Thoracic Cancer
Open Access
Thoracic Cancer ISSN 1759-7706

ORIGINAL ARTICLE

Impact of tumor infiltrating lymphocytes and lymphoid follicle formation on patient survival following surgery for lung squamous cell carcinoma

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Keywords
Lung cancer; lymphoid follicle; squamous cell carcinoma; tumor-infiltrating lymphocyte.

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Received: 15 October 2018;
Accepted: 16 November 2018.

doi: 10.1111/1759-7714.12935

Thoracic Cancer **10** (2019) 219–225

Abstract

Background: Tumor infiltrating lymphocytes (TILs) are known to correlate with the prognosis of patients affected by a variety of cancer types. We evaluated TILs in patients who underwent surgery for lung squamous cell carcinoma (SCC).
Methods: Specimens obtained from patients during resection of lung SCC were examined for TIL density, lymphoid follicle formation, PD-L1 expression, and the appearance of regulatory T cells (Tregs).
Results: We enrolled 72 patients who underwent surgery for SCC (TIL grades 0, 1, and 2: 29, 18, and 25, respectively). Lymphoid follicles were observed in 13 (18.1%) patients and 8 were positive for Tregs, which were always observed in association with lymphoid follicles ($P < 0.001$). Multivariate analysis revealed that lymphoid follicle formation, the appearance of Tregs, pathological stage, and pleural invasion were independent prognostic factors related to overall survival, whereas TIL density and PD-L1 expression were not.
Conclusion: SCC patients with lymphoid follicle formation accompanied by Tregs show poor survival following lung resection surgery.

Introduction

In the past decades, most of the advances made in the treatment of non-small cell lung cancer (NSCLC) have focused on adenocarcinoma or non-squamous cell carcinoma, while there has been no breakthrough in the treatment of squamous cell carcinoma (SCC). Recently, the use of immune checkpoint inhibitor (ICI) therapy has drastically changed treatment options for patients with NSCLC, particularly SCC. ICI administration blocks tumor immunoediting and induces tolerance between cancer cells and the immune system, thus affecting interaction between the PD-1 pathway and its ligand (PD-L1). Several reports have suggested that tumor-infiltrating lymphocytes (TILs) are correlated with the prognosis of patients with various types of cancer, such as melanoma,[1] colon,[2] ovarian,[3] breast,[4] and pancreatic cancers.[5] Furthermore, TILs play a complementary role for tumor node metastasis (TNM) classification, and TIL density or distribution is associated with lung cancer prognosis.[6] A study found that the appearance of lymphoid follicles in tumor stroma is a possible prognostic factor;[2] however, the study also noted that some studies reported favorable prognosis for patients with lymphoid follicles in tumor stroma, while others did not.[2]

In the present study, we focused on patients with resectable lung SCC and retrospectively analyzed the relationship between pathological factors, including TIL density, lymphoid follicles, PD-1/PD-L1 expression in tumor stroma, and patient prognosis in order to find prognostic markers to guide treatment.

Methods

Specimens obtained from consecutive lung SCC patients who underwent complete resection from January 2010 to December 2012 at our institution were analyzed. Informed consent for the use of materials was obtained from each patient and the Ethical Committee of Dokkyo Medical

University Hospital approved this retrospective study (#R-5-8). Follow-up examinations were completed for all patients by January 2018.

Resected specimens were fixed in 10% neutral buffered formalin at room temperature and then embedded in paraffin. Sections (2 μm thick) were obtained from a block including the largest cut surface of the tumor, stained with hematoxylin and eosin (H&E), and examined. Next, 4 μm thick sections were cut from the same blocks, deparaffinized in xylene, and dehydrated in graded alcohol solutions. A standard avidin-biotin complex peroxidase technique was then used for immunohistochemical staining of primary antibodies against CD3, CD4, CD8, CD20, and CD25. Immunohistochemical analysis of PD-L1 was performed by LSI Medience Corp. (Tokyo, Japan) using a commercially available antibody (22C3). A trained observer and a pathologist reviewed each slide in detail. When there was disagreement, other pathologists were introduced into the discussion.

The TILs were divided into three grades based on intensity and distribution: grade 0, low or focal intensity; grade 1, medium or multifocal intensity; and grade 2, high or diffuse intensity (Fig 1a–c). Lymphoid follicles were identified as CD20-positive B cell accumulations with a germinal center (Fig 1d–f). The T cell:B cell ratio of TILs was determined based on a CD3:CD20 ratio. CD4 and CD25 double-positive T cells in TILs were considered to be infiltration by regulatory T cells (Tregs) (Fig 1g–i).

Statistical analysis of the groups was performed using chi-square or Fischer's exact tests to compare variables. Survival curves were obtained using the Kaplan–Meier method and comparisons within each group were performed using a log-rank test. Risk factors for overall survival were evaluated via univariate and multivariate analyses using the Cox regression method. Statistical calculations were performed using SPSS version 25 (IBM Corp., Armonk, NY, USA). Significance was considered at $P < 0.05$.

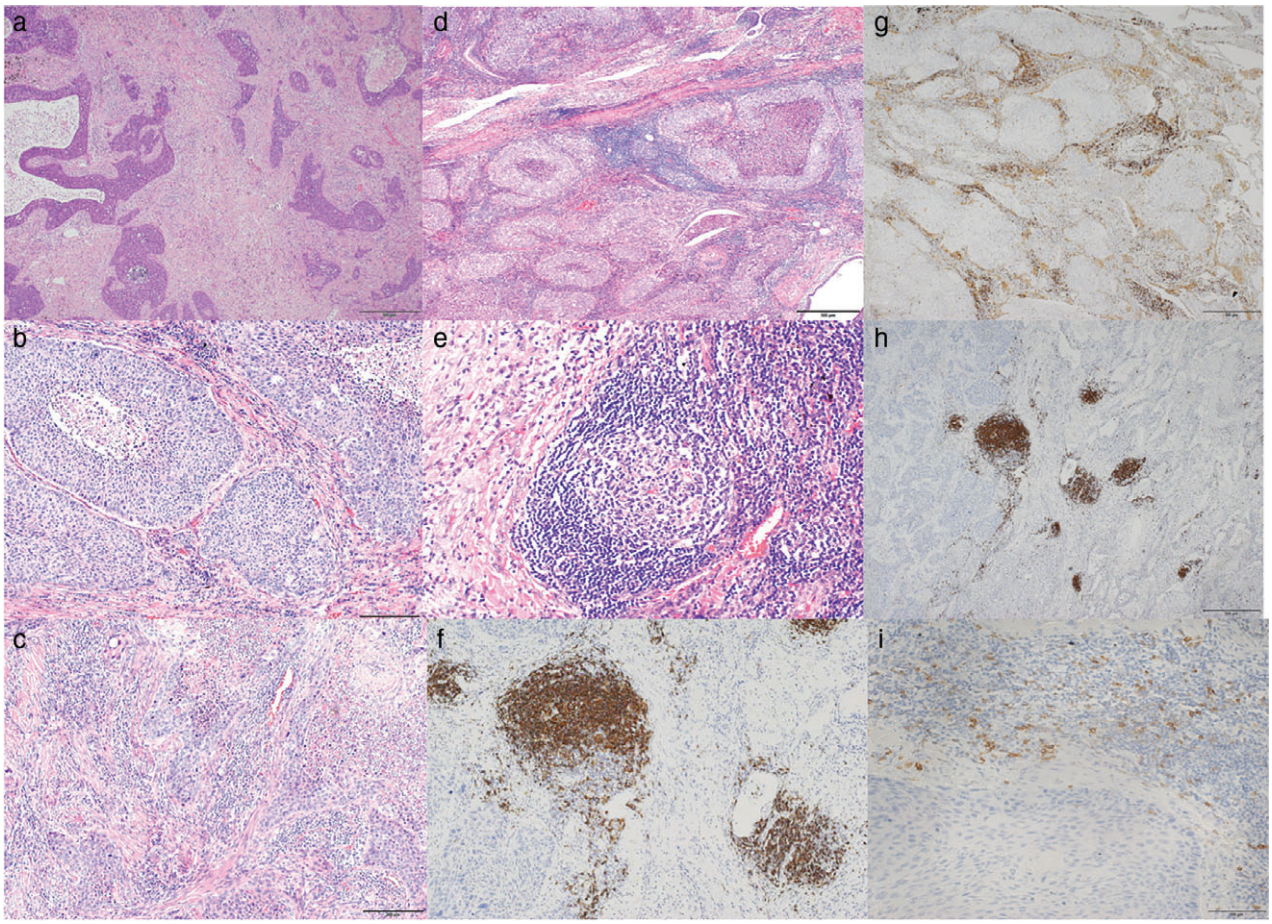

Figure 1 Representative images showing tumor-infiltrating lymphocytes (TILs): (**a**) grade 0 (hematoxylin and eosin [H&E] stain, ×40), (**b**) grade 1 (HE stain, ×100), and (**c**) grade 2 (HE stain, ×100). Lymphoid follicles (**d**) in tumor stroma (HE stain, ×40), (**e**) with a germinal center (HE stain, ×200) and (**f**) composed of B cells (CD20 stain, ×100). (**g**) T cells (CD4 stain, ×40). (**h**) B cells (CD20 stain, ×40). (**i**) Regulatory T cells (CD25 stain, ×200).

Results

A total of 72 patients with SCC underwent lung resection during the study period. Patient characteristics are shown in Table 1. Nine out of 72 patients were administered neoadjuvant therapy before surgery, 5 were administered chemoradiotherapy, and 4 were administered chemotherapy. PD-L1 expression was measured in all patients, with data from 63 available, which showed 0% in 19 and ≥ 1% in 44. Twenty-four patients experienced postoperative complications: respiratory morbidity in 19 (pneumonia in 7, prolonged air leakage in 6, acute exacerbation of interstitial pneumonia in 4, and bronchopleural fistula in 2); cardiovascular complications in 4 (arrhythmia in 2 and heart failure in 2); and surgical site infection in 1. During the five-year follow-up period, 31 patients died: 18 as the result of lung cancer and 13 to other causes (pneumonia in 4, cardiovascular disease in 2, empyema in 2, interstitial pneumonia in 1, liver cirrhosis in 1, and unknown in 3).

Among the 72 patients, the TIL grades were: 0 in 29, 1 in 18, and 2 in 25. Lymphoid follicles were observed in 13 (18.1%) of the 72 cases. Tregs were positive in eight cases and were exclusively observed in tumors of patients with lymphoid follicles ($P < 0.001$) (Table 2). The relationship between lymphoid follicles and PD-L1 expression is shown in Table 2, although the correlation was not statistically significant ($P = 0.16$).

Univariate analysis was performed using each factor (Table 3). The results revealed that pathological stage, pleural invasion, vascular invasion, and lymphoid follicles were associated with overall survival after lung resection, while neoadjuvant therapy, postoperative complications, TIL grade, and PD-L1 were not correlated with survival. Multivariate analysis was conducted with a P of < 0.05 in univariate analysis, and revealed that pleural invasion and lymphoid follicles were independent prognostic factors related to overall survival in patients who underwent lung resection (Table 3). The five-year survival rates of patients positive and negative for pleural invasion were 42.3% and 57.8%, respectively, representing a statistically significant difference ($P = 0.008$) (Fig 2a). Furthermore, the five-year survival rates for patients positive and negative for lymphoid follicles were 19.2% and 60.5%, respectively ($P = 0.002$) (Fig 2b). The five-year survival rates of patients positive and negative for Tregs were 18.8% and 58.3%, respectively ($P = 0.003$) (Fig 2c).

Table 1 Patient characteristics

Characteristics	N = 72
Gender	
Male	67
Female	5
Smoking index (packs/year)	
< 30	10
≥ 30	62
Interstitial pneumonia	
Absent	50
Present	22
SCC	
≤ 1.6	39
> 1.6	32
Unknown	1
Neoadjuvant therapy	
+	9
–	63
Type of resection	
Sublobar	9
Lobectomy or more	63
Pleural invasion	
0	51
1	8
2	3
3	10
Lymphatic invasion	
0	60
1	12
Vascular invasion	
0	28
1	44
Pathological stage	
0	1
IA	20
IB	15
IIA	11
IIB	8
IIIA	17
PD-L1 expression	
0%	19
1–49%	36
≥ 50%	8
Unknown	9
Postoperative complication	
+	24
–	48
Status	
Alive	39
Died	31
Unknown	2

SCC, squamous cell carcinoma-related antigen.

Discussion

In the present study, we evaluated TIL density and lymphoid follicles in tumor stroma to determine their effectiveness as prognostic factors in patients with lung SCC who have undergone surgery. Our results indicate that lymphoid follicles, the appearance of Tregs, pathological stage, and pleural invasion are independent prognostic factors related to overall survival following resection of lung SCC, while TIL density and PD-L1 expression are not

Table 2 Relationship between lymphoid follicles and regulatory T cells

		Tregs			PD-L1		
		Absent	Present	*P*	0%	≥ 1%	*P*
Lymphoid follicle	Absent	59	0		13	37	
	Present	5	8	< 0.001	6	7	0.16

Tregs, regulatory T cells.

Table 3 Univariate and multivariate analyses of overall survival

	Univariate				Multivariate			
Characteristics	HR	95% CI		*P*	HR	95% CI		*P*
Gender								
Male/female	0.87	0.27	4.75	0.87				
PS								
0/≥ 1	2.26	0.79	6.49	0.13				
BI								
< 600/≥ 600	5.59	0.76	41.0	0.091				
IP								
–/+	1.90	0.92	3.94	0.082				
SCC								
≤ 1.6/> 1.6	1.65	0.81	3.35	0.17				
pStage								
I/II + III	2.54	1.19	5.41	0.012	1.51	0.66	3.47	0.33
Neoadjuvant therapy								
–/+	0.63	0.19	2.07	0.45				
Operation								
limited/lobectomy or more	0.94	0.33	2.71	0.91				
Postoperative complication								
–/+	1.48	0.72	3.02	0.29				
Pleural invasion								
–/+	2.54	1.24	5.22	0.011	2.26	1.08	4.74	0.031
Lymphatic invasion								
–/+	1.64	0.70	3.84	0.28				
Vascular invasion								
–/+	2.43	1.08	5.46	0.031	1.60	0.68	3.79	0.28
TIL grade								
0/1–2	1.03	0.49	2.14	0.94				
0–1/2	0.97	0.45	2.06	0.93				
T/B lymphocyte ratio								
< 1/≥ 1	0.94	0.46	2.17	0.99				
PD-L1								
0%/≥ 1%	0.78	0.34	1.77	0.55				
0–49%/≥ 50%	1.97	0.74	5.20	0.17				
Lymphoid follicles								
–/+	3.33	1.50	7.43	0.003	2.61	1.11	6.01	0.026

BI, Brinkman index (smoking index); CI, confidence interval; HR, hazard ratio; IP, interstitial pneumonia; PS, performance status; pStage, pathological stage; SCC, squamous cell carcinoma-related antigen; TIL, tumor-infiltrating lymphocyte.

associated with survival. In the study cohort, Tregs were exclusively observed in cases with the presence of lymphoid follicles.

Tumor-infiltrating lymphocytes exist around cancer cells and play an important role in the mechanism of cancer immunity.[7] Several studies have reported relationships between TIL subsets, such as CD3-, CD8-, and FOXP3-positive cells, and prognosis.[8–11] Downregulation of the Fas/Fas-ligand pathway leads to cancer growth as cancer cells escape from the immune checkpoint system as a result of the inactivation of CD4- and CD8-positive lymphocytes, and subsequently avoid cytotoxic T cell attacks and apoptosis.[12] We evaluated CD4 and CD8 density as parameters of TIL subsets in the present specimens, but did not find their density or distribution to be a prognostic factor, with the exception of the existence of Tregs.

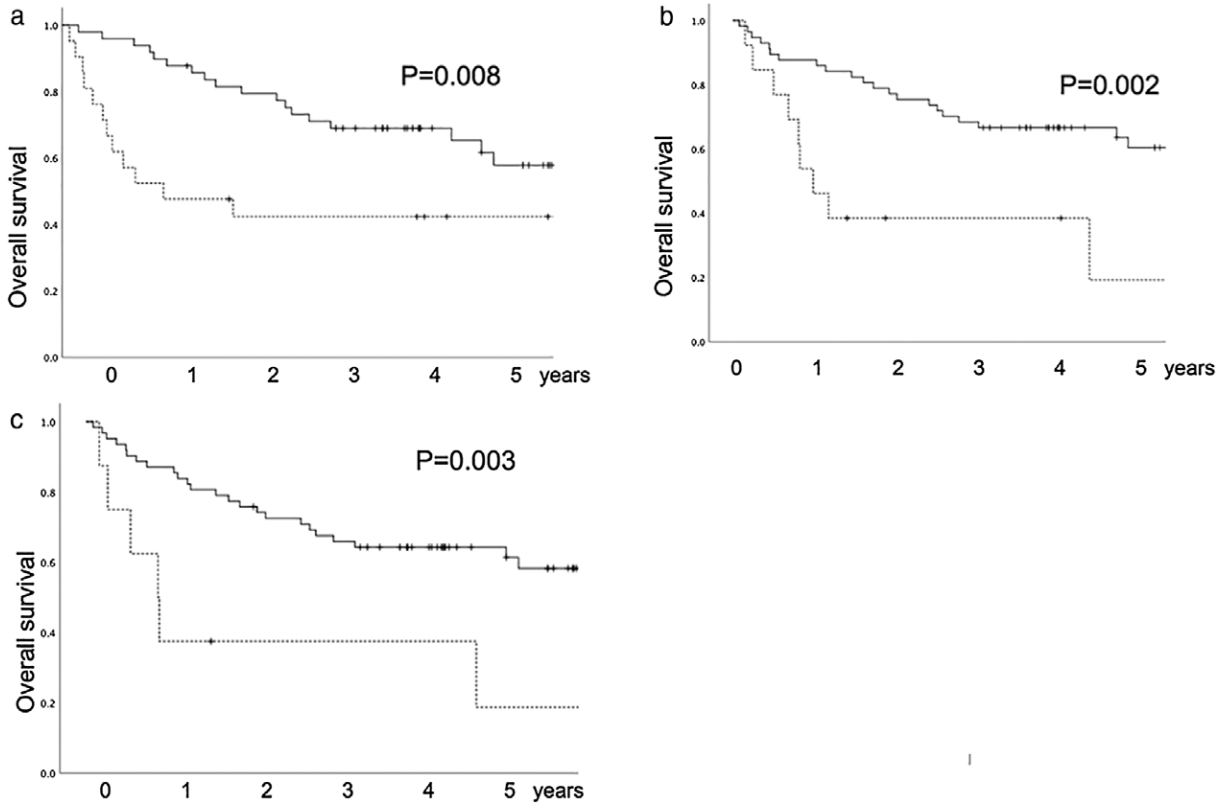

Figure 2 Overall survival curves. (**a**) Relationship between pleural invasion and overall survival. Solid line, negative for pleural invasion; dotted line, positive for pleural invasion. (**b**) Relationship between existence of lymphoid follicles and overall survival. Solid line, negative for lymphoid follicles; dotted line, positive for lymphoid follicles. (**c**) Relationship between the existence of regulatory T cells and overall survival. Solid line, negative for regulatory T cells; dotted line, positive for regulatory T cells.

Hasegawa *et al*. reported the prognostic value of Tregs in NSCLC.[13] We speculate that the existence of Tregs is more important to prognosis than TIL quantity, as shown in CD4- and CD8- positive cells.

Studies related to colon cancer have observed lymphoid follicle formation in advanced stage patients and superior prognosis in patients with lymphoid follicles compared to those without.[14–16] The presence of lymphoid follicles has been termed a Crohn's-like lymphoid reaction and a germinal center occurs with lymphocyte accumulation. The existence of these follicles has been proposed to be a prognostic factor independent of stage and TIL.[17–19] In the present study, we considered that CD20-positive B lymphocyte accumulation with a germinal center was evidence of lymphoid follicles and their existence in patients with lung SCC indicated poor prognosis compared to those without follicles. In the present study, all Treg positive patients had lymphoid follicles in their specimens. Because Treg existence is an indicator of poor prognosis in resected cancer,[13] Tregs around lymphoid follicles may have affected the survival of our study cohort. Further case accumulation and analysis is required to explain the difference between our data and other references.

FoxP3 is a specific marker of CD4 and CD25 double-positive Tregs, and Tregs play a role in the suppression and regulation of immune response, as well as the prevention of autoimmune disease.[20] The existence of Tregs in TILs has been reported to be correlated with poor survival of patients with melanoma,[21] kidney,[22] breast,[23] and ovarian cancers,[24] while those with Hodgkin lymphoma showed superior survival.[25] In colon cancer, the role of Tregs has not been consistently elucidated.[26] For example, a study found that tumor-infiltrating Tregs were associated with recurrence in pathologic stage I NSCLC patients,[27] while another reported that lung cancer patients with a high density of tumor-infiltrating Tregs had better prognosis compared to those without Tregs.[28] In the present study, we defined CD4 and CD25 double-positive lymphocytes as Tregs. These were only found around lymphoid follicles and all patients with Tregs had lymphoid follicle formation. We speculate that Tregs may suppress the immune response of lymphoid follicles, leading to poor prognosis for patients with such follicles.

Patients who undergo resection of advanced lung cancer often experience recurrence. Unfortunately, therapy options for recurrent disease are limited, especially for lung SCC. Recently, the utility of ICIs targeting PD-1 or PD-L1 has been reported, and PD-L1 expression seems to be a biomarker of the effects of ICI. In the present series, the enrolled patients underwent surgery from 2010 to 2012, and were not administered ICI therapy during the postoperative course. We conducted our study to clarify whether PD-L1 expression itself affected survival in patients that were not administered ICI therapy. Elevated PD-L1 expression is associated with a poor outcome in patients with bladder and ovarian cancers,[29,30] while NSCLC patients with PD-1 expression have also been shown to have a poor prognosis.[31,32] In our study, PD-L1 expression itself was not a prognostic factor in patients with lung SCC following surgery and was not correlated with lymphoid follicle formation.

Limitations of this study include its retrospective study design, performance by a single institution, and the small number of cases. Additional case accumulation is needed to analyze the effects of TILs and lymphoid follicles on tumor-immune interaction in cases of lung SCC.

In conclusion, lymphoid follicle formation, the appearance of Tregs, and pleural invasion were independent prognostic factors related to survival following resection of lung SCC, while TIL density and PD-L1 expression were not.

Acknowledgment

We thank Masaru Kojima and Hajime Kuroda for their diagnostic assistance.

Disclosure

No authors report any conflict of interest.

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