Neoadjuvant Chemotherapy Increases PD-L1 Expression and CD8⁺ Tumor-infiltrating Lymphocytes in Esophageal Squamous Cell Carcinoma

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Abstract. *Background/Aim:* The aim of this study was to investigate PD-L1 expression and its association with prognosis in esophageal squamous cell carcinoma (ESCC) before and after neoadjuvant chemotherapy (5-fluorouracil and cisplatin, NAC-FP). *Patients and Methods:* Using a database of 69 ESCC patients, we analyzed PD-L1 expression on tumor cells (TCs) and immune cells (ICs), as well as the density of CD8⁺ tumor-infiltrating lymphocytes (TILs) in pretreatment biopsy specimens-versus-surgical specimens after resection. We determined the prognostic significance of these factors. *Results:* The fraction of ESCC containing ICs expressing PD-L1 and having a high CD8⁺ TIL density was significantly increased after neoadjuvant treatment. However, PD-L1 expression on TCs or ICs, and CD8⁺ TIL density, was not significantly associated with patient survival in ESCC patients. *Conclusion:* NAC-FP induced PD-L1 expression on ICs and CD8⁺ TILs in ESCC patients. This finding suggests that PD-1/PD-L1 blockade could be combined with NAC-FP to treat ESCC patients.

Esophageal cancer is the sixth most common cause of cancer-related death, with an estimated 572,000 new cases per year, worldwide (1). It is of two main histological types, esophageal squamous cell carcinoma (ESCC) or esophageal adenocarcinoma. In Asia, incidence rates of squamous cell carcinoma are much higher than adenocarcinoma (2).

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Neoadjuvant chemotherapy (NAC) or chemoradiotherapy followed by esophagectomy has become the standard treatment worldwide for patients with ESCC. The Japan Clinical Oncology Group 9907 study showed that NAC consisting of 5-fluorouracil and cisplatin (FP) significantly extended survival in clinical stage II/III ESCC, but 5-year survival in patients treated with NAC and surgery was still only 54.8% (3). Therefore, additional treatment strategies are needed to improve the poor prognosis for ESCC patients.

Recently, immune checkpoint inhibitors (ICI) have yielded breakthrough treatments in several different solid cancers (4-8), including esophageal cancer (9). In order to reliably achieve cures in a greater fraction of patients, perioperative ICI immunotherapy is now being considered. In preclinical models, ICI was shown to be more effective in neoadjuvant, than in the adjuvant form (10). More recently, in the clinical setting, preoperative treatment with a single dose of anti-PD-1 ICI has been shown to be effective in melanoma (11) and non-small-cell lung cancer (NSCLC) (12). In addition, in order to enhance therapeutic efficacy, combination therapies are being enthusiastically tested (13). In ESCC, an *in vitro* study revealed that FP chemotherapy induced PD-L1 expression (14). Based on these findings, it is hypothesized that a combination of FP and anti-PD-1/PD-L1 ICI is a promising preoperative treatment for ESCC. In this context, in order to study the action of this combination therapy, it is important to analyze the immune tumor microenvironment (iTME) after NAC. Previous studies have shown that the status of the iTME as classified by PD-L1 expression and TIL density, might be predictive of anti-PD-1/PD-L1 ICI efficacy (15). Some studies have documented that PD-L1 expression has prognostic value in ESCC patients undergoing surgery without any neoadjuvant therapy (16-20). However, robust data on PD-L1 expression in ESCC patients treated with NAC are limited (21, 22). Therefore, in the present study, we aimed to clarify the effect of NAC on the iTME, utilizing FP

Table I. Relationships between clinicopathological characteristics and PD-L1 expression.

Variable	PD-L1 expression (TC)			PD-L1 expression (IC)		
	Negative (n=38)	Positive (n=31)	p-Value	Negative (n=13)	Positive (n=56)	p-Value
Age			0.028			0.700
<60	11	2		3	10	
≥60 years	27	29		10	46	
Gender			0.068			0.674
Male	36	24		11	49	
Female	2	7		2	7	
Tumor location			0.516			0.356
Upper	13	7		5	15	
Middle	12	10		2	20	
Lower	13	14		6	21	
Clinical T			0.195			0.742
≤2	15	7		5	17	
≥3	23	24		8	39	
Clinical lymph node metastasis			0.244			1.000
Negative	6	9		3	12	
Positive	32	22		10	44	
Clinical stage			0.810			1.000
≤II	16	12		5	23	
≥III	22	19		8	33	
Pathological stage			0.398			0.157
I	10	6		1	15	
II	8	11		6	13	
III	20	14		6	28	
Grade of response to NAC			1.000			1.000
≤1b	32	27		11	48	
2	6	4		2	8	

(NAC-FP) and measuring PD-L1 expression and CD8⁺ TIL density in ESCC patients. We compared expression in biopsy specimens before NAC-FP and surgical specimens after NAC-FP. In addition, we also aimed to elucidate the impact of PD-L1 expression and CD8⁺ TIL density on the long-term outcomes of ESCC patients treated with NAC-FP followed by surgery with curative intent.

Materials and Methods

Patients. Of the patients who underwent esophagectomy after chemotherapy at the Kobe University Hospital between 2008 January and 2014 December, 71 met the following criteria and were included in this study: 1) histologically confirmed ESCC by biopsy; 2) R0/R1 resection performed; 3) patients completed 2 cycles of FP chemotherapy; 4) paired biopsies and surgical specimens were available. We excluded 2 cases (2.8%) because of pathological complete response. Hence, 69 cases were finally enrolled. The Union for International Cancer Control Tumor-Node-Metastasis Classification (7th edition) was used for staging. The histopathological response to NAC was determined according to the criteria of the Japanese Society for Esophageal Diseases. Grades are determined based on the percentage of remaining viable tumor cells

within the entire tumor lesion defined as follows: grade 3, no viable residual tumor cells; grade 2, less than two-thirds; grade 1, two-thirds or more; grade 0, all viable. The FP chemotherapeutic regimen consisted of two cycles of 5-fluorouracil (800 mg/m², days 1-5) and cisplatin (80 mg/m², day 1). Surgery was performed about 4-6 weeks after chemotherapy (23). Informed consent was obtained from each patient, and this retrospective study was conducted with the approval of the Institutional Review Board and the Ethics Committee of the Graduate School of Medicine, Kobe University School of Medicine.

Immunohistochemistry (IHC). IHC staining was performed on 4-μm slices. Slides were deparaffinized in xylene and rehydrated in a graded series of ethanol dilutions. Antigen retrieval was performed by heating in a pressure oven. Biopsy specimens before NAC and surgical specimens after NAC were immunostained with rabbit monoclonal anti-PD-L1 antibody (dilution 1:100, clone SP142; Spring Bioscience, Pleasanton, CA, USA) and rabbit monoclonal anti-CD8 antibody (dilution 1:100, SP57; Ventana Medical Systems, Tucson, AZ, USA). Human placental tissue was used as a positive control for PD-L1 staining.

Immunohistochemical analysis. PD-L1 expression on tumor cells (TCs) and immune cells (ICs) was evaluated in a semi-quantitative

Figure 1. Representative micrographs of sections from esophageal squamous cell carcinoma (ESCC). A. PD-L1 expression on tumor cells (TCs). B. PD-L1 expression on immune cells (ICs). C. PD-L1 expression on neither TCs nor ICs. D. CD8⁺ tumor-infiltrating lymphocytes (TILs) in tumor tissue. E. No CD8⁺ TILs in tumor tissue. Magnification: 200 $\times$.

fashion according to a previously reported scoring system (24). PD-L1 expression on TCs was categorized into four groups according to the percentage of PD-L1-positive TCs, namely TC0<1%, 1%<TC1<5%, 5%<TC2<50%, and TC3 $\geq$ 50%. PD-L1 expression on IC was categorized into four groups according to the percentage of PD-L1-positive ICs within all ICs in the tumor area as follows: IC0<1%, 1%<IC1<5%, 5%<IC2<10%, and TC3 $\geq$ 10%. CD8⁺ TIL density was categorized in the same manner as PD-L1 expression on ICs. The immunohistochemical staining was independently evaluated by two of the authors who were blinded to the clinical data. PD-L1 positivity was defined as $\geq$ 5% of TCs or ICs (Figure

1). To be scored as positive, the density of CD8⁺ TILs was defined as $\geq$ 10% of ICs.

Statistical analysis. Statistical analyses were conducted using JMP[®] 11 (SAS Institute Inc., Cary, NC, USA). Pearson's Chi-square test or Fisher's exact test was used to analyze the relationship between immune markers and patient characteristics. McNemar's test was used to analyze changes of PD-L1 expression and CD8⁺ TILs in surgical specimens relative to the values measured in the biopsy specimens. Overall survival (OS) and recurrence-free survival (RFS) were calculated using the Kaplan–Meier method and analyzed using

the log-rank test. A p -value of <0.05 was considered to be statistically significant.

Results

Patient characteristics and immunohistochemistry. Patients at diagnosis ranged in age from 44 to 82 years (median=68 years) and were predominantly male (60 of 69, 87.0%). The location of the tumor was the upper esophagus in 20 patients (29.0%), the middle esophagus in 22 (31.9%), and the lower esophagus in 27 (39.1%). The response to NAC was grade 2 in 10 patients (14.5%), grade 1 in 51 (73.9%), and grade 0 in the remaining 8 (11.6%). A comparison of the clinicopathological characteristics according to PD-L1 expression on TCs and ICs in surgical specimens is shown in Table I. PD-L1 was expressed on TCs in 44.9% of patients (31/69), and was associated with older age (≥ 60) ($p=0.028$) (Table I). On the other hand, PD-L1 expression on ICs was observed in 81.2% of patients (56/69). No relationship was found between PD-L1 expression on ICs and any clinicopathological characteristic.

PD-L1 expression and CD8⁺ TIL density before and after NAC. We compared PD-L1 expression on TCs and ICs, and the density of CD8⁺ TILs in biopsy specimens before NAC and surgical specimens after NAC for each ESCC patient according to the scoring system described above (24). The distribution of scores was then examined (Figure 2). Although not statistically significant, the percentage of patients with TCs expressing PD-L1 increased from 34.8% before NAC to 44.9% after NAC ($p=0.114$) (Figure 2A). On the other hand, the percentage of patients with ICs expressing PD-L1 increased from 60.9% before NAC to 81.2% after NAC, and this was significant ($p=0.004$) (Figure 2B). The percentage of patients with a high density of CD8⁺ TILs also increased significantly from 21.7% before NAC to 66.7% after NAC ($p<0.001$) (Figure 2C).

Association of PD-L1 expression and CD8⁺ TIL density with survival. We assessed OS and RFS according to PD-L1 expression on either TCs or ICs, and the density of CD8⁺ TILs but found no significant difference between groups for the entire cohort of patients (Figure 3). Next, we stratified patients into pathological stage I-II-vs.-stage III. In stage I-II, PD-L1 expression on ICs was indeed associated with improved OS and RFS ($p=0.022$ and $p=0.033$, respectively) (Figure 4A and B), but in patients with stage III disease was not associated with OS or RFS ($p=0.482$ and $p=0.502$, respectively) (Figure 4C, D). On the other hand, PD-L1 expression on TCs as well as CD8⁺ TIL density was also not associated with survival if the pathological stages were stratified (data not shown).

Figure 2. Distribution of PD-L1 expression and CD8⁺ tumor-infiltrating lymphocyte (TIL) density before and after neoadjuvant chemotherapy (NAC). Each sample of designated esophageal squamous cell carcinoma (ESCC) was evaluated by the scoring system described previously (24). A. PD-L1 expression on tumor cells (TCs), B. PD-L1 expression on immune cells (ICs), C. CD8⁺ TIL density in ESCC.

Univariate and multivariate analyses of OS and RFS. Univariate and multivariate analyses of OS are summarized in Table II. In the former, clinical T, clinical stage, and pathological stage were prognostic factors for OS ($p=0.046$, $p=0.031$, and $p<0.001$, respectively). Pathological stage remained an independent prognostic factor for OS in the

Figure 3. Survival analysis for all 69 esophageal squamous cell carcinoma (ESCC) patients classified according to PD-L1 expression on tumor cells (TCs) or immune cells (ICs), and CD8⁺ TIL density.

multivariate analysis ($p<0.001$). Univariate and multivariate analyses of RFS are summarized in Table III. Again, in the univariate analysis, clinical T, clinical stage, and pathological stage were prognostic factors for RFS ($p=0.021$, $p=0.038$, and <0.001 , respectively). Also, pathological stage remained the only independent prognostic factor for RFS in the multivariate analysis ($p<0.001$). Thus, neither PD-L1

expression on TCs or ICs, nor CD8⁺ TIL density were associated with OS or RFS in the present study.

Discussion

Here, we showed that PD-L1 expression on ICs, as well as CD8⁺ TIL density, increased significantly following NAC-

Figure 4. Survival analyses based on pathological stage of esophageal squamous cell carcinoma (ESCC) according to PD-L1 expression on immune cells (ICs). A. Pathological stage I, II patients with PD-L1 expression on ICs had significantly longer overall survival ($p=0.022$). B. Pathological stage I, II patients with PD-L1 expression on ICs had significantly longer relapse-free survival ($p=0.033$). C and D. In patients with pathological stage III disease, PD-L1 expression on IC was not associated with OS and RFS ($p=0.482$ and $p=0.502$, respectively).

Table II. Analysis of overall survival.

Variables	Patients (n=69)	Univariate Analysis		Multivariate analysis	
		HR (95%CI)	p-Value	HR (95%CI)	p-Value
Age (year)					
$\geq 60 / < 60$	56/13	0.850 (0.409-1.992)	0.688		
Gender					
Male/Female	60/9	2.063 (0.742-8.568)	0.183		
Clinical T					
$\geq 3 / \leq 2$	47/22	2.058 (1.012-4.620)	0.046	1.039 (0.295-3.260)	0.949
Clinical N					
Positive/negative	54/15	1.491 (0.696-3.688)	0.321		
Clinical stage					
$\geq \text{III} / \leq \text{II}$	41/28	2.069 (1.066-4.257)	0.031	0.971 (0.347-3.211)	0.959
Pathological stage					
$\geq \text{III} / \leq \text{II}$	34/35	4.112 (2.075-8.726)	<0.001	4.104 (1.822-9.803)	<0.001
Grade of response to NAC					
2 / $\leq 1b$	10/59	0.508 (0.152-1.277)	0.163		
PD-L1 expression (TC)					
Positive/negative	31/38	0.930 (0.484-1.760)	0.824		
PD-L1 expression (IC)					
Positive/negative	56/13	0.673 (0.323-1.579)	0.340		
CD8					
Positive/negative	46/23	0.764 (0.400-1.517)	0.431		

Table III. Analysis of recurrence-free survival.

Variables	Patients (n=69)	Univariate Analysis		Multivariate analysis	
		HR (95%CI)	p-Value	HR (95%CI)	p-Value
Age (year)					
≥60/<60	56/13	0.844 (0.410-1.965)	0.673		
Gender					
Male/Female	60/9	1.751 (0.701-5.854)	0.252		
Clinical T					
≥3/≤2	47/22	2.212 (1.120-4.766)	0.021	2.061 (0.630-6.059)	0.221
Clinical N					
Positive/negative	54/15	1.197 (0.595-2.668)	0.629		
Clinical stage					
≥III/≤II	41/28	1.955 (1.039-3.845)	0.038	0.511 (0.184-1.643)	0.247
Pathological stage					
≥III/≤II	34/35	4.264 (2.231-8.582)	<0.001	4.396 (2.017-10.009)	<0.001
Grade of response to NAC					
2/≤1b	10/59	0.447 (0.134-1.117)	0.090	0.443 (0.130-1.132)	0.094
PD-L1 expression (TC)					
Positive/negative	31/38	0.894 (0.476-1.653)	0.722		
PD-L1 expression (IC)					
Positive/negative	56/13	0.697 (0.347-1.553)	0.356		
CD8					
Positive/negative	46/23	0.798 (0.425-1.566)	0.499		

FP, but that neither PD-L1 expression on ICs or TCs nor CD8⁺ TIL density was associated with survival in ESCC patients after this neoadjuvant chemotherapy (Figures 2 and 3). This study is novel, in that we strictly selected pathological samples from curative esophagectomy following completion of two cycles of NAC-FP and were able to compare biopsy specimens before NAC with matched surgical specimens from the same patients after NAC.

Presently, reliable data on PD-L1 expression in ESCC tumors from patients treated with NAC are limited (21, 22). In the current study, PD-L1 expression on ICs, as well as CD8⁺ TIL density, was found to be significantly increased in NAC-FP-treated ESCC (Figure 2B, C), whereas although PD-L1 expression was also increased on TCs, this failed to reach significance (Figure 2A). To the best of our knowledge, this is the first study to assess PD-L1 expression solely in NAC-FP-pre-treated ESCC patients. PD-L1 up-regulation on TCs is consistent with a previous report that patients with tumors expressing PD-L1 were more likely to have received NAC (22). The finding of increased CD8⁺ TILs after NAC is also consistent with a previous report showing that the density of CD8⁺ lymphocytes in the stroma was significantly greater in NAC-treated ESCC patients relative to those not receiving NAC (25).

In ESCC patients with no preoperative therapy, most studies have suggested that PD-L1 expression on TCs was associated with poor survival (16, 18, 20). In contrast, some

other reports indicated that PD-L1 expression on TCs was associated with improved survival (19, 26). On the other hand, in terms of PD-L1 expression on ICs, a few reports have shown that it was not associated with survival (18, 19).

Our study in ESCC patients after NAC-FP showed no positive or negative associations of either PD-L1 expression on ICs or TCs, or CD8⁺ TIL density, with survival (Figure 3). This is consistent with a previous report that PD-L1 expression on TCs was not significantly associated with survival following preoperative treatment (chemotherapy or chemoradiotherapy) (27). However, PD-L1 expression on ESCC TCs in patients treated with NAC combining cisplatin and 5-FU with adriamycin or docetaxel was reported to be associated with poor survival (22). In addition, PD-L1 expression on ICs was a poor prognostic factor in a study of a ESCC population where most of cases received neoadjuvant treatment (21). Thus, these disparities may be the result of differential treatments and further studies stratifying patients according to each chemotherapy regimen will be required to elucidate the relationship between PD-L1 expression and prognoses of ESCC patients after neoadjuvant treatments.

The present study did show that PD-L1 expression on IC was associated with an improved prognosis in patients with pathological stage I, II disease, but not stage III (Figure 4). The association between PD-L1 expression on ICs and a better clinical outcome has been observed in some types of

solid cancers (28, 29), but the mechanism by which high PD-L1 expression on IC contributes to a better clinical outcome remains still unclear. However, our findings may indicate that, in advanced stage ESCC, there is an immune suppression different from that in the early one. Further studies are needed to elucidate the mechanism in the future.

The anti-PD-L1 antibody (clone SP142) used in this study is the reagent used for the complementary diagnostic test for atezolizumab. Previous reports showed that high PD-L1 expression on TCs or ICs (TC3 or IC3) was associated with response to atezolizumab in patients with non-small cell lung cancer (30-32). In that study, PD-L1 TC3 or IC3 in surgical specimens was seen in 66.6% of patients (46/69). This suggests that ESCC patients pretreated with NAC-FP would be good candidates for responding to atezolizumab treatment. Additionally, a previous report showed that the presence of CD8⁺ TILs before anti-PD-1 ICI predicts the response in melanoma (33). These findings suggest that pre-treatment of ESCC patients with NAC-FP would increase their responsiveness to anti-PD-1/PD-L1 ICI. Indeed, a phase I clinical trial of atezolizumab in combination with fluorouracil and oxaliplatin for esophageal cancer is ongoing and looks promising. Further studies are needed to develop more efficient neoadjuvant strategies in combination with anti-PD-1/PD-L1 ICI using biomarkers to predict responses.

A limitation of this study is the potential discordance between biopsy specimens and surgical specimens regarding the estimation of PD-L1 expression. Ilie et al. have reported that results from biopsy specimens underestimated PD-L1 expression as assessed in surgical specimens from patients with advanced non-small cell lung cancer (34).

In conclusion, NAC-FP increased PD-L1 expression and CD8⁺ TIL density in ESCC patients and PD-L1 expression on TCs or ICs, and CD8⁺ TIL density, was not significantly associated with patient survival in ESCC patients. Further studies are needed to develop more effective neoadjuvant strategies in combination with anti-PD-1/PD-L1 ICI to improve the clinical outcome of ESCC.

Conflicts of Interest

The Authors declared that they have no conflict of interest in regard to this study.

Authors' Contributions

Conception and design: Yoshihiro Kakeji, Kimihiro Yamashita, Mitsugu Fujita, Eiji Fukuoka; Acquisition of data: Eiji Fukuoka, Tomoko Tanaka, Ryuichiro Sawada, Yutaka Sugita, Akira Arimoto, Gosuke Takiguchi, Takeru Matsuda, Taro Oshikiri, Tetsu Nakamura, Satoshi Suzuki; Analysis and interpretation of data: Eiji Fukuoka; Writing, review, and revision of manuscript: Eiji Fukuoka, Kimihiro Yamashita, Yoshihiro Kakeji.

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