Original Study

Immune Microenvironment Differences Between Squamous and Non-squamous Non–small-cell Lung Cancer and Their Influence on the Prognosis

Xiangjiao Meng,[1] Yongsheng Gao,[2] Lian Yang,[1] Haiyan Jing,[3] Feifei Teng,[1] Zhaoqin Huang,[4] Ligang Xing[1]

Abstract

The study aims to elucidate the possible difference in immune microenvironment between squamous non–small-cell lung cancer (SQ-NSCLC) and non-SQ-NSCLC. Cluster of differentiation 8 (CD8), cluster of differentiation 4, transcription factor forkhead box P3, and programmed death-ligand 1 expression were examined on 197 non-SQ-NSCLC samples. More CD8+ tumor infiltrating lymphocytes were detected in the cancer nests from patients with SQ-NSCLC. The different cCD8+ tumor infiltrating lymphocyte profile indicates that SQ-NSCLC and non-SQ-NSCLC are likely different cancer types with respect to their immune microenvironments.

Introduction: Checkpoint blockades have entered routine clinical use for non–small-cell lung cancer (NSCLC). However, there were some differences in efficacy and response predictors for anti-programmed cell death protein 1 (PD-1) antibodies between squamous (SQ) and nonsquamous (non-SQ) NSCLC. The study aims to elucidate the possible difference in immune microenvironment between SQ-NSCLC and non-SQ-NSCLC and their influence on the prognosis. **Patients and Methods:** A total of 197 patients with stages I to III NSCLC were included. cluster of differentiation 8 (CD8), cluster of differentiation 4 (CD4), transcription factor forkhead box P3 (FOXP3), and programmed death-ligand 1 (PD-L1) expression were examined in cancer nest and stroma on 85 SQ-NSCLC and 112 non-SQ-NSCLC samples using immunohistochemistry. **Results:** More CD8+ tumor infiltrating lymphocytes (TILs) were detected in the cancer nests (cCD8) from patients with SQ-NSCLC than those with non-SQ-NSCLC (56% vs. 34%; $P = .002$). There were no significant differences between the SQ and non-SQ groups in terms of other TIL markers or PD-L1 expression. Multivariate analysis showed that the degree of cCD8+ TIL infiltration was an independent positive predictor for overall survival (OS) in the SQ-NSCLC group ($P = .003$) and in the non-SQ-NSCLC group ($P = .024$). In the univariate analysis, CD8+ TILs in the stroma, CD4+ TILs in the cancer nest and stroma, and FOXP3+ TILs in the cancer stroma associated with different prognoses for patients with either non-SQ-NSCLC or SQ-NSCLC. Using a 10% cutoff, PD-L1 expression was a poor prognostic factor in total NSCLC ($P = .011$), stage I ($P = .037$), SQ-NSCLC ($P = .097$), and non-SQ-NSCLC ($P = .051$). **Conclusion:** The different cCD8+ TIL profile and different prognostic value with certain TILs indicates that SQ-NSCLC and non-SQ-NSCLC are likely different cancer types with respect to their immune microenvironments.

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Introduction

Lung cancer is one of the most common cancers worldwide. Approximately 85% of lung cancers are classified as non–small-cell lung cancer (NSCLC), which consists of 2 major histology subtypes: squamous (SQ) and non-squamous (non-SQ) carcinoma.[1] Newer therapeutic modalities for NSCLC have focused on targeting the immune system.[2] The programmed cell death protein 1 (PD-1) signaling pathway and its ligands (PD-L1/PD-L2) play an important role suppressing T-cell function and restricting tumor cell killing.[3,4] The human IgG4 PD-1 checkpoint inhibitors nivolumab

[1]Department of Radiation Oncology
[2]Department of Pathology, Shandong Cancer Hospital Affiliated to Shandong University, Shandong Academic of Medical Science, Jinan, China
[3]Department of Pathology
[4]Department of Radiology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, China

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Address for correspondence: Ligang Xing, MD, PhD, Department of Radiation Oncology, Shandong Cancer Hospital Affiliated to Shandong University, Shandong Academic of Medical Science, 440 Jiyan Road, Jinan, Shandong, China, 250117
E-mail contact: sdcancerhospital@163.com

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and pembrolizumab disrupt PD-1 with PD-L1/PD-L2 interactions and activate anti-tumor immunity.[5-8] Overall survival (OS) improvement was demonstrated for patients treated with anti-PD-1 therapy in comparison with docetaxel in several multicenter randomized trials .[5,7,8]

Although PD-1 checkpoint inhibitors improved OS for patients with NSCLC in the above trials,[5,7,8] SQ-NSCLC and non-SQ-NSCLC showed differences in anti-PD-1 efficacy and response. Compared with docetaxel, nivolumab demonstrated a delayed OS benefit but no statistically significant improvement in progression-free survival (PFS) for non-SQ-NSCLC.[7] PFS and OS were, however, significantly better with nivolumab than with docetaxel among patients with SQ-NSCLC.[5] Pembrolizumab demonstrated a significant OS benefit compared with docetaxel for non-SQ-NSCLC, whereas the difference was not statistically significant for SQ-NSCLC.[8] In terms of the predictive power of PD-L1, high PD-L1 expression is associated with a better response rate and survival for patients treated with pembrolizumab.[8] In non-SQ-NSCLC, all PD-L1 expression levels were strongly associated with clinical outcomes for all efficacy endpoints.[5] However, no association was observed between PD-L1 expression and any efficacy endpoint for SQ-NSCLC.[5]

Recent studies indicated that tumor infiltrating lymphocyte (TIL) status predicts the response to PD-1 or PD-L1 checkpoint blockades.[9,10] By detecting tumor biopsies before and during anti-PD-1 treatments for melanoma, a predictive model based on cluster of differentiation 8 (CD8) expression was established.[9] Patients with high T-effector-interferon-γ-associated gene expression showed improved OS with anti-PD-L1 therapy.[10] In a mouse model, Tang et al showed that sufficient T cell infiltration but not PD-L1 expression was essential for tumor responses to checkpoint blockades.[11]

We propose that TILs may alter checkpoint blockade efficacy between SQ-NSCLC and non-SQ-NSCLC. A recent study demonstrated that different TIL densities exist in squamous cell carcinoma and adenocarcinoma (the main component of non-squamous NSCLC).[12] TILs can be divided into lymphocytes within cancer cell nests and lymphocytes within the cancer stroma,[13-16] although the above research assessed densities in intratumoral and peritumoral compartments. In this study, CD8, cluster of differentiation 4 (CD4), forkhead box transcription factor (FOXP3), and PD-L1 expression were analyzed in samples of resected NSCLC tumors to identify immune microenvironment differences between SQ- and non-SQ-NSCLC.

Materials and Methods

Patients

Patients diagnosed with pathologic stage I to IIIA NSCLC at Shandong Cancer Hospital Affiliated to Shandong University or Shandong Provincial Hospital Affiliated to Shandong University from January 2009 to July 2011 were included in this retrospective study. Patients were excluded if they received neoadjuvant therapies or presented with synchronous tumors or with a history of other malignant tumors, or if they lacked follow-up information. Tumor characteristics, such as histology subtype, differentiation, tumor size, invasive depth, and lymph node metastatic status were assessed and re-confirmed by pathologists. Tumor staging was assessed according to the staging system from the seventh International Association for the Study of Lung Cancer (IASLC).[17] Tissue collection and analysis methods were conducted in accordance with the Declaration of Helsinki and were approved by the Scientific Review and the Institutional Ethics Committee of Shandong Cancer Hospital Affiliated to Shandong University and Shandong Provincial Hospital Affiliated to Shandong University.

Immunohistochemistry Staining

Resected tumor specimens were reexamined by the pathologist (Y.G.). Both the tumor specimens from the margin and the center of each patient were selected if there were enough viable tumor cells without necrosis. Slides with 5−μm-thick sections were deparaffinized and rehydrated. Antigen retrieval was performed under high pressure for 2 minutes. Non-specific binding was blocked by normal serum (Zhongshan Golden Bridge Biotechnology Company, Beijing, China). The sections were incubated with following primary antibodies: monoclonal rabbit anti-human CD4 antibody, CD8 antibody (Zhongshan Golden Bridge Company), monoclonal mouse anti-FOXP3 antibody or monoclonal rabbit anti-PD-L1 antibody (28-8, Abcam) in a humidified chamber at 37°C for 60 minutes. Secondary goat anti-rabbit or anti-mouse antibody was incubated at 37°C for 30 minutes (Zhongshan Golden Bridge Company). Finally, 3,3′-diaminobenzidine was added to visualize the staining.

Immunohistochemistry Scoring

The staining results were evaluated by 2 experienced pathologists (Y.G. and H.J.) who were blinded to patient characteristics. When they gave discrepant results to some sections, the final score was determined together under a multi-head microscope. For CD8+ cells, the cancer nest infiltrate was scored low if $\leq$ 5% were positive or high if $>$ 5% were positive of total cells, and the stromal infiltrate was scored low if $\leq$ 50% or high if $>$ 50% of total cells were positive.[14,18] CD4+ cells were scored high for $>$ 5% or $>$ 25% of total cells in the epithelial and stromal cores were positive, respectively.[14-18] For FOXP3, immunohistochemical (IHC) nuclear staining was graded high or low with a cutoff value of 20% in the stroma[18] and 2% in the cancer nest. Tumor PD-L1 expression was divided into a low-expression group and a high-expression group according to the percentage of positive cells, for 1%, 5%, 10%, and 50%, respectively.[5,7,8]

Statistical Analysis

The statistical analysis was performed with Statistical Product and Service Solutions (SPSS 17.0), and the results were considered statistically significant when $P < .05$. The χ^2 test was used to assess the association between markers and clinicopathologic variables. The Spearman correlation analysis was performed to assess the correlation of TIL density with PD-L1 expression. The Kaplan-Meier method was used for univariate survival analysis. The Cox proportional hazards model was used for the multivariate analysis of prognostic factors. Variables were selected in a final multivariate model using backward elimination with a threshold of $P = .05$.[19]

Results

Baseline Patient and Tumor Characteristics

In total, 197 patients were included: 85 (43%) had SQ-NSCLC and 112 (57%) had non-SQ disease (Table 1). A greater proportion

Table 1 Clinicopathologic Parameters

Clinicopathologic Parameters	Cases (n = 197), n (%)	SQ-NSCLC (n = 85), n (%)	Non-SQ-NSCLC (n = 112), n (%)	*P* Value
Age, y				
<60	108 (55)	42 (49)	66 (59)	.247
≥60	89 (45)	43 (51)	46 (41)	
Gender				
Male	129 (65)	74 (87)	55 (49)	<.001
Female	68 (35)	11 (13)	57 (51)	
Smoking history				
Non-smoking	84 (43)	11 (13)	73 (65)	<.001
Smoking	113 (57)	74 (87)	39 (35)	
Differentiation				
Low	75 (38)	28 (33)	47 (42)	.033
Moderate	84 (43)	45 (53)	39 (35)	
High	38 (19)	12 (14)	26 (23)	
Pathologic tumor status				
T1	46 (23)	17 (20)	29 (26)	.066
T2	128 (65)	53 (62)	75 (67)	
T3	23 (12)	15 (18)	8 (7)	
Pathologic node status				
N0	127 (64)	53 (62)	74 (66)	.447
N1	32 (16)	17 (20)	15 (13)	
N2	38 (19)	15 (18)	23 (21)	
Stage				
I	112 (57)	45 (53)	67 (60)	.456
II	45 (23)	23 (27)	22 (20)	
III	40 (20)	17 (20)	23 (20)	
Postoperative treatment				
No treatment	96 (49)	40 (47)	56 (50)	.472
Chemotherapy	58 (29)	23 (27)	35 (31)	
Chemoradiotherapy	43 (22)	22 (26)	21 (19)	

Abbreviations: Non-SQ-NSCLC = Non-squamous non–small-cell lung cancer; SQ-NSCLC = squamous non–small-cell lung cancer

of female patients was in the non-SQ group than in the SQ group (51% vs. 13%; $P < .001$). More patients in the SQ group had a smoking history than did those in the non-SQ group (87% vs. 35%; $P < .001$). There were more low differentiation cases in the non-SQ group than in the SQ group (33% vs. 42%; $P = .033$). There were 112 patients with stage I disease, 45 patients with stage II disease, and 40 patients with stage III disease. Among all patients, 58 (29%) patients received postoperative chemotherapy, and 43 (22%) patients received postoperative chemoradiotherapy.

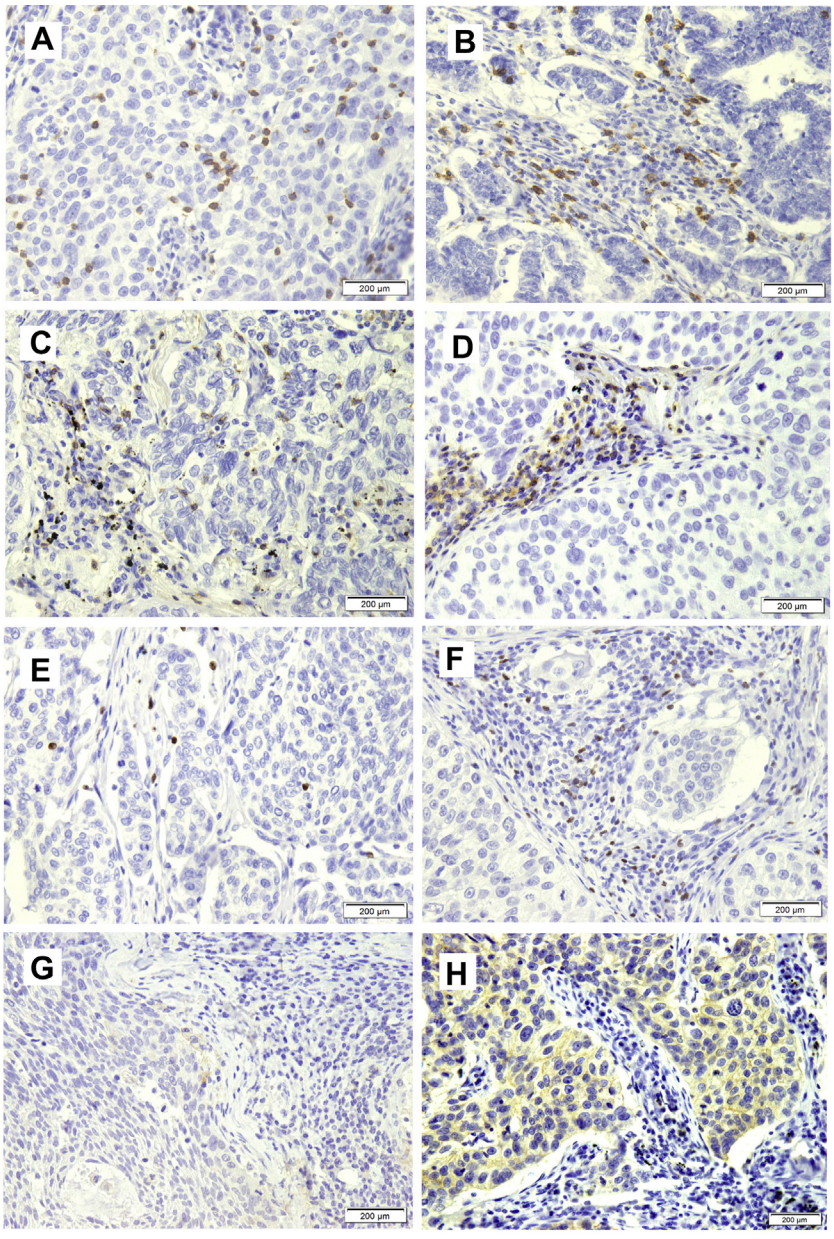
The Frequency and Distribution of CD4+, CD8+, and FOXP3+ TILs

The expression and frequency of CD4+, CD8+, and FOXP3+ TILs in cancer nests (Figure 1A, C and E) and stromal regions (Figure 1B, D and F) were quantified and compared (Table 2). A large majority of CD8+, CD4+, and FOXP3+ TILs were located within the stroma. The mean percentage of CD8+, CD4+, and FOXP3+ TILs in the stroma was 3- to 7-fold greater than the percentage of TILs in the cancer nest (Figure 2A). Further stratifying the cases based on histology revealed that high CD8+ TILs in the cancer nest (cCD8+) were more abundant in the SQ group than in the non-SQ group (56% vs. 34%; $P = .002$) at a 5% cutoff (Table 2). There was no significant difference between the SQ group and the non-SQ group for CD8+ TILs in the stroma (sCD8+), CD4+ TILs in the cancer nest (cCD4+), CD4+ TILs in the stroma (sCD4+), FOXP3+ TILs in the cancer nest (cFOXP3+), or FOXP3+ TILs in the stroma (sFOXP3+) (Table 2). The smoking history showed no correlation with CD8+, CD4+, and FOXP3+ T cells infiltration (Table 2).

PD-L1 Expression

Tumor PD-L1 expression was evaluated based on IHC staining in the cytoplasm and membrane of nucleated cells (Figure 1G and H). For the total patients, 35% scored as PD-L1 positivity < 1%, 20% scored as 1% ≤ PD-L1 < 5%, 15% scored as 5% ≤ PD-L1 < 10%, 19% scored as 10% ≤ PD-L1 < 50%, and 11% scored as

Figure 1 TIL Markers and PD-L1 Expression by Immunohistochemistry. A, CD8+ T Cell Infiltration in the Cancer Nest; B, CD8+ T Cell Infiltration in the Stroma; C, CD4+ T Cell Infiltration in the Cancer Nest; D, CD4+ T Cell Infiltration in the Stroma; E, FOXP3+ T Cell Infiltration in the Cancer Nest; F, FOXP3+ T Cell Infiltration in the Stroma; G, Low Expression of PD-L1; and H, High Expression of PD-L1



Abbreviations: CD4 = cluster of differentiation 4; CD8 = cluster of differentiation 8; FOXP3 = forkhead box transcription factor; PD-L1 = programmed death-ligand 1; TIL = tumor-infiltrating lymphocytes.

50% ≤ PD-L1 (Figure 2B). Utilizing a 1% cutoff, PD-L1 positivity was 60% in the SQ group and 70% in the non-SQ group. At a 5% cutoff, 44% and 46% of tumor cells were positive for PD-L1 in the SQ and non-SQ groups, respectively. Using a 10% cutoff, 31% of tumors in the SQ group and 30% of tumors in the non-SQ group were classified with high PD-L1 expression. In the SQ and non-SQ patients, 9% and 13% of tumor samples were scored ≥ 50% PD-L1-positive. There was no significant difference in PD-L1 expression at any cutoff value between the SQ group and non-SQ group by the χ^2 test.

Correlation of TIL Density With PD-L1 Expression

The correlations between PD-L1 expression and the CD4+, CD8+, and FOXP3+ TIL subgroups were assessed by Spearman

Table 2 Comparison of TILs Between SQ-NSCLC and Non-SQ-NSCLC

	Total Cases, n (%)							Stage I, n (%)			
	N	SQ	Non- SQ	*P* Value	Smoker	Non-smoker	*P* Value	N	SQ-	Non- SQ	*P* Value
CD8											
Cancer nest											
Low	111 (56)	37 (44)	74 (66)	.002	63 (55)	48 (58)	.720	63 (56)	19 (42)	44 (66)	.014
High	86 (44)	48 (56)	38 (34)		51 (45)	35 (42)		49 (44)	26 (58)	23 (34)	
Stromal											
Low	108 (55)	43 (51)	65 (58)	.298	60 (53)	48 (58)	.469	67 (60)	26 (58)	41 (61)	.718
High	89 (45)	42 (49)	47 (42)		54 (47)	35 (42)		45 (40)	19 (42)	26 (39)	
CD4											
Cancer nest											
Low	117 (59)	46 (54)	71 (63)	.189	66 (58)	51 (61)	.616	57 (51)	19 (42)	38 (57)	.133
High	80 (41)	39 (46)	41 (37)		48 (42)	32 (39)		55 (49)	26 (58)	29 (43)	
Stromal											
Low	114 (58)	47 (55)	67 (60)	.524	69 (61)	45 (54)	.376	56 (50)	22 (49)	34 (48)	.847
High	83 (42)	38 (45)	45 (40)		45 (39)	38 (46)		56 (50)	23 (51)	33 (49)	
FOXP3											
Cancer nest											
Low	128 (65)	50 (59)	78 (70)	.602	72 (63)	56 (67)	.480	70 (63)	29 (64)	41 (66)	.728
High	69 (35)	35 (41)	34 (30)		42 (37)	27 (33)		42 (37)	16 (36)	26 (34)	
Stromal											
Low	118 (60)	48 (56)	70 (63)	.392	65 (57)	53 (64)	.334	59 (53)	27 (60)	32 (48)	.203
High	79 (40)	37 (44)	42 (38)		49 (43)	30 (36)		53 (47)	18 (40)	35 (52)	

Abbreviations: CD4 = Cluster of differentiation 4; CD8 = cluster of differentiation 8; FOXP3 = transcription factor forkhead box P3; SQ = squamous non–small-cell lung cancer; non-SQ = squamous non–small-cell lung cancer.

correlation analysis. PD-L1 positivity was significantly associated with decreased cCD8+ TILs in the total population ($r = -0.301$; $P < .001$) (Figure 2C), in the SQ-NSCLC ($r = -0.290$; $P = .007$) (Figure 2D), and in the non-SQ-NSCLC group ($r = -0.291$; $P = .002$) (Figure 2E). sCD8+ TILs correlated negatively with PD-L1 expression in the total population ($r = -0.282$; $P < .001$) (Figure 2F), in the SQ-NSCLC ($r = -0.270$; $P = .012$) (Figure 2G), and in the non-SQ-NSCLC group ($r = -0.294$; $P = .002$) (Figure 2H). There were no significant correlations between PD-L1 expression and cCD4+ TILs, sCD4+ TILs, cFOXP3+ TILs, and sFOXP3+ TILs.

Univariate Analyses of Prognostic Value of CD4, CD8, FOXP3, and PD-L1 Expression

The log-rank univariate analysis between clinicopathologic parameters and survival is summarized in Table 3. There were 83 stage I patients without treatment analyzed as a subgroup for survival analysis. In total population, patients with high CD8+ and CD4+ TILs exhibited significantly better disease-free survival (DFS) and OS than those with low CD8+ TILs and CD4+ TILs. Neither FOXP3 expression in the cancer nest nor in the stroma showed significant correlation with DFS or OS.

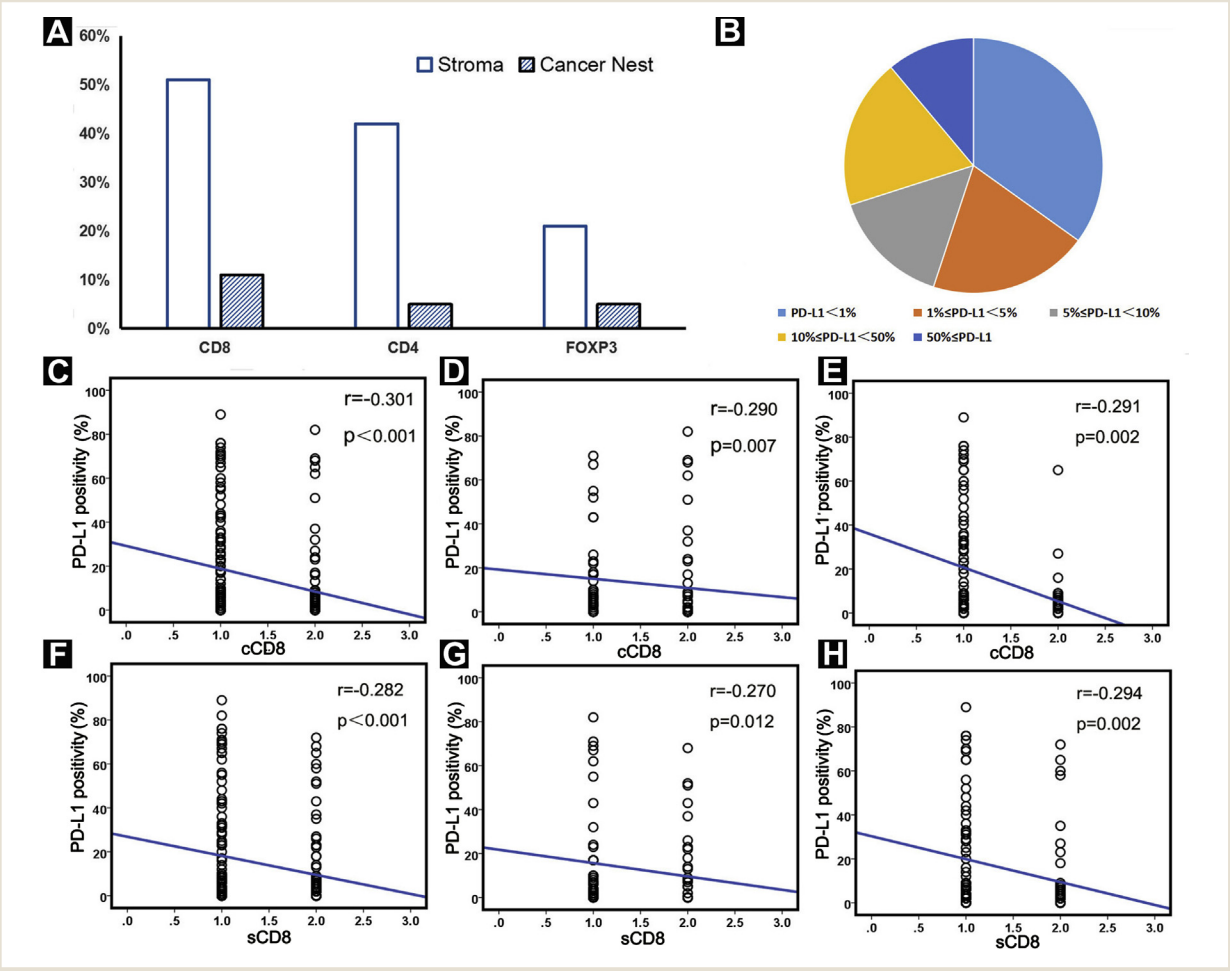
Upon subgroup analysis, cCD8+ TILs were a significant positive predictor of OS for patients with SQ-NSCLC ($P < .001$) (Figure 3A), those with non-SQ-NSCLC ($P = .007$) (Figure 3B), and those with stage I NSCLC ($P < .018$) (Figure 3C). High stromal CD8+ cells infiltration was significantly associated with prolonged OS in patients with SQ-NSCLC ($P = .031$) (Figure 3D) but not in patients with non-SQ-NSCLC ($P = .160$) (Figure 3E), a tendency in stage I NSCLC ($P = .053$) (Figure 3F). In patients with SQ-NSCLC, OS was significantly associated with high cCD4+ TILs and low sFOXP3+ TILs ($P = .028$ and $P = .013$, respectively). In addition to cCD8+ TILs, sCD4+ TILs showed a tendency with better OS ($P = .064$) in the patients with non-SQ-NSCLC.

Using a 10% cutoff, PD-L1 expression was a poor prognostic factor for OS in total NSCLC ($P = .011$) (Figure 4A) and stage I NSCLC without treatment ($P = .037$) (Figure 4B). A tendency of worse OS with high PD-L1 expression was observed in the SQ group ($P = .097$) (Figure 4C) and in the non-SQ group ($P = .051$) (Figure 4D). However, no correlation of PD-L1 expression with survival was observed in the total NSCLC population, the SQ group, or the non-SQ group at the cutoff values of 1%, 5%, and 50%.

Multivariate Analyses of Immune Markers

All variables with $P < .10$ from the univariate analyses were entered into the multivariate Cox regression analysis (Table 4). High cCD8+ TIL numbers were an independent significant prognostic factor for OS in patients with SQ-NSCLC ($P = .002$) and non-SQ-NSCLC ($P = .024$). sFOXP3 + TILs were

Figure 2 A, Comparison of CD4+, CD8+, and FOXP3+ T Cells in the Cancer Nest and in the Stroma of Overall NSCLC; B, The Distributions of PD-L1 Positivity; C, D, E, The PD-L1 Expression Correlated Negatively With cCD8 TILs in the Total Population, SQ-NSCLC, and Non-SQ-NSCLC. F, G, H, The PD-L1expression Correlated Negatively With sCD8 TILs in the Total Population, SQ-NSCLC, and Non-SQ-NSCLC



Abbreviations: c = cancer nest; CD4 = cluster of differentiation 4; CD8 = cluster of differentiation 8; FOXP3 = forkhead box transcription factor; NSCLC = non–small-cell lung cancer; PD-L1 = programmed death-ligand 1; s = stromal; SQ = squamous; TIL = tumor-infiltrating lymphocytes.

independent prognostic factors for OS in the SQ group and stage I NSCLC ($P = .019$ and $P = .046$, respectively).

Discussion

In this study, the increased infiltration of cCD8+ lymphocytes was observed in SQ tumors. PD-L1 expression was significantly associated with decreased cCD8+ TILs, with different cutoffs for the SQ group and the non-SQ-group, respectively. The level of cCD8+ TILs was a significant positive predictor for survival, both in the SQ group and in the non-SQ group. cCD4+ TILs, sCD8+TILs, and sFOXP3+ TILs were differently correlated with OS between the SQ group and the non-SQ group, demonstrating that the immune microenvironment is partly different between SQ and non-SQ NSCLC.

We examined PD-L1 expression by IHC and analyzed it using various cutoffs. No difference in PD-L1 expression was observed between the SQ-NSCLC and non-SQ-NSCLC groups, and these results are consistent with the studies of nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer and non-squamous non-small-cell lung cancer.[5,7] In clinical trials, not all PD-L1-positive patients responded well to anti-PD-1 therapy, and patients with negative PD-L1 expression also showed a response to PD-1 pathway inhibitors.[5,7,8,20,21] Thus, PD-L1 expression may not be the best predictive factor, and it is not likely to be the key difference between SQ-NSCLC and non-SQ-NSCLC. Therefore, we compared TILs in resected tumor samples.

Our study indicated that high cCD8+ TILs were more abundant in the SQ group than in the non-SQ group, which agrees with previous studies that showed more than twice the number of cCD8+ in SQ-NSCLC than in adenocarcinoma (the main component of non-SQ-NSCLC).[15,22] Checkpoint blockades target immune cells, and the interaction between tumors and immune

Table 3 Univariate Analysis Using Log-rank Test Between Clinicopathologic Parameters and Survival

	SQ-NSCLC		Non-SQ-NSCLC		Stage I[a]	
Parameters	**5-Year OS, %**	***P* Value**	**5-year OS, %**	***P* Value**	**5-Year OS, %**	***P* Value**
Age, y						
<60	73.0	.802	58.8	.359	86.1	.823
≥60	74.4		64.5		84.6	
Gender						
Male	72.9	.634	68.7	.080	87.3	.568
Female	81.8		51.8		82.9	
Smoking history						
Non-smoking	80.8	.577	59.4	.999	86.5	.777
Smoking	72.9		59.8		84.2	
Differentiation						
Low	67.9	.488	61.7	.081	92.6	.468
Moderate	75.2		58.2		81.6	
High	83.3		75.4		82.4	
Tumor status						
T1	94.1	.003	79.3	.057	92.9	.366
T2	73.5		53.9		81.2	
T3	51.3		50.0		–	
Node status						
N0	86.6	.002	74.1	<.001	–	
N1	52.9		52.5		–	
N2	53.3		26.1		–	
Cancer CD8						
Low	56.0	<.001	50.3	.007	76.4	.018
High	87.4		78.9		95.0	
Stromal CD8						
Low	62.1	.031	54.7	.160	78.9	.053
High	85.7		72.3		94.3	
Cancer CD4						
Low	64.9	.028	54.3	.106	84.7	.915
High	84.3		70.7		86.0	
Stromal CD4						
Low	65.7	.09	53.5	.064	86.8	.739
High	84.0		73.0		84.2	
Cancer FOXP3						
Low	77.9	.335	59.7	.731	89.2	.292
High	68.8		61.2		80.6	
Stromal FOXP3						
Low	85.2	.013	59.6	.431	92.6	.076
High	95.5		64.0		78.6	
PD-L1						
1% cutoff						
<1%	73.1	.851	52.9	.354	85.7	.969
≥1%	74.2		63.8		85.3	
5% cutoff						
<5%	76.9	.291	65.5	.144	88.9	.365
≥5%	69.8		53.3		81.2	
10% cutoff						
<10%	77.8	.097	64.4	.051	91.1	.037

Table 3 Continued

Parameters	SQ-NSCLC		Non-SQ-NSCLC		Stage I[a]	
	5-Year OS, %	*P* Value	5-year OS, %	*P* Value	5-Year OS, %	*P* Value
≥10%	64.5		47.1		73.1	
50% cutoff						
<50%	76.5	.124	61.7	.402	87.6	.172
≥50%	50.0		50		80.0	

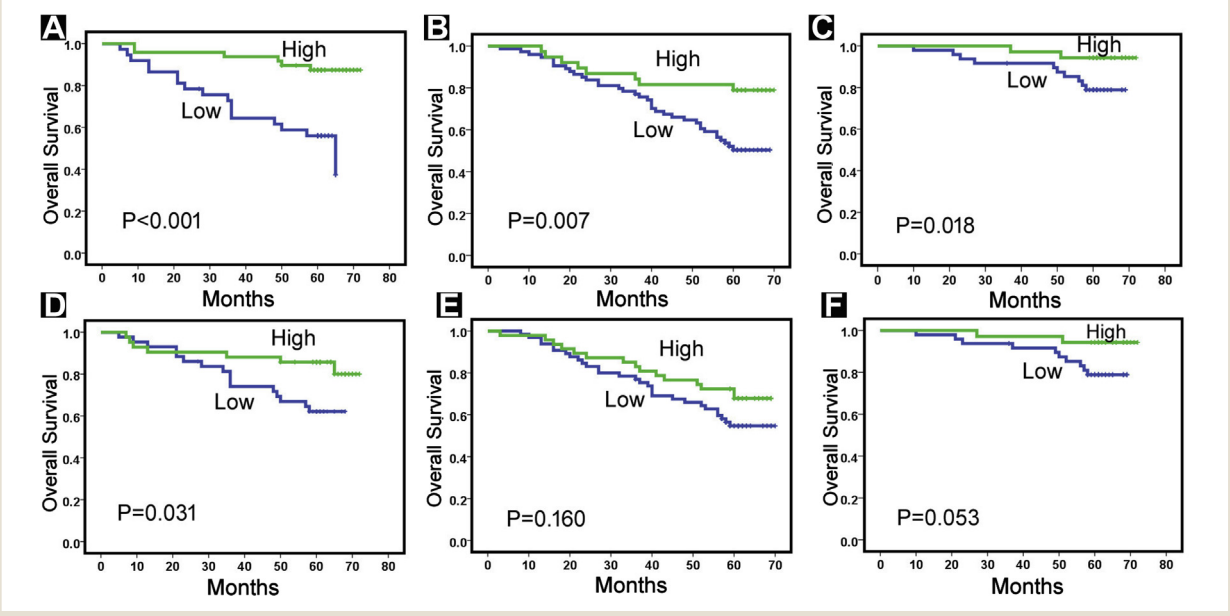
Abbreviations: CD4 = cluster of differentiation 4; CD8 = cluster of differentiation 8; DFS = disease-free survival; FOXP3 = transcription factor forkhead box P3; OS = overall survival; PD-L1 = programmed death ligand-1.
[a]Stage I patients without treatment.

system is essential for cancer therapy. Previous studies suggested that immune checkpoint blockades may not be sufficient in patients with decreased TILs.[11,20,23,24] A PD-L1+ tumor with insufficient CD8+ TILs does not respond to anti-PD-L1 immunotherapy, whereas a PD-L1+ tumor with sufficient CD8+ TILs can be well-controlled by checkpoint blockades.[11] Furthermore, CD8+ T cells accumulate in the tumor mass of patients who respond to PD-L1 blockade, whereas in the fraction of patients who are resistant to anti-PD-L1 therapy, these cells localize to the edges of tumors.[9,20] Lymphocytes must infiltrate the tumor tissue to interact with target cancer cells and trigger functional activity. At this point, the cCD8+ TILs may be a marker to predict the anti-PD-1 therapy response, and different levels of cCD8+ TILs may be a possible mechanism for the varied efficacy of anti-PD-1 antibodies in SQ-NSCLC and non-SQ-NSCLC.

No significant differences in sCD8+, cCD4+, sCD4+, cFOXP3+, or sFOXP3+ TILs were observed between the SQ group and the non-SQ group. This result is inconsistent with a previous study that compared TIL density between different NSCLC histologies.[12] Our study did not detect any differences, whereas the study by Parra showed a significant difference of CD4 and FOXP3 expression between the different histologic types. These discordant results may be because of several reasons. First, in the study by Parra, the tumors were divided into peritumoral and intratumoral compartments, whereas we analyzed TILs in the stroma and cancer cell nest, both of which were in the intratumoral compartment. Second, the definition for high expression was different between the 2 studies.

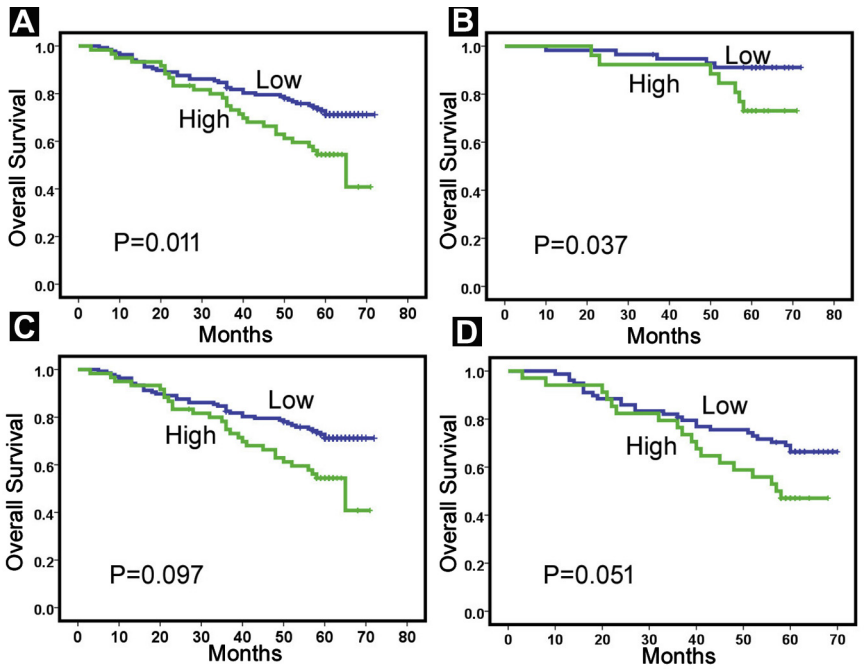
The association between PD-L1 and TILs has been assessed in several studies.[25-27] In this study, PD-L1 expression was

Figure 3 Kaplan-Meier Analysis for Overall Survival With TILs. The Patients With High cCD8+ TILs Showed a Better Overall Survival SQ-NSCLC (A), Non-SQ-NSCLC (B), and Stage I NSCLC Without Treatment (C). The Significant Difference of sCD8+ TILs With Survival was Limited to SQ-NSCLC (D), but it was Not Observed in Non-SQ-NSCLC (E) and Stage I NSCLC Without Treatment (F)



Abbreviations: c = cancer nest; CD8 = cluster of differentiation 8; NSCLC = non–small-cell lung cancer; s = stromal; SQ = squamous; TIL = tumor-infiltrating lymphocytes.

Figure 4 Overall Survival Curves for PD-L1 Expression. For Total Patients With NSCLC, High PD-L1 Expression Correlates With a Poorer Overall Survival at the 10% Cutoff Value in the Total Population (A) and in Patients With Stage I NSCLC Without Treatment (B). Patients With High PD-L1 Expression Exhibited a Tendency of Worse Overall Survival in the SQ Group (C) and in the Non-SQ Group (D)



Abbreviations: NSCLC = non–small-cell lung cancer; PD-L1 = programmed death-ligand 1; SQ = squamous.

significantly associated with decreased cCD8+ TILs for 2 histologic subgroups and stage I NSCLC, further confirming that PD-L1 expression contributes to the negative regulation of TILs in NSCLC.[26]

Intense tumor lymphocytic infiltration was validated as a favorable prognostic marker for survival in a large cohort of resected NSCLC.[28] But the lymphocyte infiltration just was assessed on hematoxylin and eosin-stained representative section, and there was no correlation analysis of certain kind of TILs with prognosis in details. In the current research, cCD8+ and sCD8+ TILs correlated strongly with a good clinical outcome overall for resected NSCLC, which is consistent with a previous study that showed higher epithelial and stromal CD8+ TILs were a significant positive predictor for disease-specific survival.[14] By subgroup analysis, the predictive significance of sCD8 was limited to SQ-NSCLC and not in non-SQ-NSCLC. Al-Shibli et al also found that higher sCD8+ was correlated with a better outcome in SQ lung cancer but not in adenocarcinoma.[14] In multivariate analysis, only cCD8+ was an independent prognostic factor for DFS and OS. The finding indicates that cCD8+ TILs may be directly involved in immunity against lung cancer cells. A previous study demonstrated that a higher number of cCD8+ T cells were associated with tumor cell apoptosis in NSCLC.[29]

Our data showed that cCD4+ TILs were correlated with a favorable OS in SQ-NSCLC but not in the non-SQ group, which only partially overlaps with previous reports. Wakabayashi et al and Al-Shibli et al observed that only sCD4+ TILs levels were associated with a better prognosis.[13,14] On the other hand, Hiraoka et al found that patients with high numbers of both cCD8+ and cCD4+ cells exhibited better survival.[30] Further stratifying the cases based on histology revealed that the significant correlation of cCD4+ TILs and prognosis was limited to the SQ group, whereas a similar significance for sCD4 was limited to the non-SQ group, further demonstrating the differences in the immune microenvironment between SQ- and non-SQ-NSCLC.

Expression of the transcription factor FOXP3 characterizes regulatory T cells (Tregs) that normally function to maintain immunologic self-tolerance.[31] Previous studies showed that high FOXP3+ T-cell infiltration conferred a worse survival.[16,32] Our study showed that accumulation of FOXP3+ TILs was associated with worse DFS and OS in SQ-NSCLC, but no correlation was observed in non-SQ-NSCLC. These results indicate that the function of FOXP3+ TILs may be more relevant to SQ-NSCLC, although the underlying mechanism requires further investigation.

PD-L1 expression was a poor prognostic factor for OS in total NSCLC and stage I NSCLC at the 10% cutoff. The results indicate that 10% may be an appropriate cutoff value for assessing the prognostic function of PD-L1. We must note that no significant correlation was observed in the SQ group ($P = .097$), and the non-SQ group exhibited worse OS ($P = .051$). The difference between the total population and the subgroups may be caused by differences in sample size. Furthermore, PD-L1 alone may not be sufficient to

Table 4 Multivariate Analysis for Survival

Parameters	HR	95% CI	*P* Value
5-year OS (SQ-NSCLC)			
T	3.309	1.382-7.925	.007
N	2.179	1.285-3.695	.004
Cancer CD8	0.150	0.044-0.516	.003
Stromal CD8	0.762	0.283-2.048	.590
Cancer CD4	0.938	0.312-2.816	.909
Stromal CD4	0.478	0.151-1.512	.209
Stromal FOXP3	6.422	2.275-18.13	<.001
PD-L1 at 10% cutoff	1.773	0.693-4.537	.232
5-year OS (non-SQ-NSCLC)			
Gender	1.990	1.062-3.732	.032
Differentiation	0.861	0.576-1.287	.466
T	1.792	0.999-3.216	.051
N	2.075	1.484-2.901	<.001
Cancer CD8	0.388	0.171-0.882	.024
Stromal CD4	0.685	0.297-1.154	.122
PD-L1 at 10% cutoff	1.170	0.598-2.288	.647
5-year OS (Stage I and no treatment)			
Cancer CD8	0.205	0.037-1.140	.070
Stromal CD8	0.567	0.107-3.014	.506
Stromal FOXP3	4.011	1.026-15.69	.046
PD-L1 at 10% cutoff	1.781	0.524-6.051	.355

Abbreviations: CD4 = cluster of differentiation 4; CD8 = cluster of differentiation 8; DFS = disease-free survival; FOXP3 = transcription factor forkhead box P3; N = node; non-SQ-NSCLC = non-squamous non–small-cell lung cancer; OS = overall survival; PD-L1 = programmed death ligand-1; SQ-NSCLC = squamous non–small-cell lung cancer; T = tumor.

predict NSCLC outcome. A recently published study showed that combining low PD-L1 levels with high CD8+ TIL density was correlated with longer survival in stage III NSCLC.[33] Therefore, whether 10% is the appropriate cutoff value for PD-L1 or whether PD-L1 levels should be combined with other immunology markers to be a significant predictive factor should be further explored in large cohort studies.

In conclusion, high cCD8+ TILs were more abundant in the SQ group than in the non-SQ group. In survival analysis, high cCD8+ T cell infiltration was the only independent positive predictor for DFS and OS. The difference in cCD8 infiltration and prognostic effects of certain TILs indicate that SQ-NSCLC and non-SQ-NSCLC are likely different kinds of cancer in terms of their immune microenvironments. Therefore, cCD8 TILs may be a candidate marker for response to immunotherapy and good prognosis. It may be necessary to recruit patients with SQ-NSCLC and non-SQ-NSCLC separately for clinical trials about immunotherapy.

Clinical Practice Points

- There were some differences in efficacy and response predictors for anti-PD-1 antibodies between SQ-NSCLC and non–SQ-NSCLC. Better understanding of PD-L1 expression profile and TILs will provide important insight into the immune microenvironment of SQ-NSCLC and non–SQ-NSCLC, and assist in the development of predictive biomarkers for PD-1/PD-L1 pathway blockades immunotherapy.
- We compared the expression of CD8, CD4, FOXP3, and PD-L1 between SQ-NSCLC and non–SQ-NSCLC. The profile of different frequency of cCD8+ T cells infiltration, different correlations of PD-L1 with TILs, and the different prognostic effects of certain TILs indicates that SQ-NSCLC and non–SQ-NSCLC are probably different kinds of cancer in the immune microenvironment.
- It is necessary to identify different biomarkers to definitively predict response to PD-1 pathway inhibitors for patients with SQ-NSCLC or non-SQ-NSCLC in clinical practice.

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Disclosure

The authors have stated that they have no conflicts of interest.

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