ORIGINAL ARTICLE

Cancer Science WILEY

Prognostic impact of count of extratumoral lymphatic permeation in lung adenocarcinoma and its relation to the immune microenvironment

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Funding information
This study was supported by in part by the National Cancer Center Research Fund (2020-A-9) and JSPS KAKENHI (21H02931).

Abstract

Extratumoral lymphatic permeation (ly-ext) has been reported as an independent poor prognostic factor for lung adenocarcinoma, but whether or not the number of ly-ext foci is associated with prognosis and its relationship to the immune microenvironment is unclear. We counted the number of ly-ext foci on pathological slides from patients with completely resected lung adenocarcinoma with ly-ext, and divided them into two groups: a group with a high number of ly-ext foci (ly-ext high) and one with a low number of ly-ext foci (ly-ext low). Among the patients with ly-ext, only a high number of ly-ext foci was an independent poor prognostic factor. The 3-year recurrence-free survival (RFS) rate of the ly-ext high group was significantly lower than that of the ly-ext low group (14.7% vs. 50.0%, $P < 0.01$). Then, we analyzed the immune microenvironment of pT1 lung adenocarcinoma with ly-ext (13 cases of ly-ext high and 11 cases of ly-ext low tumor) by immunohistochemistry using antibodies for stem cell markers (aldehyde dehydrogenase 1 A1 and CD44), tumor-promoting mucin (MUC1), tumor-infiltrating lymphocytes (CD4, CD8, FOXP3, and CD79a), and tumor-associated macrophages (CD204). The number of CD8+ TILs within the primary lesion was significantly lower and the number of FOXP3+ TILs within the primary lesion was significantly higher in the ly-ext high group ($P < 0.05$ and $P < 0.01$, respectively). Our results indicated that a high number of ly-ext foci was an independent poor prognostic factor. Moreover, tumors with high numbers of ly-ext foci had a more immunosuppressive microenvironment.

Abbreviations: ALDH1A1, aldehyde dehydrogenase 1 A1; CD, cluster of differentiation; CI, confidence interval; FOXP3, Forkhead boxprotein P3; HE, hematoxylin-eosin; HR, hazard ratio; IL, interleukin; LN, lymph node; ly-ext, extratumoral lymphatic permeation; MUC, mucin; NSCLC, non-small-cell lung cancer; OS, overall survival; RFS, recurrence-free survival; TAM, tumor-associated macrophage; TGF, transforming growth factor; TIL, tumor infiltrating lymphocyte; TNM, Tumor-Node-Metastasis; VEGF, vascular endothelial growth factor; VVG, Verhoeff-Van Gieson.

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1 | INTRODUCTION

Lung cancer is one of the most lethal cancers worldwide, and adenocarcinoma is the most common histological type of lung cancer. Surgery is still the most effective treatment for locally advanced NSCLC, but some patients who undergo complete resection experience relapse. There have been various studies on the clinicopathological factors associated with postoperative recurrence of NSCLC.[1-5]

Many studies have highlighted the prognostic importance of lymphovascular invasion.[6] In lung cancer, vascular invasion and lymphatic permeation are assessed only by its presence or absence, and their location and quantity have not been noted in the current TNM classification.

We previously reported that lymphatic permeation of lung cancer has a different prognostic impact depending on whether it is inside or outside the primary lesion. Saijo et al.[7] reported that the RFS time of patients with ly-ext was significantly shorter than that of patients with intratumoral lymphatic permeation. Matsumura et al.[8] found that the rates of OS and RFS of patients with T1 and T2 tumors with ly-ext were comparable to those of patients with T3 tumors. However, the impact of the number of ly-ext foci on prognosis was unclear.

Cancer tissue comprises not only cancer cells but also different kinds of stromal cells, such as TILs or TAMs, which are commonly found in association with cancer cells. TAMs promote cancer progression by producing cytokines involved in angiogenesis, matrix remodeling, tumorigenesis, and immunosuppression.[9] TILs regulate cancer progression by playing two conflicting roles: suppressing tumor growth and promoting tumor progression. Recently, regulatory T cells in cancer stroma were reported to enable cancer cells to escape host immune surveillance in several cancers by inhibiting cytotoxic T cells and inducing an immunosuppressive environment.[10]

In this study, we examined whether the number of ly-ext foci is a prognostic factor among patients with ly-ext. Furthermore, we aimed to identify the differences in the immune microenvironment associated with high or low number of ly-ext foci.

2 | MATERIALS AND METHODS

2.1 | Patients

We retrospectively reviewed a total of 1865 patients who underwent lung resection for primary lung adenocarcinoma between January 2010 and December 2017 in our hospital. Patients who had undergone incomplete resection, sublobar resection, and preoperative therapies, including chemotherapy or radiotherapy, were excluded from this study. The remaining 1458 patients were finally enrolled in this study. A flowchart of the case selection is shown in Figure S1. This study was approved by the Institutional Review Board of the National Cancer Center (approval numbers 2020-453) and informed consent was obtained from all patients and conformed to the provisions of the Declaration of Helsinki.

2.2 | Evaluation of clinicopathological factors

We reviewed the clinicopathological characteristics of the patients from the available medical records. The following clinicopathological factors were investigated retrospectively to assess their prognostic effect: age, sex, smoking history, pT status, pN status, vascular invasion, lymphatic permeation, pleural invasion, and intrapulmonary metastasis.

2.3 | Histological examination

All specimens were fixed with 10% formalin via infusion through the bronchial tree and then embedded in paraffin. The tumors were sliced at approximately 5 mm intervals and serial 4 μm sections were stained with HE. VVG staining was performed to evaluate blood vessel and pleural invasion in all cases. Lymphatic permeation was determined via sections stained with HE based on the following criteria: more than one tumor cell floating in vessels without supporting smooth muscles or elastic fibers within the bronchovascular bundle, subpleural, and intralobular pleural space. The slides were reviewed by two pathologists (TN and GI). We classified lymphatic permeation into the following three categories: ly0, absence of lymphatic permeation; ly-int, presence of intratumoral lymphatic permeation; and ly-ext, presence of extratumoral lymphatic permeation (Figure S2). Tumors with both ly-int and ly-ext were classified as ly-ext. Histological typing was based on the 5th revised World Health Organization histological classifications. All the tumors were pathologically staged using the 8th edition of the TNM classification of lung cancer published by the Union for International Cancer Control.

2.4 | Counting the number of ly-ext foci

All HE-stained slides were scanned using the Aperio scan system (Leica Biosystems). We counted the total number of ly-ext foci in all slides made from a slice that contained the maximum cut surface of tumor. If there were no ly-ext foci in a slice of the maximum cut surface of the tumor, we searched for ly-ext foci in the slices that were directly above or below that slice. We measured the area outside the tumor where extratumoral lymphatic permeation was located (Figure S3). We counted the total number of ly-ext foci and calculated the total number of ly-ext foci per square centimeter in the slice.

2.5 | Antibodies and immunohistochemical staining

The primary antibodies used in this study are listed in Table S1. We took 4 μm sections from the blocks and stained them with antibodies using a BenchMark ULTRA automated immunohistochemical slide staining system (Ventana Medical Systems).

2.6 | Calculation of immunohistochemical scores

Immunostaining scores of CD44, ALDH1A1, and MUC1 were calculated from the staining-intensity scores and percentages of positively stained cells. The staining intensity scores were as follows: 0, negative, total absence of staining; 1+, weak staining; and 2+, strong staining. These were multiplied by the percentages of immunohistochemically stained tumor cells per section (0%–100%), resulting in scores ranging from 0 to 200. The numbers of CD4+ T

cells, CD8+ T cells, FOXP3+ T cells, CD79a+ B cells, and CD204+ tumor-associated macrophages were counted on virtual slides using high-power fields (0.0625 mm²/field). Lymphocytes and macrophages were counted in the stromal area between cancer nests within primary lesion and in ly-ext foci. For the primary lesion, the averages of the number of positive cells in five high-power fields selected at random were recorded as their scores. About in ly-ext foci, we chose the lymphatic vessels with the highest number of tumor cell infiltrations for evaluation. All the immunohistochemical slides were assessed by two pathologists (TN and GI). Both were unaware of the clinical and pathological information.

2.7 | Statistical analysis

Fisher's exact test and chi-square test were used to compare the clinicopathological factors between the two variables. The OS and

TABLE 1 Clinicopathological characteristics according to lymphatic permeation status

Characteristics	ly0 (%) n = 1288	ly-int (%) n = 78	ly-ext (%) n = 92	P ly0 vs. ly-ext	P ly-int vs. ly-ext
Age (year)					
Median (range)	69 (33–93)	69(41–86)	65(30–88)	<0.01	0.01
Sex					
Male	685 (53)	40 (51)	59 (64)	0.051	0.12
Female	603 (47)	38 (49)	33 (36)		
Smoking					
Ever	744 (58)	48 (62)	59 (64)	0.27	0.75
Never	544 (42)	30 (38)	33 (36)		
pT status					
Tis	58 (5)	0	0	<0.01*	0.19*
T1	837 (65)	35 (45)	29 (32)		
T2	306 (24)	30 (38)	40 (43)		
T3	58 (5)	9 (12)	16 (17)		
T4	29 (2)	4 (5)	7 (8)		
pN status					
N0	1110 (86)	42 (54)	17 (18)	<0.01**	<0.01**
N1	90 (7)	19 (24)	26 (28)		
N2	88 (7)	17 (22)	49 (53)		
Vascular invasion					
v−	955 (74)	41 (53)	25 (27)	<0.01	<0.01
v+	333 (26)	37 (47)	67 (73)		
Pleural invasion					
pl−	1003 (78)	47 (60)	45 (49)	<0.01***	0.17***
pl+	285 (22)	31 (40)	47 (51)		
PM					
pm−	1260 (98)	74 (95)	77 (84)	<0.01****	0.03****
pm+	28 (2)	4 (5)	15 (16)		

Note: *pTis-2 vs pT3-4, **pN0 vs pN1-2, ***pl+ vs pl−, ****pm+ vs pm−.

Abbreviations: ly-ext, extratumoral lymphatic permeation; ly-int, intratumral lymphatic permeation; PM, intrapulmonary metastasis.

RFS rates were estimated using the Kaplan–Meier method and compared using the log-rank test. Univariate and multivariate Cox regression analyses were performed to assess the impact of clinicopathological factors on OS and RFS. We divided patients with ly-ext into two groups with the median number of ly-ext foci as a cut-off value, and the impact of the number of ly-ext foci on RFS of patients with ly-ext were assessed. The Mann–Whitney U test was used to evaluate the immunostaining scores. All statistical calculations were performed using JMP Pro software (version 12.2.0, SAS Institute). Results were considered significant when the P value was <0.05.

3 | RESULTS

3.1 | Clinicopathological characteristics of all patients

The median age was 69 years (range 30–93), and 784 patients (54%) were men. A total of 851 patients (58%) had a history of smoking. A total of 289 patients (20%) had lymph node metastasis. Vascular invasion, pleural invasion, and lymphatic permeation were detected in 437 (30%), 363 (25%), and 170 (12%) patients, respectively (Table S2).

3.2 | Differences in clinicopathological characteristics between patients with ly-ext and those with ly-int

The number of patients classified as ly0 (absence of lymphatic permeation), ly-int (presence of intratumoral lymphatic permeation), and ly-ext (presence of extratumoral lymphatic permeation) were 1288 (88%), 78 (5%), and 92 (6%), respectively. Patients with ly0 or ly-int were significantly younger than those with ly-ext (ly0 vs. ly-ext $P < 0.01$ and ly-int vs. ly-ext $P < 0.05$), but the sex and smoking history of the patients were not significantly different among the groups (Table 1). Patients with ly-ext had significantly advanced nodal disease (pN1-2; 81% (ly-ext) vs. 46% (ly0) and 14% (ly-int); $P < 0.01$ and $P < 0.01$, respectively) and intrapulmonary metastasis (pm1 and pm2;16% (ly-ext) vs. 2% (ly0) and 5% (ly-int); $P < 0.01$ and $P < 0.05$, respectively) compared to the others. Compared to patients without lymphatic permeation, patients with ly-ext had more advanced pT status (pT3 and pT4; 25% (ly-ext) vs. 7% (ly0); $P < 0.01$) and pleural invasion (pl+; 51% (ly-ext) vs. 22% (ly0); $P < 0.01$). The median number of extratumoral infiltrated lymphatic vessels in the ly-ext patients was 5 (range 1–210; Figure 1A).
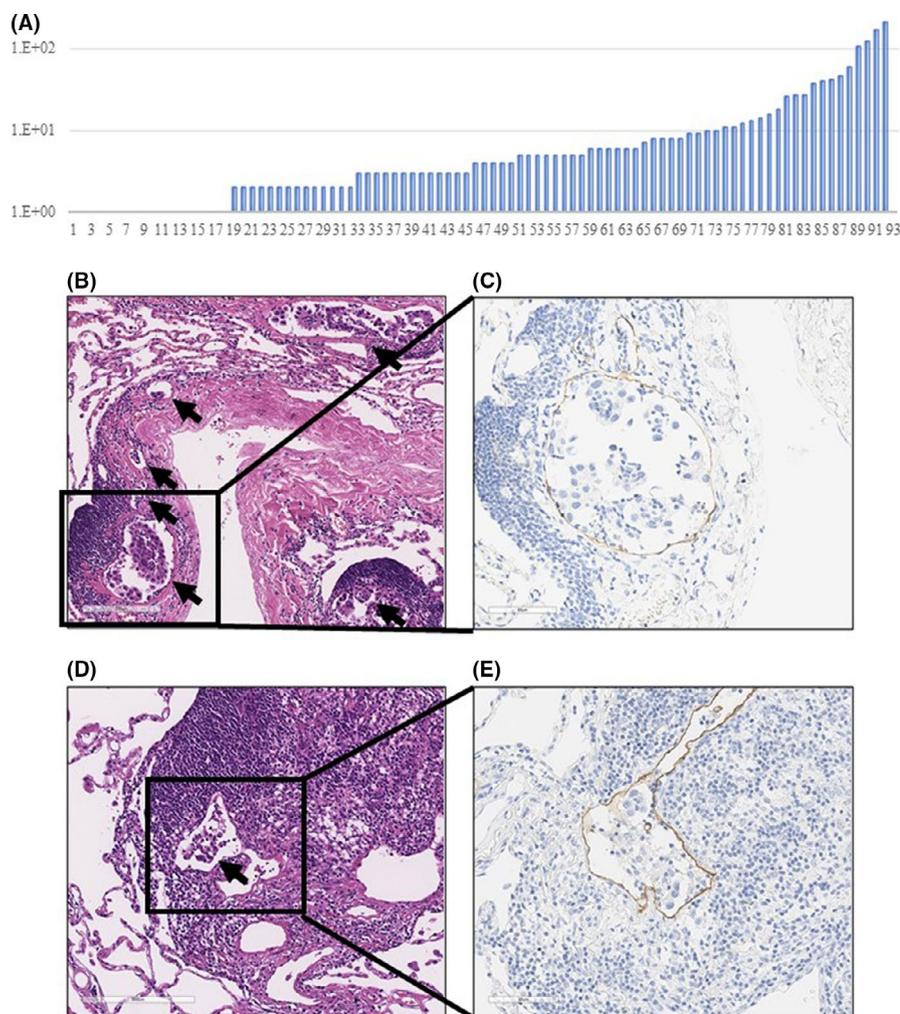

FIGURE 1 Representative hematoxylin-eosin (HE) and anti-D2-40 staining according to the number of extratumoral lymphatic permeation (ly-ext). (A) The x axis represents each case and the y axis represents the logarithmic scale of ly-ext foci numbers. The histogram shows that median number of ly-ext foci was 5 (range 1–210) in all cases with ly-ext. (B) Example of an HE staining slide of ly-ext foci in tumors of patients from the ly-ext high group. Arrows indicate ly-ext foci. (C) Example of a D2-40 staining slide of ly-ext foci in tumors of patients from the high group. (D) Example of a HE staining slide of ly-ext foci in tumors of patients from the ly-ext low group. Arrows indicate ly-ext foci. (E) Example of a D2-40 staining slide of ly-ext foci in tumors of patients from the ly-ext low group

TABLE 2 Univariate and multivariate analysis of clinicopathological factor associated with recurrence-free survival

Variables	Univariate			Multivariate		
	HR	95% CI	P value	HR	95% CI	P value
Age						
<65	1.00					
65≥	1.13	0.92–1.39	0.26			
Sex						
Female	1.00			1.00		
Male	1.56	1.28–1.90	<0.01	1.17	0.90–1.52	0.24
Smoking						
Never	1.00			1.00		
Ever	1.52	1.25–1.86	<0.01	0.97	0.74–1.27	0.83
pT status						
Tisor1or2	1.00			1.00		
T3or4	4.52	3.55–5.69	<0.01	1.59	1.15–2.15	<0.01
pN status						
–	1.00			1.00		
+	6.43	5.30–7.80	<0.01	2.82	2.22–3.57	<0.01
Vascular invasion						
–	1.00			1.00		
+	5.25	4.32–6.40	<0.01	2.38	1.87–3.03	<0.01
Pleural invasion						
–	1.00			1.00		
+	3.61	2.98–4.37	<0.01	1.55	1.25–1.93	<0.01
PM						
–	1.00			1.00		
+	4.89	3.44–6.74	<0.01	1.36	0.88–2.10	0.16
ly-ext						
–	1.00			1.00		
+	5.18	3.97–6.65	<0.01	1.52	1.14–2.02	<0.01

Abbreviations: CI, confidence interval; HR, hazard ratio; ly-ext, extratumoral lymphatic permeation; PM, intrapulmonary metastasis.

3.3 | Differences in prognosis between patients with ly-ext and those with ly-int

The median follow-up duration was 60 months (range 1–129 months). During the study period, 269 patients died. Cancer recurrence was detected in 336 patients. The 3-year OS rates for the ly0, ly-int, and ly-ext groups were 92.1%, 83.3%, and 76.1%, respectively. The OS of the ly-ext group was significantly inferior to that of the ly0 group ($P < 0.01$), but there was no significant difference in the OS of the ly-int and ly-ext groups. The 3-year RFS rates for the ly0, ly-int, and ly-ext groups were 82.3%, 62.8%, and 31.3%, respectively, and the RFS of the ly-ext group was significantly inferior to that of the ly0 group ($P < 0.01$) and the ly-int group ($P < 0.01$; Figure S4). Table 2 shows the result of univariate and multivariate Cox regression analyses of RFS. pT status (pT3 and pT4; $P < 0.01$, HR 1.59, 95% CI 1.15–2.15), pN status (N1 and N2; $P < 0.01$, HR 2.82, 95% CI 2.22–3.57), vascular invasion (positive; $P < 0.01$, HR 2.38, 95% CI 1.87–3.03), pleural invasion (positive; $P < 0.01$, HR 1.55, 95% CI 1.25–1.93), and ly-ext ($P < 0.01$, HR 1.52, 95% CI 1.14–2.02) were independent poor prognostic factors. In multivariate Cox regression analysis of OS, ly-ext was not a significant prognostic factor (Table S3).

3.4 | Clinicopathological characteristics and prognosis of patients with high and low numbers of ly-ext foci

Since the median number of ly-ext foci in ly-ext patients was 5, we classified ly-ext high group as 5 or more than 5 and ly-ext low group as less than 5. Figure 1B–E shows representative HE and anti D2-40 staining of the ly-ext high and low groups. Table S4 shows the clinicopathological characteristics of patients with high (≥5) and low (<5) numbers of ly-ext foci. The ly-ext high group tended to

have more patients with lymph node metastasis or solid or micro-papillary predominant tumor. In pT1 patients with ly-ext, the ly-ext high group tended to have more patients with vascular invasion (Table S5). Figure 2 shows the OS and RFS curves of patients with high and low numbers of ly-ext foci. There was no significant difference in 3-year OS rate between the ly-ext high and low groups (70.5% vs. 81.4%, $P = 0.11$; Figure 2A), but the 3-year RFS rate of the ly-ext high group was significantly lower than that of the ly-ext low group (14.7% vs. 50.0%, $P < 0.01$; Figure 2B). Moreover, there was no significant difference in 3-year OS between ly-ext foci/cm^2 high and low groups (Figure 2C), but the 3-year RFS of patients with a high number of ly-ext foci/cm^2 was significantly shorter than that of patients with a low number of ly-ext foci/cm^2 (Figure 2D). Table 3 shows the result of univariate and multivariate Cox regression analyses on RFS of the patients with ly-ext. A high number of ly-ext foci was an independent poor prognostic factor ($P < 0.01$, HR 2.38, 95% CI 1.44–4.00). When the number of ly-ext foci/cm^2 was used, the number of ly-ext foci/cm^2 was also an independent prognostic factor (Table S6). Even in the analysis limited to stage I patients, the 3-year RFS rate of patients in the ly-ext high group was significantly lower than that of patients in the ly-ext low group (15.4% vs. 75.0%, $P < 0.05$; Figure S5).

3.5 | Correlations between the number of ly-ext foci and immunohistochemical staining scores

Figure 3 shows the comparison of immunohistochemical staining scores between the two groups, and representative slides of anti-CD8 and FOXP3 staining are shown in Figure 4. The expression levels of CD44, ALDHA1, and MUC1 in tumor cells within the primary lesion and extratumoral lymphatic vessels were not significantly different between the two groups. In tumor stroma within the primary lesion, the number (median [interquartile range]) of CD8+ T cells in the ly-ext high group was significantly lower than that in the ly-ext low group (13.6 [9.7, 18.4] vs. 21.8 [17.6, 25.2], $P < 0.05$), and the number of FOXP3+ T cells in the ly-ext high group was significantly higher than that of the ly-ext low group (17.6 [8.5, 22.4] vs. 5.8 [2.6, 15.8], $P < 0.01$). The CD8/FOXP3 ratio of the ly-ext high group was significantly lower than that of the ly-ext low group (1.2 ± 0.7 vs. 6.4 ± 10.1, $P < 0.01$). There was no significant difference in the number of both CD4+ T cells and CD79a+ B cells within the primary lesions and in the extratumoral lymphatic vessels between the ly-ext high group and the ly-ext low group. The number of CD204+ TAMs in the primary tumor stroma tended to be higher in the ly-ext high group than in the ly-ext low group. Within extratumoral lymphatic vessels, the number of
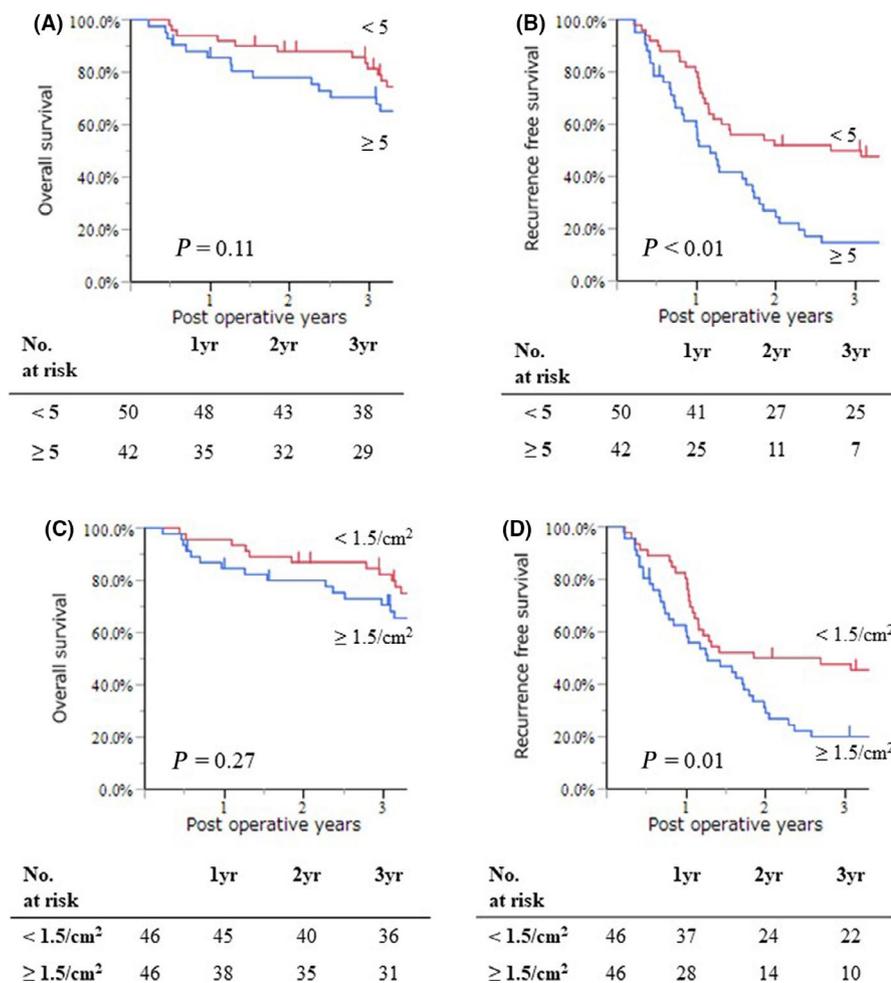

FIGURE 2 Overall survival (OS) and recurrence-free survival (RFS) curves of patients with completely resected lung adenocarcinoma with extratumoral lymphatic permeation (ly-ext). (A) OS curves of patients with high and low numbers of ly-ext foci. The red line represents the group with a low number of ly-ext foci and the blue line represents the group with a high number of ly-ext foci. (B) RFS curves of patients with high and low numbers of ly-ext foci. The red line represents the group with a low number of ly-ext foci and the blue line represents the group with a high number of ly-ext foci. (C) OS curves of patients with high and low numbers of ly-ext foci/cm^2. The red line represents the group with a low number of ly-ext foci/cm^2 and the blue line represents the group with a high number of ly-ext foci/cm^2. (D) RFS curves of patients with a high and low number of ly-ext foci/cm^2. The red line represents the group with a low number of ly-ext foci/cm^2 and the blue line represents the group with a high number of ly-ext foci/cm^2.

TABLE 3 Univariate and multivariate analysis of clinicopathological factor associated with recurrence-free survival of ly-ext patients

Variables	Univariate			Multivariate		
	HR	95% CI	P value	HR	95% CI	P value
Age						
<65	1.00					
≥65	0.83	0.51–1.32	0.42			
Sex						
Female	1.00					
Male	1.16	0.71–1.94	0.56			
Smoking						
Never	1.00					
Ever	1.08	0.66–1.74	0.74			
Adjuvant CTx						
No	1.00					
Yes	0.79	0.50–1.27	0.33			
EGFR mutation						
Negative	1.00					
Positive	1.35	0.84–2.15	0.21			
pT status						
T1or2	1.00			1.00		
T3or4	1.61	0.93–2.69	0.09	1.53	0.51–3.71	0.41
pN status						
–	1.00			1.00		
+	1.69	0.92–3.42	0.10	1.17	0.60–2.46	0.65
Vascular invasion						
–	1.00					
+	1.15	0.69–2.02	0.61			
Pleural invasion						
–	1.00					
+	1.32	0.83–2.11	0.25			
PM						
–	1.00			1.00		
+	1.77	0.94–3.11	0.08	1.03	0.37–3.32	0.96
No. of ly–ext						
<5	1.00			1.00		
≥5	2.45	1.52–4.00	<0.01	2.38	1.44–4.00	<0.01

Abbreviations: CI, confidence interval; CTx, chemotherapy; HR, hazard ratio; PM, intrapulmonary metastasis.

FOXP3+ T cells in the ly-ext high group was significantly higher than that in the ly-ext low group (5 [1, 7.5] vs. 0 [0, 2], $P < 0.05$), but there was no significant difference in the number of CD8+ T cells, CD8/FOXP3 ratio, and CD204+ TAMs between the groups.

4 | DISCUSSION

In the present study, we retrospectively evaluated the prognostic impact of the number of ly-ext foci in patients with completely resected lung adenocarcinoma. The ly-ext high group had a remarkably shorter RFS than the ly-ext low group. In addition, we compared differences in the immune microenvironment between the ly-ext high and low groups and found that the ly-ext high group had a more suppressive immune microenvironment. This is the first study to investigate the prognostic impact of the number of ly-ext foci and the differences in the microenvironment based on the number of ly-ext foci.

In many types of cancers, such as gastric, esophagus, colorectal, hepatic, and breast cancers, the frequency of blood vessel and lymphatic permeation has been reported as an independent prognostic factor.[11-16] However, in lung cancer, the prognostic impact
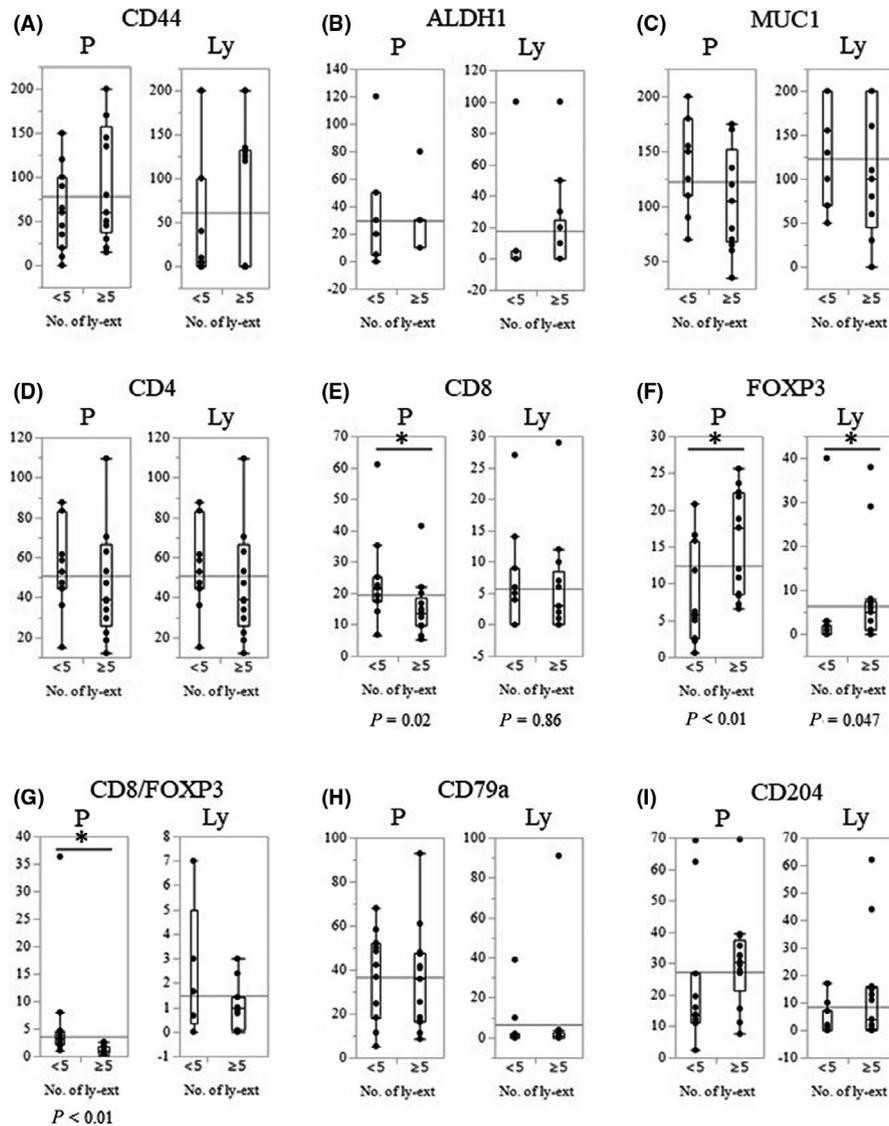
FIGURE 3 Comparison of the immunohistochemical staining score of primary tumors and tumors in extratumoral lymphatic vessels between the extratumoral lymphatic permeation (ly-ext) high and low groups. (A) Comparison of the CD44 score of primary tumors and tumors in intralymphatic regions between the ly-ext high and low groups. (B) Comparison of the ALDH1 score of primary tumors and tumors in intralymphatic regions between ly-ext high and low group. (C) Comparison of the MUC1 score of primary tumors and tumors in intralymphatic regions between the ly-ext high and low groups. (D) Comparison of the number of CD4+ T cells in primary tumor stroma and intralymphatic regions between the ly-ext high and low groups. I Comparison of the number of CD8+ T cells in primary tumor stroma and intralymphatic regions between the ly-ext high and low groups. (F) Comparison of the number of FOXP3+ T cells in primary tumor stroma and intralymphatic regions between the ly-ext high and low groups. (G) Comparison of CD8+/FOXP3+ T cells ratio in primary tumor stroma and intralymphatic regions between the ly-ext high and low groups. (H) Comparison of the number of CD79a+ B cells in primary tumor stroma and intralymphatic regions between the ly-ext high and low groups. (I) Comparison of the number of CD204+ macrophages in primary tumor stroma and intralymphatic regions between the ly-ext high and low groups

of the number of blood vessel invasion is still controversial. Kaseda et al. have reported that the frequency of vessel invasion is not a predictor of recurrence after resection, while others have argued that frequency of vessel invasion is a more important prognostic factor than the presence of vessel invasion.[17,18] Few studies have examined the significance of the number of lymphatic permeation foci on prognosis.[18] Lymphatic permeation and blood vessel invasion have different recurrence patterns sin resected lung cancer. Lung cancer with lymphatic permeation significantly recurs in the

mediastinal lymph nodes, while lung cancer with vascular invasion is more likely to recur in the distant organs, including the brain, liver, and adrenal glands.[19,20] In other words, tumor cells that invade blood vessels enter the systemic bloodstream, whereas those that invade lymphatic vessels are trapped in the lymph nodes that they flow in. This difference may be one of the reasons why the number of lymphatic invasions is associated with recurrence.

In this study, the ly-ext high group had fewer CD8+ T cells and more FOXP3+ T cells in the tumor stroma within the primary lesion
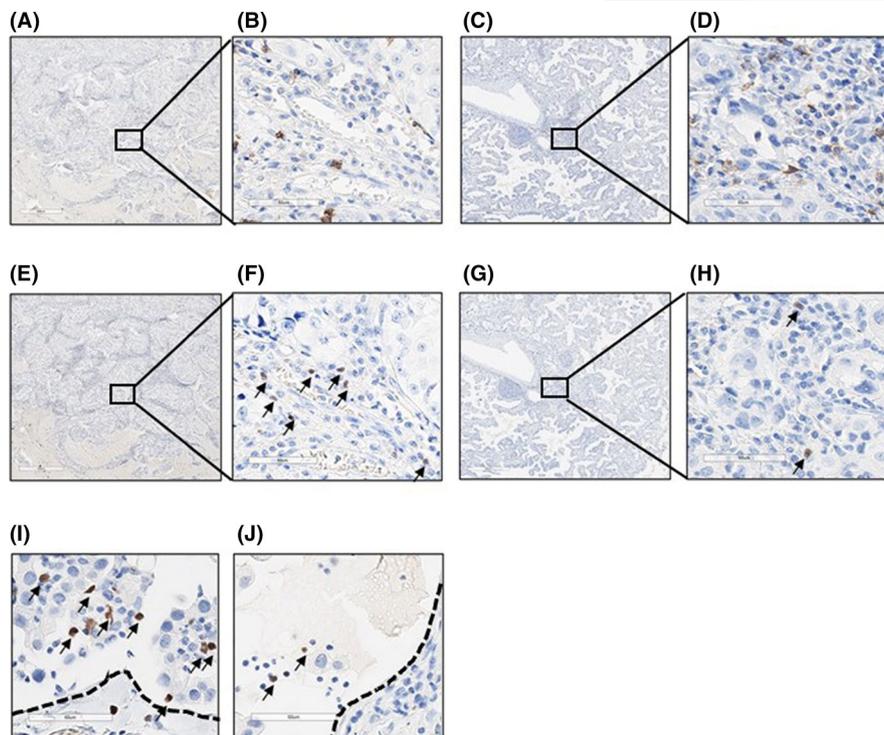
FIGURE 4 Representative immunohistochemical staining images. (A) Example of a low power field of CD8 staining of tumor stroma within a primary lesion of the extratumoral lymphatic permeation (ly-ext) high group. (B) High power field of square of (A). (C) Example of a low power field of CD8 staining of tumor stroma within a primary lesion of the ly-ext low group. (D) High power field of square of (C). (E) Example of a low power field of FOXP3 staining of tumor stroma within a primary lesion of the ly-ext high group. (F) High power field of square of (E). Arrows indicate FOXP3 positive lymphocytes. (G) Example of a low power field of FOXP3 staining of tumor stroma within a primary lesion of the ly-ext low group. (H) High power field of square of (G). Arrows indicate FOXP3 positive lymphocytes. (I) Example of a high power field of FOXP3 staining of intralymphatic regions of the ly-ext high group. Arrows indicate FOXP3 positive lymphocytes. (J) Example of a high power field of FOXP3 staining of intralymphatic regions of the ly-ext high group. Arrows indicate FOXP3 positive lymphocytes

than the ly-ext low group. The tumor microenvironment plays a critical role in cancer progression, and TILs are considered to be biomarkers of the host immune reaction to tumor antigens. In previous studies, a high number of stromal CD8+ T cells was reported as a favorable prognostic factor for lung cancer, whereas a high number of stromal FOXP3+ T cells was considered to be a poor prognostic factor.[21,22] In breast cancer, as in our study, a high number of FOXP3+ T cells in the primary tumor stroma was reported to be significantly associated with the presence of lymphatic vessel invasion.[23] FOXP3+ regulatory T cells produce cytokines such as TGF-β and IL-10 and inhibit the function of cytotoxic T cells against cancer cells.[24] Additionally, TGF-β increases the invasiveness of cancer cells by reducing the adhesion of cancer cells and increasing their motility.[25] TGF-β also promotes tumor-associated lymphangiogenesis by inducing VEGF-C expression.[26] These findings support the results of the present study.

In present study, the number of CD204+ TAMs in primary tumor stroma tended to be higher in the ly-ext high group than in the ly-ext low group. TAMs stimulate angiogenesis via expressing factors such as VEGF. TAMs also produce various growth factor and chemokines and contribute to the migration of tumor cells towards vessels.[27] This may be one of the reasons for our finding that the number of CD204+TAMs is associated with the number of ly-ext foci.

In present study, there were no differences in the expression levels of ALDH1 and CD44 in cancer cells between two groups. Matsumura et al. reported that tumor cells that form multiple small nests have high expression levels of CD44 and a higher frequency of intrapulmonary metastasis.[28] Kirita et al. showed that low ALDH1 expression in cancer cells is independent predictive factors for LN metastasis.[29] In their study, the expression level of ALDH1 and CD44 in cancer cells in the primary lesion was not a significant predictive factor for LN and intrapulmonary metastasis. Taken together with the results of the present study, the expression of CD44 and ALDH1 in tumor cells in the primary lesion may not be related to the frequency of lymphatic invasion.

There were some limitations in the current study. This was a retrospective study carried out at a single institution. The total number of patients with ly-ext was relatively small and the follow-up period was short.

To our knowledge, this is the first report to examine the association between the number of ly-ext foci and the prognosis, and between the number of ly-ext foci and the immune microenvironment. This study clearly indicates that the frequency of extratumoral lymphatic permeation is an independent prognostic factor, as is its presence, and suggests that tumors with a suppressive immune

microenvironment in the primary lesion are more likely to develop lymphatic vessel invasion than those without it.

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REFERENCES

1. Maeda R, Yoshida J, Ishii G, et al. Long-term survival and risk factors for recurrence in stage I non-small cell lung cancer patients with tumors up to 3 cm in maximum dimension. *Chest*. 2010;138:357-362.
2. Maeda R, Yoshida J, Ishii G, et al. Poor prognostic factors in patients with stage IB non-small cell lung cancer according to the seventh edition TNM classification. *Chest*. 2011;139:855-861.
3. Naito Y, Goto K, Nagai K, et al. Vascular invasion is a strong prognostic factor after complete resection of node-negative non-small cell lung cancer. *Chest*. 2010;138:1411-1417.
4. Li Z, Yu Y, Lu J, et al. Analysis of the T descriptors and other prognosis factors in pathologic stage I non-small cell lung cancer in China. *J Thorac Oncol*. 2009;4:702-709.
5. Poncelet AJ, Cornet J, Coulon C, et al. Intra-tumoral vascular or perineural invasion as prognostic factors for long-term survival in early stage non-small cell lung carcinoma. *Eur J Cardiothorac Surg*. 2008;33:799-804.
6. Kelsey CR, Marks LB, Hollis D, et al. Local recurrence after surgery for early stage lung cancer: an 11-year experience with 975 patients. *Cancer*. 2009;115:5218-5227.
7. Saijo T, Ishii G, Ochiai A, et al. Evaluation of extratumoral lymphatic permeation in non-small cell lung cancer as a means of predicting outcome. *Lung Cancer*. 2007;55:61-66.
8. Matsumura Y, Hishida T, Shimada Y, et al. Impact of extratumoral lymphatic permeation on postoperative survival of non-small-cell lung cancer patients. *J Thorac Oncol*. 2014;9:337-344.
9. Takeya M, Komohara Y. Role of tumor-associated macrophages in human malignancies: friend or foe? *Pathol Int*. 2016;66:491-505.
10. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. 2011;331:1565-1570.
11. Ishigami S, Natsugoe S, Hokita S, et al. Carcinomatous lymphatic invasion in early gastric cancer invading into the submucosa. *Ann Surg Oncol*. 1999;6:286-289.
12. Moriya H, Ohbu M, Kobayashi N, et al. Lymphatic tumor emboli detected by D2–40 immunostaining can more accurately predict lymph-node metastasis. *World J Surg*. 2011;35:2031-2037.
13. Akagi Y, Adachi Y, Ohchi T, et al. Prognostic impact of lymphatic invasion of colorectal cancer: a single-center analysis of 1,616 patients over 24 years. *Anticancer Res*. 2013;33:2965-2970.
14. Shinto E, Tsuda H, Ueno H, et al. Prognostic value of the number and size of venous invasions in pT3 colorectal cancer: a prospective observational study. *World J Surg*. 2014;38:3257-3264.
15. Fujita N, Aishima S, Iguchi T, et al. Histologic classification of microscopic portal venous invasion to predict prognosis in hepatocellular carcinoma. *Hum Pathol*. 2011;42:1531-1538.
16. Uematsu T, Kasami M, Watanabe J, et al. Is lymphovascular invasion degree one of the important factors to predict neoadjuvant chemotherapy efficacy in breast cancer? *Breast Cancer*. 2011;18:309-313.
17. Kaseda K, Ishii G, Aokage K, et al. Identification of intravascular tumor microenvironment features predicting the recurrence of pathological stage I lung adenocarcinoma. *Cancer Sci*. 2013;104:1262-1269.
18. Hashizume S, Nagayasu T, Hayashi T, et al. Accuracy and prognostic impact of a vessel invasion grading system for stage IA non-small cell lung cancer. *Lung Cancer*. 2009;65:363-370.
19. Sugita Y, Kinoshita T, Shima T, et al. Lymphatic permeation and vascular invasion should not be integrated as lymphovascular invasion in lung adenocarcinoma. *Gen Thorac Cardiovasc Surg*. 2021;69(7):1070-1078.
20. Shimizu R, Kinoshita T, Sasaki N, et al. Clinicopathological factors related to recurrence patterns of resected non-small cell lung cancer. *J Clin Med*. 2020;9:2473.
21. Al-Shibli KI, Donnem T, Al-Saad S, et al. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. *Clin Cancer Res*. 2008;14:5220-5227.
22. Geng Y, Shao Y, He W, et al. Prognostic role of tumor-infiltrating lymphocytes in lung cancer: a meta-analysis. *Cell Physiol Biochem*. 2015;37:1560-1571.
23. Chen Y, Klingen TA, Aas H, et al. Tumor-associated lymphocytes and macrophages are related to stromal elastosis and vascular invasion in breast cancer. *J Pathol Clin Res*. 2021;7(5):517-527. doi:10.1002/cjp2.226
24. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. *Oncogene*. 2008;27:5904-5912.
25. Elliott RL, Blobe GC. Role of transforming growth factor Beta in human cancer. *J Clin Oncol*. 2005;23:2078-2093.
26. Pak KH, Park KC, Cheong JH. VEGF-C induced by TGF-beta1 signaling in gastric cancer enhances tumor-induced lymphangiogenesis. *BMC Cancer*. 2019;19:799.
27. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer*. 2004;4:71-78.
28. Matsumura Y, Ishii G, Aokage K, et al. Morphophenotypic characteristics of intralymphatic cancer and stromal cells susceptible to lymphogenic metastasis. *Cancer Sci*. 2012;103:1342-1347.
29. Kirita K, Ishii G, Matsuwaki R, et al. Identification of biological properties of intralymphatic tumor related to the development of lymph node metastasis in lung adenocarcinoma. *PLoS One*. 2013;8:e83537.

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