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Original

Roles of FoxP3-positive Regulatory T Cells in Lymphoid Follicle Formation Associated with Lung Squamous Cell Carcinoma

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SUMMARY

Background : We previously reported that lymphoid follicle formation by tumor infiltrating lymphocytes (TILs) is a negative predictor of prognosis in patients with lung squamous cell carcinoma (SCC) following surgery. However, the roles of $FoxP3^+/CD4^+/CD25^+$-regulatory T cells (Tregs) in formation of lymphoid follicles as well as survival remain unclear.

Methods : Specimens obtained from patients during resection of lung SCC were examined for lymphoid follicle formation and subjected to immunohistochemistry analysis for the presence of TILs.

Results : The appearance of Tregs was correlated with lymphoid follicle formation ($p=0.001$). Univariate analysis also showed that Tregs tended to be correlated with overall survival ($p=0.097$), whereas multivariate analysis revealed that lymphoid follicle formation ($p=0.042$) and pleural invasion ($p=0.031$) were independent prognostic factors related to overall survival, while the appearance of Tregs was not.

Conclusion : Treg appearance was correlated with lymphoid follicle formation. That lymphoid follicle formation, rather than appearance of Tregs, is a predictor of patients survival following surgery for lung SCC.

Key Words : lung cancer, squamous cell carcinoma, regulatory T cell, lymphoid follicle

INTRODUCTION

Introduction of immune checkpoint inhibitor (ICI) therapy has drastically increased treatment options for patients with non-small cell lung cancer (NSCLC). Treatment with an ICI blocks tumor immune tolerance between cancer cells and the immune system by affecting their interactions, for example, that of the programmed death (PD)-1 pathway and its ligand (PD-L1). Several reports have also suggested that tumor-infiltrating lymphocytes (TILs) are correlated with prognosis of patients with various types of cancer[1~6], including findings showing that the appearance of lymphoid follicles in the tumor stroma is a possible prognostic factor[2]. Recently, we reported that lymphoid follicle formation by TILs is a negative predictor of prognosis in patients with lung squamous cell carcinoma (SCC) following surgery, and showed findings suggesting that $CD4^+/CD25^+$-T cells, a

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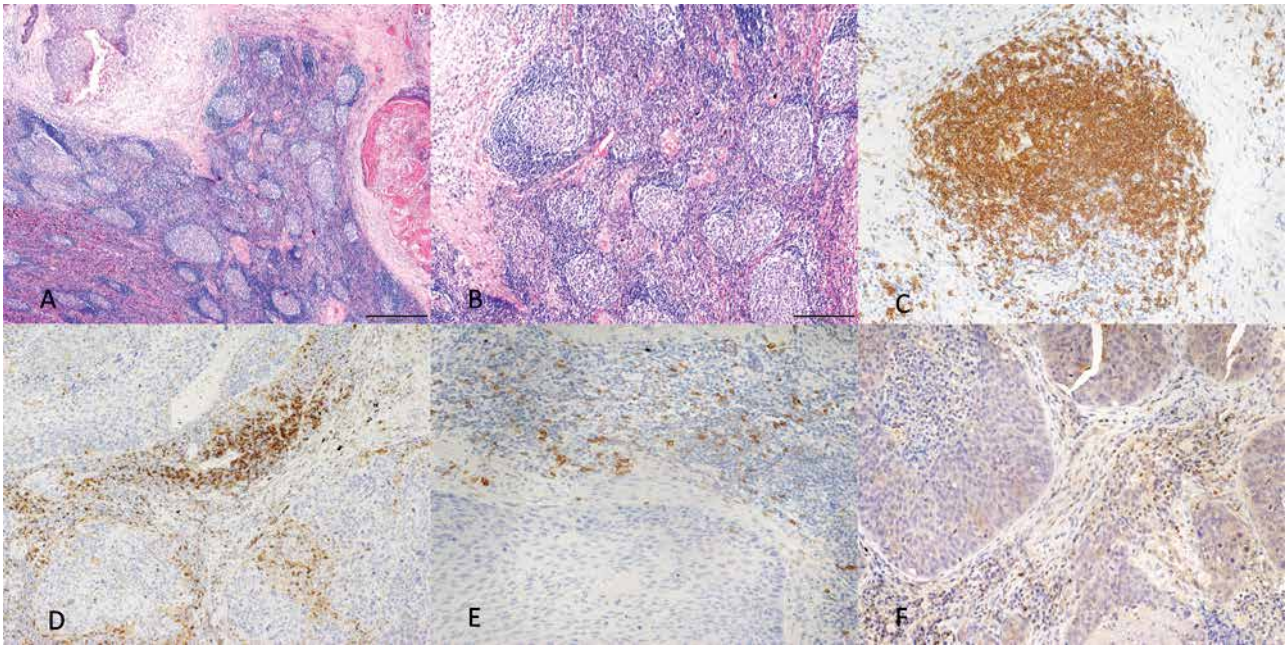

Figure 1 Representative images showing lymphoid follicle formation in TILs
Lymphoid follicles (**A**) in tumor stroma (HE stain, ×40), (**B**) with a germinal center (HE stain, ×200), and (**C**) composed of B cells (CD20 stain, ×100). (**D**) T cells (CD4 stain, ×100), (**E**) CD25-positive cells (CD25 stain, ×200), and (**F**) Tregs (FoxP3 stain, ×200).

cohort including regulatory T cells (Tregs), may play a role in negative outcomes of patients with lymphoid follicle formation[7]. However, the roles of $FoxP3^+/CD4^+/CD25^+$-T cells, true Tregs, on lymphoid follicle formation and survival remain unclear. In the present study, we focused on the effects of $FoxP3^+/CD4^+/CD25^+$-Tregs on formation of lymphoid follicles in patients with resectable lung SCC.

METHODS

Patients with lung SCC who underwent a complete resection at Dokkyo Medical University Hospital from January 2010 through December 2012 were enrolled and specimens obtained during surgery were examined. Informed consent for use of the materials was obtained from each patient. The Ethical Committee of Dokkyo Medical University Hospital granted approval for this retrospective study (#R-5-8).

Pathological examinations were conducted as described in our previous report[7]. Briefly, resected specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Next, 2-μm thick sections were obtained from the block that included the largest cut surface of the tumor, and stained with hematoxylin and eosin (HE). Sections were then cut (4-μm thick) from the same block, and a standard avidin-biotin complex peroxidase technique was used for immunohistochemical staining of primary antibodies against CD4, CD20, CD25, and FoxP3. Both a trained observer (MN) and pathologist (YN) reviewed each slide in detail. When there was disagreement, another pathologist (HK) was consulted and the final determination was made based on majority decision.

Lymphoid follicles were identified as CD20-positive B cell accumulation with a germinal center (Figure 1A-C). TILs were classified as 3 different 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. $FoxP3^+/CD4^+/CD25^+$-T cells among TILs were considered to be evidence of infiltration by Tregs when the grade was 1 or greater (Figure 1D-F).

A chi-square test was used for statistical analysis between two groups. Survival curves were obtained using the Kaplan-Meier method and comparisons within each group were performed with a log-rank

Table 1 Relationship between regulatory T cells and lymphoid follicles

		Tregs ($CD4^+CD25^+FoxP3^+$)			
		Absent	Present	Total	P value
Lymphoid follicle	Absent	50	0	50	
	Present	9	4	13	0.001
Total		59	4	63	

Tregs, regulatory T cells

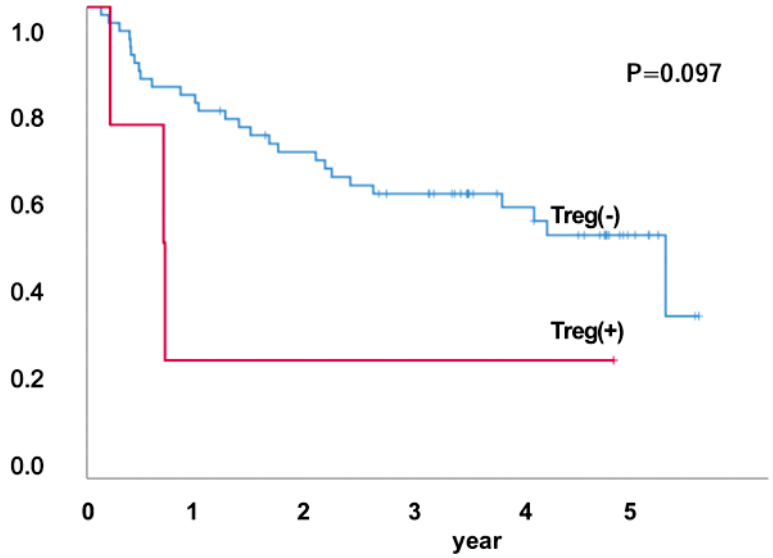

Figure 2 Overall survival
Blue line, Treg-negative ; red line, Treg-positive

test. Risk factors for overall survival were evaluated with univariate and multivariate analyses using the Cox regression method. Factors for multivariate analysis in the present study were the same as examined in our former report[7] because the analyses were conducted with the same database, including pleural and vascular factors, lymphoid follicle formation, pathological stage, and appearance of Tregs. Statistical calculations were performed using the SPSS statistics version 25 software program (IBM Corp., NY, USA), with $p<0.05$ considered to indicate a significant difference and borderline significance shown by $p<0.10$.

RESULTS

A total of 72 patients with SCC underwent a lung resection procedure during the study period. From those cases, total of 63 specimens were obtained and subjected to FoxP3 staining. $FoxP3^+/CD4^+/CD25^+$-Treg appearance was positive in 4 cases and consistently correlated with lymphoid follicle formation ($p=0.001$) (Table 1).

Univariate analysis of cases possessing $FoxP3^+/CD4^+/CD25^+$-Tregs showed a tendency for correlation with overall survival ($p=0.097$) (Figure 2). The 5-year survival rate of Treg-negative cases was 51.6 %, while that of Treg-positive cases was 25.0%. Multivariate analysis revealed that lymphoid follicle formation and pleural invasion were statistically significant independent prognostic factors related to overall survival, whereas the appearance of $FoxP3^+/CD4^+/CD25^+$-Tregs was not (Table 2).

DISCUSSION

To clarify the role of Tregs among TILs in patients with lung SCC, we conducted FoxP3 staining in addition to CD4/CD25 staining, as we have previously

Table 2 Multivariate analysis of overall survival

	HR	95% CI		P value
Pl (−/+)	2.21	1.04	4.70	0.040
V (−/+)	1.70	0.70	4.12	0.24
Lymphoid follicles (+/−)	2.85	1.04	7.79	0.042
pStage (I/II+III)	0.92	0.41	2.07	0.85
Tregs (+/−)	1.08	0.26	4.48	0.92

Pl, pleural invasion ; V, vascular invasion ; pStage, pathological stage ; Tregs, regulatory T cells ; HR, hazard ratio ; CI, confidence interval

reported[7]. In that prior study, we regarded $CD4^+$/$CD25^+$-T cells as Tregs and concluded that the existence of $CD4^+$/$CD25^+$-Tregs around lymphoid follicles indicated increased risk to the prognosis of worse survival. However, $CD4^+$/$CD25^+$-T cells actually consist of Tregs and some helper T cells, thus they should not be considered as a pure Treg cohort. In the present study, appearance of $FoxP3^+$/$CD4^+$/$CD25^+$-Tregs was positive in 4 cases and each of those cases included lymphoid follicles in the obtained specimens, as we previously described[7], while the existence of Tregs among the TILs had an association with overall survival.

Tregs have roles in suppression and regulation of immune response, as well as prevention of autoimmune disease development[8]. The existence of Tregs among TILs has been reported to be correlated with poor survival of patients with a various types of cancer[9~12], though survival of those with Hodgkin disease was found to be superior as compared to others[13]. As for lung cancer, the role of Tregs has not been clearly elucidated. For example, tumor-infiltrating Tregs were reported to be associated with recurrence in pathologic stage I NSCLC patients[14], while another study found that lung cancer patients with a high density of tumor-infiltrating Tregs had better prognosis as compared to those without the presence of Tregs[15]. In the present results, Tregs in lung SCC specimens had a tendency to be associated with worse prognosis as compared to specimens without Tregs. On the other hand, our multivariate analysis revealed that lymphoid follicle formation was an independent prognostic factor, whereas the presence of Tregs was not. Therefore, we consider that lymphoid follicle formation itself is a prognostic factor, although Treg development is correlated with that.

In a recent report, Shalapour and colleagues noted that immunosuppressive plasma cells, found to express IgA, interleukin (IL)-10, and PD-L1, impeded T-cell-dependent immunoreactions[16]. Unfortunately, we have no findings in regard to IgA, IL-10, or PD-L1 from the present study, though speculate that plasma cells among lymphoid follicles in lung SCC may have an immunosuppressive role. Additional research is necessary to more fully elucidate the functions of B cells and lymphoid follicles in TILs.

Important limitations of this study include its retrospective design and the small group of subjects from a single institution.

CONCLUSION

The appearance of Tregs was found to be correlated with lymphoid follicle formation in the present subjects. However, lymphoid follicle formation, rather than possession of Tregs, was shown to be a predictor of patient survival following surgery for lung SCC.

Conflict of interest

All authors declare that they have no conflicts of interest.

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