Ann Surg Oncol (2020) 27:2102–2109
https://doi.org/10.1245/s10434-019-08029-9

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Combined Evaluation of Tumor-Infiltrating CD8 + and FoxP3 + Lymphocytes Provides Accurate Prognosis in Stage IA Lung Adenocarcinoma

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ABSTRACT

Background. Immunotherapy has become a standard treatment option for non-small cell lung cancer (NSCLC), with the tumor microenvironment attracting significant attention. CD8 + and forkhead box protein P3 + (FoxP3 +) tumor-infiltrating lymphocytes (TILs) influence the tumor microenvironment, but the clinical significance of CD8 + and FoxP3 + TILs in stage IA lung adenocarcinoma (LAD) is poorly understood.

Methods. We analyzed 203 patients with stage IA primary LAD who had undergone surgery at Kyushu University from January 2003 to December 2012. We evaluated CD8 + and FoxP3 + TILs by immunohistochemistry. We set the cutoff values at 50 cells/0.04 mm^2 for CD8 + TILs and 20 cells/0.04 mm^2 for FoxP3 + TILs, respectively. We divided the patients into four groups: CD8-Low/FoxP3-Low; CD8-High/FoxP3-Low; CD8-Low/FoxP3-High; and CD8-High/FoxP3-High. We compared clinical outcomes among them. Programmed cell death ligand-1 (PD-L1) expression by tumor cells was also evaluated as previously reported.

Results. Respectively, 104 (51.2%), 46 (22.7%), 22 (10.8%), and 31 (15.3%) patients were classified as CD8-Low/FoxP3-Low, CD8-High/FoxP3-Low, CD8-Low/FoxP3-High, and CD8-High/FoxP3-High. Both disease-free survival (DFS) and overall survival (OS) were significantly worse in the CD8-Low/FoxP3-High group than the other groups (5-year DFS: 66.3% vs. 90.5%; $P = 0.0007$, 5-year OS: 90.9% vs. 97.0%; $P = 0.0077$). In the multivariate analysis, CD8-Low/FoxP3-High and PD-L1 expression were independent prognostic factors of DFS, and lymphatic invasion, surgical procedure, and PD-L1 expression were independent prognostic factors of OS.

Conclusions. CD8-Low/FoxP3-High was an independent prognostic factor of DFS (hazard ratio: 3.22; 95% confidence interval: 1.321–7.179; $P = 0.0121$) in stage IA LAD. Immunosuppressive conditions were associated with poor prognosis in stage IA LAD.

Recently, a treatment paradigm shift for patients with lung cancer has occurred with the development of immune checkpoint inhibitors, such as nivolumab, pembrolizumab, atezolizumab, and durvalumab.[1–5] Also, the immune mechanisms within the tumor microenvironment have attracted much attention. Tumor-infiltrating lymphocytes (TILs) play a central role in the tumor microenvironment, and there have been several studies analyzing the significance of TILs in lung cancer.[6–12] TILs showed an association with survival, recurrence, and malignancy of

Electronic supplementary material The online version of this article (https://doi.org/10.1245/s10434-019-08029-9) contains supplementary material, which is available to authorized users.

First Received: 23 August 2019;
Published Online: 26 November 2019

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lung cancer.[6–10] Furthermore, TILs predict therapeutic responses to immunotherapy in non-small cell lung carcinoma (NSCLC).[11, 12]

Cluster of differentiation 8 + (CD8 +) lymphocytes, known as cytotoxic T lymphocytes, are activated via major histocompatibility complex class I antigen, and lyse target cells, such as tumor cells or virus infected cells, through the release of perforin and granzymes.[13] Forkhead box protein P3 (FoxP3) is a transcription factor specific to regulatory T lymphocytes. FoxP3 + lymphocytes exert their immunosuppressive effects through various mechanisms: consumption of interleukin 2, cytotoxic T lymphocyte antigen 4 signal, and production of immune inhibitory cytokines.[14] In NSCLC, past meta-analyses have shown that a high density of CD8 + TILs indicated good prognosis for overall survival (OS), disease-free survival (DFS), and recurrence-free survival (RFS), and high levels of FoxP3 + TILs had unfavorable prognostic effects for OS and RFS.[6, 7] However, in lung adenocarcinoma (LAD), there are several studies that show that a high density of CD8 + TILs was associated with poor prognosis for death and recurrence.[8–10] Based on these findings, we considered that the histologic type of lung cancer and patient characteristics had a strong effect on the significance of TILs. Therefore, in this study, to eliminate the bias of the histologic type of lung cancer and patient characteristics, we selected patients with stage IA LAD and elucidated the significance of CD8 + and FoxP3 + TILs in stage IA LAD, exclusively.

METHODS

Study Patients

A total of 459 patients with LAD who had undergone surgery between January 2003 and December 2012 at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University were enrolled in the study. Of them, 229 patients were pathologically diagnosed with stage IA adenocarcinoma according to the 7th edition of the TNM Classification.[15] Finally, 203 formalin-fixed paraffin-embedded specimens were available for immunohistochemical staining. After surgery, routine follow-up, including physical examination, blood tests, and chest radiographs, were performed at 3-month intervals for the first 3 years and 6-monthly thereafter. Clinicopathological characteristics, DFS, and OS were retrospectively analyzed. Clinicopathological characteristics assessed included age, sex, smoking history, vascular invasion (v), lymphatic invasion (ly), histological subtypes, surgical procedure, and *EGFR* mutation. This study was approved by our institutional review board (Kyushu University, IRB No. 29-402).

Immunohistochemical Staining

Immunohistochemical staining was performed in 203 surgically resected stage IA LADs. Sections were cut at 4-μm thickness from formalin-fixed and paraffin-fixed tissue blocks, then dewaxed with xylene, and rehydrated through a graded concentration series of ethanol. After inhibition of endogenous peroxidase activity with 3% hydrogen peroxide in methanol for 30 min, the sections were pretreated with Target Retrieval Solution (Dako) in a decloaking chamber at 121 °C for 15 min and then incubated with primary antibodies at 4 °C overnight. The primary antibodies were mouse monoclonal anti-human CD8 antibody (clone #C8/144B, dilution 1:100, Dako) and mouse monoclonal anti-human FoxP3 antibody (clone #236A/E7, dilution 1:100, eBioscience). The immune complex was detected with a DAKO EnVision Detection System (Dako). The sections were finally reacted in 3,30-diaminobenzidine, counterstained with hematoxylin, and mounted. Sections of tonsil were used as positive controls for CD8 and FoxP3. Stained slides were scanned using the NanoZoomer (Hamamatsu Photonics KK). In this study, all hematoxylin–eosin images and immunohistochemical images were reviewed by at least two investigators, including a pathologist, and TILs were distinguished from other cancer cells by their morphology.

The density of CD8 + and Foxp3 + TILs was evaluated by counting the number of CD8 + and Foxp3 + TILs per 0.04 mm^2 over 5 fields, then averaging the cell counts. Samples were evaluated by at least two investigators, including a pathologist. The cutoff values of the number of CD8 + and FoxP3 TILs were 50 (cells/0.04 mm^2) and 20 (cells/0.04 mm^2), defined by ROC curve analysis (Supplementary Fig. 1).

Programmed cell death ligand-1 (PD-L1) expression was detected by immunohistochemical staining with rabbit monoclonal anti-human PD-L1 antibody (clone #SP142, dilution 1:100, Spring Bioscience), as described previously.[16] In this study, more than 1% tumor membrane staining was considered to denote PD-L1 positivity.[16]

Statistical Analysis

Fisher's exact test was used to analyze patients' characteristics. DFS was defined as the period between surgery and the date of the last follow-up, recurrence or death, and OS as the period between surgery and the date of last follow-up or death. Survival curves were estimated by using the Kaplan–Meier method with the log-rank test. Cox proportional hazards regression analysis was performed to

estimate the hazard ratios for the positive risk factors with the backward elimination method. All results were considered as statistically significant at $P < 0.05$. JMP pro 13.0 software (SAS Institute) was used for all statistical analyses.

RESULTS

Clinicopathological Characteristics in Patients with Stage IA LAD

The study cohort comprised 203 patients with stage IA LAD who had undergone surgical resection (Table 1). The mean age was 68 (range 34–85) years, and 115 patients (56.7%) were female. On histological examination of resected tumors, 22 patients (10.8%) had tumors of non-invasive (adenocarcinoma in situ or minimally invasive adenocarcinoma), and 181 were invasive adenocarcinomas (89.2%). The surgical procedures included sublober resection performed on 74 patients (36.5%). Seventy (53.8%) patients had *EGFR* mutation, and 48 (23.6%) patients showed PD-L1 positivity.

The mean numbers of CD8 + and FoxP3 + TILs were 39.2 (2.6–114.2; cells/0.04 mm^2) and 12.4 (0–9.4; cells/0.04 mm^2), respectively. Seventy-eight (38.4%) and 52 (25.6%) patients were classified as having high infiltrations of CD8 + (CD8-High) and FoxP3 + (FoxP3-High) TILs, respectively. Representative images with CD8 and FoxP3 staining with CD8-Low, CD8-High, FoxP3-Low, and FoxP3-High are shown in Figs. 1a–d, respectively.

We examined the relationship between clinicopathological characteristics and CD8 + and FoxP3 + TILs (Supplementary Table 1). Patients with CD8-High were significantly associated with vascular invasion positivity, lobectomy and increased infiltration of FoxP3 + cells. Conversely, patients with FoxP3-High were significantly associated with vascular invasion positivity, invasive subtypes, PD-L1 positivity and CD8-High status.

Prognosis Analysis of Patients with Stage IA LAD According to CD8 + and FoxP3 + TILs

Prognostic analysis in relation to CD8 + and FoxP3 + TILs was performed using the Kaplan–Meier method. There was no significant difference between CD8-High and CD8-Low groups in both DFS (5-year DFS: 93.0% vs. 84.4%; P =0.2833; Fig. 2a) and OS (5-year OS: 97.4% vs. 95.6%; P =0.9583; Fig. 2b). In FoxP3 + TILs, the DFS was not significantly different in patients with FoxP3-High and FoxP3-Low groups (5-year DFS: 81.7% vs. 89.9%; P =0.0775; Fig. 2c); however, the OS was significantly worse in the FoxP3-High group than the FoxP3-Low group (5-year OS: 92.2% vs. 97.8%; P =0.0192; Fig. 2d).

TABLE 1 Clinicopathological characteristics of patients with resected stage IA LAD

Characteristic	*n* = 203	
	Mean (range)/number (%)	
Age	68	(34–85)
Sex		
Female	115	(56.7%)
Male	88	(43.3%)
Smoking[a]		
Never smoked	109	/53.7%)
Smoker	92	(46.3%)
Vascular invasion		
Negative	177	(87.2%)
Positive	26	(12.8%)
Lymphatic invasion		
Negative	194	(95.6%)
Positive	9	(4.4%)
Histological subtype		
Noninvasive	22	(10.8%)
Invasive	181	(89.2%)
Surgical procedure		
≥Lobectomy	129	(63.5%)
Sublobar resection	74	(36.5%)
EGFR mutation[a]		
Wild-type	60	(46.2%)
Mutant	70	(53.8%)
PD-L1 expression		
Negative (< 1%)	155	(76.4%)
Positive (≥ 1%)	48	(23.6%)
CD8 + lymphocytes		
Low (< 50 cells/0.04 mm^2)	125	(61.6%)
High (≥ 50 cells/0.04 mm^2)	78	(38.4%)
FoxP3 + lymphocytes		
Low (< 20 cells/0.04 mm^2)	151	(74.4%)
High (≥ 20 cells/0.04 mm^2)	52	(25.6%)

[a]Available data were counted, excluding unknown data

LAD lung adenocarcinoma; *EGFR* epidermal growth factor receptor; *PD-L1* programmed cell death ligand-1; *CD8* cluster of differentiation 8; *FoxP3* Forkhead box protein P3

Combined Evaluation of CD8 + and FoxP3 + TILs in Stage IA LAD and Survival Analysis

We further conducted combinatory analysis of CD8 + and FoxP3 + TILs. Patients were categorized into the following four groups: CD8-Low/FoxP3-Low, CD8-High/

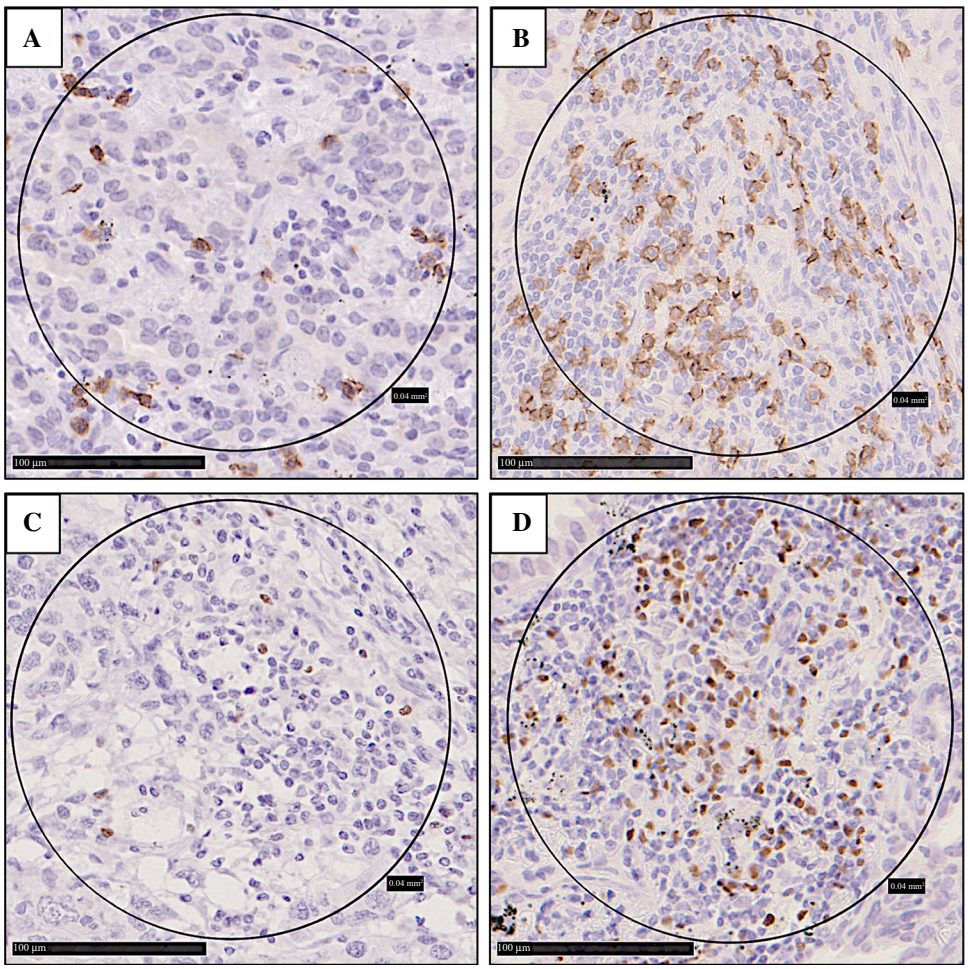

FIG. 1 Representative images of immunohistochemical staining of CD8 and FoxP3 in surgically resected specimens from patients with stage IA LAD. Typical CD8 staining of LAD with CD8-Low (**a**) and CD8-High (**b**), and typical FoxP3 staining of LAD with FoxP3-Low (**c**) and FoxP3-High (**d**). Scale bars: 100 mm. *LAD* lung adenocarcinoma; *CD8* cluster of differentiation 8; *FoxP3* Forkhead box protein P3

FoxP3-Low, CD8-Low/FoxP3-High, and CD8-High/FoxP3-High. The DFS and OS among the four groups had significant differences (P =0.0096 and P = 0.0463, respectively). In particular, the CD8-Low/FoxP3-High group had significantly worse prognosis in DFS and OS than the other groups (5-year DFS: 66.3% vs. 90.5%; P =0.0007; Fig. 3A, 5-year OS: 90.9% vs. 97.0%; P =0.0077; Fig. 3B). In our multivariate analysis, CD8-Low/FoxP3-High remained an independent predictor of DFS (hazard ratio: 3.22; 95% confidence interval: 1.321–7.179; P =0.0121; Table 2). We verified the association between the CD8-Low/FoxP3-High group and clinicopathological characteristics, and there were no characteristics associated with CD8-Low/FoxP3-High (Supplementary Table 2). There was an association between CD8-Low/FoxP3-High and high PD-L1 expression; however, it was not significant (P = 0.0607).

DISCUSSION

In this study, we described the significance of CD8 + and FoxP3 + TILs in stage IA LAD. The density of CD8 + TILs did not have a significant effect on prognosis. In contrast, the high levels of FoxP3 TILs were associated with worse prognosis of OS but were not independent predictive factors for poor survival. However, the combined evaluation of CD8 + and FoxP3 + TILs elucidated that CD8-Low/FoxP3-High was significantly associated with poor prognosis in both OS and DFS and was an independent predictive factor for DFS. These results demonstrated that the combined evaluation of CD8 + and Foxp3 + TILs provides accurate prognosis for stage IA LAD.

Several reports mentioned the significance of CD8 + TILs in lung cancer. Past meta-analyses indicated that a high density of CD8 + TILs was associated with good prognosis in NSCLC.[6,7] However, especially in LAD, some papers described the high infiltration of CD8 + TILs as an unfavorable prognostic factor.[8–10] Thus, we thought that the significance of CD8 + TILs in lung cancer was not yet

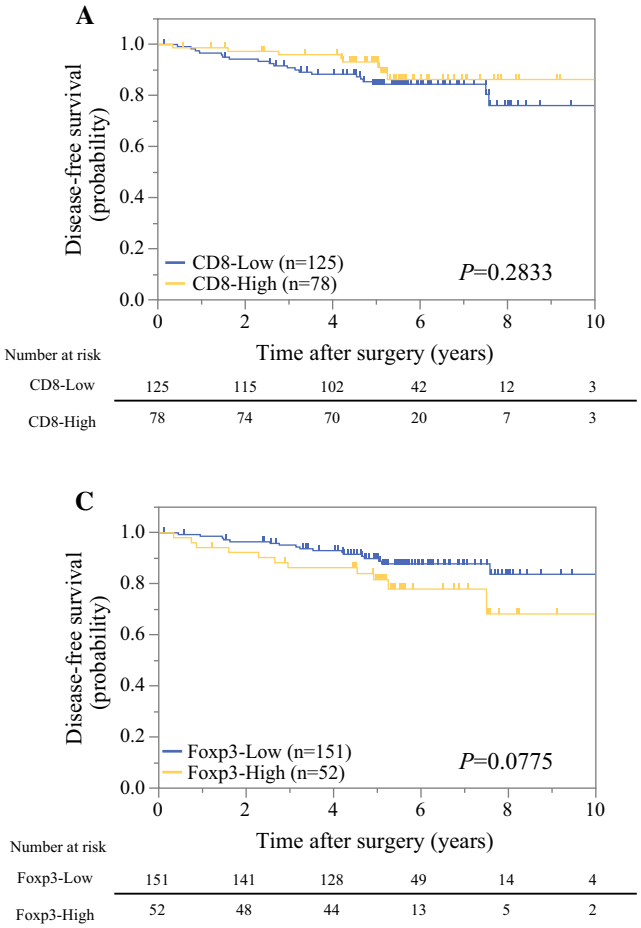

FIG. 2 Kaplan-Meier curves showing survival of patients with stage IA LAD according to CD8 + and FoxP3 + TILs. (**a**) Disease-free survival and (**b**) overall survival of CD8-Low and CD8-High groups. (**c**) Disease-free survival and (**d**) overall survival of FoxP3-Low and FoxP3-High groups. *LAD* lung adenocarcinoma; *CD8* cluster of differentiation 8; *FoxP3* Forkhead box protein P3; *TILs* tumor-infiltrating lymphocytes

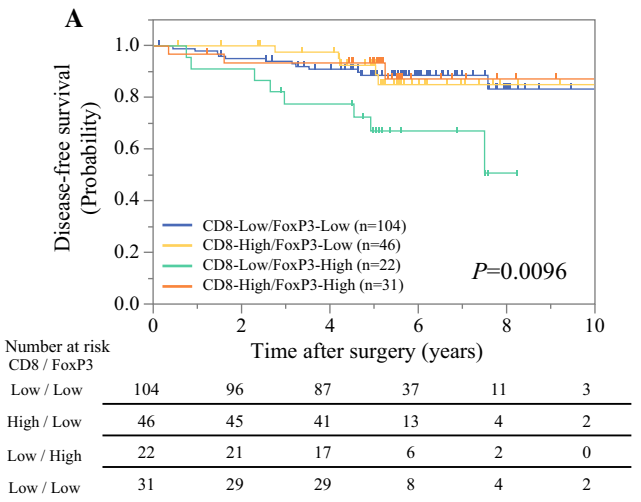

FIG. 3 Kaplan-Meier curves showing survival of patients with stage IA LAD among the following four groups; CD8-Low/FoxP3-Low, CD8-High/FoxP3-Low, CD8-Low/FoxP3-High, and CD8-High/FoxP3-High. (**a**) Disease-free survival and (**b**) overall survival of CD8-Low/FoxP3-Low, CD8-High/FoxP3-Low, CD8-Low/FoxP3-High, and CD8-High/FoxP3-High groups. *LAD* lung adenocarcinoma; *CD8* cluster of differentiation 8; *FoxP3* Forkhead box protein P3

TABLE 2 Univariate and multivariate analyses of DFS and OS in patients with stage IA LAD

Characteristic	DFS						OFS					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value	HR	95%CI	*P* value	HR	95%CI	*P* value
Age												
≥70	1.48	0.702–3.140	0.2978				3.90	1.128–17.830	0.0310			
Sex												
Male	2.34	1.109–5.159	0.0256				2.61	0.788–9.988	0.1164			
Smoking[a]												
Smoker	2.14	1.010–4.741	0.0470				2.46	0.739–9.446	0.1426			
Lymphatic invasion												
Positive	1.54	0.516–3.746	0.4050				2.54	0.557–8.799	0.2027	11.30	1.466–88.800	0.0207
Vascular invasion												
Positive	2.59	0.616–7.382	0.1686				1.65	0.090–8.658	0.6572			
Histological subtypes												
Invasive	1.55	0.464–9.634	0.5231				$5e^{8}$	0.615–0.615	0.1180			
Surgical procedure												
Sublobar resection	2.10	0.996–4.479	0.0512				4.96	1.432–22.630	0.0109	16.30	2.668–154.200	0.0012
EGFR mutation[a]												
Mutant	0.30	0.084–0.883	0.0280				0.24	0.012–1.597	0.1470			
PD-L1 expression												
Positive	2.90	1.359–6.113	0.0066	2.58	1.196–5.473	0.0165	7.77	2.241–35.540	0.0011	7.36	2.056–35.520	0.0019
CD8 + lymphocytes												
High	0.64	0.265–1.403	0.2735				1.03	0.270–3.439	0.9584			
FoxP3 + lymphocytes												
High	1.96	0.887–4.131	0.0932				3.76	1.128–13.060	0.0317			
CD8 + and FoxP3+												
CD8-Low/FoxP3-High	3.74	1.546–8.220	0.0049	3.22	1.321–7.179	0.0121	4.59	1.200–15.260	0.0282			

[a]Available data were counted, excluding unknown data

DFS disease-free survival; *OS* overall survival; *LAD* lung adenocarcinoma; *HR* hazard ratio; *CI* confidence interval; *EGFR* epidermal growth factor receptor; *PD-L1* programmed cell death ligand-1; *CD8* cluster of differentiation 8; *FoxP3* Forkhead box protein P3

fully clarified. Shimizu et al. demonstrated that the high levels of CD8 + TILs were significantly related with PD-L1 expression positivity.[17] Additionally, in the evaluation of prognosis of LAD by combining CD8 + TILs with PD-L1 expression, patients with CD8-Low and high PD-L1 expression had poor prognosis; conversely, patients with CD8-High and low PD-L1 expression had good prognosis.[17] Furthermore, Kim et al. also described that high levels of CD8 + TILs and low PD-L1 expression together were associated with favorable prognosis in NSCLC.[18] Koh et al.[19] reported that in LAD, the high density of CD8 + TILs was a good prognostic factor; however, the high ratio of PD-1 + TILs to CD8 + TILs was correlated with a poor prognosis. Furthermore, Kinoshita et al. elucidated that high levels of CD8 + TILs were associated with poor prognosis in LAD, particularly in non-smokers, and further analysis showed that immunoregulatory CD8 + lymphocytes co-expressed FoxP3 and immunodysfunctional CD8 + lymphocytes co-expressed GATA-binding protein 3 were increased in the LADs of non-smokers.[10] Based on these past studies, the significance of CD8 + TILs might fluctuate by histology, patient characteristics, and immune environment surrounding CD8 + TILs.

The methods for evaluating FoxP3 + TILs were diverse, such as ratio of FoxP3/CD8, FoxP3/CD4 and FoxP3/CD3.[11,20,21] However, almost all past studies reported that high infiltration of FoxP3 + TILs was associated with poor prognosis in NSCLC.[6,7,20,21] Furthermore, FoxP3 + TILs are important to the field of immunotherapy where an association between FoxP3 + TILs and PD-L1 expression was demonstrated.[22] A low ratio of FoxP3/CD8 was reported as a therapeutic predictor of PD-1 inhibitor.[11]

As described above, CD8 + and FoxP3 + TILs were closely connected with the prognosis of lung cancer and the therapeutic effect of immunotherapy. However, while the significance of FoxP3 + TILs was consistent, the significance of CD8 + TILs was still controversial. One of the reasons was that past studies on TILs included patients with advanced as well as early stage lung cancer. We considered that the cancer stage was associated with changes in the immune microenvironment of lung cancer. Another reason was that the evaluation method of TILs varied widely. Our study analyzed whole tumor sections and assessed TILs evenly in the tumor tissue. In contrast, several studies were analyzed by tissue microarray or evaluated separately as cancer stoma and nests.[8,18,21,23,24] Furthermore, the functions of CD8 + TILs were controlled by many factors, such as FoxP3 + TILs or PD-1/PD-L1 signal, and it was difficult to elucidate the significance of CD8 + TILs only by assessment of the number. Therefore, the analysis of only CD8 + TILs might be insufficient for assessing the significance of CD8 + TILs in lung cancer. Thus, we analyzed the combination of cytotoxic CD8 + and immunosuppressive FoxP3 + TILs in stage IA LAD.

Our study showed that the prognosis of the CD8-Low/FoxP3-High group was worse than other groups; however, there was no significant difference among the CD8-Low/FoxP3-Low, CD8-High/FoxP3-Low, and CD8-High/FoxP3-High groups. Our study cohort included only stage IA LAD and the prognosis was relatively good. Therefore, only the CD8-Low/FoxP3-High group with worse immune status might show significant differences with other groups. We propose that a larger study is needed to elucidate the difference between CD8-Low/FoxP3-Low, CD8-High/FoxP3-Low, and CD8-High/FoxP3-High groups.

We analyzed PD-L1 expression in tumor cells in addition to CD8 + and FoxP3 + TILs. In a multivariate analysis, PD-L1 expression was an independent prognostic predictor for both DFS and OS. While not significant, CD8-Low/FoxP3-High group tended to have higher PD-L1 expression. This trend is possibly one of the reasons why the CD8-Low/FoxP3-High group had a poor prognosis.

The standard therapy for stage IA LAD is surgical resection and patients with pathological stage IA disease tend to have a long survival time after complete surgical resection. However, the survival rate after recurrence is very poor.[25] Therefore, it is important to identify survival-associated factors for stage IA lung cancer. If we could predict the stage IA lung cancer patients with poor prognosis, adjuvant chemotherapy would be one of the treatment options for the patients after surgical resection.

One limitation of our study is that we could not evaluate all factors that had effects on the function of CD8 + TILs. Furthermore, the design of this study was retrospective, and the study cohort was relatively small due to the selection of patients with stage IA LAD. In addition, the PD-L1 analysis was performed using a specific antibody against PD-L1 (SP142). According to the report by the Blueprint Working Group, the detection rate for the SP142 clone was low compared with other antibodies, such as 28-8, 22C3, and SP263.[26] Thus, we should investigate PD-L1 expression using other antibodies in future studies.

CONCLUSIONS

We showed the importance of analyzing the combination of CD8 + and Foxp3 + TILs in stage IA LAD. We consider that combinational analysis of TILs was required to further elucidate the significance of TILs in lung cancer.

ACKNOWLEDGMENT The authors thank S. J. Win, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

DISCLOSURE The authors have no conflicts of interest to declare.

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