence. However, in the metastatic lymph node tumors, high clusterin and ZEB2 expressions in the cancer cells and high podoplanin expression in the CAFs were significantly correlated with recurrence (P = 0.03, 0.04, and 0.007, respectively). In a multivariate analysis, only podoplanin expression in the CAFs in metastatic lymph node tumors was identified as a significantly independent predictive factor of recurrence (P = 0.03). Our study indicated that the immunophenotypes of both cancer cells and CAFs in metastatic lymph node tumors, but not primary tumors, provide useful information for predicting the recurrence of pathological N2 lung SqCC.

Many studies on predictive factors of recurrence have been carried out in NSCLC of various pathological stages. In particular, the pathological N factor, especially mediastinal lymph node metastasis (N2), has been considered an important predictor of recurrence.[1] The risk of distant metastasis and recurrence in patients with N2 in NSCLC is extremely high, and patients with N2 have a poor prognosis. The 5-year survival rate for pathological N2 NSCLC is reportedly 33.4%.[2]

Adenocarcinoma is the most common type of NSCLC, and a number of articles have discussed predictive factors of recurrence and prognosis. Squamous cell carcinoma is the second most common type, and the prognosis of patients with SqCC is more unfavorable than that for patients with adenocarcinoma because few anticancer drugs are available for treatment and the effects of these drugs are insufficient if the patients develop recurrence after surgery.[3,4] Moreover, information about predictive factors of recurrence is very limited. For this reason, the clinicopathological factors influencing recurrence in SqCC, particularly in the pathological N2 group which has a high risk of recurrence, need to be investigated.

Cancer tissue is composed of not only cancer cells, but also different kinds of stromal cells that are known as CAFs, tumor-associated macrophages, and immunoregulatory cells. The malignancy of cancer is not defined only by cancer cells. Biological analyses of non-cancer cells surrounding the cancer cells are also required, and their importance has been supported by many articles in recent years.[5,6]

To gain insight into the mechanism of cancer progression, the microenvironment of cancer at metastatic sites, in addition to primary sites, needs to be understood to determine the molecular mechanisms of cancer progression. At metastatic sites as well, cancer tissue is composed of not only cancer cells, but also the surrounding CAFs and other stromal cells such as lymphocytes and monocytes/macrophages. We previously reported that the presence of podoplanin-positive CAFs in metastatic lymph nodes, but not in primary tumors, predicted poor prognosis in pathological N2 stage III lung adenocarcinoma, suggesting that the biological characteristics of the cancer tissue in the metastatic lymph nodes may be more predictive of recurrence than that in the primary cancer tissue.[7]

The aim of this study was to identify how the immunophenotypic features of cancer cells and infiltrating CAFs in primary tumors and metastatic lymph node tumors could be correlated with recurrence for patients with pathological N2 SqCC. As for cancer cells, we focused on the cancer-initiating cell/cancer

stem cell and EMT-related molecules. In addition, we investigated the presence of CAFs with a tumor-promoting phenotype.

Materials and Methods

Subjects. A total of 546 consecutive patients with primary lung SqCC underwent surgical complete resection between July 1992 and December 2009 at the National Cancer Center Hospital East (Chiba, Japan). We excluded patients who did not undergo a standard operation or who had other cancers from the analyses. The number of pathological N0, N1, and N2 cases was 357 (65.4%), 125 (22.9%), and 64 (11.7%), respectively. The 3-year recurrence-free survival (RFS) rate and the 3-year overall survival rate of each group were significantly different ($P < 0.01$) (Table S1). Sixty-four cases with pathological N2 disease were enrolled in this study, and the median follow-up time was 5.3 years. The study was approved by the Ethics Committee of our institution.

Histological studies. The surgical specimens were fixed in 10% formalin or 100% methyl alcohol and embedded in paraffin. The tumors were cut into 5–10-mm thick slices, and serial 4-μm sections were stained using H&E. We counted the number of metastatic lymph nodes in the N2 area and measured the area of maximum metastatic lymph node tumors under a light microscope.

Immunofluorescence staining. Immunostaining was carried out using 4-μm paraffin-embedded tissue serial sections. The slides were deparaffinized in xylene and dehydrated in a graded ethanol series, and endogenous peroxidase was blocked with 3% hydrogen peroxide in 100% methyl alcohol. After epitope retrieval, the slides were incubated with mouse anti-AE1/3 antibody (Leica Biosystems, Newcastle Upon Tyne, UK) for cancer cells and rabbit polyclonal anti-α-SMA antibody (Lab Vision, Fremont, CA, USA) for CAFs. Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) were used as the secondary antibody. Before mounting, all the sections were stained with DRAQ5TM (Alexis Biochemical, Lausen, Switzerland) to identify nucleated cells. After mounting, the fluorescent signals were analyzed using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Antibodies and immunohistochemical staining. Information regarding the antibodies used in this study is shown in Table S2. Caveolin (clone D46G3; Cell Signaling, Danvers, MA, USA),[8,9] clusterin (clone 1A11; Acris Antibodies, Herford, Germany),[10,11] E-cadherin (clone 36; BD Biosciences, San Jose, CA, USA),[12,13] and ZEB2 (Novus Biologicals, Littleton, CO, USA)[14,15] were used as EMT-related markers. To evaluate the expression of cancer stem cell-related molecules, we used ALDH-1 (clone 44/ALDH; BD Biosciences),[16,17] CD44 variant 6 (clone VFF-7; Acris Antibodies),[18] and podoplanin (clone D2-40; Signet Antibodies, Princeton, NJ, USA).[19–22] To evaluate tumor-promoting CAFs, we used caveolin,[23] clusterin,[24] CD90 (Atlas Antibodies, Stockholm, Sweden),[25] and podoplanin.[5,7,26–28] After epitope retrieval, immunohistochemical staining was carried out as previously reported.[5–7]

Immunohistochemical scoring. All the stained tissue sections were semiquantitatively scored and evaluated independently under a light microscope by two pathologists (R.M. and G.I.) who had no knowledge of the patients' clinicopathological data. The labeling scores for cancer cells were calculated by multiplying the percentage of positive cancer cells per lesion (0–100%) by the staining intensity level (0, negative; 1, weak; 2, strong). Staining intensity 2 (strong) was defined as intensity level equal to positive control. Staining intensity 1 (weak) was defined as intermediate staining. We selected the median score to define high and low staining. A high staining score was defined as a score above the median value; a low score was defined as a score below the median value.

Cancer-associated fibroblasts were defined as stromal spindle cells that were morphologically identified as fibroblasts. As for the CAFs, cases with positive-stained spindle-shaped cells accounting for more than 10% of the cells in the cancer stroma were identified as the high expression group.

Statistical analysis. Recurrence-free survival was defined as the time from surgery until the time of the tumor recurrence or the date of the last follow-up. The survival curves were estimated using the Kaplan–Meier method, and the differences in survival between the subgroups were compared using the log–rank test. A multivariate analysis was carried out using the Cox proportional hazard model. The significance level was set at $P < 0.05$. Statistical analysis software (Stat View, version 5.0, SAS Institute Inc., Cary, NC, USA) was used to carry out the analyses.

Results

Patient characteristics and pathological factors of primary tumors. Univariate analyses of the clinical factors and the pathological factors in the primary tumors were carried out. A higher smoking index (>1000) was significantly correlated with a shorter interval until recurrence (Table 1). The other pathological factors were unrelated to recurrence.

Pathological factors of metastatic lymph node tumors. We carried out univariate analyses of pathological factors, the number of metastatic lymph nodes, and the station of N2. In addition, we measured the area of the metastatic lymph node tumor under a light microscope and univariate analysis was carried out (Table 2). However, the differences were not significant.

Cancer-associated fibroblasts in metastatic lymph node tumors. We confirmed that spindle cells had infiltrated the area around the cancer cells in the metastatic lymph node tumors, similar to the situation for the primary tumors (Fig. 1a,b). Double immunofluorescence staining revealed that the cancer cells were positive for AE1/3 (green) and that the spindle cells

Table 1. Univariate analysis of clinicopathological factors for recurrence-free survival (RFS) in patients with resected pathological N2 squamous cell carcinoma of the lung (n = 64)

Factor	No.	3-Year RFS, %	*P*-value
Gender			
Male/female	60/4	35.9/50.0	0.70
Age, years			
<65/≥65	29/35	40.6/33.2	0.68
Smoking index			
<1000/≥1000	39/25	49.2/22.2	0.01†
Pathological T status			
T1/T2–T4	42/22	40.0/33.9	0.71
Vascular invasion			
v(−)/v(+)	11/53	40.0/33.5	0.37
Lymphatic pemeation			
ly(−)/ly(+)	35/29	47.2/29.6	0.58
Pleural invasion			
pl(−)/pl(+)	28/36	42.3/30.6	0.25

†Significance.

Table 2. Univariate analysis of pathological factors in metastatic lymph node tumors for recurrence-free survival (RFS) in patients with resected pathological N2 squamous cell carcinoma of the lung (*n* = 64)

Factor	No.	3-Year RFS, %	*P*-value
No. of metastatic lymph nodes of N2 area			
1/>1	10/54	48.1/30.3	0.22
Station of N2			
Single/multiple	46/18	41.6/32.4	0.62
Area of the metastatic foci, mm^2			
<84/≥84	31/33	31.9/28.9	0.46

were positive for α-SMA (red), indicating that these cells were myofibroblasts (Fig. 1c,d). From these results, we confirmed that CAFs had also infiltrated the metastatic lymph node tumors, similar to the results of our previous study.[7]

Correlation between immunohistochemical staining of cancer cells and CAFs in primary tumors and prognostic impact. As for the cancer cells, we evaluated the expressions of ALDH-1, caveolin, CD44 variant 6, clusterin, E-cadherin, podoplanin, and ZEB2 (Table 3). In addition, the expressions of caveolin, CD90, clusterin, and podoplanin were analyzed in the CAFs. None of the expressions of any of the examined molecules in the primary tumors were related to recurrence.

Correlation between immunohistochemical staining of cancer cells and CAFs in metastatic lymph node tumors and prognostic impact. We carried out univariate analyses in the metastatic lymph node tumors (Table 4). A high clusterin expression level in cancer cells was observed in 24 cases (38%) (Fig. 2a,b). The 3-year RFS rate of cases with a high clusterin expression level was 28.6%, whereas that of cases with a low clusterin expression level was 45.2%. The difference between the two groups was significant ($P = 0.04$; Fig. 3a).

A high ZEB2 expression level in cancer cells was observed in 16 cases (25%) (Fig. 2c,d). Figure 3(b) shows the Kaplan–Meier curve for RFS in patients with pathological N2 SqCC according to the expression status of ZEB2 in the cancer cells. The 3-year RFS rate of cases with a high ZEB2 expression level was 15.6%, while that of cases with a low ZEB2 expression level was 46.3%. High ZEB2 expression in cancer cells in metastatic lymph node tumors was significantly correlated with a shorter interval until recurrence, compared with low ZEB2 expression in the cancer cells ($P = 0.03$; Fig. 3b).

A high podoplanin expression level in the CAFs was observed in 27 cases (42%) (Fig. 2e,f). The 3-year RFS rate of cases with a high podoplanin expression level was 19.8%, while that of cases with a low podoplanin expression level was

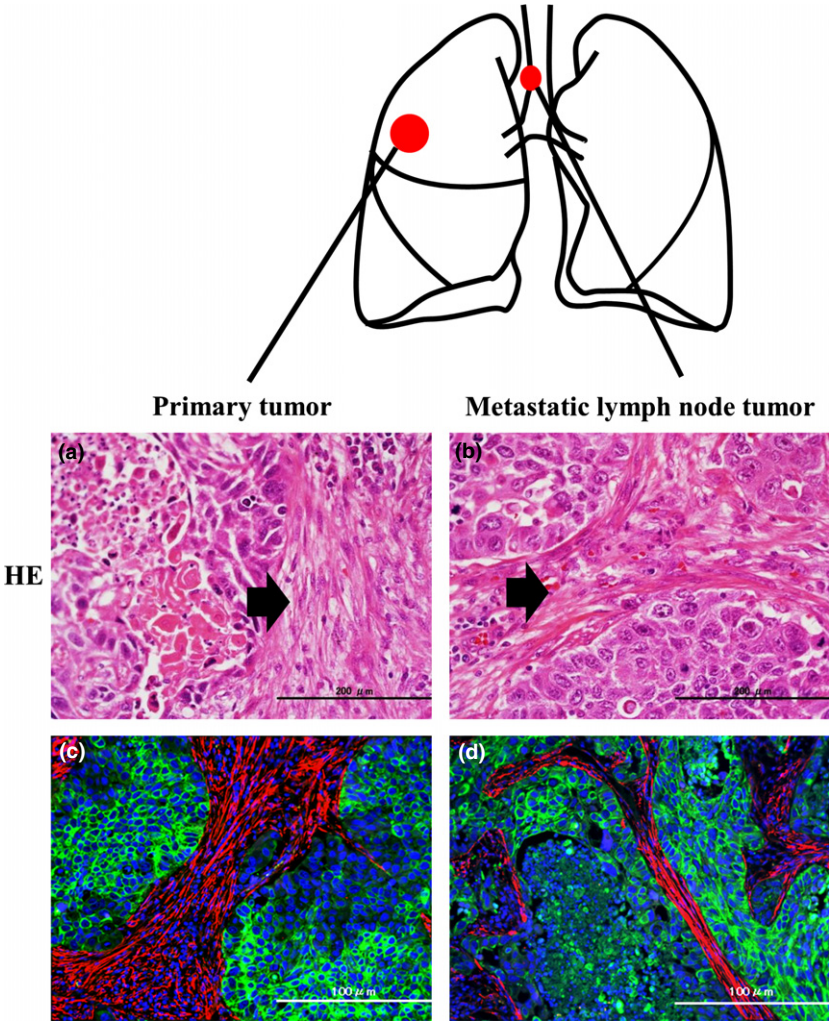

Fig. 1. Staining with H&E and double immunofluorescence staining of tumor cells in a primary and a metastatic lymph node tumor in a patient with squamous cell carcinoma of the lung. (a, b) H&E staining in the primary tumor (a) and the metastatic lymph node tumor (b). Arrows indicate cancer-associated fibroblasts (CAFs). (c) Double immunofluorescence staining in the primary tumor. Blue, nucleus; green, AE1/3-positive cancer cells; red, α-smooth muscle actin-positive myofibroblasts (CAFs). (d) Double immunofluorescence staining in the metastatic lymph node tumor. Blue, nucleus; green, AE1/3-positive cancer cells; red, α-smooth muscle actin-positive myofibroblasts (CAFs).

Table 3. Univariate analysis of immunochemical staining of (a) cancer cells and (b) cancer-associated fibroblasts in primary tumors in patients with resected pathological N2 squamous cell carcinoma of the lung (*n* = 64)

Antibodies	Median score	High	Low	3-Year RFS, %	*P*-value
(a)					
EMT-related molecules					
Caveolin	0	29	34	High, 47.2 Low, 32.0	0.34
Clusterin	10	34	30	High, 33.3 Low, 45.2	0.12
E-cadherin	48	32	32	High, 39.9 Low, 34.4	0.87
ZEB2	0	33	31	High, 40.1 Low, 36.1	0.79
Stem cell-related molecules					
ALDH-1	123	32	32	High, 32.3 Low, 45.1	0.21
CD44 variant 6	65	32	32	High, 32.5 Low, 41.4	0.60
Podoplanin	10	30	34	High, 48.0 Low, 28.9	0.15

Antibodies	High	Low	3-Year RFS, %	*P*-value
(b)				
Cancer-associated fibroblasts				
Caveolin	29	35	High, 35.6 Low, 39.3	0.98
CD90	55	9	High, 35.8 Low, 44.4	0.23
Clusterin	45	18	High, 38.8 Low, 31.0	0.88
Podoplanin	47	17	High, 33.8 Low, 52.3	0.12

EMT, epithelial–mesenchymal transition; RFS, recurrence-free survival; RFS, recurrence-free survival.

Table 4. (a) Univariate analysis of immunochemical staining of cancer cells in metastatic tumors in patients with resected pathological N2 squamous cell carcinoma of the lung (*n* = 64); (b) Univariate analysis of immunochemical staining of cancer-associated fibroblasts in metastatic lymph node tumors in patients with resected pathological N2 squamous cell carcinoma of the lung

Antibodies	Median score	High	Low	3-Year RFS, %	*P*-value
(a)					
EMT-related molecules					
Caveolin	0	21	43	High, 45.4 Low, 32.8	0.60
Clusterin	0	24	40	High, 28.6 Low, 45.2	0.04†
E-cadherin	30	31	33	High, 44.7 Low, 32.7	0.30
ZEB2	0	16	48	High, 15.6 Low, 46.3	0.03†
Stem cell-related molecules					
ALDH-1	128	32	32	High, 34.4 Low, 45.1	0.20
CD44 variant 6	30	33	31	High, 32.3 Low, 42.3	0.35
Podoplanin	0	17	47	High, 43.0 Low, 34.7	0.60

Antibodies	High	Low	3-Year RFS,%	*P*-value
(b)				
Cancer-associated fibroblasts				
Caveolin	3	61	High, 33.3 Low, 39.2	0.750
CD90	32	32	High, 37.5 Low, 39.1	0.260
Clusterin	24	40	High, 28.7 Low, 43.7	0.210
Podoplanin	27	37	High, 19.8 Low, 52.6	0.007†

†Significance. EMT, epithelial–mesenchymal transition; RFS, recurrence-free survival; RFS, recurrence-free survival.

52.6%. High podoplanin expression in the CAFs in metastatic lymph node tumors was significantly correlated with a shorter interval until recurrence, compared with low podoplanin expression in the CAFs ($P = 0.007$, Fig. 3c).

The expressions of clusterin and ZEB2 in cancer cells and the expression of podoplanin in CAFs in metastatic lymph node tumors were significantly correlated with those in the primary tumors (Table S3).

Multivariate analyses to identify factors significantly associated with recurrence. A multivariate analysis using the Cox proportional hazard model was carried out to determine the recurrence of conventional clinicopathological factors (Table 5). Only podoplanin expression in CAFs in metastatic lymph node tumors was identified as a significantly independent predictor of RFS ($P = 0.03$).

Discussion

This is the first report to discuss the prognostic importance of the tumor microenvironment of metastatic lymph node tumors. In this study, we identified clusterin and ZEB2 expression in cancer cells and podoplanin expression in CAFs in metastatic lymph node tumors as significant predictive factors of recurrence in patients with pathological N2 SqCC. However, none of the expression levels of the molecules examined in the primary tumors were significantly correlated with recurrence. Few studies to date have examined prognostic significance by considering the biological characteristics of both the primary tumors and the metastatic lymph node tumors in advanced-stage cases with lymph node metastasis.[29] Fukuse *et al.* reported that a high expression level of proliferating cell nuclear antigen in both the primary tumors and metastatic lymph node tumors was a significant predictor of a poor prognosis in pathological N2 NSCLC.[30] In addition, CAFs also reportedly exist in metastatic lymph node tumors, and the EMT is influenced by CAFs.[31,32] We previously reported that the presence of podoplanin-positive CAFs in metastatic lymph node tumors, but not in primary tumors, predicted poor prognosis in patients with pathological N2 stage III lung adenocarcinoma.[7] Taken together, predictive factors of recurrence in patients with lymph node metastasis should be analyzed with due consideration given to the metastatic tumor microenvironment.

We previously reported that the presence of podoplanin-positive CAFs in primary tumor is correlated with poorer prognosis in stage I SqCC, which was inconsistent with the results of our current study.[28] This would also support the biological impor-

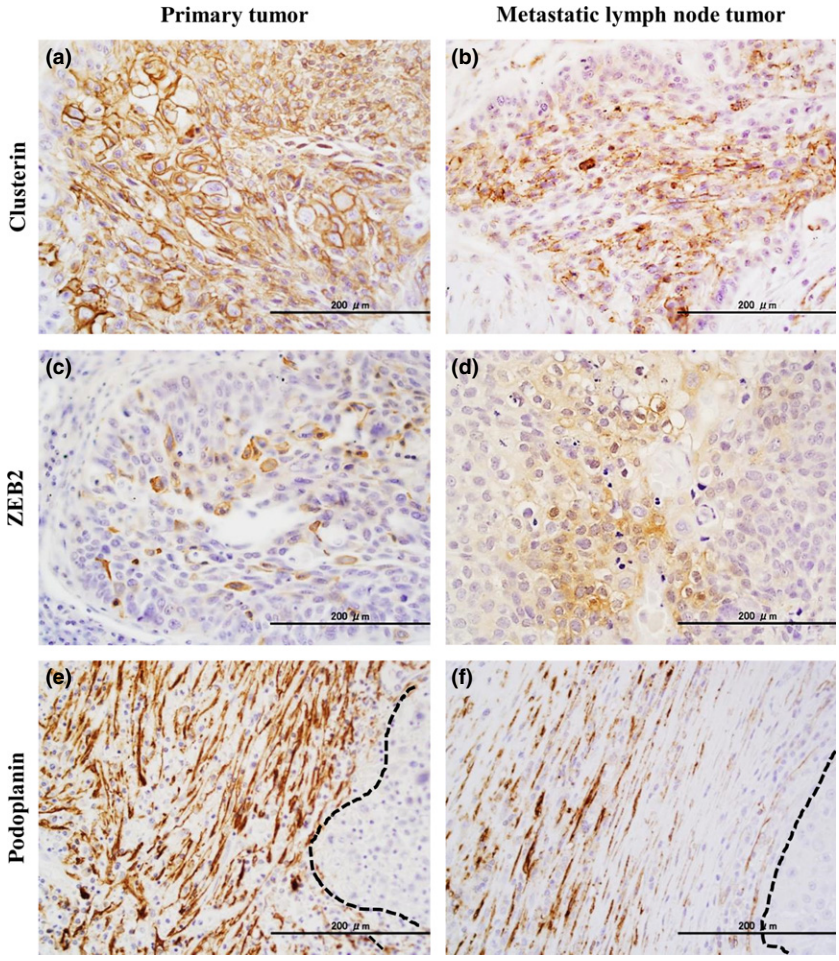

Fig. 2. Immunohistochemical staining of tumor cells in a primary tumor and a metastatic lymph node tumor in a patient with squamous cell carcinoma of the lung. (a,b) Clusterin expression of cancer cells in the primary tumor (a) and the metastatic lymph node tumor (b). (c,d) ZEB2 expression of cancer cells in the primary tumor (c) and the metastatic lymph node tumor (d). (e,f) Podoplanin expression of cancer-associated fibroblasts in the primary tumor (e) and the metastatic lymph node tumor (f). Dotted lines show the margin of the cancer cell nest.

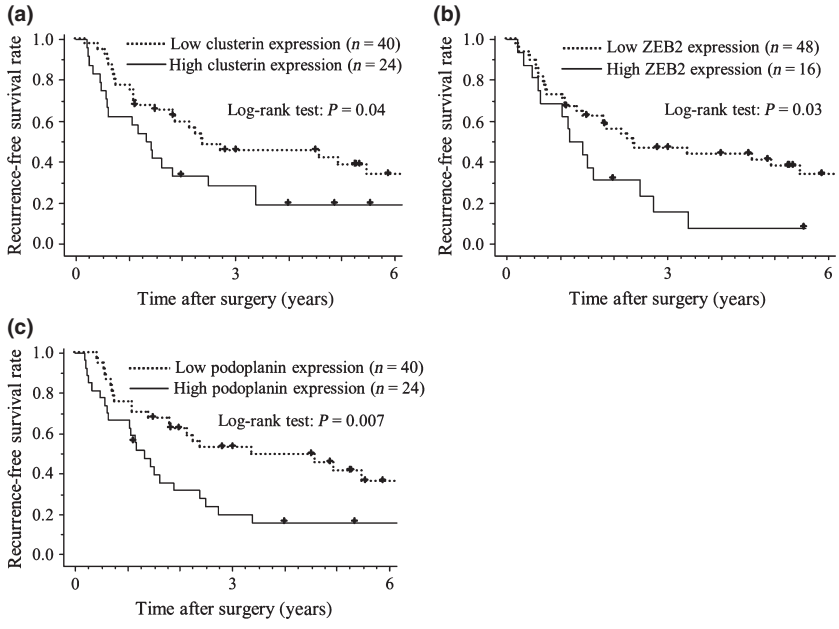

Fig. 3. Kaplan–Meier recurrence-free survival (RFS) curve for patients with resected pathological N2 squamous cell carcinoma of the lung according to immunohistochemical staining. (a) Kaplan–Meier RFS curve according to clusterin expression of cancer cells in metastatic lymph node tumors. (b) Kaplan–Meier RFS curve according to ZEB2 expression of cancer cells in metastatic lymph node tumors. (c) Kaplan–Meier RFS curve according to podoplanin expression of cancer-associated fibroblasts in metastatic lymph node tumors.

tance of cancer tissue in metastatic sites of advanced cancer (N2 disease).

Tumor metastasis has been postulated to start with EMTs, a process through which a small number of tumor cells at the primary site acquire a more invasive and metastatic phenotype. After engraftment at metastatic sites, tumor cells with subsequent mesenchymal–epithelial transitions, the reverse phenomenon of EMTs, develop metastatic tumors and recruit certain sorts of CAFs. Thus, the microenvironment of metastatic tumors created by cancer cells and surrounding CAFs might differ from that of the primary tumors. This difference could explain why the biological characteristics of metastatic lymph

Table 5. Multivariate analysis of clinicopathological factors for recurrence-free survival in patients with resected pathological N2 squamous cell carcinoma of the lung (n = 64)

Factor	Hazard ratio	(95% CI)	P-value
Smoking index			
≥1000/<1000	1.92	(0.83–2.92)	0.17
Clusterin expression of cancer cells in metastatic lymph node tumors			
High/low	1.55	(0.74–2.58)	0.30
ZEB2 expression of cancer cells in metastatic lymph node tumors			
High/low	1.39	(0.96–3.82)	0.06
Podoplanin expression of CAFs in metastatic lymph node tumors			
High/low	2.00	(1.08–3.72)	0.03†

†Significance. CAF, cancer-associated fibroblasts; CI, confidence interval.

node tumors were more strongly predictive of recurrence than those of the primary tumors.

Podoplanin is 40-kD glycoprotein for type I transmembrane sialomucin participating in platelet aggregation, invasion, and metastasis of cancer. Recent studies, including some by our group, have identified podoplanin as a marker of tumor-promoting CAFs in lung adenocarcinoma, SqCC, and breast cancer.[26–28,33] Our current study showed that the presence of podoplanin-positive CAFs in metastatic lymph node tumors, but not in primary tumors, participated in recurrence, similar to the results observed for adenocarcinoma with N2 disease.[7] The metastatic microenvironment created by both podoplanin-expressing CAFs and cancer cells may confer an additional malignant potential to metastasized cancer cells, such as effects on migration, proliferation, and survival. Moreover, podoplanin expression was the most significant predictor of RFS. Thus, consideration of the biological characteristics of CAFs in metastatic lymph node tumors might be very important for determining the likelihood of recurrence after surgery.

Clusterin, a stress-activated and apoptosis-associated molecular chaperone that confers survival and a proliferative advantage to cancer cells, is an important mediator of the transforming growth factor-β-induced EMT.[11] Clusterin overexpression in cancer cells upregulates metastasis and is related to chemoresistance.[10,34] ZEB2 is one of the transcription factors that regulates the expression of E-cadherin and mediates the EMT. ZEB2 overexpression in the cancer cells of primary tumors was reportedly correlated with a poor prognosis in several types of cancers.[14,35] Kurahara *et al.*[15] reported that pancreatic cancer cells in metastatic lymph node tumors expressed high levels of ZEB1 and ZEB2, suggesting that these cancer cells were associated with the EMT phenotype. In the current study, high expression levels of the EMT-related markers, clusterin and ZEB2 in cancer cells at metastatic lymph node tumors were significantly correlated with a shorter time until recurrence. These findings suggest that the EMT phenotypes of cancer cells that have detached from the primary tumors are likely to be an important determinant of the development of remote metastasis.

The conversion to the EMT phenotype of cancer cells is mediated by several factors, and E-cadherin is known to be an EMT-related marker. In this study, a low E-cadherin expression level in cancer cells at metastatic lymph node tumors was not correlated with recurrence. No inverse correlations between clusterin or ZEB2 expression and E-cadherin expression in metastatic lymph node cancer cells was seen (data not shown). This discrepancy may be explained by the fact that the expression of E-cadherin is regulated not only by numerous EMT-related transcription factors such as ZEB1, ZEB2, Twist, and Snail, but also by epigenetic mechanisms.

In conclusion, we found that clusterin and ZEB2 expression in cancer cells and podoplanin expression in CAFs in metastatic lymph node tumors were significant predictive factors of cancer recurrence. The prognostic importance of the microenvironment in primary tumors has already been reported for early-stage cases, but the current study also suggests the need to examine the microenvironment in metastatic lymph node tumors in advanced-stage cases. Although a prospective study with a larger number of patients and a multicenter study are warranted, this study has important implications for investigations focusing on the microenvironment in metastatic lymph node tumors, and should provide a significant indicator to future directionality.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

CAFs	cancer-associated fibroblasts
EMT	epithelial–mesenchymal transition
MET	mesenchymal–epithelial transition
NSCLC	non-small-cell lung cancer
RFS	recurrence-free survival
SMA	smooth muscle actin
SqCC	squamous cell carcinoma

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Overall survival and recurrence-free survival in 546 patients with resected squamous cell carcinoma.

Table S2. Antibodies used in the immunohistochemical staining.

Table S3a. Correlation of clusterin expression between cancer cells in primary tumors and metastatic lymph node tumors.

Table S3b. Correlation of ZEB2 expression between cancer cells in primary tumors and metastatic lymph node tumors.

Table S3c. Correlation of podoplanin expression between cancer-associated fibroblasts in primary tumors and metastatic lymph node tumors.