Programmed death-ligand 1 expression at tumor invasive front is associated with epithelial-mesenchymal transition and poor prognosis in esophageal squamous cell carcinoma

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Key words
Epithelial-mesenchymal transition, esophageal cancer, immune system, malignant potential, tumor invasion

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Funding Information
None.

Received January 27, 2017; Revised March 3, 2017; Accepted March 8, 2017

Cancer Sci 108 (2017) 1119–1127
doi: 10.1111/cas.13237

Esophageal cancer is the eighth most common cancer worldwide and ranks sixth among all cancers in mortality. Esophageal cancer has two major histological types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. ESCC is the dominant type in Asia. In Japan, ESCC remains the predominant type among all esophageal cancers; the ratio of squamous cell carcinoma to adenocarcinoma is 26:1. Programmed death 1 (PD-1), an inhibitory costimulatory molecule, is induced on activated T cells, B cells, and NK cells, and plays a crucial role in regulating peripheral tolerance. Two PD-1 ligands have been identified, programmed death ligand 1 (PD-L1, also known as CD274) and programmed death ligand 2 (PD-L2); both are members of the B7 family. PD-L1 is expressed by various human tumors and plays an essential role in evasion of the host immune system in cancer. A high level of PD-L1 expression in severe malignancies has been associated with a poor prognosis. Current clinical trials show that anti-PD-1 and anti-PD-L1 therapies that inhibit the interaction between PD-1 and PD-L1 show potential for the treatment of cancer patients by generating measurable clinical activity with minimal toxicities.

Epithelial-mesenchymal transition (EMT), a process whereby epithelial cells lose their cell polarity and cell–cell adhesion ability and acquire migratory and invasive properties to gain mesenchymal phenotype, is important for invasion and metastasis of carcinoma. In the case of many cancers, EMT-inducing signals emanating from the tumor-associated stroma, including those mediated by HGF, EGF, PDGF, and TGF-β, appear to be responsible for the induction or functional activation in cancer cells of a series of EMT-inducing transcription factors, including Snail, Slug, zinc finger E-box binding homeobox 1 (ZEB1), Twist, and FOXC2.

We previously reported that PD-L1 expression is correlated with malignancy and prognosis in ESCC. However, the mechanism and pattern of PD-L1 expression in ESCC has not been fully revealed. A recent study reported that TGF-β1 induced ZEB1 binding site, we speculated that PD-L1 expression might be regulated by the ZEB1 transcription

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factor. The aim of this study was to clarify the relationships between PD-L1 expression and EMT, and clinicopathological features in ESCC.

Materials and Methods

Patients. This study included 90 patients (mean age 62.7 years) who had undergone esophageal resection for ESCC without preoperative treatment at the Department of Surgery, Kyushu University Hospital, between April 1997 and March 2005. This study was approved by the Ethics Committee of Kyushu University (Approval Number: 27-397).

Immunohistochemistry and evaluation of staining. Tumor sections were assessed by immunohistochemistry (IHC) using rabbit polyclonal antibodies against PD-L1 (1:200; Lifespan Bioscience, Seattle, WA, USA), IgG mouse monoclonal antibodies against ZEB1 (1:150; Origene, Rockville, MD, USA), CD8 (1:100; DAKO, Santa Clara, CA, USA), and E-cadherin (1:100, DAKO, Santa Clara, CA, USA). Briefly, 4-μm sections were deparaffinized in xylene and dehydrated in an ethanol series. For antigen retrieval, the specimens were pretreated in an autoclave for PD-L1 (121°C, 10 min, in 0.01 M citrate buffer; pH 6.0) and ZEB1 (121°C, 20 min, in Target EDTA, pH 9.0). E-cadherin (121°C, 15 min, in 0.01 M citrate buffer; pH 6.0), and CD8 (121°C, 15 min, in 0.01 M citrate buffer; pH 6.0). The sections were incubated for 30 min in 0.3% hydrogen peroxidase in absolute methanol to deactivate endogenous peroxidases. After blocking nonspecific binding of antibodies, the specimens were incubated at room temperature with primary antibodies against PD-L1, ZEB1, CD8, and E-cadherin overnight. IHC staining was performed using an EnVision system and DAB kits (DAKO). The levels of expression of PD-L1, ZEB1, CD8, and E-cadherin were evaluated by two investigators, including one general pathologist. The expression of PD-L1 was evaluated according to the staining of the cell membrane and/or in the cytoplasm of tumor cells (TCs) at tumor invasive front as previously reported.10 We evaluated the tumor invasive front at the deepest layer of tumor, based on findings of hematoxylin-eosin staining. In this study, PD-L1 positivity was defined as tumor, based on findings of hematoxylin-eosin staining. In this study, the cut-off value for ZEB1 and CD8 was set as isotype controls, respectively. For cell membrane staining, cells were incubated with PD-L1 antibody and isotype control antibody (BD Biosciences). We added 7-aminoactinomycin D (Thermo Fisher Scientific) before analysis. For intracellular staining, cells were fixed with Fixation and Permeabilization Solution (BD Biosciences, Franklin, MA, USA) and treated according to the manufacturer’s instructions. Briefly, cells were fixed for 20 min at 4°C, washed two times with Perm/Wash buffer (BD Biosciences). Cells were stained with ZEB1 antibody and isotype control for 30 min at 4°C. Cells were washed with Perm/Wash buffer and re-suspended with FACS buffer. The fluorescence data were collected using the Cell Sorter SH800 (Sony, Tokyo, Japan), and were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis. For in vitro studies, quantitative data are presented as means SD (unless indicated otherwise). Differences between two groups were estimated using Student’s t test. For TGF-β1 treatment, TE5, TE6, and TE11 cells were seeded in a 6-well plate (0.25 x 10^5 cells per well) and cells were treated for 96 h with recombinant TGF-β1 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 20 ng/mL.

Quantitative real-time PCR. We determined mRNA expression levels using TaqMan qPCR Technology. Total RNA was isolated from cells and converted into cDNA using the SuperScript III First-strand Synthesis SuperMix (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was performed using the StepOnePlus (Applied Biosystems, Foster City, CA, USA). Reactions were run in three independent experiments. The geometric mean of the housekeeping gene β-actin was used as an internal control to normalize the variability in expression levels.

Western blot analysis. Cells cultured in 6-well dishes were scraped into 300 μl ice-cold RIPA buffer (Nacalai Tesque, Kyoto, Japan). Samples were clarified by centrifugation at 12,000 g for 30 min at 4°C. We used the iBind Western Blotting Reagents (Pierce, Thermo Fisher Scientific) to isolate protein from cells. The total protein was used for Western blot analysis. We used the iBind Western Blotting Reagents (Pierce, Thermo Fisher Scientific) to isolate protein from cells. The total protein was used for Western blot analysis.

Flow cytomtery analysis. Cells were stained with mouse monoclonal antibodies to human PD-L1 (Biolegend, San Diego, CA, USA) and ZEB1 (Novusbio, Littleton, CO, USA), and mouse IgG2b, κ (Biolegend) and IgG1 (Novusbio) were used as isotype controls, respectively. For cell membrane staining, cells were incubated with PD-L1 antibody and isotype control for 30 min at 4°C. We added 7-aminoactinomycin D (Thermo Fisher Scientific) before analysis. For intracellular staining, cells were fixed with Fixation and Permeabilization Solution (BD Biosciences, Franklin, MA, USA) and treated according to the manufacturer’s instructions. Briefly, cells were fixed for 20 min at 4°C, washed two times with Perm/Wash buffer (BD Biosciences). Cells were stained with ZEB1 antibody and isotype control for 30 min at 4°C. Cells were washed with Perm/Wash buffer and re-suspended with FACS buffer. The fluorescence data were collected using the Cell Sorter SH800 (Sony, Tokyo, Japan), and were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis. For in vitro studies, quantitative data are presented as means SD (unless indicated otherwise). Differences between two groups were estimated using Student’s t test.
Expression of PD-L1 at invasive front and correlation with clinicopathological findings and prognostic outcomes. IHC evaluation showed that PD-L1 was expressed in the cell membrane and cytoplasm of TCs in ESCC specimens (Fig. 1a,b). Among the 90 ESCC cases, positive expression of PD-L1 at the invasive front was observed in 57 cases (63.3%). High expression of CD8+ lymphocyte infiltration was observed in 40 cases (44%) (Fig. 1c,d). E-cadherin positive case is shown in Figure 1(e) and ZEB1 positive case is shown in Figure 1(f). In this report, we defined EMT when mesenchymal morphological changes and low E-cadherin expression were found at the invasive front of tumor. A comparison of clinicopathological features according to PD-L1 expression is shown in Table 1. In the PD-L1-positive expression group, lymph node metastasis and lymphatic invasion tended to be more frequent ($P = 0.0906$ and $P = 0.0815$, respectively). Greater depth of tumor invasion ($P = 0.0021$), EMT and less CD8+ lymphocyte infiltration were also more frequent ($P = 0.0013$ and $P = 0.0053$, respectively) in the PD-L1-positive group (Table 1).

In prognostic analysis, PD-L1-positive cases were associated with worse overall survival rate (OS) and relapse-free survival (RFS) rates. The 5-year OS rate in the patients with PD-L1 positive expression was significantly poorer than those with

| Table 1. Clinicopathological features according to PD-L1 expression in patients with esophageal squamous cell carcinoma who underwent esophagectomy |
|---|---|---|---|
| Factor | PD-L1 | PD-L1 | $P$-value |
| | negative | positive |  |
| Age (mean) | 62.1 | 63.0 | 0.4382 |
| Sex (male/female) | | | |
| Male | 31 (93.9) | 49 (85.9) | 0.2460 |
| Female | 2 (6.1) | 8 (14.1) |  |
| Differentiation of squamous cell carcinoma | | | |
| Well/moderately | 27 (81.8) | 46 (80.7) | 0.8960 |
| Poorly | 6 (18.2) | 11 (19.3) |  |
| Pathological depth of tumor invasion | | | |
| $T < 3$ | 26 (78.7) | 26 (45.6) | 0.0021 |
| $T \geq 3$ | 7 (21.3) | 31 (54.3) |  |
| Pathological lymph node metastasis | | | |
| pN(-) | 20 (60.6) | 42 (41.2) | 0.0906 |
| pN(+) | 13 (39.4) | 33 (58.8) |  |
| ly | | | |
| (-) | 19 (57.5) | 22 (38.5) | 0.0815 |
| (+) | 14 (42.5) | 35 (61.5) |  |
| v | | | |
| (-) | 24 (72.7) | 37 (65.0) | 0.4446 |
| (+) | 9 (27.3) | 20 (35.0) |  |
| EMT | | | |
| (-) | 26 (78.7) | 25 (43.8) | 0.0013 |
| (+) | 7 (21.3) | 32 (56.2) |  |
| CD8 | | | |
| Low | 12 (36.4) | 38 (66.7) | 0.0053 |
| High | 21 (63.6) | 19 (33.3) |  |
| ZEB1 | | | |
| Low | 24 (72.7) | 29 (50.9) | 0.0397 |
| High | 9 (27.3) | 28 (49.1) |  |

The numbers in parentheses indicate percentages. EMT, epithelial mesenchymal transition; ly, lymphatic permeation; PD-L1, programmed death-ligand 1; v, venous permeation.
negative expression (39.2% vs 67.0%, P = 0.0112). The 5-year RFS rate in the patients with PD-L1 positive expression was also worse than those with negative expression (22.4% vs 57.8%, P = 0.0040) (Fig. 2a, b). Univariate and multivariate analyses of RFS and OS in all patients are shown in Table S1. In multivariate analyses, ly (P = 0.032) and PD-L1 (P = 0.015) were multivariate prognostic factors in RFS, and pathological lymph node metastasis (P = 0.028) was a multivariate prognostic factor in OS.

Correlation between ZEB1 expression and PD-L1 expression. ZEB1 expression was evaluated by counting positive staining of the cell nucleus of TCs at the same invasive front area as PD-L1 (Fig. 1f). High expression of ZEB1 was observed in 37 cases (41.1%). A comparison of clinicopathological features according to ZEB1 is shown in Table S2. ZEB1 high expression was related to depth of tumor invasion (P = 0.005) and EMT (P = 0.0006). In prognostic analysis, ZEB1 high expression was associated with worse OS and tended to be associated with worse RFS. The 5-year OS rate in patients with ZEB1 high and low expression was 36.2% and 60.1%, respectively (P = 0.0271). The 5-year RFS rate in patients with ZEB1 high and low expression was 32.0% and 38.3%, respectively (P = 0.1836) (Fig. S1). In the ZEB1 high expression group, PD-L1 positive and negative expression was observed in 76% and 24% of patients, respectively, indicating a positive correlation between ZEB1 and PD-L1 expression (P = 0.0397) (Table 1). We divided patients into four groups according to expression of PD-L1 and ZEB1, and the patient group with PD-L1 positive and ZEB1 high expression showed the worst prognosis in OS (P = 0.0240) and RFS (P = 0.0328) (Fig. 2c, d).

Suppression of PD-L1 expression by siRNA for ZEB1. We measured PD-L1, ZEB1, E-cadherin, Vimentin, and TGF-β1 mRNA expression and protein expression in TE5, TE6, TE8 and TE11 cell lines (Fig. 3a–e). The levels of PD-L1 mRNA expression were almost same in all cell lines (data not shown), ZEB1 and TGF-β1 mRNA expression were higher in TE8 cells than the other cell lines. TE8 cells exhibited an EMT characteristic, spindle-like mesenchymal morphology with high expression of the mesenchymal marker Vimentin and low expression of the epithelial
marker E-cadherin. In contrast, TE5, TE6, and TE11 cells exhibited epithelial characteristic, cobblestone-like epithelial morphology with high expression of the epithelial marker E-cadherin and low expression of the mesenchymal marker Vimentin.

We next tested whether mRNA expressions of PD-L1 and E-cadherin were affected by EMT inducing transcriptional factors ZEB1, Twist and Snail by q-PCR in the TE8 cell line. We used siRNAs to target ZEB1 (siZEB1-1, siZEB1-2), Twist (siTwist-1, siTwist-2) and Snail (siSnail1, siSnail-2). In TE8 cells, siZEB1 effectively suppressed ZEB1 and PD-L1 mRNA expression and promoted E-cadherin mRNA expression compared with non-targeting (NT) siRNA (Fig. 4a–c). On the other hand, siTwist and siSnail suppressed Twist and Snail mRNA expression (*P < 0.001), respectively, but did not suppress PD-L1 mRNA expression (Fig. S2a–d).

We next measured the surface and intracellular expression of PD-L1 and ZEB1 by FACS. The PD-L1 surface expression in TE8 cells was low; thus to investigate the influence of ZEB1 on PD-L1 expression more clearly, TE8 cells transfected by siZEB1 were treated with INF-γ (5 ng/mL). FACS analysis confirmed that siZEB1 suppressed intracellular expression of ZEB1 (Fig. 4d). Surface expression of PD-L1 was also suppressed by siZEB1 (Fig. 4e). siZEB1 promoted E-cadherin expression and did not change vimentin expression (Fig. 4f). These results suggest that ZEB1 regulates PD-L1 expression in ESCC cell lines.

**EMT and PD-L1 expression induced by TGF-β1.** Transforming growth factor-β is a potent inducer of EMT in epithelial cancers. As mentioned above, TE5, TE6 and TE11 cells show epithelial characteristics. We treated these cells with TGF-β1 for 96 h and confirmed that their cobblestone-like epithelial morphology changed to a spindle-like mesenchymal morphology (Fig. 5a). WB analysis revealed low expression of E-cadherin, high expression of Vimentin and ZEB1 (Fig. 5b). We measured the surface expression of PD-L1 by FACS and found that PD-L1 expression was clearly increased in TE5, TE6 and TE11 cells (Fig. 5c–e). These results revealed that TGF-β1-induced ZEB1 expression resulted in EMT phenotype and high surface expression of PD-L1 in ESCC cell lines having cobblestone-like epithelial phenotype.

**Discussion**

Many reports have demonstrated PD-L1 expression in many types of human cancer. However, the mechanism and pattern of PD-L1 expression in ESCC has been poorly understood. In the present study, patients with PD-L1 positive expression had significantly poorer prognosis than those with negative expression. A previous study reported that prognosis was not related to PD-L2 expression in ESCC. Our previous study reported that among patients with high expression of HLA class I, high PD-L1 expression was correlated with significantly poorer RFS and OS in ESCC. These results
implied that compared with patients with low HLA class I expression, patients with high HLA class I expression might be more likely to be affected by the PD-1/PD-L1 interaction that inhibits the CD8+ T cell response and permits tumor progression. In the current study, we focused on PD-L1 expression at the invasive front of ESCC. Our data showed that overexpression at the invasive front was associated with advanced stage and poor survival in ESCC, suggesting that PD-L1 expression status at the deep invasive site might be a critical predictor of malignant potential of ESCC.

For patients in whom overexpression of tumor PD-L1 was observed prior to treatment, anti-PD-1/PD-L1 directed therapy could lead to improved clinical outcomes. Thus, high PD-L1 expression may be a predictive marker for efficacy of PD-1/PD-L1 directed immune therapy. We evaluated CD8+ expression to evaluate the immune response at the invasive front of ESCC. One mechanism that limits the host immune response in cancer tissues is via upregulation of PD-L1 and the combining of PD-L1 to PD-1 on antigen-specific CD8+ T cells, which are controlled by two mechanisms: innate immune resistance and adaptive immune resistance. Several reports showed that the relationships between CD8+ T cell and PD-L1 expression were either an equilateral correlation or inverse correlation. This controversy may be a result of the differences in fields focused on the histological specimens. In this report, CD8+ T cell infiltration was reversely correlated with PD-L1 expression, because we focused on the invasive front of cancer tissues. Besides these statistical results, in some cases, high PD-L1 expression on TCs without EMT changes were also observed to be associated with CD8+ lymphocyte infiltration (data not shown). These findings suggest at least two different pathways for PD-L1 expression under the control of adaptive immune resistance in ESCC: one is infiltrating CD8+ lymphocyte-mediated IFN-γ-induced PD-L1 upregulation, and the other is TGF-β-mediated PD-L1 expression associated with EMT. Our in vitro data showing that both IFN-γ and TGF-β upregulated PD-L1 expression of TE cell lines support this hypothesis. Therefore, adaptive immune resistance around PD-L1 expression in cancer cells might accelerate tumor progression at the invasive front of ESCC.

Epithelial-mesenchymal transition is related with metastasis and invasion of cancer. In ESCC, EMT status was significantly associated with invasion, metastasis and prognosis. We previously reported that the transcriptional factor Ets-1 upregulates the expression of c-Met, and consequently confers on cells a highly motile phenotype leading to an EMT-like form. Some studies have reported a relationship between PD-L1 expression and EMT in various cancers. Ock et al. reported that EMT with PD-L1 expression was an independent upstream pathway distinct from human papilloma virus/p16 association in head and neck squamous cell carcinoma. PD-L1 was reported to function in the promotion of
EMT via downregulation of E-cadherin and upregulation of Slug and Twist in skin epithelial cells. Another report showed that the bidirectional effect between EMT status and PD-L1 expression, especially in the Claudin-low subtype of breast cancer cells, was mainly dependent on the activation of the PI3K/AKT pathway. In this study, we proposed a novel ZEB1-PD-L1 pathway and relationship with EMT at the invasive front of ESCC. The ZEB1 transcription factor strongly induces EMT in cancer metastasis, acting through transcriptional repression of E-cadherin. Because the PD-L1 promoter region contains a ZEB1 binding site, we hypothesized that ZEB1 possibly affected PD-L1 expression. We herein report that ZEB1 expression was correlated with PD-L1 expression at the invasive front of ESCC. In ESCC cell lines, siZEB1 suppressed PD-L1 expression through the ZEB1-PD-L1 pathway and TGF-β1 inducing EMT. Our data implied that ZEB1 transcription factor is upstream of the PD-L1 signal pathway and regulates PD-L1 expression, which simultaneously induces EMT and avoidance of the immune system. Chen et al. reported that microRNA-200 (miR-200) targeted PD-L1 and ZEB1 relieved miR-200 repression and PD-L1 on tumor cells leading to CD8+ T-cell immunosuppression and metastasis in lung cancer. Similarly, Noman et al. reported that the selective upregulation of PD-L1 was dependent on the ZEB1/miR-200 axis in breast cancer. In this article, we designed our study focus based on the PD-L1 gene promoter region containing a binding site for ZEB1. No report has previously investigated the PD-L1 gene promoter region for ZEB1 binding so far. We first showed relationship of PD-L1 and ZEB1 by focusing on the gene promoter region. However, we consider that ZEB1 might have two signal pathways in the tumor microenvironment: one by direct regulation through the gene promoter region, and another by indirect regulation through miR-200.

Several factors, such as IFN-γ, TLR, JAK/STAT and viruses, regulate PD-L1 expression. PD-L1 is induced under inflammatory conditions, triggered by several cytokines, especially IFN-γ, and exogenous stimuli delivered by pathogen-associated molecular patterns. Receptor-mediated signaling molecules that affect the cell cycle, proliferation, apoptosis, and survival (including NF-κB, MAPK, PI3K, mTOR, and JAK/STAT) are involved in PD-L1 induction. Upregulation of PD-L1 in immune cells and several cancer cells is heavily dependent on TLR- or IFN-γ-mediated signaling pathways. Several reports have shown that TGF-β1 induces ZEB1 expression and EMT. Our data showed that TGF-β1 induced ZEB1, and subsequently induced PD-L1 expression in ESCC, which is consistent with several reports showing PD-L1 induction by TGF-β1.

In conclusion, PD-L1 expression at the invasive front was related to ZEB1 expression, EMT and poor prognosis in ESCC. Whether the ZEB1-PD-L1 signal pathway could be a target in treatment for ESCC requires further investigation.

Acknowledgments
The authors thank fellowship researcher Koji Teraishi and are grateful for technical assistance from Saori Tsurumaru and Yuko Kubota.
Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Prognosis according to ZEB1 expression in the patients with esophageal squamous cell carcinoma. (a) Overall and (b) relapse free survival rates of patients with esophageal squamous cell carcinoma in relation to ZEB1 expression status.

**Fig. S2.** PD-L1 expression after siTwist and siSnail transfection. TE8 cells were transfected by siTwist (a, b) and siSnail (c, d) or nontargeting (NT) siRNA for 48 h, Two different siRNAs directed against Twist and Snail were used. (a) Twist, (b) PD-L1, (c) Snail, and (d) PD-L1 mRNA expression levels were analyzed by quantitative real-time PCR. The results are expressed as fold mRNA expression levels of NT siRNA treated cells (arbitrarily defined as 1) (*P < 0.001).

**Table S1.** Univariate and multivariate analyses of RFS and OS in patients with esophageal squamous cell carcinoma who underwent esophagectomy.

**Table S2.** Clinicopathological features according to ZEB1 expression in patients with esophageal squamous cell carcinoma who underwent esophagectomy.