Original article

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

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Running title: Impact of lymphoid follicle formation for lung SCC

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Abstract

Purpose: 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 resected from patients during resection of lung SCC were examined for TIL density, lymphoid follicle formation, expression of PD-L1, and appearance of regulatory T cells (Tregs).

Results: We enrolled 72 patients who underwent surgery for SCC (TIL grade 0, 1, and 2; 29, 18, and 25, respectively). Lymphoid follicles were observed in 13 (18.1%) 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, 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 worse survival following lung resection surgery.

Key words: lung cancer. Squamous cell carcinoma, tumor-infiltrating lymphocyte, lymphoid follicle.

Introduction

In the pat decades, most of the advances in the treatment of non-small cell lung cancer (NSCLC) have focused on adenocarcinoma or non-squamous cell carcinoma, while there have been no breakthrough in the treatment of squamous cell carcinoma (SCC). Recently, use of immune checkpoint inhibitor (ICI) therapy has drastically changed treatment options for patients with NSCLC, especially SCC. ICI administration blocks tumor immunoediting, and induces tolerance between cancer cells and the immune system, thus affecting the interaction between the programmed death (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 (5). Furthermore, TILs play a complementary role for TNM classification, and TIL density or distribution is associated with lung cancer prognosis (6). Another study found that the appearance of lymphoid follicles in tumor stroma is a possible prognostic

factor (2), though also noted that some reports reported favorable prognosis for patients with lymphoid follicles in tumor stroma, while others did not (2).

In the present study, we focused on the patients with resectable lung SCC, and retrospectively analyzed the relationship between pathological factors, including TIL density, lymphoid follicles and the expression of PD1/PDL1 in tumor stroma, and prognosis of the patients in order to find prognostic markers to guide treatment.

Methods

Specimens resected from consecutive lung SCC patients who underwent a complete resection procedure from January 2010 through December 2012 at our institution were investigated. Informed consent for use of the 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, then embedded in paraffin. Sections (2 μm thick) were obtained from a block including the largest cut surface of the tumor, then stained with hematoxylin and eosin (H&E), and examined. Next, 4-μm thick sections were cut from the same blocks, and 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. Trained observer (MN) and pathologists (YN) reviewed each slide in detail. When there is any disagreement, other pathologists (MK and HK) were introduced in. PD-L1 analysis of the blocks was performed by LSI Medience Corp. (Tokyo, Japan) using a commercially available antibody (22C3).

Grading of TIL was divided into 3 grades based on intensity and distribution, with low or focal intensity given a grade of 0, medium or multi-focal intensity a grade of 1, and high or diffuse intensity a grade of 2 (Figure 1A-C). Lymphoid follicles were identified as CD20-positive B cell

accumulations with a germinal center (Figure 1D-F). The T cell/B cell ratio of TILs was determined based on CD3/CD20 ratio. CD4 and CD25 double-positive T cells in TILs were considered to be infiltration by regulatory T cells (Tregs) (Figure 1G-I).

Statistical analysis of the groups was performed using a chi-square test or Fischer's exact test 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 with univariate and multivariate analyses using the Cox regression method. Statistical calculations were performed using the SPSS statistics version 25 software program (IBM Corp., NY, USA). Significance was considered at $p<0.05$.

Results

A total of 72 patients with SCC underwent a lung resection procedure during the study period. Patient characteristics are shown in Table 1. Nine

out of 72 patients underwent neo-adjuvant therapy before surgery, chemoradiotherapy in 5 and chemotherapy in 4. PD-L1 expression was measured in all, with data from 63 available, which showed 0% in 19 and ≥1% in 44. Twenty-four patients had a postoperative complication, respiratory morbidity was in 19, pneumonia in 7, prolonged air leakage in 6, acute exacerbation of interstitial pneumonia in 4, and broncho-pleural fistula in 2; cardiovascular was in 4, arrhythmia in 2 and heart failure in 2; and surgical site infection was in 1. During the 5-year follow-up period, 31 of the patients died, 18 due to lung cancer and 13 to other causes, including 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, a TIL grade of 0 was noted in 29, a grade of 1 was noted in 18, and a grade of 2 was noted in 25. Lymphoid follicles were observed in 13 (18.1%) of the 72 cases. Tregs were positive in 8 cases and 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, though the correlation was not statistically significant (p=0.16).

Univariate analysis was performed using each factor (Table 3). Those results revealed that pathological stage, pleural invasion, vascular invasion, and lymphoid follicles were associated with overall survival after lung resection, while neo-adjuvant therapy, postoperatibe complication, TIL grade, and PD-L1 were not correlated with survival. Multivariate analysis was conducted with a p-value 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 5-year survival rate for patients positive for pleural invasion was 42.3%, while that was 57.8% for those negative for that factor (Figure 2A), a statistically significant difference (p=0.008). Furthermore, the 5-year survival rate for patients positive for lymphoid follicles was 19.2% and 60.5% for patients without those follicles (Figure 2B), p=0.002. As for Tregs, the

5-year survival rate was 18.8% and 58.3% in patients positive and negative, respectively (p=0.003) (Figure 2C).

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 after undergoing surgery. Our results indicate that lymphoid follicles, appearance of Tregs, pathological stage, and pl factor are independent prognostic factors related to overall survival following resection of lung SCC, while TIL density and PD-L1 expression were not associated with survival. In the present patients, Tregs were exclusively observed in cases with the presence of lymphoid follicles.

TILs exist around cancer cells and play an important role in the mechanism of cancer immunity (7), while several reports have pointed out relationships of TIL subsets, such as CD3-, CD8-, and FOXP3-positive cells, with prognosis (8-11). Cancer cells that escape from the immune check-point

system because of down-regulation of the Fas/Fas-ligand pathway (12) lead to cancer growth due to inactivation of CD4- and CD8-positive lymphocytes, and avoid cytotoxic T cell attacks and apoptosis. 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 except Treg existence. Hasegawa and colleague reported prognostic value of Treg in NSCLC (13). We speculate that the existence of Treg is more important to the prognosis than quantity of TIL as shown in CD4- and CD8- positive cells.

Studies related to colon cancer have noted observations of lymphoid follicle formation in advanced stage patients and that the prognosis of those with lymphoid follicles was superior as 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 as compared to those without those follicles. In present study, all Treg positives had lymphoid follicles in their specimens. Because Treg existence is an indicator of worse prognosis in resected cancer (x), Treg existence around lymphoid follicle may affect the survival in present series. Further case accumulation and analysis will be necessary to explain the difference between our data and other references.

FoxP3 is a specific marker of CD4 and CD25 double-positive Tregs, while Tregs have a role in suppression and regulation of immune response, as well as 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 (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 as compared to those without Tregs (28). In the present study, we defined CD4 and CD25 double-positive lymphocytes as Tregs. Those were only found to exist 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 worse prognosis for patients with those follicles present.

Patients who undergo resection of advanced lung cancer often experience recurrence. Unfortunately, therapy options for recurrent disease are limited, especially lung SCC. Recently, the utility of ICI targeting PD-1 or its ligand PD-L1 has been reported, and PD-L1 expression seems to be a biomarker of the effects of ICI. In present series, enrolled patients were operated from 2010 to 2012, and they did not undergo ICI therapy during postoperative course. We considered whether PDL1 expression itself affected survival without ICI therapy or not. An elevated expression of PD-L1 is associated with worse outcome of patients with bladder and ovarian cancer

(29, 30), while NSCLC patients with PD-1 expression have also been shown to have a worse prognosis (31, 32). In our study, PD-L1 expression itself was not a prognostic factor for patients with lung SCC following surgery and also 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 for analyzing the effects of TILs and lymphoid follicles on tumor-immune interaction in cases of lung SCC.

In conclusion, lymphoid follicle formation, 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.

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Disclosure statement

None was declared.

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Figure legends

Figure 1. Representative images showing tumor-infiltrating lymphocytes (TILs). A. Grade 0 (HE stain, x40). B. Grade 1 (HE stain, x100). C. Grade 2 (HE stain, x100). D. Lymphoid follicles in tumor stroma (HE stain, x40). E. Lymphoid follicles with a germinal center (HE stain, x200). F. Lymphoid follicles composed of B cells (CD20 stain x100). G. T cells (CD4 stain, x40). H. B cells (CD20 stain, x40). I. Regulatory T cells (CD25 stain, x200).

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 existence of regulatory T cells and overall survival. Solid line, negative for regulatory T cells; dotted line, positive for regulatory T cells.

Table 1. Patient characteristics

Total		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
Neo-adjuvant therapy		
	+	9
	-	63
Type of resection		
	sublobar resection	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
	dead	31
	unknown	2

SCC, squamous cell carcinoma related antigen; PD-L1, programmed death-ligand 1

Table 2. Relationship between lymphoid follicles and regulatory T cells

		Tregs			PD-L1		
		absent	present	P value	0	0	P value
Lymphoid follicle	absent	59	0		13	13	
	present	5	8	<0.001	6	6	0.16

Tregs, regulatory T cells; PD-L1, programed death-ligand 1

Table 3. Univariate and multivariate analysis of overall survival

	Univariate			Multivariate			
	HR	95% CI	P-value	HR	95%CI		P-value
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
Neo-adjuvant 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

HR, hazard ratio; CI, confidential interval; PS, performance status; BI, Brinkman index (smoking index); IP, interstitial pneumonia; SCC, squamous cell carcinoma related antigen; pStage, pathological stage; TIL, tumor-infiltrating lymphocyte; PD-L1, programmed death ligand 1