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CTLA-4 Expression in Tumor-infiltrating Lymphocytes Is Irrelevant to PD-L1 Expression in NSCLC

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Abstract. *Background/Aim: Treatments containing ipilimumab have shown a good outcome in patients with non-small cell lung cancer (NSCLC) regardless of the PD-L1 tumor proportion score (TPS). However, the association between PD-L1 TPS and the expression of CTLA-4 in tumor-infiltrating lymphocytes is unknown. Patients and Methods: Fifty-five NSCLC patients who underwent surgery in our hospital were included in this study. We measured the proportions of CTLA-4$^+$ regulatory T cells, and CTLA-4$^+$ CD8 T cells, and statistically analyzed their correlations with the PD-L1 TPS. Results: Statistical correlations were found neither between the proportion of CTLA-4$^+$ regulatory T cells to CD8 T cells and the PD-L1 TPS (p=0.2859) nor between the proportion of CTLA-4$^+$ cells in CD8 T cells and the PD-L1 TPS (p=0.1919). Conclusion: The proportions of CTLA-4$^+$ regulatory T cells to CD8 T cells and CTLA-4$^+$ cells in CD8 T cells were irrelevant to the PD-L1 TPS in NSCLC patients.*

The combination therapies with anti-PD-1/PD-L1 antibody and chemotherapy have progressed in recent years in the treatment of advanced or recurrent non-small cell lung cancer (NSCLC) (1, 2). However, they have not met the needs of NSCLC patients with PD-L1 tumor proportion score (TPS) <1%. Ipilimumab, an anti-CTLA-4 antibody, + nivolumab ± chemotherapy showed a good outcome regardless of PD-L1 TPS in CheckMate 227 trial and CheckMate 9LA trial of first-line treatment of advanced NSCLC (3, 4). On the other hand, treatment with ipilimumab can increase the incidence of immune-related adverse events (3). Biomarkers are required for appropriate treatment selection for each patient.

Tumor mutation burden has been attracting attention as a biomarker for ipilimumab (5), but it is currently considered inadequate (3). It has been reported that patients with higher levels of CTLA-4 expression in the tumor microenvironment of metastatic melanoma have clinical benefit from treatment with anti-CTLA-4 antibody (5). CTLA-4 on regulatory T cells as well as CD8 T cells are thought to be therapeutic targets of an anti-CTLA-4 antibody (6). However, it is still unknown whether the proportion of CTLA-4$^+$ regulatory T cells to CD8 T cells and CTLA-4$^+$ cells in CD8 T cells are associated with the PD-L1 TPS in NSCLC patients.

We conducted this study in order to clarify the association between the proportion of CTLA-4$^+$ cells in tumor-infiltrating lymphocytes and the PD-L1 TPS. Additionally, we investigated the association between absolute lymphocyte counts in peripheral blood and the PD-L1 TPS. Absolute lymphocyte counts in peripheral blood were reported to be associated with the clinical outcome of nivolumab or ipilimumab in patients with melanoma (7, 8).

Patients and Methods

Patients. This study was approved by the Human Ethics Committee at Fukushima Medical University. This study was performed in accordance with the Declaration of Helsinki and in compliance with the Japanese Ethical Guidelines for Medical and Health Research involving Human Subjects. Key eligibility criteria were as follows: surgically resected NSCLC, no prior systemic therapy, no sensitizing *EGFR* mutations (exon 19 deletion or exon 21 L858R) or known *ALK* alterations. Patient inclusion flowchart is shown in Figure 1. We enrolled 55 NSCLC patients who gave informed consent. Disease staging was evaluated according to the eighth edition of the TNM classification for NSCLC. The PD-L1 TPS was evaluated using PD-L1 IHC 22C3 pharmDX on the DAKO autostainer Link 48 (SRL, Inc. Tokyo, Japan). The PD-L1 TPS was defined as the percentage of viable tumor cells with partial or

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Key Words: Ipilimumab, CTLA-4 antigen, regulatory T cells, non-small cell lung cancer, immune checkpoint inhibitors.

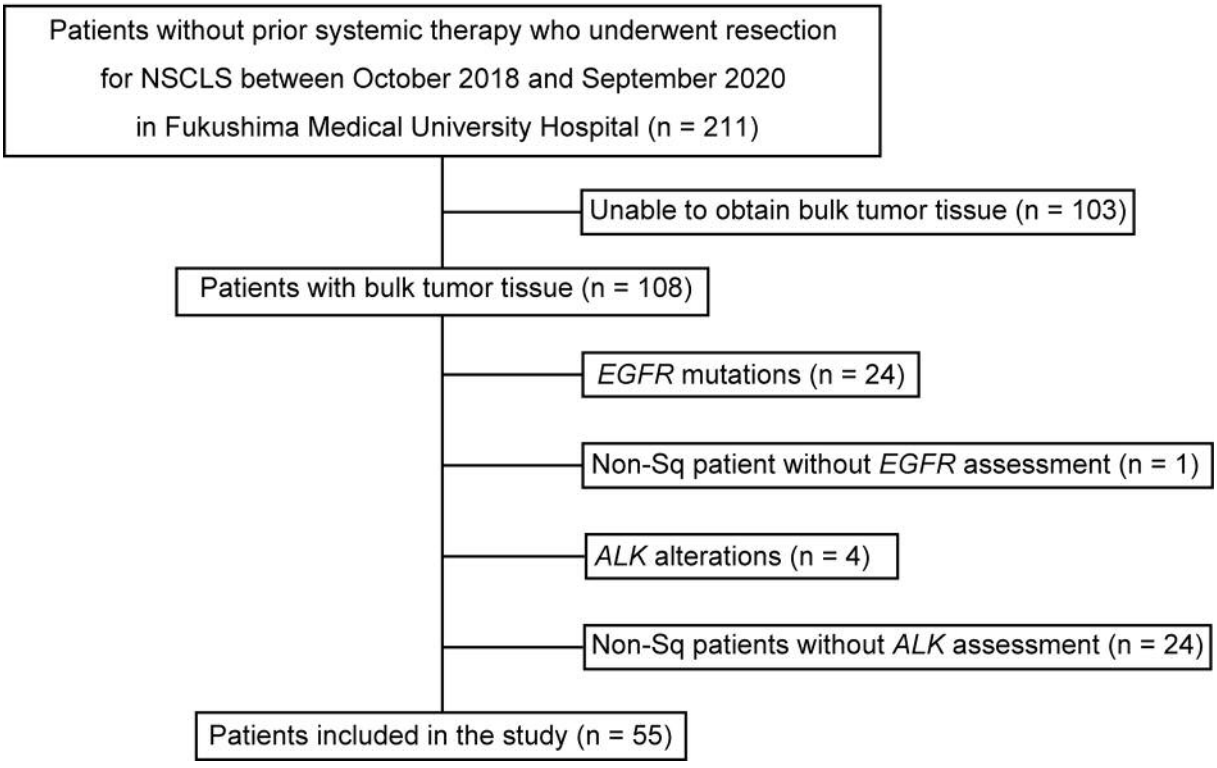

Figure 1. *Patient inclusion flowchart.*

complete membrane staining (9). Absolute lymphocyte counts in peripheral blood were calculated from complete blood cell count, obtained two days before surgery.

TIL flow cytometry analysis. Bulk tumor tissues were dissociated into single-cell suspensions using gentleMACS™ Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and tumor-infiltrating cells were collected using CD45 microbeads (cat. no. 130-045-801, Miltenyi Biotec) and autoMACS® (Miltenyi Biotec). Cell surface staining was performed by using 5 μl of the following fluorescein-conjugated antibody in 100 μl of phosphate-buffered saline containing 1% bovine serum albumin for 20 min on ice: CD3 (PerCP-Cy5.5, clone UCHT1, cat. no. 300430, BioLegend, Inc., San Diego, CA, USA), CD4 (FITC, clone RPA-T4, cat. no. 555346, BD Biosciences, Franklin Lakes, NJ, USA), CD8 (PE-Cy7, clone SK1, cat. no. 344712, BioLegend, Inc.), CD45RA (APC, clone HI100, cat. no. 561884, BD Biosciences), CTLA-4 (PE, clone BNI3, cat. no. 369604, BioLegend, Inc.) and its isotype control mouse IgG2a, κ (BioLegend, Inc.). The cells were fixed and permeabilized using eBioscience FoxP3 transcription factor staining buffer set (cat. no. 00-5523-00, ThermoFisher Scientific, Waltham, MA, USA) and stained using anti-FoxP3 antibody (Pacific Blue, clone 206D, cat. no. 320116, BioLegend, Inc) according to the manufacturer's instructions. Dead cells were also dyed using Zombie NIR Fixable Viability Kit (1:100, cat. no. 423105, BioLegend, Inc.). Data acquisition was performed using FACS CantoII (BD Biosciences) and analyzed using FlowJo software version 10.7.1 (FlowJo LLC, Ashland, OR, USA). Firstly, single and live cells were gated and then each subset of CD3 T cells was analyzed (Figure 2). CD4 T cells were divided into subsets by the expression of FoxP3 and CD45RA. $CD45RA^{-}$ $FoxP3^{++}$ CD4 T cells were defined as regulatory T cells in this study (10). The proportions of $CTLA\text{-}4^{+}$ regulatory T cells to CD8 T cells and $CTLA\text{-}4^{+}$ cells in CD8 T cells were analyzed.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software v.8.4.3 (GraphPad Software, Inc., San Diego, CA, USA). The patients were divided into three groups according to the PD-L1 TPS (<1%, 1-49%, ≥50%), and clinical characteristics were compared between the groups using one-way ANOVA or χ^2 test. The proportion of $CTLA\text{-}4^{+}$ regulatory T cells to CD8 T cells was compared between the three groups. The same comparison was conducted for the proportion of $CTLA\text{-}4^{+}$ cells in CD8 T cells and for absolute lymphocyte counts in peripheral blood. *p*-Values were obtained by comparing the three groups with the Kruskal-Wallis test. *p*-Values <0.05 were considered statistically significant.

Results

Clinical characteristics of the patients are presented in Table I. There were 46 men and 9 women with a mean age of 72.6±5.7 (SD) years. Thirty-five patients (64%) had squamous cell carcinoma and 20 (36%) had non-squamous cell carcinoma. The number of patients in stage I, II, III, and

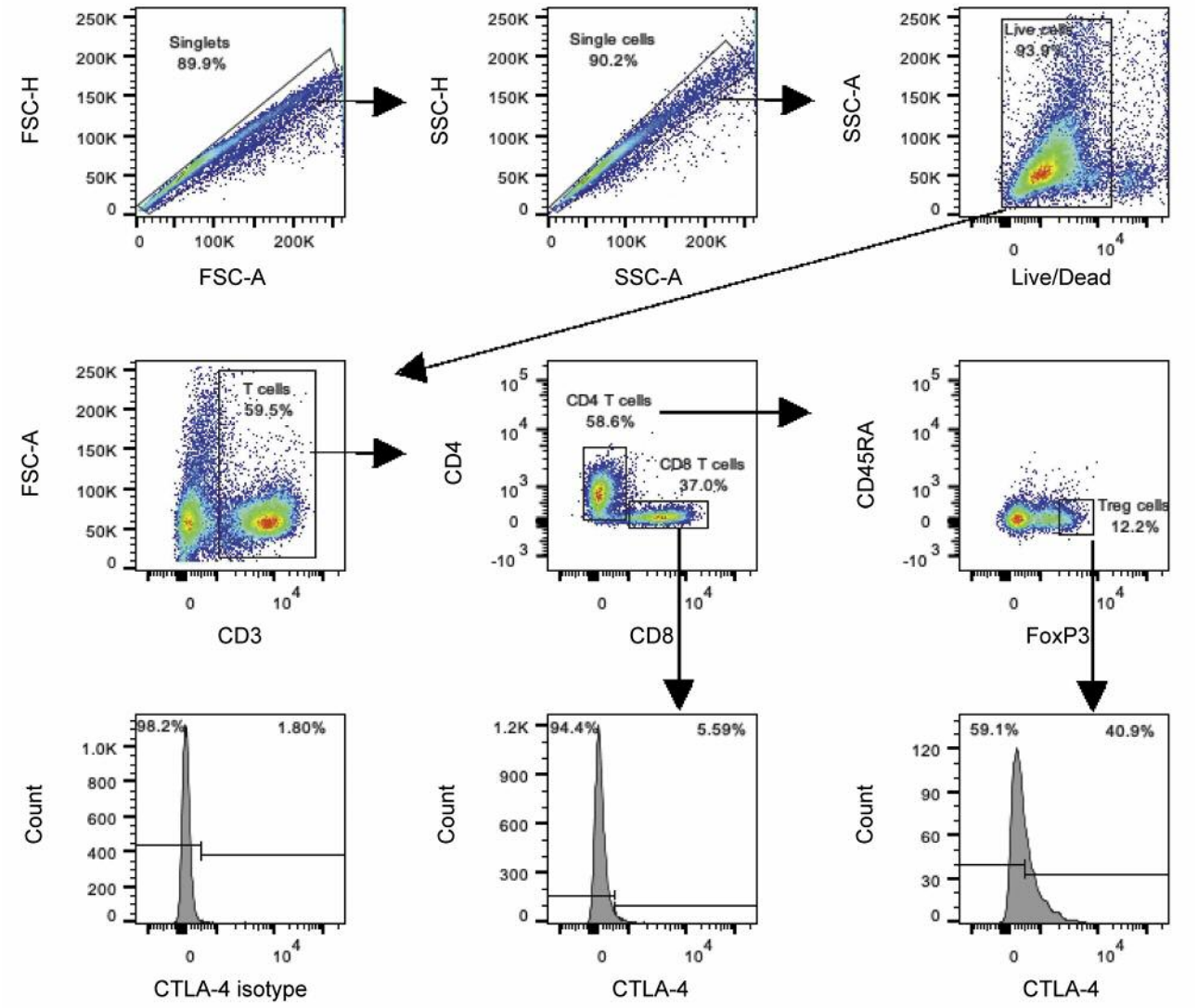

Figure 2. *Plots and gating for flow cytometry. Single live cells were gated and CD3 T cells were selected. CD4 T cells were divided into subsets, and CD45RA- FoxP3^{++} CD4 T cells were defined as regulatory T cells in the study. The proportion of CTLA4^{+} cells in regulatory T cells or in CD8 T cells was analyzed.*

Table I. *Clinical characteristics of patients*.

Variable	Total (N=55)	PD-L1 TPS <1% (N=19)	PD-L1 TPS 1-49% (N=16)	PD-L1 TPS ≥50% (N=20)	*p*-Value
Age (years)	72.6±5.7	71.4±5.6	74.6±5.9	72.2±5.4	0.2347
Male	46 (84%)	14 (74%)	15 (94%)	17 (85%)	0.2728
Female	9 (16%)	5 (26%)	1 (6%)	3 (15%)	
Sq	35 (64%)	9 (47%)	11 (69%)	15 (75%)	0.1764
Non-Sq	20 (36%)	10 (53%)	5 (31%)	5 (25%)	
Stage I	23 (42%)	6 (32%)	8 (50%)	9 (45%)	0.6026
Stage II	16 (29%)	7 (37%)	3 (19%)	6 (30%)	
Stage III	13 (24%)	5 (26%)	3 (19%)	5 (25%)	
Stage IV	3 (5%)	1 (5%)	2 (13%)	0 (0%)	

Data are mean±SD or n (%). One-way ANOVA or χ^2 test was used to compare the three groups. *p*-Values <0.05 were considered statistically significant. Sq: Squamous cell carcinoma.

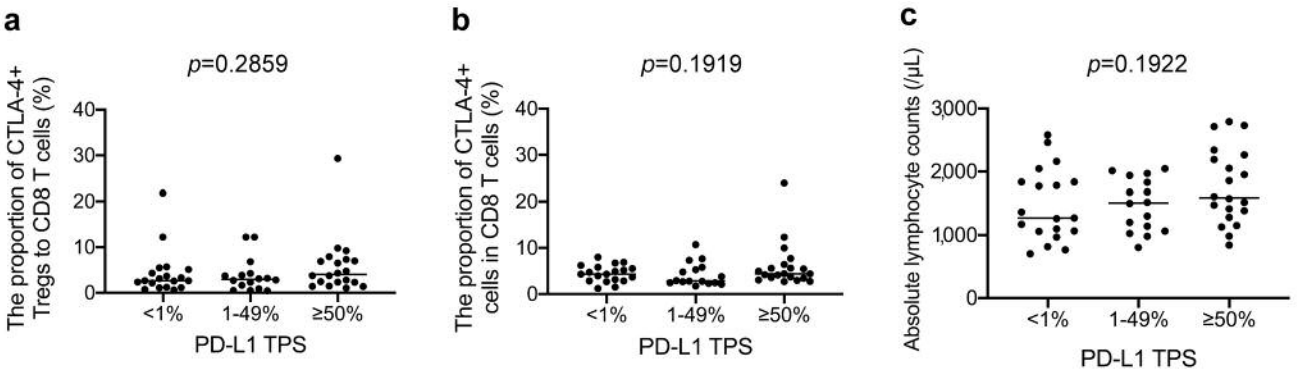

Figure 3. *The association between PD-L1 TPS and the following variables: (A) the proportion of CTLA-4+ regulatory T cells to CD8 T cells; (B) the proportion of CTLA-4+ cells in CD8 T cells; and (C) absolute lymphocyte counts in peripheral blood. PD-L1 tumor promotion score (TPS) is presented as a categorical variable. p-Values were obtained by comparing the three groups with the Kruskal-Wallis test. p-Values <0.05 were considered statistically significant. Tregs: Regulatory T cells.*

IV was 23 (42%), 16 (29%), 13 (24%), and 3 (5%), respectively. There were no significant differences in clinical characteristics between the three groups of PD-L1 TPS.

The proportion of CTLA-4+ regulatory T cells to CD8 T cells did not correlate to the PD-L1 TPS (p=0.2859, Figure 3A). The proportion of CTLA-4+ cells in CD8 T cells did not show correlation to the PD-L1 TPS, either (p=0.1919, Figure 3B). Absolute lymphocyte counts in peripheral blood also showed no correlation to the PD-L1 TPS (p=0.1922, Figure 3C).

Discussion

We showed that the proportion of CTLA-4+ regulatory T cells to CD8 T cells in tumor-infiltrating lymphocytes was irrelevant to the PD-L1 TPS in surgically resected NSCLC. The proportion of CTLA-4+ cells in CD8 T cells in tumor-infiltrating lymphocytes was also irrelevant to the PD-L1 TPS. In addition, it has been reported that nivolumab prolonged survival in advanced NSCLC patients irrespective of the PD-L1 TPS (11). These data support the results of CheckMate 227 and CheckMate 9LA showing survival benefit regardless of the PD-L1 TPS (3, 4). CTLA-4 on regulatory T cells and CD8 T cells are thought to be therapeutic targets of anti-CTLA-4 antibody (6). However, it is still unclear whether the expression of CTLA-4 is associated with the therapeutic effect of ipilimumab in NSCLC. The clinical benefit of anti-CTLA-4 antibody treatment has been reported in patients with metastatic melanoma who had higher levels of CTLA-4 expression in the tumor microenvironment (5). Some reports have described the association between CTLA-4 levels and clinical benefits of ipilimumab in various cancers (7, 12-15). A prospective study is desired to determine whether the expression of CTLA-4 could be a biomarker of ipilimumab in NSCLC.

Additionally, we showed that absolute lymphocyte counts in peripheral blood were irrelevant to the PD-L1 TPS. Topalian *et al*. reported that absolute lymphocyte counts were associated with the clinical outcome of nivolumab in CheckMate 003 clinical trial, which included patients with advanced melanoma, renal cell carcinoma or NSCLC (15). More interestingly, it was reported that ipilimumab increases absolute lymphocyte counts in melanoma (8). According to these reports, ipilimumab may increase the effect of nivolumab through expansion of absolute lymphocyte counts. The trajectory of absolute lymphocyte counts is considered to be a candidate for predictive biomarkers in NSCLC patients treated with nivolumab + ipilimumab.

There are two limitations in this study. One is that most of the patients in this study were at an early stage of NSCLC. This was because we conducted this study using tumor tissue obtained by surgery. Only three of the included 55 patients were at stage IV. Although the tumor microenvironment might be different in advanced NSCLC, the PD-L1 TPS distribution in patients in the current study was almost equal to that in advanced NSCLC in clinical trials (1). Further study is needed to compare the immune evading mechanisms in the tumor microenvironment between early and advanced NSCLC. The other limitation is that we did not examine immune cells in tumor draining lymph nodes. CTLA-4 mediated immune tolerance is thought to occur between CTLA-4 expressed on activated T cells or regulatory T cells and B7 expressed on antigen presenting cells (16). Therefore, findings on immune cell profiles in tumor draining lymph nodes will lead to further understanding of cancer immunology.

In conclusion, we revealed that the proportions of CTLA-4+ regulatory T cells to CD8 T cells and CTLA-4+ cells in CD8 T cells in tumor-infiltrating lymphocytes and absolute

lymphocyte counts in peripheral blood were independent of the PD-L1 TPS. These results give us a new perspective on CheckMate 227 and CheckMate 9LA trials in which ipilimumab containing treatment provided clinical benefits to patients irrespective of the PD-L1 TPS.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

All Authors contributed to the study conception and design. Material preparation and data collection were performed by SM, SI, HY, HM, HT, YO, MW, TI, TY, MF, NO, YM, TH, JO, MH, MH and YS. Data analysis was performed by SM. The first draft of the manuscript was written by SM. Review and editing were performed by HS. All Authors read and approved the final manuscript.

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