Chemotherapy enhances programmed cell death 1/ligand 1 expression via TGF-β induced epithelial mesenchymal transition in non-small cell lung cancer

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Abstract. In cancer immunology, the programmed cell death 1-programmed cell death 1/ligand 1 (PD-1/PD-L1) pathway plays a major role. Anti-PD-1 and anti-PD-L1 antibodies provide reliable immunotherapy when given as treatment for various types of malignancy including lung cancer. PD-L1 expression in cancer cells has been reported to be a predictive factor for the therapeutic effects of immunotherapy. However, the mechanism of PD-L1 expression remains unclear. Another key process in cancer progression is epithelial-mesenchymal transition (EMT). In the present study, we investigated the mechanism of PD-L1 expression as well as changes in its expression during the EMT process in non-small cell lung cancer (NSCLC). In this study, A549 cells underwent EMT by treatment with TGF-β or chemotherapeutic agents and then PD-L1 expression was evaluated. The alterations of PD-L1 expression was also examined during the reverse EMT process; mesenchymal-epithelial transition (MET). The relationship between for PD-L1 expression and EMT status in clinical specimens with NSCLC after induction chemotherapy were analyzed by immunohistochemical staining. We found that PD-L1 expression was upregulated following TGF-β induction; in contrast, it was downregulated by TGF-β receptor-kinase inhibitors and the MET process. Furthermore, chemo-treatment increased TGF-β expression and enhances PD-L1 expression via autocrine TGF-β induced EMT. Analysis of clinical samples revealed a significant relationship between PD-L1 expression and EMT status ($P<0.05$). In conclusion, our results suggest that PD-L1 expression is regulated by TGF-β induced EMT and enhanced by chemo-treatment via the chemo-induced TGF-β signaling. The anti-PD-1/PD-L1 blockade may provide more effective anticancer activities in combination with chemotherapy in NSCLC.

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Key words: non-small cell lung cancer, PD-1/PD-L1, epithelial-mesenchymal transition, induction chemotherapy, TGF-β

Introduction

Non-small cell lung cancer (NSCLC) is a leading cause of cancer-related death in most of the industrial countries. Chemotherapy, radiation therapy and surgery are the main therapeutic options. Despite these therapies, the prognosis for NSCLC has not improved as expected, and the mortality rate remains high. Therefore, additional therapeutic options are needed.

Recently, cancer immunotherapy has been developed and shown promising results against some malignancies in clinical trials (1-4). One of immuno-check-point proteins, the programmed cell death 1/programmed cell death 1/ligand 1 (PD-1/PD-L1) pathway has attract attention as playing a main role in cancer immunology (5). PD-1 (also known as CD279) is a receptor expressed on immune cells, and when combined with its ligand, PD-L1 (also known as CD274) expressed on cancer cells, induces the immunosuppression of cancer cells and blocks the attack by host immunity (5). Anti-PD-1/PD-L1 immunotherapies have recently been developed and has shown promising results against several malignancies (1-4). The expression of PD-L1 in cancer cells reportedly is a useful predictive factor for the therapeutic effect of anti-PD-1 or anti-PD-L1 antibody immunotherapies (6).

However, the mechanism of PD-L1 expression in cancer cells remains unclear. In addition, immunotherapy has other problems to be solved (e.g. the selection of suitable patients and the timing of administration of the immunotherapy). Elucidation of the mechanism of PD-L1 expression may provide useful information to solve those problems.

Epithelial-mesenchymal transition (EMT) is a key process in cancer progression and is induced by several factors, including transforming growth factor (TGF-β). We previously showed that chemo-treatment increased TGF-β expression in human adenocarcinoma cell lines, and this autocrine TGF-β lead to higher malignant characteristics of cancer cells via EMT process (7,8). We also revealed the clinical significance of the EMT in NSCLC after induction chemotherapy (7,8). In the present study, we explored the mechanism by which

TGF-β controls PD-L1 expression and elucidated the clinical relationships between the PD-L1 expression and EMT status in NSCLC after induction chemotherapy.

Materials and methods

Cell culture, reagents and antibodies. The human lung adenocarcinoma cell lines A549 and NCI-H358 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS) and streptomycin and penicillin. Carboplatin was purchased from Sigma-Aldrich (St. Louis, MO, USA; cat, no. C2538) and TGF-β1 was from R&D Systems (Minneapolis, MN, USA; cat. no. 240-B). SB 431542, a specific and selective inhibitor of TGF-β1 receptor kinase inhibitor, was purchased from Tocris Bioscience (St. Louis, MO, USA; cat. no. 1614).

Reversion assay [mesenchymal-epithelial transition assay (MET)]. To determine whether there is a causal relationship between PD-L1 expression EMT, reversion assay was performed. We analyzed the gene expression of PD-L1 and N-cadherin in A549 cells under MET (EMT reverse process). First, we induced EMT in the cells by using culture medium including TGF-β1 (1 ng/ml) for 3 days, and then we promoted MET by changing to culture medium without TGF-β1.

Immunohistochemical staining analysis of EMT status and PD-L1 expression in clinical samples. IHC staining was performed as follows. Formalin-fixed paraffin-embedded tissue sections of non-small cell lung cancer from patients who underwent surgical resection after induction chemotherapy were deparaffinized and rehydrated. For antigen retrieval, the sections were brought to boil in 1 mM EDTA pH 8.0 and then maintained for 15 min at a sub-boiling temperature. EMT status was evaluated according to N-cadherin, E cadherin and TGF-β1 staining intensities. The evaluation of EMT status and PD-L1 were based on a previous study (9). In brief, the stained specimens were scored in a semi-quantitative manner (H score): the staining percentages (0-100%) and the intensity 0 (no staining), +1 (weak staining), +2 (distinct staining), +3 (very strong staining) H score was calculated by multiplying the percentage by the intensity. In addition, H score was classified as 0 (score <10), +1 (≥10 or <30), or +2 (≥30 or <70), +3 (>70). We defined a positive change in EMT status as either or both H score classifications decrease in staining for E-cadherin and increase in the staining for N-cadherin, respectively, between the biopsy samples before induction chemotherapy and the surgical samples after induction chemotherapy (CT). The PD-L1 immunohistochemistry was evaluated based on the method described by Koh *et al* (9). Briefly, the intensity and proportion of membranous and/or cytoplasmic staining in tumor cells were scored as follows: 0, negative; 1, weak or moderate in <10% of tumor cells; 2, moderate in ≥10% of tumor cells; 3, strong (more intense than alveolar macrophages for PD-L1) in ≥10% of tumor cells. Cases with scores of 2 or 3 were deemed positive for PD-L1 expression. The antibody used for immunohistochemistry (IHC) were as follows: monoclonal mouse anti-human N-cadherin (6G11/M3613,1/50; Dako, Glostrup, Denmark), monoclonal mouse anti-human E-cadherin (NCH-38/M3612; Dako), polyclonal rabbit anti-human TGF-β1 (ab66043; Abcam, Cambridge, UK) and polyclonal rabbit anti-human PD-L1 (EIL3N/13684, 1/200; Cell Signaling Technology, Inc., Danvers, MA, USA).

Table I. Patients characteristics.

Variables	N
Age (mean years)	63.1
Sex	
Male	22
Female	6
Clinical stage	
II	3
III	25
Pathological stage	
I	9
II	6
III	13
Histopathology	
Adenocarcinoma	14
Squamous cell carcinoma	12
Others	2
Chemotherapy	
1st	
CDDP	13
CBDCA	15
2nd	
ETP	1
PTX	10
TXT	9
VDS	6
VNR	2
Surgical procedure	
Lobectomy	23
Pneumonectomy	2
Bi-lobectomy	3

CDDP, cisplatin; CBDCA, carboplatin; ETP, etoposide; PTX, paclitaxel; TXT, docetaxel; VDS, vindesine; VNR, vinorelbine.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from cell lines by using an RNeasy Mini kit (Qiagen, Tokyo, Japan). Real-time RT-PCR was conducted with a TaqMan assay; the relative expression levels were calculated by the comparative C_t method. The TaqMan gene assays (Applied Biosystems, Carlsbad, CA, USA) for GAPDH (Hs02758991_g1), E-cadherin (Hs01023894_m1), N-cadherin (Hs00983056_m1), PD-L1 (Hs01125301_m1) and TGF-β (Hs00998133_m1) were used. All experiments were performed in triplicate and the results are presented as means ± SD. The significance of differences between the untreated cells and the treated cells was tested with the Mann-Whitney U test.

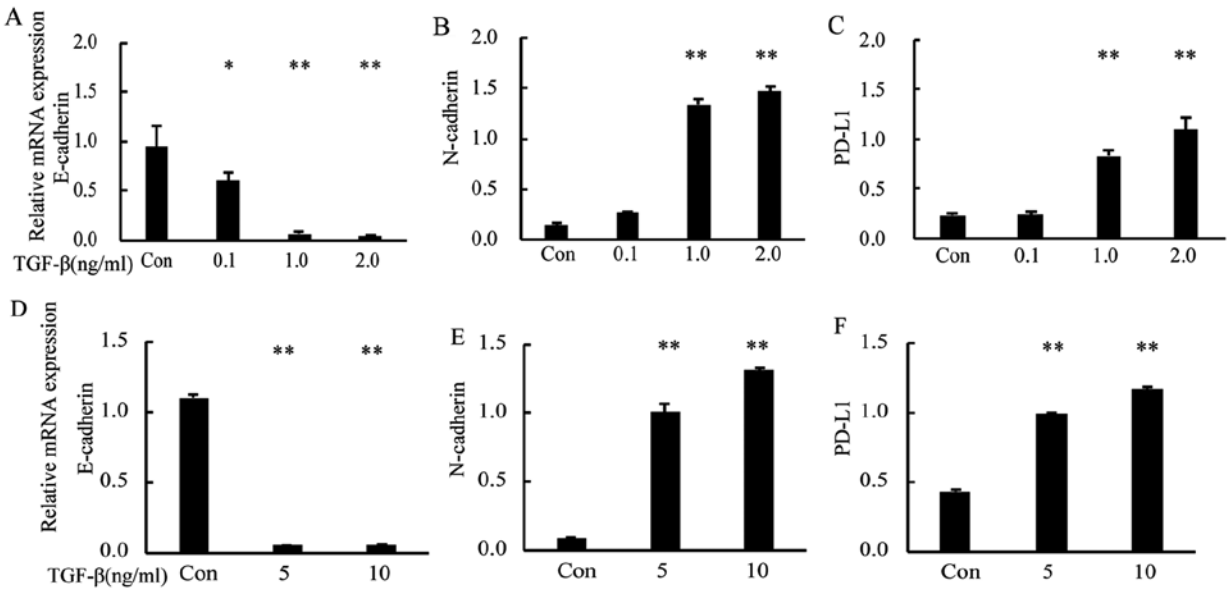

Figure 1. Effect of TGF-β1 treatment on gene expression. (A-C) RT-PCR analysis of expression of the genes encoding E-cadherin (E-cad), N-cadherin (N-cad) and PD-L1 in A549 human lung adenocarcinoma cells following RPMI alone (Con; control) and TGF-β1 treatment; mRNA levels were normalized to the levels of GAPDH mRNA; 0.1, 1.0 and 2.0 indicate treatment with TGF-β1 at 0.1, 1.0 and 2.0 ng/ml, respectively. (*P<0.05 vs. Con and **P<0.01 vs. Con). (D-F) Human Caucasian bronchioalveolar carcinoma H 358 (NCI-H358) cells were not treated (Con) or treated with TGF-β1 (5-10 ng/ml) for 2 days. Then, total RNA was extracted and real-time RT-PCR was performed to detect E-cadherin, N-cadherin and PD-L1 mRNA. (**P<0.01 vs. Con).

Western blot analysis. Monolayers of cultured cells were treated with TGF-β or carboplatin, and the proteins were extracted with RIPA buffer (Cell Signaling Technology; cat. no. 9806). Cell extracts were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted as previously described (10,11). Western blotting after extraction of membrane proteins was performed by using a Cell Surface Protein Isolation kit (P74008; Takara Bio, Tokyo, Japan). The following antibodies were used for detection: mouse anti-human E-cadherin (monoclonal, M106, 2 μg/ml; Takara), mouse anti-human N-cadherin (monoclonal, sc-59987; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-human GAPDH (monoclonal, G9545; Sigma-Aldrich) and rabbit anti-human TGF-β (polyclonal ab66043; Abcam).

Study population. Twenty-eight patients underwent induction chemotherapy and pulmonary resection between 1996 and 2010 at the Osaka University Hospital. Osaka University Hospital Review Board approved this retrospective study, and written informed consent for this retrospective study and surgery was obtained from each patient. Each patient received two cycles of cisplatin- or carboplatin-based chemotherapy every 4 weeks in one of three regimens: cisplatin at 80 mg/m^2 on day 1 and vindesine at 3 mg/m^2 on days 1 and 8, with or without mitomycin at 8 mg/m^2 on day 1 (PV(M) regimen), cisplatin at 80 mg/m^2 on day 1 and vinorelbine at 20 mg/m^2 on days 1 and 8 (nPV regimen), or cisplatin at 80 mg/m^2 on day 1 and docetaxel at 60 mg/m^2 on day 1 (DP regimen) (Table I). A surgical resection was performed 6-8 weeks after induction chemotherapy.

Statistical design and data analysis. A χ^2 test, Mann-Whitney U test, or repeated-measures analysis of variance were used to compare the RT-PCR results. The correlations between PD-L1 IHC status and EMT markers were analyzed by the Pearson's Chi-squared test. Disease-free survival (DFS) and overall survival (OS) were analyzed by using the Kaplan-Meier method, and the log-rank test was used to compare the survival distributions of subgroups. All statistical analyses were performed by using the JMP version 11 for Windows (SAS Institute, Inc., Cary, NC, USA). P<0.05 is considered to be statistically significant.

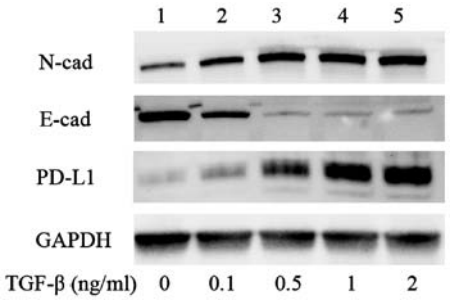

Figure 2. Western blot analysis PD-L1 and EMT marker protein after TGF-β1 treatment. Western blot analysis of the indicated proteins following TGF-β1 treatment; 0.1, 0.5, 1.0 and 2.0 indicate treatment with TGF-β1 at 0.1, 0.5, 1.0 and 2.0 ng/ml, respectively.

Results

PD-L1 is enhanced in TGF-β induced EMT and decreased in MET induced by removal of TGF-β stimulation. To examine the relationship between PD-L1 and EMT status, we induced EMT in A549 and NCI-H358 cells by using TGF-β1 at 0.1-2 ng/m. RT-PCR analysis showed that, after TGF-β1 treatment, the gene expression of E-cadherin (as the epithelial marker) was downregulated compared with that in control

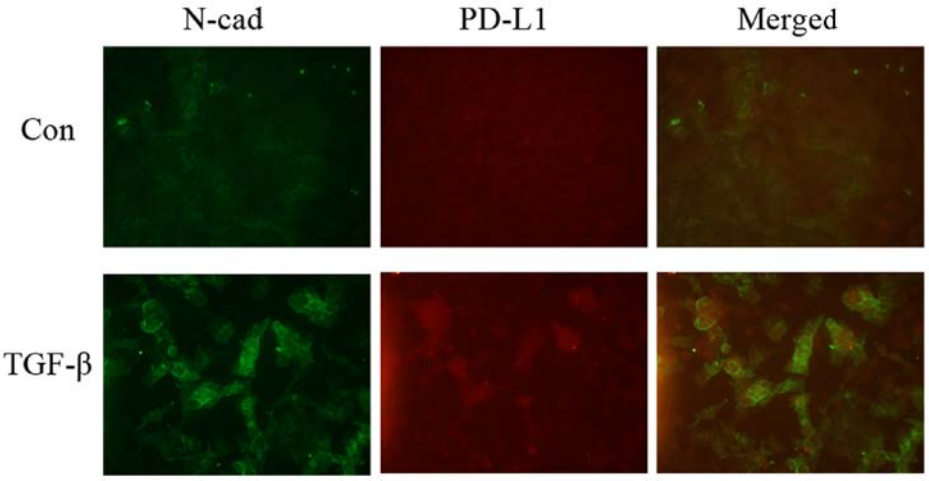

Figure 3. Immunofluorescence staining of TGF-β treated A549 cells. Representative images of fluorescent immunohistochemical staining of N-cadherin (GFP) and PD-L1 (red) in control and TGF-β treated A549 cells.

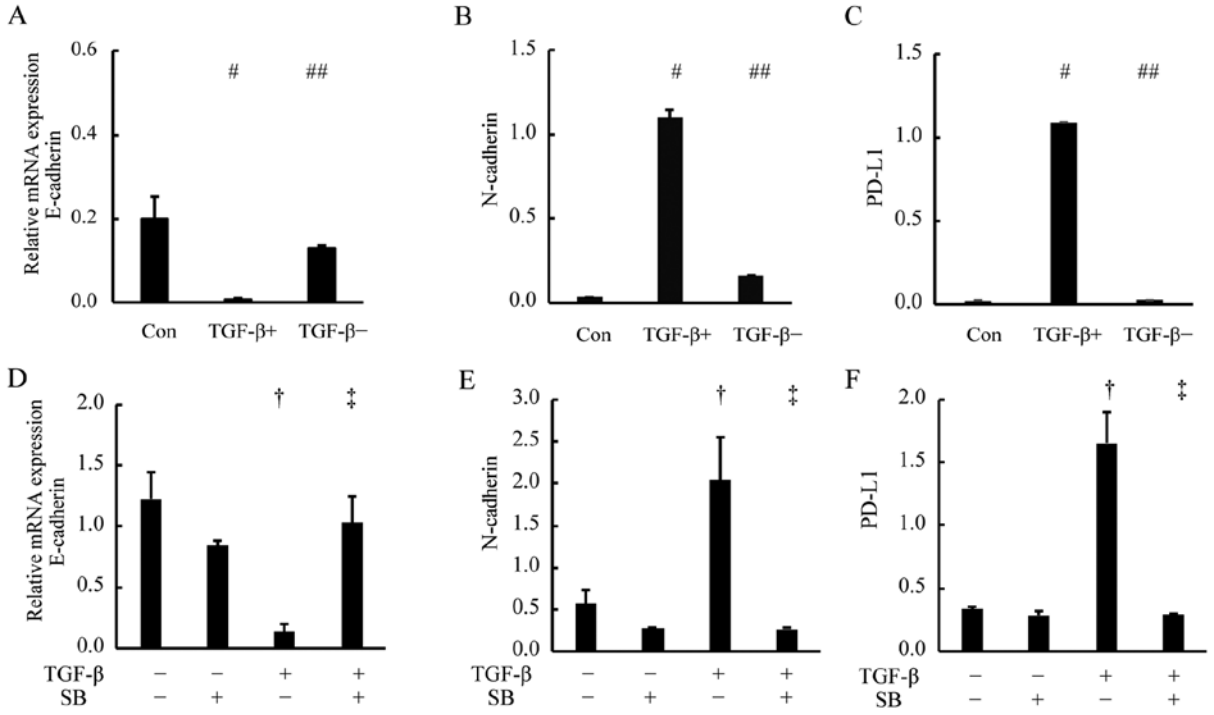

Figure 4. The PD-L1 expression is regulated by TGF-β signaling. (A-C) The gene expression analysis of reversion assay. mRNA expression of A549 cells after TGF-β1 treatment (TGF-β$^+$, 1 ng/ml) and A549 cells following removal of TGF-β1 from the medium (TGF-β$^-$). The mRNA levels were normalized to the levels of GAPDH mRNA. ($^{\#}$P<0.01 vs. Con, $^{\#\#}$P<0.01 vs. TGF-β$^+$). (D-F) A549 cells were not treated (Con), treated with TGF-β1 (TGF-β$^+$, 2 ng/ml), or treated with SB43152 (SB$^+$, 10 μM) plus TGF-β1 (2 ng/ml) for 3 days. Then, total RNA was extracted and real-time RT-PCR was performed to detect E-cadherin (E-cad), N-cadherin (N-cad) and PD-L1. (†P<0.01 vs. TGF-β$^-$/SB$^-$, ‡P<0.01 vs. TGF-β$^+$/SB$^-$).

cells, whereas that of N-cadherin (as the mesenchymal marker) and PD-L1 were upregulated in a dose-dependent manner (Fig. 1A-C; P<0.05 and P<0.01 vs. Con). We also analyzed the PD-L1 gene expression in the human Caucasian bronchioalveolar carcinoma cell line H358 and obtained similar results (Fig. 1D-F; P<0.01 vs. Con). Western blot analysis of A549 with TGF-β1 revealed that equivalent differences were observed at the protein level (Fig. 2). Immunofluorescence analysis showed that the expression levels of PD-L1 and N-cadherin were enhanced by TGF-β treatment and those on the cell surface became co-localized (Fig. 3).

To determine whether there is a causal relationship between the PD-L1 expression and TGF-β induced EMT, reversion assay was performed. We used RT-PCR assays to analyze the gene expression of PD-L1, E-cadherin and N-cadherin in the cells under EMT and MET (Fig. 4A-C). The gene expression levels of PD-L1, E-cadherin and N-cadherin changed with significant difference as compared with the pre-treatment levels (Con; control) (P<0.01 vs. Con) and reverted to pre-treatment (TGF-β$^-$) levels after the change to culture medium without TGF-β ($^{\#\#}$P<0.01 vs. TGF-β$^+$). These results suggest that the PD-L1 expression

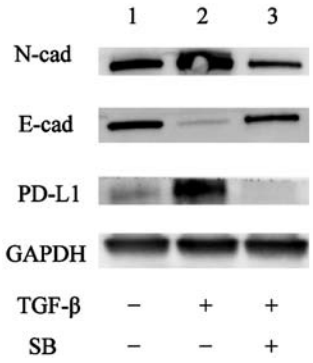

Figure 5. Western blot analysis of PD-L1 and EMT marker protein after TGF-β treatment and TGF-β1 receptor kinase inhibitor. A549 cells were not treated or treated with TGF-β1 or TGF-β1 plus SB 42152 for 3 days and then analyzed through western blotting for E-cadherin, N-cadherin, PD-L1 and GAPDH.

is regulated by the TGF-β signaling and changes in parallel with the EMT status.

TGF-β inhibitors block the expression of PD-L1. Next, to examine whether the PD-L1 expression was regulated through the TGF-β signal pathway, we treated A549 cells for 2 days with medium alone (Fig. 4D-F, Con; TGF-β^-/SB^-), the TGF-β1 receptor kinase inhibitor; SB 431542 (Fig. 4D-F, TGF-β^-/SB^+), or TGF-β1 plus SB 431542(Fig. 4D-F, TGF-β^+/SB^+). Next, we examined PD-L1, E-cadherin and N-cadherin expression by RT-PCR and western blot analysis. The RT-PCR results showed that the upregulation of PD-L1 and N-cadherin mRNA expression after TGF-β1 treatment was suppressed by SB 431542 (Fig. 4D-F; P<0.01 vs. TGF-β^-/SB^-, P<0.01 vs. TGF-β^+/SB^-) and the western blot analysis demonstrated that the PD-L1 protein level was suppressed by SB 431542 (Fig. 5, lane 2 vs. 3). Taken together, these results suggest that the PD-L1 expression is regulated through the TGF-β signal pathway.

PD-L1 expression was enhanced by chemo-induced TGF-β pathway. We previously reported that chemo-treatment increased the expression of TGF-β and the autocrine TGF-β induced EMT in A549 cells (12,13). In this study, we hypothesized that chemo-treatment enhanced PD-L1 expression through the TGF-β pathway. We evaluated the TGF-β, EMT markers and PD-L1 mRNA and protein expression in the A549 cells treated with carboplatin (25 μM) for 4 days. The RT-PCR results indicated that, for E-cadherin, N-cadherin and PD-L1, the expression patterns of the control versus treatment subline (Fig. 6A-C) were similar to those for control vs. TGF-β treatment (P<0.05; vs. Con). Next, to examine whether PD-L1 upregulation under chemo-treatment was via TGF-β pathway, we examined PD-L1 expression of the cells after treatment with carboplatin alone (CB) and carboplatin plus SB 43152 (CB+SB), by RT-PCR and western blot analysis. Fig. 6E showed the results of RT-PCR that PD-L1 gene expression after chemo-treatment (CB) increased significantly as compared with no-treated cell lines (Con). In contrast, the upregulation of the mRNA levels of PD-L1 and N-cadherin was attenuated by SB 431542 (CB+SB) (Fig. 6D-E, P<0.05 vs. Con, P<0.01 vs. CB). While, TGF-β mRNA expression was upregulated by carboplatin as previously described (12) (Fig. 6F; P<0.05 vs. Con); however, TGF-β upregulation with carboplatin treatment was not attenuated by SB 431542. Fig. 6F shows that there was no significant difference in TGF-β mRNA levels between carboplatin alone (CB) and carboplatin plus SB 431542 treatment (CB+SB). Moreover, in western blot analysis, the expression of PD-L1 was upregulated by chemo-treatment in

Table II. Univariate and multivariate analyses of disease-free survival.

A, Univariate analysis of disease-free survival

Factors	Hazard ratio	95% CI	P-value
Sex			
Male	1		
Female	0.59	0.03-2.93	0.58
Histology			
Adeno	1		
Sq	0.79	0.28-2.32	0.66
other	0.42	0.02-2.31	0.36
p-Stage			
I+II	1		
III+IV	2.29	0.83-6.27	0.1
Down-stage			
Positive	1		
Negative	2.27	0.73-10.03	0.16
PD-L1			
Low	1		
High	2.97	1.13-8.54	0.02

B, Multivariate analysis of disease-free survival

Factors	Hazard ratio	95% CI	P-value
PD-L1			
Low	1		
High	2.8	1.00-8.41	0.04
Histology			
Adeno	1		
Sq	0.8	0.26-2.42	0.7
other	0.7	0.1-3.17	0.69
p-Stage			
I+II	1		
III+IV	2.95	0.83-11.7	0.09
Down-stage			
Positve	1		
Negative	1.07	0.32-3.47	0.9

Adeno, adenocarcinoma; Sq, squamous cell carcinoma; CI, confidence interval.

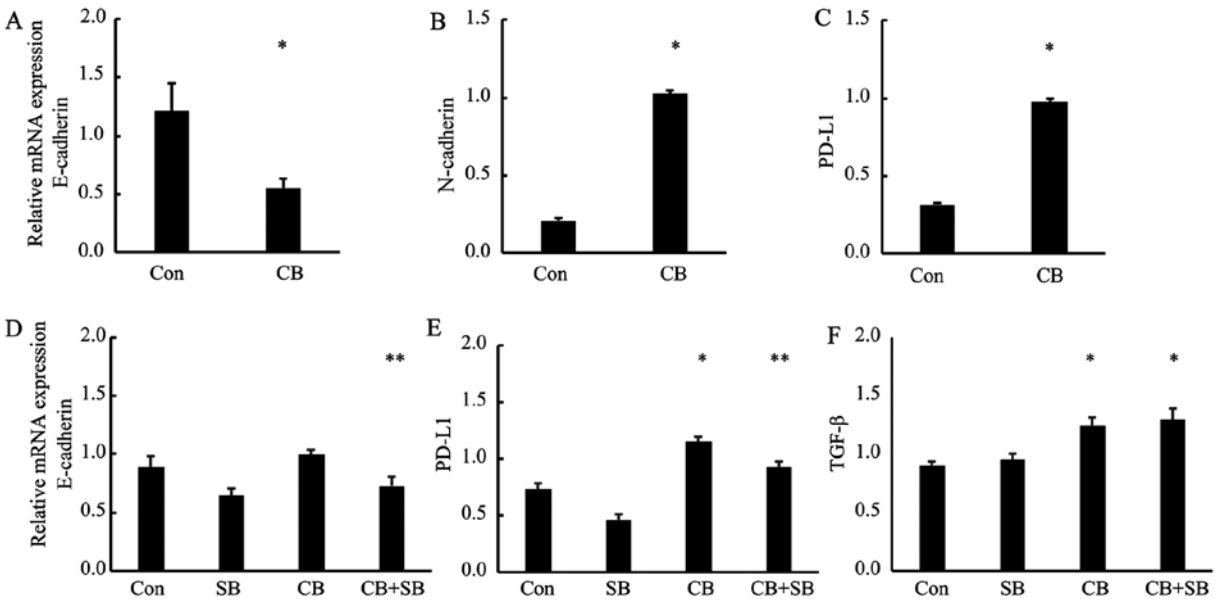

Figure 6. Chemo-treatment enhances PD-L1 expression via TGF-β signaling. RT-PCR analysis of untreated A549 cells (Con) and those cells treated with carboplatin (25 μM) for 4 days (CB). Total RNA was extracted from the untreated A549 cells (Con) and the cells treated with carboplatin (CB), and E-cadherin, N-cadherin, PD-L1 and GAPDH transcripts were quantified by RT-PCR analysis (A-C). (*P<0.05 vs. Con). (D-F) Control A549 cells (Con), the cells treated with carboplatin alone (CB), and the cells treated with carboplatin and SB 43152 (SB; 10 μM) (CB+SB) underwent RT-PCR analysis for quantification of TGF-β, N-cadherin, PD-L1 and GAPDH mRNA levels. (N-cad; *P<0.05 vs. CB, PD-L1; *P<0.05 vs. Con, **P<0.01 vs. CB, TGF-β; *P<0.05 vs. Con).

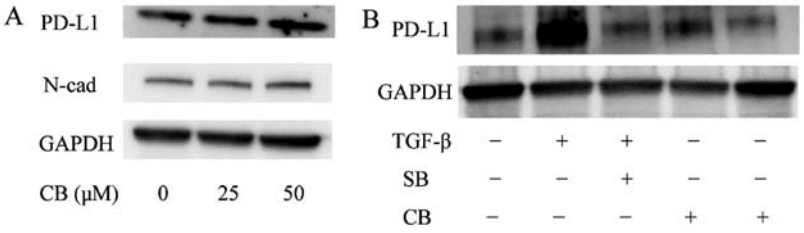

Figure 7. Chemo-treatment enhances PD-L1 protein expression via TGF-β signaling. (A) Western blot analysis of the indicated proteins following carboplatin treatment (CB). Twenty-five and 50 indicate treatment with carboplatin at 25 and 50 μM, respectively. (B) Control A549 cells, the cells treated with carboplatin alone (CB^{+}), and cells treated with carboplatin and SB 43152 (10 μM) (SB^{+}) underwent western blot analysis for quantification of PD-L1 and GAPDH levels.

a dose-dependent manner (Fig. 7A), and this upregulation was abolished by SB 431542 (Fig. 7B; lane 4 vs. 5). These results suggest that the PD-L1 gene expression is upregulated through the chemo-induced TGF-β pathway.

High expression of PD-L1 in non-small cell lung cancer cases after induction chemotherapy. To elucidate the relationship between PD-L1 and EMT status in clinical samples of NSCLC, we performed IHC staining of clinical specimens obtained from samples resected from 28 patients with NSCLC after induction chemotherapy.

The patient characteristics are listed in Table I. IHC staining was used to analyze the expression of EMT markers, TGF-β and PD-L1. Fig. 8A shows the representative images of low expression of PD-L1 and Fig. 8B shows those of high expression of PD-L1. Fig. 8C-E shows the representative images of E-cadherin, N-cadherin and TGF-β positive IHC. A total of 28 patients with NSCLC underwent induction chemotherapy followed by complete surgical resection. All cases underwent carboplatin or cisplatin-based doublet chemotherapy. The patients comprised 22 men and 6 women with a mean age of 63.1.

Eight cases (28.6%) showed a positive EMT change. Of them, 7 (87.5%) showed positive results for PD-L1 staining. The proportion of EMT-change positive cases among PD-L1-high expression cases was significantly higher than that among PD-L1-low expression cases (Fig. 9A, Pearson's chi-square test; P=0.01). In TGF-β IHC analysis, there were also significant relationships between PD-L1 expression and TGF-β expression (Fig. 9B, Pearson's chi-square test; P=0.04). The DFS (measured as no recurrence of cancer) in PD-L1-high expression cases was significantly worse than that of PD-L1-low expression cases (Fig. 9C, P=0.02; log-rank test); however, the OS rate was not significantly related to PD-L1 status (Fig. 9D, P=0.2; log-rank test).

Univariate and multivariate analyses showed that high PD-L1 expression was an independent prognostic factor for NSCLC surgically resected after induction chemotherapy (Table II).

Discussion

In the present study, we showed that PD-L1 was regulated by TGF-β, and that chemo-treatment enhanced the PD-L1 expres-

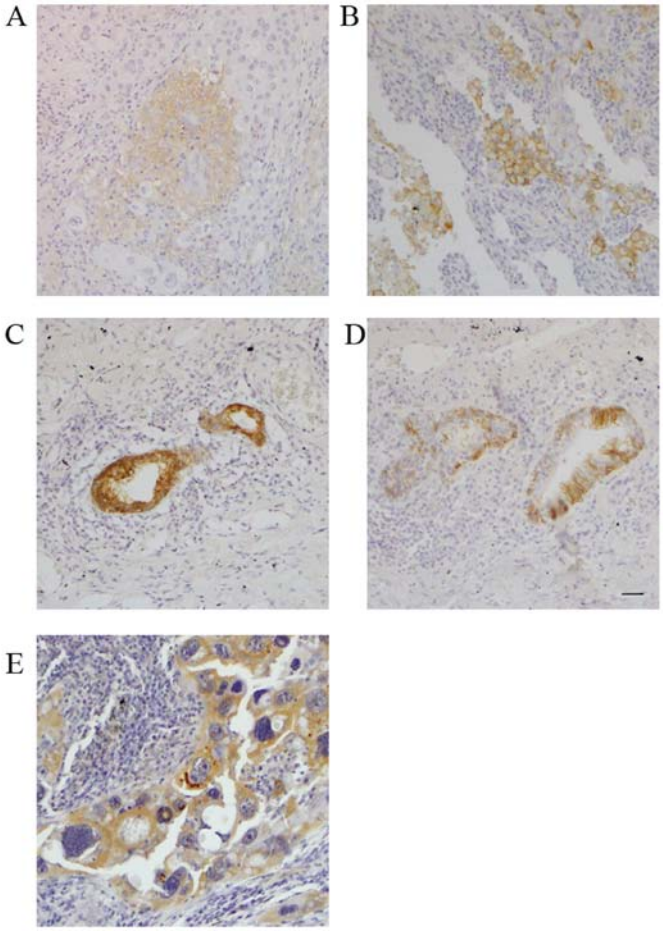

Figure 8. Immunohistochemical staining analysis of PD-L1 and EMT markers. Representative images of immunohistochemical staining of PD-L1 and EMT marker. (A) Weak staining intensity of PD-L1. (B) Strong staining intensity of PD-L1. The representative images of positive E-cadherin, N-cadherin and TGF-β IHC staining (C; E-cadherin, D; N-cadherin, E; TGF-β). Scale bar, 100 μm.

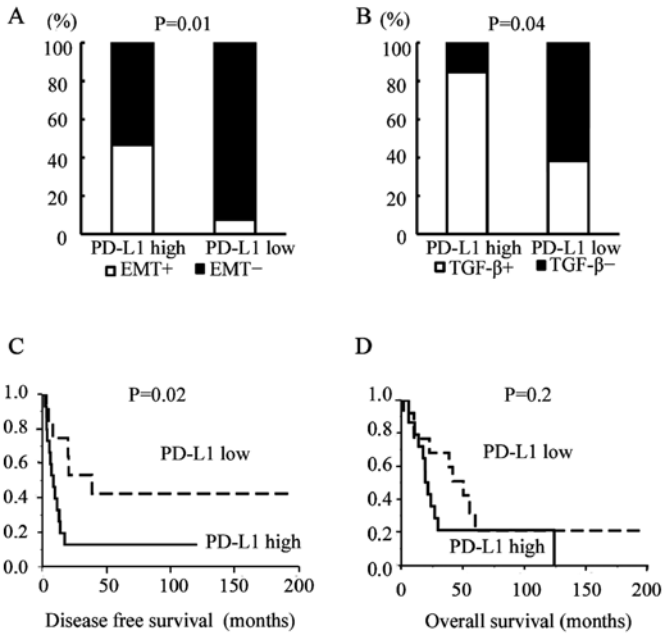

Figure 9. The relationships between PD-L1 expression and EMT markers, and Kaplan-Meier survival curve according to PD-L1 expression. (A) Proportions of cells staining for EMT status in PD-L1 expression intensity (Pearson's chi-square test). (B) Proportion of cells staining for TGF-β according to PD-L1 expression intensity (Pearson's chi-square test). (C) Disease-free survival rates and (D) overall survival rates according to tumor expression of PD-L1.

sion through the chemo-induced TGF-β signal pathway in NSCLC cell lines. We also performed IHC staining of surgically resected NSCLC after induction chemotherapy and thus revealed a significant relationship between PD-L1 expression and EMT status.

To overcome advanced NSCLC, surgery and chemotherapy are the most important therapeutic options; however, their results are far from satisfactory. Immunotherapy has been recently developed as another option; in particular, anti-PD-1/PD-L1 blockade agents have exhibited dramatic antitumor efficacy in clinical trials for patients with a variety of cancer types (14). However, immunotherapy likewise is associated with several problems to be solved, as outlined below.

The first problem is the selection of suitable patients. The current selection method depends on PD-L1 IHC results as a predictor of therapeutic effect. PD-L1 expression by IHC has been also reported to be a useful prognostic indicator (15); however, staining intensity and sensitivity vary according to the type of PD-L1 IHC antibody (15,16). The next problem is when to start this immunotherapy. Understanding the mechanism of PD-L1 expression will yield important information regarding potential solutions to these problems. To elucidate this mechanism, we focused on EMT.

EMT plays a crucial role in cancer progression, and this phenomenon can be found in this cancer microenvironment. EMT is promoted by TGF-β secreted from not only the cancer cell itself but also from other cell types, and it gives cancer cells the invasive and metastatic abilities necessary for successful metastasis (7,8,12,17). In the present study, we demonstrated that PD-L1 expression in cancer cells was regulated by TGF-β signal pathway. Other groups showed a significant relationship between PD-L1 expression and EMT status by IHC staining analysis on several malignancies including NSCLC (18,19). Together, these data support our notion that the TGF-β pathway is an important regulator of PD-L1 expression, and EMT markers may be useful for selecting patients that would likely benefit from immunotherapy.

We previously reported that chemo-treatments increased TGF-β production and autocrine TGF-β induced EMT in human lung adenocarcinoma cell lines (7,12). Based on our previous results, we showed that chemo-treatment enhanced PD-L1 expression through the chemo-induced TGF-β signal pathway. Moreover, from the IHC analysis, we revealed the significant relationship between PD-L1 expression and EMT status of resected samples after induction chemotherapy. Zhang *et al* (20) demonstrated that chemo-preventive agents induced PD-L1 in human breast cancer cells and promoted PD-L1-mediated INF-γ by T-cell apoptosis. Hecht *et al* (21) also showed that PD-L1 expressing cells in rectal adenocarcinoma were upregulated after chemoradiotherapy (CRT) as compared with before CRT. Other groups have shown that several stimuli, such as hypoxia and radiation induction, enhance PD-L1 expression (22-24). Those results suggested that PD-L1 is enhanced by chemo-treatment and the PD-L1 blockade after chemotherapy or in combination with chemo-

therapy could be a more effective anticancer activity than the monotherapy.

This study has some limitations. The limitation is the small sample size analyzed. In our hospital, adjuvant chemoradiation therapy was usually performed in most of advanced cases. As mentioned above, the radiation, like chemotherapy, enhances the expression of PD-L1 (22-24). Since the effect of radiation cannot be ignored, we analyzed only cases after induction chemotherapy alone. Moreover, the predictive role of PD-L1 expression is still controversial. Our results showed the PD-L1 expression was a prognostic indicator. However, our sample size was small as mentioned. Therefore, further investigation will be needed.

In conclusion, the present study provides new and important information regarding the mechanism of PD-L1 expression. The results suggest that EMT markers could be a surrogate marker for the selection of patients for immunotherapy and that a combination of immunotherapy and chemotherapy could be a new therapeutic option to overcome NSCLC.

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