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PD-L1 and PD-L2 expression status in relation to chemotherapy in primary and metastatic esophageal squamous cell carcinoma

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Abstract

Immune checkpoint inhibitors have shown efficacy in various cancers. Although programmed death ligand 1/2 (PD-L1/L2) expressions have been demonstrated as predictive biomarkers of response to immune checkpoint inhibitors and prognostic markers, whether PD-L1/L2 expression is altered in esophageal squamous cell carcinoma during the therapeutic course is unclear. Whether PD-L1/L2 expression in metastatic or recurrent lesions is consistent with that in primary tumors is also unknown. This study included 561 surgically resected esophageal squamous cell carcinomas and PD-L1/L2 expression was evaluated by immunohistochemistry. We investigated the influence of chemotherapeutic drugs (cisplatin and fluorouracil) on PD-L1/L2 expression and PD-L1/L2-related pathways in vitro. We also examined PD-L1/L2 expression in 18 surgically resected lymph node metastases and 10 recurrent lesions compared with primary lesions. The positive rate of PD-L1 was significantly higher in patients with preoperative chemotherapy than in those without preoperative therapy. The positive rate of PD-L2 expression showed no significant difference between patient groups. Cisplatin increased PD-L1 expression in cancer cell lines in vitro, but decreased PD-L2 in some cell lines. The effects of cisplatin on phosphorylated signal transducer and activator of transcription 1/3 (pSTAT1/3) also differed depending on cell lines. Fluorouracil increased PD-L1 and PD-L2 expression. PD-L1/L2 expression in lymph node metastases and recurrent lesions did not always match expression in primary lesions. PD-L1/L2 expression may be altered by preoperative chemotherapy, and PD-L1 /L2 expression in primary lesions does not always match that of metastatic/

Abbreviations: 5-FU, fluorouracil; CDDP, cisplatin; EGFR, epidermal growth factor receptor; HIF-1, hypoxia-inducible factor-1; IHC, immunohistochemistry; JAK, Janus kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PD-1, programmed death 1; PD-L1, programmed death ligand 1; PD-L2, programmed death ligand 2; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; STAT, signal transducer and activator of transcription; YAP/TAZ, Yes-associated protein/transcriptional co-activator with PDZ-binding motif.

recurrent lesions. Thus, one-time evaluation is not sufficient to evaluate PD-L1/L2 expression as a biomarker in esophageal cancer.

KEYWORDS
chemotherapy, esophageal cancer, metastasis, PD-L1, PD-L2

1 | INTRODUCTION

Despite the development of multimodal therapies, including surgery, chemotherapy, and chemoradiotherapy, the prognosis of esophageal cancer patients, including those who undergo complete resection, remains poor. The poor prognosis of esophageal cancer is because of the aggressive nature of this cancer type and the poor survival rate.[1] The limited improvement in treatment outcome from conventional therapies has prompted the search for innovative strategies, especially immunotherapy treatments.[2] Immune checkpoint inhibitors (ICIs), such as PD-1 antibody, have been reported to have therapeutic effects and have been clinically used for the treatment of various types of cancers, including esophageal cancer.[3,4]

The PD-1 pathway serves as a checkpoint to limit the T-cell-mediated immune response. Two ligands, PD-L1 and PD-L2, engage the PD-1 receptor and induce PD-1 signaling and the associated T-cell exhaustion, a reversible inhibition of T-cell activation and proliferation.[5,6] Both PD-L1 and PD-L2 expression have been reported to be predictive biomarkers for the response to PD-1 antibody and are also prognostic factors for several types of cancers.[7-10] Several studies showed that PD-L1 expression is altered in response to various chemotherapeutic drugs and radiation therapies.[11-13] In contrast, only a few reports have examined the effect of these treatments on PD-L2.[14,15] Given the efforts to develop combination treatments of ICIs with conventional chemotherapeutic drugs,[16-18] clarifying the change of PD-L1/L2 expression in response to chemotherapy is clinically important.

We previously reported that cancer cells expressing PD-L1 do not always express PD-L2.[10] Furthermore, PD-L1 and PD-L2 exhibited differences in expression timing and response to chemotherapeutic drugs. Therefore, it is critical to evaluate PD-L2 as well as PD-L1. In addition, preoperative therapy may show different influences on PD-L1/L2 expression. However, no reports have examined whether chemotherapy has different effects on PD-L1 and PD-L2 expression.

We have noticed that PD-L1/L2 expression in the biopsy does not always match expression in the resected samples from esophageal cancer patients. Similar results have been reported for other type of cancers.[19,20] Although PD-L1/L2 expression is generally evaluated in the primary lesion, whether expression in the primary lesion is the same as that in metastatic or recurrent lesions is not clear, especially in esophageal cancer. Therefore, whether PD-L1/L2 expression should be evaluated in multiple samples or time points during therapeutic treatment has not been resolved.

The purpose of this study was to elucidate the effects of chemotherapy on PD-L1 and PD-L2 expression in esophageal cancer. In addition, we investigated whether PD-L1 and PD-L2 expressions in the primary lesion were consistent with their expression in lymph node metastasis or recurrent lesions.

2 | MATERIAL AND METHODS

2.1 | Patients

A total of 724 consecutive patients with esophageal cancer who were undergoing curative resection at Kumamoto University Hospital between April 2005 and January 2020 were enrolled in this study. Among the 724 patients, 50 patients who did not have assessable cancer cells, 31 patients without clinical data, and 82 patients who have not squamous cell carcinoma were also excluded. A total of 561 esophageal cancer patients was finally included in this study. Among the 561 patients, 241 patients received preoperative chemotherapy or chemoradiotherapy. Most of these patients were treated with a CDDP and 5-FU (n = 121, 50.2%) or CDDP, 5-FU, and docetaxel regimen (n = 112, 46.5%). For comparisons of the primary lesion and lymph node metastasis, we examined 18 patients who had one lymph node metastasis and who did not receive preoperative therapy. For comparisons of the primary and recurrent lesions, we examined 10 patients who underwent surgical resection for recurrence. Tumor stage was classified according to the 8th edition of the American Joint Committee on Cancer TNM classification system.[21] Written informed consent was obtained from all patients, and the study procedures were approved by the Institutional Review Board of Kumamoto University (Permission number: 1365). Our study was performed in accordance with the Declaration of Helsinki.

2.2 | PD-L1 and PD-L2 immunohistochemical staining

PD-L1/L2 staining was conducted as previously described.[9,10] We evaluated the expression of PD-L1/L2 in the most representative section and whole tumor area in all samples, including biopsy and resected samples (Figure S1). We scored PD-L1/L2 expression in terms of the percentage of tumor cells expressing PD-L1/L2 (0%–10% = score 0, 10%–30% = score 1, 30%–50% = score 2, or over

50% = score 3) and the intensity of PD-L1/L2 expression (no expression = score 0, weak expression = score 1, moderate expression = score 2, or strong expression = score 3). The IHC score was calculated as the product of the expression score and proportion score. The criteria for PD-L1/L2 positivity were previously described.[9,10] PD-L1- and PD-L2-stained tissue sections were reviewed by two pathologists (YB and YK) who were unaware of other data.

2.3 | Multiplex immunofluorescence

We used anti-PD-L1 antibody (clone E1L3N; Cell Signaling Technology), PD-L2 antibody (clone D7U8C; Cell Signaling Technology) and the Opal 4-Color fluorescent IHC kit (PerkinElmer) for multiplex immunofluorescence staining. PD-L1 staining was optimized using Opal 470 Fluorophore (red) and PD-L2 staining was optimized using Opal 520 Fluorophore (green) according to the instructions of the Opal IHC kit. VECTASHIELD mounting medium with DAPI (Vector Laboratories) was used to stain nuclei.

2.4 | Esophageal cancer cell lines

Human esophageal squamous cell carcinoma cell lines TE-1, TE-9, and TE-10 were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan. KYSE 30 cells were purchased from American Type Cell Collection. TE-1, TE-9, and TE-10 cells were grown in RPMI 1640 medium (Sigma-Aldrich) and KYSE 30 cells were grown in Dulbecco's Modified Eagle Medium (D-MEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) at 37°C in a humidified chamber supplemented with 5% CO_2. Twice weekly, the cells were harvested and re-plated.

2.5 | Cisplatin and 5-FU treatments

Cells were seeded into 30- or 60-mm plates at specific concentrations to yield 70%–80% confluence in the untreated cells at the time of harvest. At 24 h post-plating, cells were treated with various concentrations of CDDP (NICHI-IKO) or 5-FU (TOWA). The concentrations of CDDP or 5-FU were determined based on clinical doses[22]; since the effects of CDDP and 5-FU differed depending on the cancer cell line, we slightly adjusted each dose depending on the cancer cell line. In CDDP experiments, qRT-PCR was performed 24 h after treatment and flow cytometry analysis was performed 72 h after treatment (after 24 h of CDDP treatment followed by 48 h culture in medium). In 5-FU treatment experiments, qRT-PCR was performed 48 h after treatment and flow cytometry analysis was performed 72 h after treatment. Western blot analyses were performed using cell lysates obtained at 24 h after CDDP or 5-FU treatment.

2.6 | Quantitative real-time reverse transcription-polymerase chain reaction

RNA was isolated from cultured cells using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The mRNA expression levels of PD-L1 and PD-L2 were determined by qRT-PCR using TaqMan probes (Roche) and were normalized to those of β-actin mRNA. We used the Universal Probe Library (Genenet) to design the qRT-PCR primers, following the manufacturer's recommendations. Real-time PCR was performed with the following primer sequences and probes:

PD-L1 (PD-L1_#25), 5′-GGCATCCAAGATACAAACTCAA-3′, 5′-CAGAAGTTCC AATGCTGGATTA-3′; PD-L2 (PD-L2_#36), 5′-AAAGAGGGAAGTGAACAGTGC T-3′, 5′-GCTTCTTTAGATGT CATATCAGGTCA-3′; and β-actin (ACTB_#11), 5′-ATTGGCAATGA GCGGTTC-3′, 5′-CGTGGATGCCACAGGACT-3′. All qRT-PCR reactions were performed in the LightCycler 480 System II (Roche) according to the manufacturer's protocol. The recommended cycling conditions for qRT-PCR were denaturation at 95°C for 10 min followed by 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s. All data were obtained from triplicate experiments and are presented as the mean ± standard error.

2.7 | Flow cytometry analysis

Cells were adjusted to 3.0×10^5 cells/mL in Phosphate-Buffered Saline (PBS) with 2% FBS. Cells were then incubated with the phycoerythrin (PE) anti-human CD274 (PD-L1) antibody (clone 29E.2A3) and the allophycocyanin (APC) anti-human CD273 (PD-L2) antibody (clone MIH18) (both from BioLegend) for 30 min at 4°C. Flow cytometry was performed on a BD FACSVerse instrument (BD Biosciences) and data were analyzed using FlowJo v10 software (BD Biosciences).

2.8 | Western blotting

Cells were washed with ice-cold phosphate-buttered saline and lysed in Radioimmunoprecipitation assay buffer (RIPA) buffer (Thermo) containing 1% protease and phosphatase inhibitor (Thermo). Lysates were centrifuged, supernatants were collected, and protein concentrations were determined using the Bio-Rad DC protein assay kit II (Bio-Rad Laboratories). Ten micrograms of protein were separated on 7.5%–10% acrylamide gels and blotted onto Polyvinylidene fluoride (PVDF) membranes using the Trans-Blot Turbo System (Bio-Rad Laboratories) according to the manufacturer's protocol. The membranes were blocked with 5% BSA in Phosphate-Buffered Saline/Tween (PBS-T) for 1 h at room temperature and then incubated overnight at 4°C with anti-EGFR (1:1000; D38B1), anti-pEGFR (1:1000; Y1068), anti-MEK1/2 (1:1000; L38C12), anti-pMEK1/2 (1:1000; 41G9), anti-STAT1 (1:1000; D1K9Y), anti-pSTAT1 (1:1000; Y701), anti-STAT3

(1:2000; 79D7), anti-pSTAT3 (1:2000; D3A7), anti-STAT6 (1:1000), anti-pSTAT6 (1:1000; Tyr641), anti-HIF-1α (1:1000; D1S7W), anti-YAP/TAZ (1:1000; D24E4), anti-NF-kB p65 (1:1000; C22B4), anti-Phospho-NF-kB p65 (1:1000; Ser536), and anti-β-actin (1:1000) antibodies (all from Cell Signaling Technology) in PBS-T. The membranes were then incubated with secondary anti-rabbit (1:5000) or anti-mouse (1:5000) HRP-linked IgG antibodies (Cell Signaling Technology) in PBS-T for 1 h at room temperature. Bands were detected using ECL Prime (GE Healthcare UK) and the ChemiDoc Touch Imaging System (Bio-Rad Laboratories).

2.9 | Statistical analysis

All statistical calculations were performed with JMP version 13 software (SAS Institute). All *P* values were two-sided. Categorical variables were presented as numbers and percentages, and groups were compared using the χ^2 test or Fisher's exact test. Continuous variables were expressed as means and standard deviations, and means were compared using the *t*-test. The survival time distribution was evaluated by the Kaplan–Meier method and the log-rank test was used for comparisons. Variables for which the *P* value in the univariate analysis was <.05 were subjected to multivariate analysis by a stepwise backward elimination procedure using a threshold *P* value of <.05. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | PD-L1/L2 expressions and clinicopathological features in esophageal cancer

A total of 561 esophageal squamous cell carcinoma patients were included in this study. Among the 561 cases, 104 (18.5%) cases were PD-L1 positive and 97 (17.3%) cases were PD-L2 positive. There was no significant correlation between PD-L1 and PD-L2 expression ($P = .15$; Table S1). The positive rate of PD-L1 was higher in patients who received preoperative chemotherapy (24.2%; $P = .006$) or chemoradiotherapy (25.0%; $P = .016$) than in patients who did not receive preoperative therapy (14.1%; Figure 1A). The positive rate of PD-L2 was not significantly different among the three groups (17.8%, 17.8%, and 14.3% in patients with no preoperative therapy, with chemotherapy and with chemoradiotherapy, respectively).

We also evaluated PD-L1/L2 expression in pretreatment biopsy samples and surgically resected specimens in patients who underwent preoperative chemotherapy using immunofluorescence. Representative images are shown in Figure 1B and show increased PD-L1 expression in resected specimens compared with biopsy samples. These results suggest that PD-L1 expression may be increased by preoperative chemotherapy, but PD-L2 is not increased.

We next examined the relation between PD-L1 and PD-L2 expression with clinicopathological characteristics of patients with esophageal cancer (Table S2). Table S2 summarizes the clinicopathological features of all cases. PD-L1 was significantly associated with age ($P = .037$), tobacco use ($P = .015$), pathological stage ($P < .001$), and receiving preoperative therapy ($P = .0017$), while PD-L2 did not show a significant relationship with any variables.

3.2 | The influence of CDDP treatment on PD-L1 and PD-L2 expression in esophageal cancer cell lines

Among the 561 patients in this study, 241 patients received preoperative chemotherapy or chemoradiotherapy, and 96.7% of the patients were treated with the regimen including CDDP and 5-FU. CDDP and 5-FU are the most widely used chemotherapy treatments for esophageal cancer.[22,23] We thus next assessed the influence of CDDP treatment on PD-L1 and PD-L2 mRNA and protein expressions in four esophageal cancer cell lines using qRT-PCR and flow cytometry (Figure 2). PD-L1 mRNA and protein expression levels were upregulated in all four cell lines in response to CDDP treatment. In contrast, PD-L2 mRNA and protein expression were downregulated in TE-1 and TE-10 cells treated with CDDP and upregulated in TE-9 and KYSE30 cells treated with CDDP. These results suggest that while CDDP increased PD-L1 expression in all four esophageal cancer cell lines, the effect of CDDP on PD-L2 expression may differ depending on the cell line.

We performed further in vitro experiments to elucidate the molecular mechanism underlying the differences in PD-L2 response to CDDP. The EGFR and JAK/STAT pathways have been reported to play a crucial role in the regulation of PD-L1/L2 expression, and several studies have shown that these pathways are influenced by CDDP or 5-FU treatment.[14,15,24,25] Thus, we examined EGFR and JAK/STAT pathway proteins in cell lines with decreased PD-L2 in response to CDDP (TE-1 and TE-10) and those with increased PD-L2 in response to CDDP (TE-9 and KYSE30; Figure 3A). We found that pSTAT1/3 was decreased in TE-1 and TE-10 cells treated with CDDP, while pSTAT1/3 was increased in TE-9 and KYSE30 cells incubated with CDDP. In addition, pSTAT1/3 decreased in TE-10 cells and increased in KYSE30 cells in a time- and dose-dependent manner (Figure 3B). These results suggest that STAT1/3 activation may be involved in the alteration of PD-L2 expression levels triggered by CDDP treatment.

3.3 | The influence of 5-FU treatment on PD-L1 and PD-L2 expression in esophageal cancer cell lines

We also assessed the influence of 5-FU treatment on PD-L1 and PD-L2 expression in the four esophageal cancer cell lines (TE-1, TE-9, TE-10, and KYSE30) using qRT-PCR and flow cytometry (Figure 4). We found that PD-L1 and PD-L2 mRNA levels were upregulated in a dose-dependent manner in all four cell lines. We also examined PD-L1 and PD-L2 expression levels after 72 h of treatment with 5-FU by flow cytometry and found that both PD-L1 and PD-L2 expressions were upregulated compared with control cells. We also investigated the effect

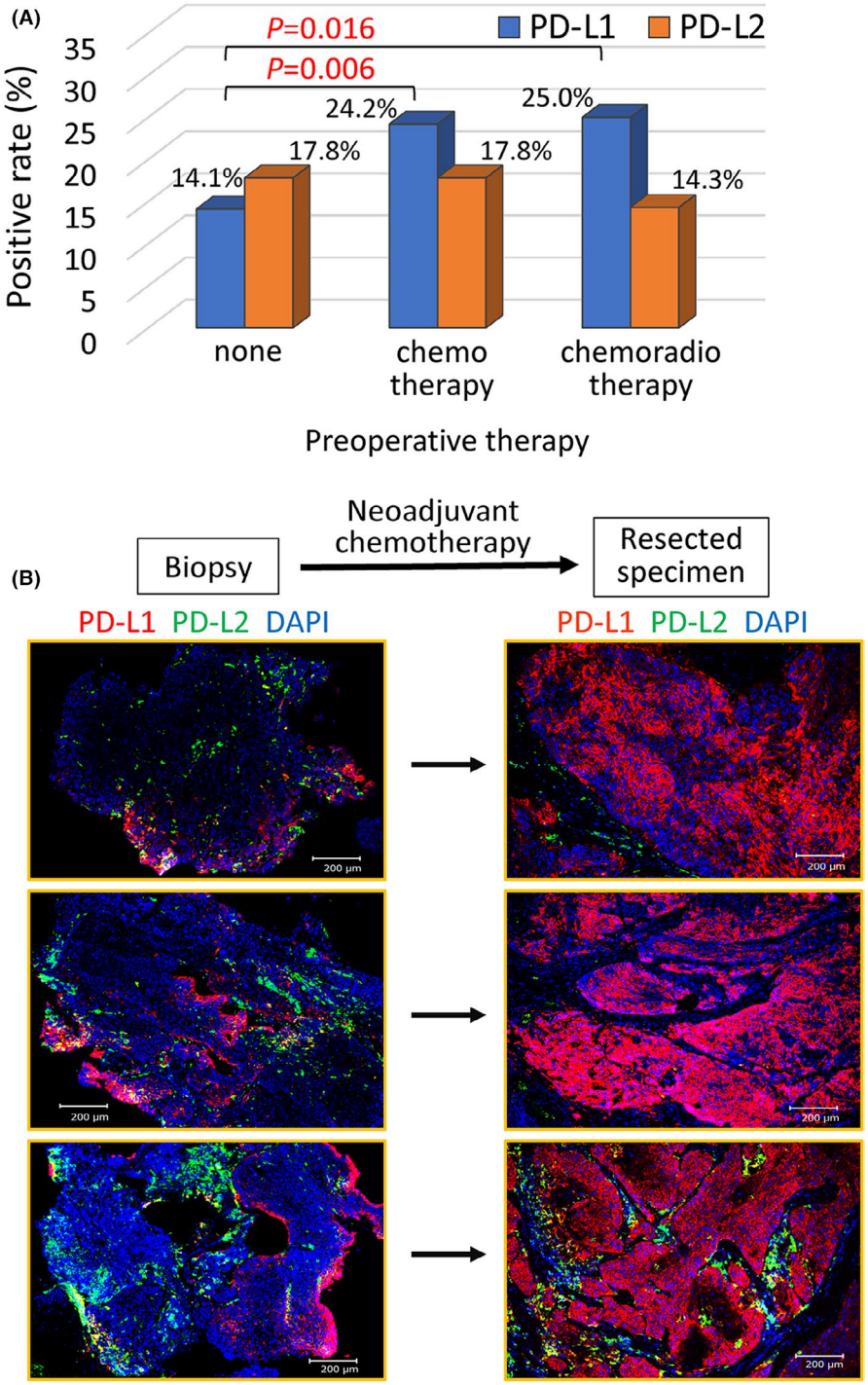

FIGURE 1 Programmed death ligand 1/2 (PD-L1/L2) positive rate is associated with preoperative therapy in esophageal cancer. A, The PD-L1-positive rates in preoperative chemotherapy (24.2%) or preoperative chemoradiotherapy patients (25.0%) were significantly higher than in patients without preoperative therapy (14.1%) ($P = .006$ and .016, respectively). The PD-L2-positive rate showed no significant difference between patients with preoperative chemotherapy (17.8%) or chemoradiotherapy (14.3%) and patients without preoperative therapy (17.8%) ($P = 1.0$ and .44, respectively). PD-L1 and PD-L2 expression status was evaluated by immunohistochemical staining. B, Immunofluorescence of biopsy and resected samples in representative cases with preoperative chemotherapy. Multiplex immunohistochemical staining of PD-L1 and PD-L2 was performed. FITC was used to visualize PD-L2 (green), Cy5 was used to visualize PD-L1 (red), and DAPI was used to visualize nuclei (blue). Scale bar, 200 μm

of 5-FU treatment on the EGFR and JAK/STAT pathway (Figure S2), and HIF-1, YAP/TAZ, and NF-kB pathways (Figure S3) using western blot analysis but did not observe any noticeable effects.

3.4 | Expression of PD-L1/L2 in the primary lesion and lymph node metastasis in esophageal cancer

Previous studies have shown that PD-L1 and PD-L2 have heterogeneity in cancer lesions.[26,27] We have also confirmed that not all cancer cells express PD-L1 or PD-L2, even in cases that strongly express PD-L1 or PD-L2. Whether PD-L1/L2 expression status is the same in the primary lesion and lymph node metastasis in esophageal cancer has not been clarified. Therefore, we compared PD-L1/L2 expression status in primary lesions and lymph node metastases in 18 patients with lymph node-positive cases who did not receive preoperative therapy (Figure 5). We evaluated the expression status of PD-L1 and PD-L2 (positive or negative) in the primary lesion as well as the lymph node metastasis according to the criteria of this study and found differences in PD-L1 expression in five out of 18 patients (27.8%) and differences in PD-L2 expression in six out of 18 patients

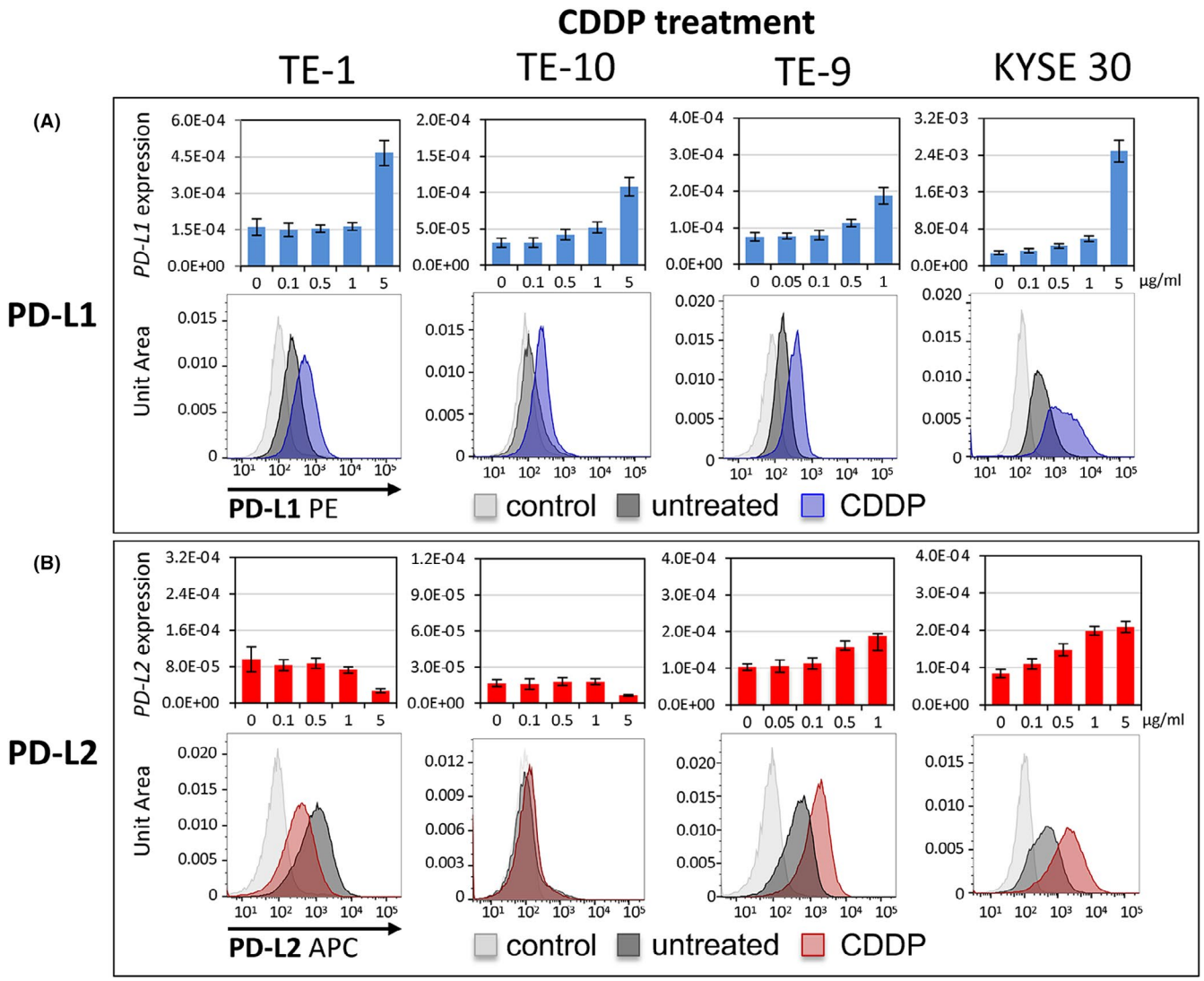

FIGURE 2 Cisplatin (CDDP) treatment influenced programmed death ligand 1 (PD-L1) and programmed death ligand 1 (PD-L2) expression in esophageal cancer cell lines. Top row: Cells were treated with CDDP and quantitative real-time reverse transcription-polymerase chain reaction was performed 24 h after treatment. Bottom row: Cells were treated with CDDP for 24 h, cultured for 48 h, and then examined by flow cytometry analysis (light gray, isotype control; gray, untreated cell lines; blue, PD-L1 expression of CDDP-treated cell lines (A); red, PD-L2 expression of CDDP-treated cell lines (B)). TE-1 and TE-10 cells were treated with 5 μg/mL CDDP, and TE-9 and KYSE30 cells were treated with 2 μg/mL CDDP in flow cytometry analysis

(33.3%). Figure 5A shows comparisons of PD-L1 expression according to positive rate (0-100), expression score (0-3), and IHC score (0-9). The PD-L1 positive rate in lymph node metastasis was significantly correlated with the PD-L1 positive rate in the primary lesion (coefficient of correlation = 0.68, $P = .0018$; Figure 5A, upper right); however, some cases showed weak PD-L1 expression in the primary lesion but high expression in the lymph node metastasis, while other cases showed high expression of PD-L1 in the primary lesion and weak expression in lymph node metastasis. In contrast, the PD-L2 positive rate did not significantly correlate between the primary lesion and lymph node metastasis (coefficient of correlation = 0.19, $P = .46$). Figure 5B shows the comparisons of the PD-L2 expression according to positive rate, expression score, and IHC score; PD-L2 expression in the primary lesion and lymph node metastasis did not appear to be same. These results indicate that PD-L1/L2 expression is not always similar between the primary lesion and the lymph node metastatic site.

3.5 | Expression of PD-L1/L2 expression in the primary lesion and recurrence site in esophageal cancer

Currently, PD-1 antibody is a therapeutic option for recurrent esophageal cancer, and previous studies indicated that PD-L1/L2 expressions may be predictive markers of the therapeutic effect of PD-1 antibody.[4] However, no reports have examined and compared PD-L1/L2 expression between primary lesions

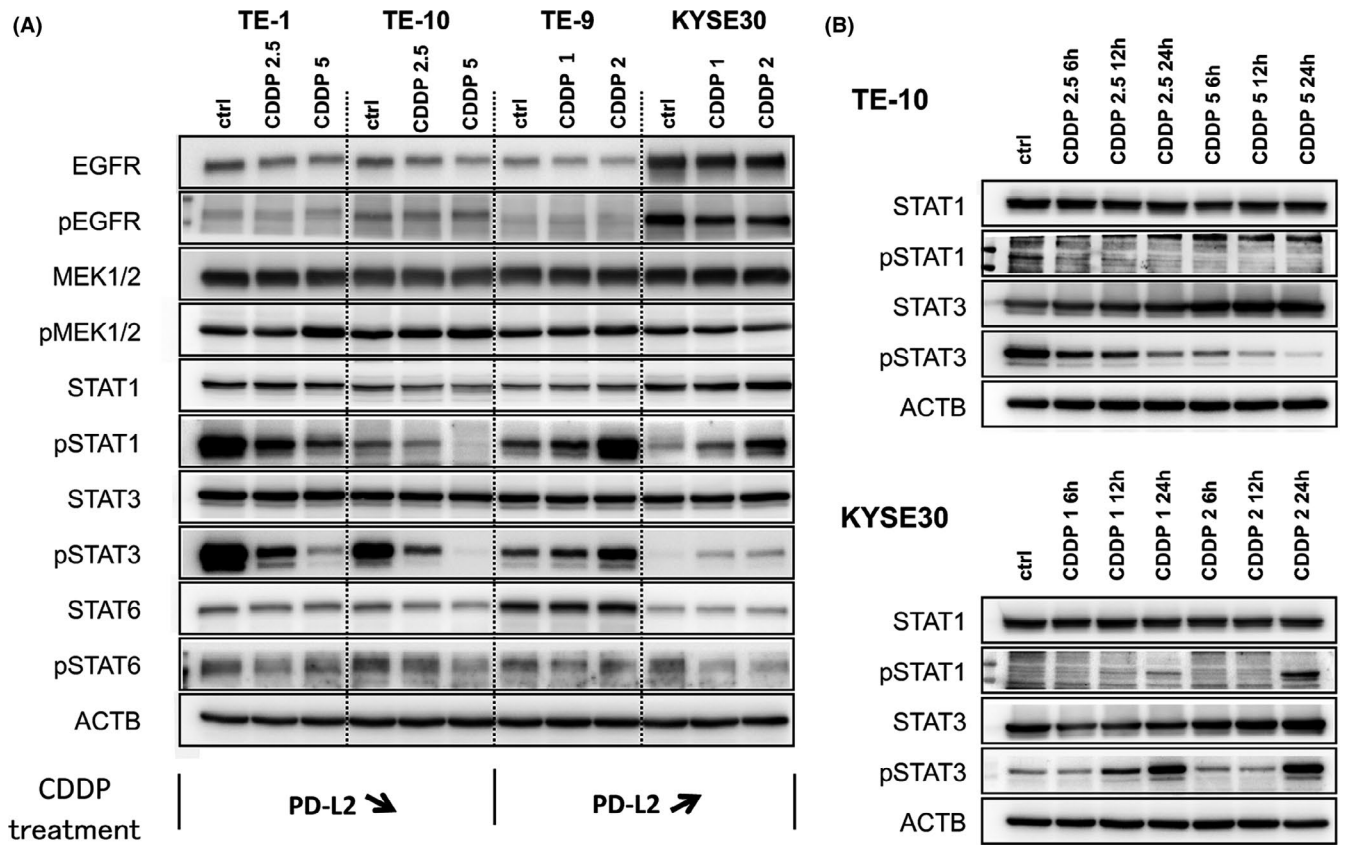

FIGURE 3 The influence of cisplatin (CDDP) treatment on epidermal growth factor receptor and Janus kinase/signal transducer and activator of transcription pathways in esophageal cancer cell lines. A, Western blot analysis of the indicated proteins in esophageal cancer cell lines treated with CDDP. TE-1 and TE-10 cells were treated with 2.5 and 5.0 μg/mL CDDP, and TE-9 and TE-11 cells were treated with 1 and 2 μg/mL CDDP for 24 h. B, Western blot of signal transducer and activator of transcription 1 (STAT1), phosphorylated signal transducer and activator of transcription 1 (pSTAT1), signal transducer and activator of transcription 3 (STAT3), and phosphorylated signal transducer and activator of transcription 3 (pSTAT3) expression in esophageal cancer cell lines treated with CDDP. TE-10 cells were treated with 2.5 or 5 μg/mL CDDP for 6, 12, and 24 h. KYSE30 cells were treated with 1 or 2 μg/mL CDDP for 6, 12, and 24 h

and recurrent lesions, and whether PD-L1/L2 expression status should be evaluated in the primary lesion or in the recurrent sites is not clear. We examined 10 recurrent esophageal cancer cases and compared PD-L1/L2 expression status in the primary lesion and recurrent lesion (Figure 6). In three out of 10 cases, both PD-L1 and PD-L2 expression status did not match between the primary and recurrent lesions. There was no significant correlation between the primary lesion and the recurrent lesion for both PD-L1 and PD-L2 positive rates ($P = .099$ and .93, respectively). Figure 6 shows comparisons of the PD-L1/L2 expression according to positive rate (0-100), expression score (0-3), and IHC score (0-9). PD-L1/L2 expression in the primary lesion and lymph node metastasis did not appear to be same. In some cases, PD-L1 and/or PD-L2 were expressed at weak levels in the primary lesion but expressed at high levels in recurrent lesions, while other cases showed high expression of PD-L1 and/or PD-L2 in the primary lesion but weak expression in recurrent lesions. Similar to our results with lymph node metastasis, these findings indicate that PD-L1/L2 expression is not always similar between the primary lesion and the recurrent lesion.

4 | DISCUSSION

In the current study, we evaluated PD-L1 and PD-L2 expression by immunohistochemistry in 561 surgically resected esophageal squamous cell carcinoma samples, as well as 18 lymph node metastases and 10 recurrent lesions. We also examined the effects of CDDP on PD-L1/L2 expression and the EGFR and JAK/STAT pathway. Our findings demonstrate that PD-L1/L2 expression is influenced by CDDP and 5-FU, and we show that the effect of CDDP treatment on PD-L2 expression is dependent on the esophageal cancer cell line through pSTAT1/3 expression. Additionally, we revealed that PD-L1 and PD-L2 expression might be different among primary lesions, lymph node metastases, and recurrent sites. Given that when and how PD-L1 and PD-L2 expression should be evaluated for esophageal cancer has not been established, our observations may have clinical implications.

We used anti-PD-L1 (clone E1L3N) and PD-L2 (clone D7U8C) antibodies to evaluate PD-L1/L2 expression, both of which were obtained from Cell Signaling Technology. There are many commercially available PD-L1 antibodies for immunohistochemical staining,

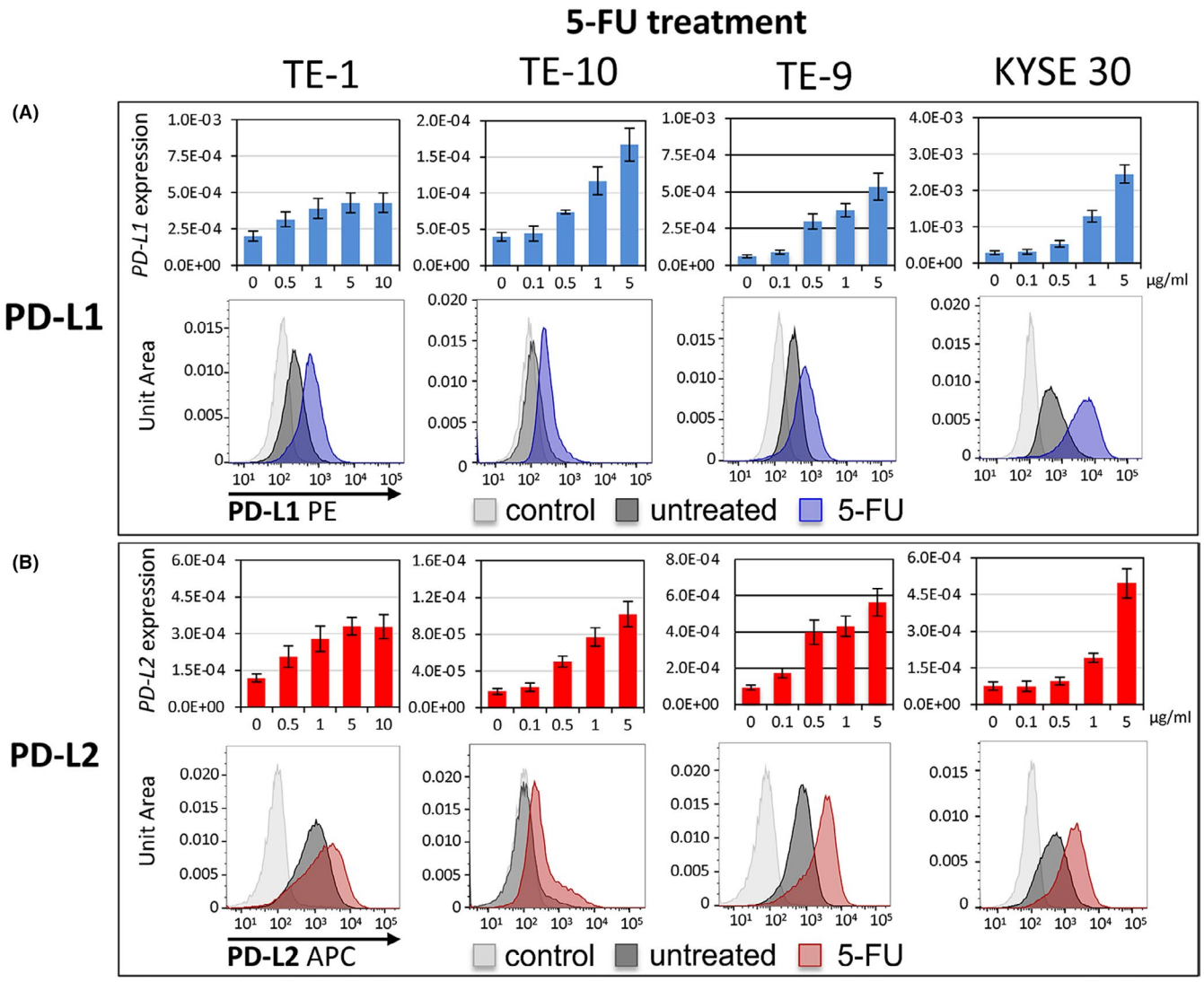

FIGURE 4 Fluorouracil (5-FU) treatment influenced programmed death ligand 1 (PD-L1) and programmed death ligand 1 (PD-L2) expression in esophageal cancer cell lines. Top row: Cells were treated with 5-FU and quantitative real-time reverse transcription-polymerase chain reaction was performed 48 h after treatment. Bottom row: Cells were treated with 5-FU for 72 h and then examined by flow cytometry analysis (light gray, isotype control; gray, untreated cell lines; blue, PD-L1 expression of 5-FU-treated cell lines (A); red, PD-L2 expression of 5-FU-treated cell lines (B)). TE-1 cells were treated with 10 μg/mL 5-FU, and TE-10, TE-9 and KYSE30 cells were treated with 5 μg/mL 5-FU in flow cytometry analysis

and the positive rates of the antibodies were reported to be slightly different. Clone E1L3N is often used for evaluation of PD-L1 in biological studies and the positive rate is similar to that of other antibodies.[28-30] In contrast, there are not many commercially available PD-L2 antibodies. Some antibodies have been used in previous studies about PD-L2, but whether the positive rate was different depending on the type of antibody has not been investigated. We used two antibodies to evaluate PD-L2 expression with IHC, and we obtained better results using clone D7U8C for evaluating esophageal cancer tissue.

Immune checkpoint inhibitors have shown significant effects on advanced or recurrent esophageal cancer patients[3,4] and are being used clinically. PD-L1 expression has been evaluated as a marker for predicting the therapeutic effect of PD-1 antibody, and PD-L2 expression may also be useful as a marker for predicting the therapeutic effects.[8] PD-L1/L2 expressions are often evaluated in the primary lesion or biopsy samples, but here we demonstrated that biopsies obtained before chemotherapy treatment, lymph node metastases, or recurrent lesions do not show similar expressions as the primary lesions. We also found that the PD-L1/L2 expression status in biopsy samples was different from expression in surgically resected samples (data are not shown). Taken together, these results indicate that PD-L1/L2 expression can change during the course of tumor treatment and tumor progression, and can vary depending on the primary lesion, metastasis or recurrence. Therefore, it is necessary to repeatedly evaluate PD-L1/L2 expression for predicting the efficacy of ICI treatment and to develop a new method for assessing PD-L1/L2 expression throughout the tumor. Considering

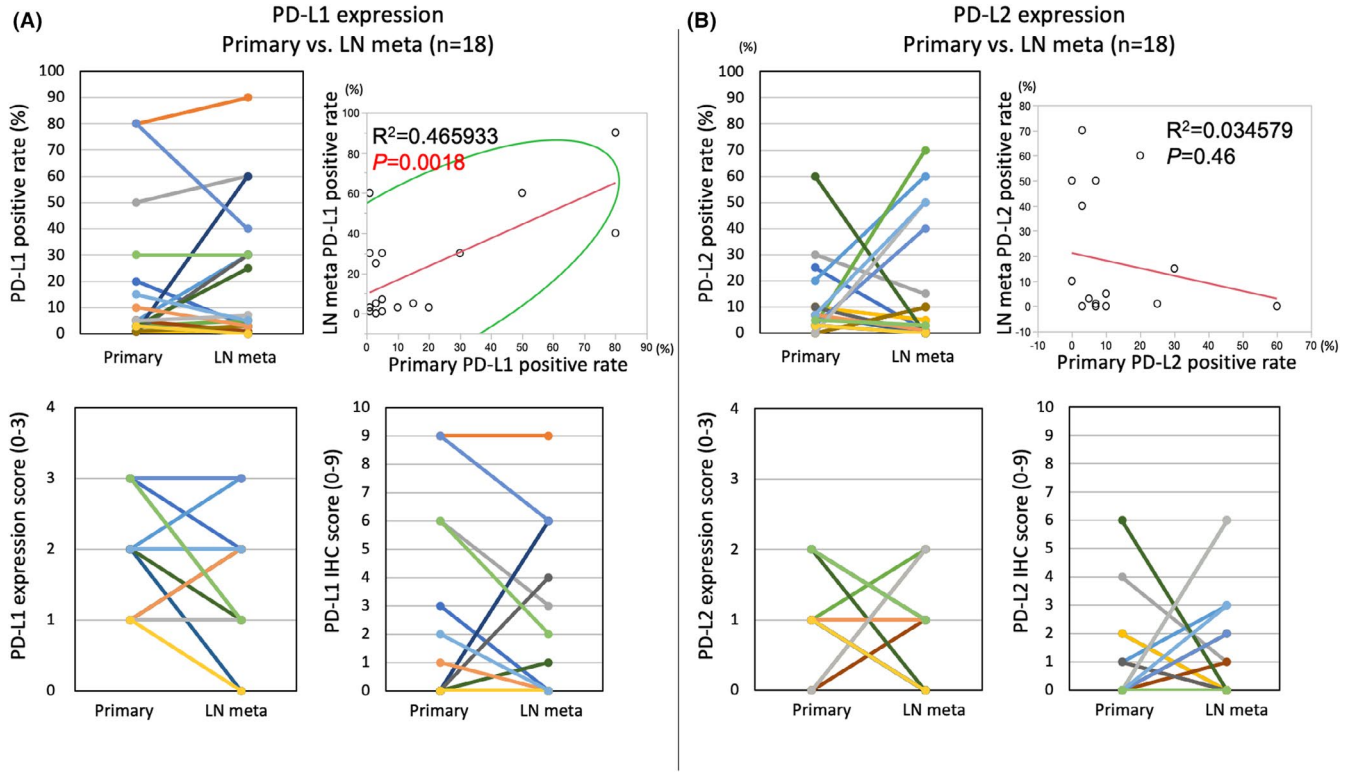

FIGURE 5 Comparison of programmed death ligand 1 (PD-L1) and programmed death ligand 1 (PD-L2) expression between primary lesion and lymph node metastasis. A, PD-L1 expression status was measured with PD-L1 positive rate (0%-100%) (upper left), PD-L1 intensity score (0-3) (lower left), and PD-L1 IHC score (0-9) (lower right). Right upper panel: There was a significant correlation of PD-L1 positive rate between the primary lesion and lymph node metastasis ($P = .0018$). B, PD-L2 expression status was measured with PD-L2 positive rate (0%-100%) (upper left), PD-L2 intensity score (0-3) (lower left), and PD-L2 IHC score (0-9) (lower right). There was no significant correlation of PD-L2 positive rate between primary lesion and lymph node metastasis ($P = .46$)

the mechanism of PD-1 inhibitors, PD-L1 positive patients are likely to respond to PD-1 inhibitors, but some clinical trials showed no significant difference in the response rate of PD-L1-positive cases and PD-L1-negative cases to PD-1 inhibitors.[3] One of the reasons for this observation may be the temporal and spatial heterogeneity of PD-L1/L2 expression during tumor progression.

One observation from our results is the relationship between PD-L1/L2 expression and chemotherapy. The precise strategy for administering chemotherapy and ICIs in combination or sequentially has been currently under debate.[18] Therefore, whether chemotherapy influences the expression status of PD-L1 and PD-L2 may be important. Previous reports suggested that the effect of chemotherapy on enhancing immunotherapy is caused not only immunogenic cell death or change of the tumor microenvironment but also upregulating PD-L1 expression levels.[11,18] Accumulating evidence suggests that preoperative therapy including 5-FU, CDDP, and radiation therapy might upregulate PD-L1 expression levels in human cancers.[12] However, the relationship between PD-L2 expression and chemotherapeutic agents has been less well studied. Only two reports have examined the effect of CDDP on PD-L2 expression. Notably, our results using esophageal cancer cell lines showed that the effect of CDDP treatment on PD-L2 differed depending on the cancer cell line. In some clinical cases, chemotherapy may increase PD-L2 expression, while in other cases, PD-L2 expression may be decreased. Based on these results, we considered that no significant relationship was found between the history of chemotherapy and PD-L2 expression. In this study, we could not clarify the mechanism of increased PD-L1 expression involving EGFR and JAK/STAT pathways by CDDP treatment. In addition to the EGFR and JAK/STAT pathways, HIF-1, YAP/TAZ, and NF-κB pathways have been also reported to be involved in PD-L1 expression changes.[13] However, we did not observe any significant changes in these pathways in response to CDDP (Figure S3). Regarding the change of PD-L2 expression by CDDP treatment, one study indicated that PD-L2 decreased via STAT6[14] while another report showed that PD-L2 increased via STAT1/3.[15] Our study showed that PD-L2 may decrease in some cases and may increase in other cases via STAT1/3. While examination of the mechanism should be pursued in future studies, we would like to emphasize that the findings in the previous reports may not apply to all cancers. PD-L1 is mainly regulated by the IFN-γ receptor pathway and PD-L2 is mainly regulated by the IFN-γ and IL-4 receptor pathways.[31,32] Since the JAK/STAT pathway is mainly involved downstream of the IFN-γ and IL-4 receptor pathways, we think it makes sense to investigate the effects of chemotherapeutic agents on the JAK/STAT pathway. Further studies are required to identify which patients will show upregulated PD-L2 expression and

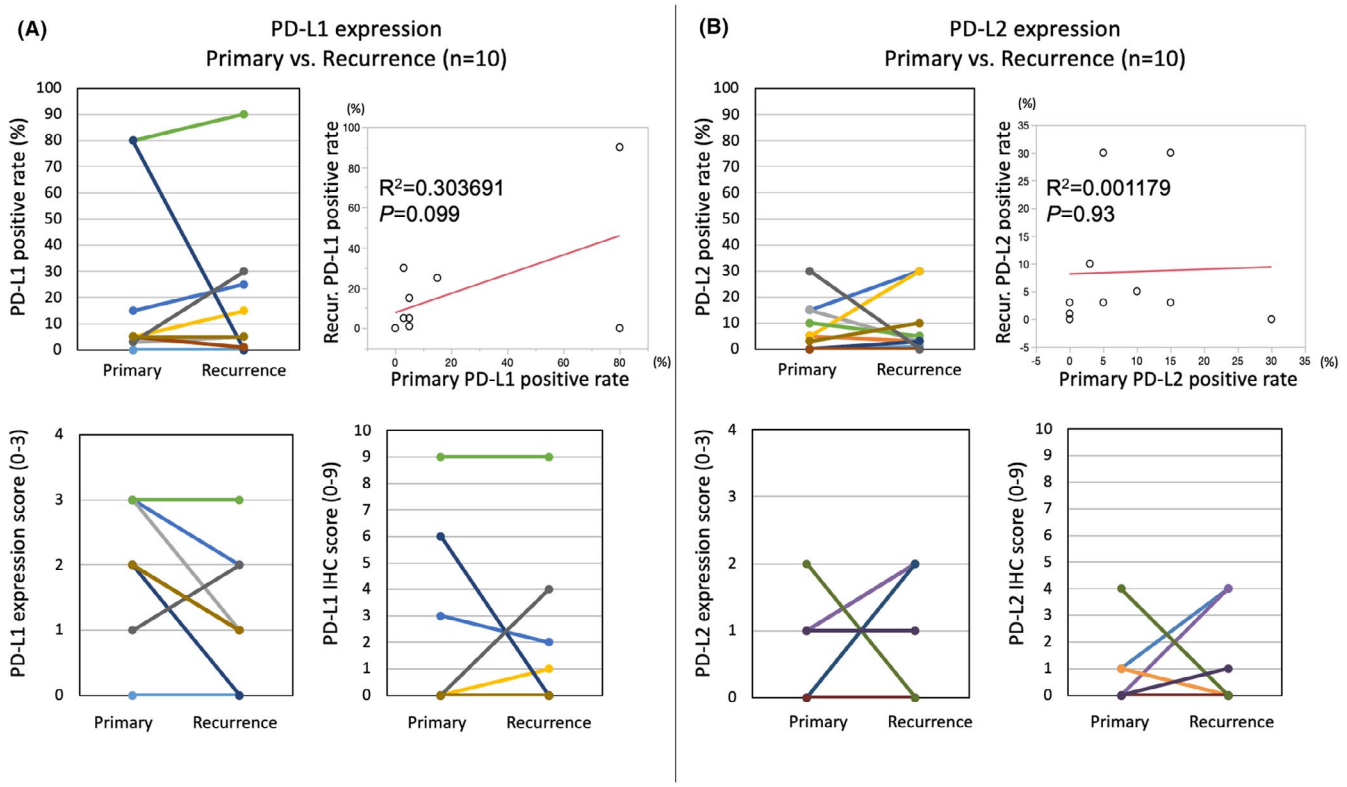

FIGURE 6 Comparison of programmed death ligand 1 (PD-L1) and programmed death ligand 1 (PD-L2) expression status between primary lesion and recurrent metastasis. A, PD-L1 expression status was measured with PD-L1 positive rate (0%-100%) (upper left), PD-L1 intensity score (0-3) (lower left), and PD-L1 IHC score (0-9) (lower right). There was no significant correlation of PD-L1 positive rate between primary lesion and recurrent metastasis ($P = .099$). B, PD-L2 expression status was measured with PD-L2 positive rate (0%-100%) (upper left), PD-L2 intensity score (0-3) (lower left), and PD-L2 immunohistochemistry score (0-9) (lower right). There was no significant correlation of PD-L2 positive rate between primary lesion and recurrent metastasis ($P = .93$)

which patients have downregulated expression with chemotherapeutic agents. Several papers have examined the relationship between CDDP and STAT1/3 expression levels. However, the effects of CDDP treatment on STAT1/3 expression remain unclear. Some papers showed STAT1/3 were activated and other papers showed STAT1/3 were inactivated by CDDP. We speculate that one possibility for how CDDP activates or inactivates STAT1/3 among cell types may be that molecules that promote or suppress the JAK/STAT pathway may undergo epigenetic changes. However, we cannot focus on specific molecule that key factor of these phenomenon at this time. We believe that it is very important to clarify the detailed mechanism of the effect of CDDP on STAT1/3 in future research.

We also examined differences in PD-L1/L2 expression in the primary lesion and lymph node metastasis as well as the recurrent lesion. Previous studies reported that PD-L1 and PD-L2 have spatial heterogeneity among tumors; even in tumors in which PD-L1 or PD-L2 is strongly expressed, there are no cases in which all cancer cells express PD-L1 or PD-L2 because of the spatial heterogeneity. This spatial heterogeneity may be present not only within tumors, but also in metastatic or recurrent lesions compared with primary lesions. Several reports examined whether PD-L1 expression in primary lesions matched metastasis[28-31] or recurrent lesions.[32,33] However, few studies have examined PD-L2 expression in metastatic or recurrent lesions. In this study, discordant PD-L1 expression status between matched primary and lymph node metastases was present in five out of 18 cases (27.8%) and discordant PD-L1 expression status between matched primary and metastatic lesions was observed in three out of 10 cases (30%); discordant PD-L2 expression status was present in six out of 18 cases (33.3%) and three out of 10 cases (30%), respectively. These results were consistent with previous reports in other types of cancers.[29,31-33] Together these results indicate that PD-L1/L2 expression in the primary lesion and lymph node metastasis or recurrent lesions in esophageal cancer are not always the same. Furthermore, our finding of the different PD-L1/L2 status between primary and metastasis/recurrent in esophageal cancer needs to be confirmed with independent cohorts in future studies.

Based on results showing heterogeneity of PD-L1/L2 expression within tumors, during tumor progression, and in response to chemotherapy, repeated evaluation for PD-L1/L2 expression is needed. Since it is difficult to evaluate PD-L1/L2 expression in tissue samples, identifying a new method for evaluating PD-L1/L2 expression, such as in liquid biopsy, is critical.[34-36] PD-L1 expression in circulating tumor cells and exosomes in liquid biopsy are reported to be associated with prognosis and the therapeutic effect of ICIs. Further investigation of whether these methods can precisely evaluate PD-L1/L2 expression in tissue samples is required.

In this study, we found that PD-L1and PD-L2 expressions in esophageal squamous cell carcinoma are altered by chemotherapy and PD-L1/L2 expression status in the primary lesion did not always match that of the metastases or recurrent lesions. Thus, it is not sufficient to evaluate the PD-L1/L2 expression status of a tumor just once, and it is necessary to consider that PD-L1/L2 expression may differ in the primary tumor compared with expression in metastases or recurrence.

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DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

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