Programmed death-ligand 1 is prognostic factor in esophageal squamous cell carcinoma and is associated with epidermal growth factor receptor

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Esophageal cancer is the 6th leading cause of cancer-related mortality and 8th most common cancer in the world, with an estimated 456,000 new cases per year worldwide.1,2 In China, esophageal cancer is the 4th leading cause of cancer-related mortality, and esophageal squamous cell carcinoma (ESCC) is the predominant histological type, accounting for more than 90% of the cases of esophageal cancer. Esophagectomy remains the standard treatment for resectable esophageal cancer.3 Although neoadjuvant chemoradiotherapy may improve survival rates, only a few studies have found long-term survival rates exceeding 40%.4 More than 50% of patients experience local-regional recurrence or distant metastasis.5,6 Thus, new prognostic and therapeutic strategies are urgently needed.

Programmed death-ligand 1 (PD-L1) expression either indicates immune inhibitory status or concurrent immune response. Although the relationship between PD-L1 and clinical outcomes has been studied widely in recent years, its role in prognosis of esophageal squamous cell carcinoma (ESCC) remains unclear. Here, we assessed the significance of PD-L1 in ESCC and its association with epidermal growth factor receptor (EGFR) and radiation response. We found that PD-L1 was present both on the surface of tumor cells and tumor-infiltrating immune cells. Patients with tumor-infiltrating immune cell PD-L1 expression had better survival. PD-L1 expression on immune cells was an independent prognostic factor for patients with ESCC. PD-L1 expression either on tumor-infiltrating immune cells or tumor cells was negatively associated with EGFR expression. EGFR/PD-L1 pairs could separate the survival between EGFR low/PD-L1 positive and EGFR high/PD-L1 negative groups. In ESCC cell lines with EGFR high expression, PD-L1 expression was induced significantly when EGFR signaling was activated by radiation and was dramatically inhibited by an EGFR tyrosine kinase inhibitor. In conclusion, tumor-infiltrating immune cell PD-L1 expression is an independent prognostic factor for ESCC, and the association between EGFR and PD-L1 is vital to determining survival. It is important to consider radiotherapy-induced imbalance of pro-tumor and anti-tumor immune response. A combination of radiotherapy and PD-L1-targeted therapy could be a promising therapeutic strategy for ESCC patients.
Programmed death-ligand 1 (PD-L1; also called B7-H1 or CD274), which is expressed on many cancer cells and tumor-infiltrating immune cells (ICs), plays an important role in tumor immune escape by binding its receptors programmed death-1 (PD-1) and B7.1 (CD80), both of which are negative regulators of T lymphocyte activation. Several studies have shown that PD-L1 expression on tumor cells (TCs) is associated with worse prognosis in patients with renal cell carcinoma and cancers of the lung, breast, pancreas, liver, and ovary. However, recent studies have reported inconsistent results. In non-small cell lung cancer, breast cancer, metatstatic melanoma, and colorectal cancer, PD-L1 expression has been associated with improved survival rates, indicating that induction of the PD-1/PD-L1 pathway may present an occurred or ongoing adaptive antitumor response. To date, the study of the role of PD-L1 and its related immune status in prognosis of human esophageal cancer is not sufficient.

Multiple studies in recent years have focused on the relationship between PD-L1 and epidermal growth factor receptor (EGFR) activation, but the results have been controversial. In studies of non-small cell lung cancer, it has been reported that PD-L1-positive status was significantly associated with the presence of EGFR mutations. Activation of an EGFR mutant gene induced PD-L1 expression that contributed to tumor immune escape. However, another study reported no significant relationship between PD-L1 and EGFR/Kirsten rat sarcoma viral oncogene homolog (KRAS) expression in lung adenocarcinoma. A recent study of head and neck cancers indicated that overexpressed EGFR mediated immune evasion by upregulating PD-L1 expression. Although EGFR is the most studied gene in esophageal cancer research, its association with PD-L1 remains to be determined.

We previously reported that patients with high EGFR expression benefited from postoperative radiotherapy; however, the survival rate in these patients was still lower than in those with low EGFR expression who received surgery only. Though radiation exposure could induce or strengthen adaptive antitumor response, PD-L1 also increased immediately in response to interferon-γ (IFN-γ) which was produced by activated lymphocytes in irradiated tissue, leading to the development of adaptive resistance and might reduce therapeutic effect. Nonetheless, the direct effects of radiotherapy on PD-L1 expression in EGFR-overexpressing ESCC tumor cells are not yet known.

This study aimed to determine the role of immunity in the prognosis of ESCC patients. We assessed the prognostic potential of PD-L1 expression in patients with ESCC. We also investigated the association between EGFR and PD-L1 in tumor tissues and the effect of radiation exposure on PD-L1 expression in ESCC cell lines with high EGFR expression. Our study provides new insight into identifying a potential predictor for prognosis of ESCC and combination of radiotherapy with PD-1/PD-L1 targeted immunotherapy.

Materials and Methods

Tissue samples. The resected tumor samples had been collected for our previous study conducted between September 1986 and December 1997. We retrieved 344 formalin-fixed and paraffin-embedded archived tissue samples, with 197 samples from patients who received surgery alone and 147 from patients who received postoperative radiotherapy. The Institutional Review Board of Cancer Hospital, Chinese Academy of Medical Science, approved the study protocol. All the experiments were conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all subjects.

Patient follow-up. Patients had been instructed to return for follow-up, including a clinical examination, barium swallow test, chest radiography, abdominal ultrasonography, and thoracic computed tomography, at 3- to 6-month intervals for 1 year, and then every 6–12 months thereafter. All patients were followed through the end of December 2010. The patient demographic and other clinical data from a prospectively maintained database. Details have been described in a previous study.

Immuochemistry. The paraffin-embedded tumor samples were sectioned to 5 μm slices. The tissue section slides were deparaffinized and rehydrated. For PD-L1 immunohistochemistry, antigen retrieval was achieved in boiled ethylenediaminetetraacetic acid buffer (pH 8.0) for 10 min. The tissue section slices were incubated with peroxidase blocking reagent (3% hydrogen peroxide solution) for 10 min and then washed in phosphate-buffered saline (PBS) solution three times to block the endogenous peroxidase. The slices were incubated with the primary anti-PD-L1 antibody (clone SP142; Spring Bioscience, Pleasanton, CA, USA) in a humidified chamber overnight at 4°C. After being washed with PBS/0.05% Tween three times, the slices were incubated with MaxVision enhanced reagent (catalog no. KIT-5020, Fuzhou Maixin Biotech, Co., Ltd., Fuzhou, China) for 20 min at room temperature and washed again in PBS/0.05% Tween. The slices were then incubated with MaxVision HR-polymer antibody (rabbit secondary antibody) (catalog no. KIt-5020, Fuzhou Maixin Biotech) chamber for 30 min. We then stained the slices with 3,3′-diaminobenzidine (catalog no. ZLI-9018; Zhongshan, Beijing, China). PD-L1 expression was evaluated on both TCs and tumor-infiltrating ICs. The scoring was performed by two experienced pathologists who were blinded to the clinical outcome data independently.

PD-L1 immunostaining was semiquantitatively evaluated as previously described both on TCs and tumor-infiltrating ICs. The proportion of PD-L1 positive cells was estimated as the percentage of total TCs or ICs: 0, 0%–1%; 1, 1%–5%; 2, 5%–10%; 3, >10%. PD-L1 expression was scored in at least five microscopic fields. PD-L1 positivity was defined as ≥5% of ICs or TCs with staining for PD-L1. The mean percentage of PD-L1 positive cells in each sample of the five fields was calculated.

The result of EGFR expression in tumor cells was from our previous study. Briefly, for EGFR score, the intensity was classified as follows: 0, negative staining; 1, weak staining; 2, moderate staining; 3, strong staining. The rate of positive cells was recorded: 1, 0%–25%; 2, 26%–50%; 3, 51%–75%; 4, greater than 75%. A final score was achieved by multiplication of the intensity (0, 1, 2, and 3) and the rate of positive cells (1, 2, 3, and 4). For data analysis, scores of less than 8 were defined as low expression and scores of 8 or more, as high expression.

Cell culture. Esophageal squamous cell lines with wild-type EGFR expression kysre30, TE1, and CaEs-17 were purchased from ATCC. All cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mmol/L L-glutamine, 100 units/mL penicillin (Hyclone, GE Healthcare Life Sciences, Logan, UT, USA), 100 μg/mL streptomycin (Hyclone), and 10% fetal calf serum (FCS) (Gibco, Thermo-Fisher Scientific, Grand Island, NY, USA). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.
and used in the experiments when in the logarithmic growth phase.

**Radiation exposure.** Complete culture media (10% FCS in RPMI 1640 media) was replaced by RPMI 1640 media w/o EGFR tyrosine kinase inhibitor AG1478 (10μM) before exposure of cells to radiation for 30 min. Then cells were exposed to 6 MV X-rays at 0, 2, 4 or 8 Gy. 24 h later, the cells were collected and tested using flow cytometry or Western blot.

**Flow cytometry.** The cells were collected and washed twice in cold flow cytometry washing buffer (pH7.4 PBS containing 0.2% (w/v) bovine serum albumin). The cells were then resuspended in cold staining buffer to a final concentration of 10⁷ cells/mL. We aliquoted 100μL of the cell suspension into each tube and added 5μL of directly conjugated primary antibodies (PE-EGFR, catalog no. 55997; APC-PD-L1, catalog no. 563741, BD Biosciences, San Jose, CA, USA). The cells were incubated for 30 min on ice in the dark, then washed twice with staining buffer to remove the unbound antibody. The samples were analyzed by flow cytometry immediately.

To test the whole expression of EGFR on both cell surface and in cytoplasm, we performed the fixation and permeabilization with a BD Cytofix/Cytoperm kit (catalog no. 554714; BD Biosciences) according to the manufacturer’s instructions after the surface staining. Then, we stained the intracellular EGFR protein.

**Western blot.** Cells were lysed and protein was extracted using mammalian protein extraction agent (ThermoFisher Scientific Pierce, Waltham, MA, USA) plus Halt protease inhibition cocktail (ThermoFisher Scientific Pierce). Protein concentrations were determined using a bicinchoninic acid assay (ThermoFisher Scientific Pierce). Aliquots of protein lysates were separated on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred onto a nitrocellulose membrane, which was blocked with 5% blotting grade milk (BioRad, Hercules, CA, USA) in PBST (0.1% Tween 20 in PBS). The membrane was then hybridized with primary antibodies to human EGFR and phospho-EGFR (Tyr1068). We also tested the level of STAT3 and phospho-STAT3 (Tyr705) as STAT3 has been identified to regulate PD-L1 transcriptionally. Then, with the corresponding secondary antibodies conjugated with horseradish peroxidase, and detected using a chemiluminescence assay (EMD Millipore, Temecula, CA, USA), membranes were exposed to X-ray film (Kodak China Investment, Shanghai, China) to visualize the bands. β-actin was used as loading control. All primary and secondary antibodies used in the Western blotting were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Statistical methodology.** Statistical analysis was performed using SPSS version 23.0 software (SPSS, Chicago, IL, USA). The survival rate was examined using the Kaplan–Meier method, and differences were assessed using the log-rank test. Associations were analyzed using Pearson’s χ² test. The multivariate analysis was performed using a Cox regression model. The survival curves were plotted using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Survival time was calculated from the date of surgery to date of death or last follow-up. The disease-free survival (DFS) rate was calculated from the date of surgery to the date of first local or distant recurrence or last date of follow-up. To compare the two PD-L1-positive groups by radiation exposure, we used one-way ANOVA followed by Least-significant Difference test, with P < 0.05 considered significant.

**Results**

**Patient characteristics and immunohistochemistry.** A total of 344 patients with ESCC were included in the study. Patient demographic and clinicopathologic characteristics are summarized in Table 1. PD-L1 staining was observed on TCs and on tumor-infiltrating ICs (Fig. 1). PD-L1-positive tumor-infiltrating ICs were more common than PD-L1-positive TCs (ICs, 85 samples [24.7%]; TCs, 50 samples [14.5%]). Low EGFR expression was observed in 86 samples (25.5%) and high EGFR expression in 258 samples (75.0%) in PD-L1 expression on both TCs and tumor-infiltrating ICs was negatively correlated with EGFR expression (ICs, P = 0.02; TCs, P = 0.01) (Table 1). There was a positive association between TC and IC PD-L1 expression (Table S1).

**Relationship between PD-L1 expression and survival.** Firstly, we analyzed the relationship between the overall survival (OS)
rate and PD-L1 expression on tumor-infiltrating ICs. For the group with positive PD-L1 expression, the 5-year OS rate was 37.6%, with a median survival time of 31.6 months; for the group with negative PD-L1 expression, the 5-year OS rate was 20.5%, with a median survival time of 17.0 months. The differences of both OS and DFS rates were greater between these two groups than between groups with low versus high EGFR-(18) or negative versus positive PD-L1 expression, which are both associated with higher survival rates, and groups with low EGFR and positive PD-L1 expression, which are both associated with higher survival rates. As shown in Figure 3, the differences in survival were greater between these two groups than between groups with low versus high EGFR-(18) or negative versus positive PD-L1 expression (Fig. 2). The results indicated that ESCC patients with low EGFR/positive PD-L1 expression had much better survival rates and durations no matter whether PD-L1 was localized on TCs or tumor-infiltrating ICs (ICs: OS, 53.3%, 62.7 months vs 22.8%, 19.5 months,  P = 0.001; DFS: 63.2%, 76.4 months vs 42.7%, 60.0 months,  P = 0.001) (Fig. 2b,d).

We also compared survival rates between the groups with positive and negative PD-L1 expression on TCs. No significant difference was found between these two groups (OS: 36.0%, 23.6 months vs 22.8%, 19.5 months,  P = 0.086; DFS: 36.0%, 18.5 months vs 21.4%, 14.7 months,  P = 0.095) (Fig. 2b,d). The results suggested that PD-L1 expressed on tumor-infiltrating ICs, but not TCs, might indicate the prognosis of ESCC patients.

**Correlation between PD-L1 and EGFR expression in patients with ESCC.** We found significant differences in OS rates for patients with different EGFR-(18) and tumor-infiltrating IC PD-L1 expression, but not TC PD-L1 expression. Additionally, PD-L1 expression both on tumor-infiltrating ICs and TCs was negatively related to EGFR expression (Table 1). We wondered whether we could improve the separation of survival rate by combining groups with high EGFR and negative PD-L1 expression, which are both associated with lower survival rates, and groups with low EGFR and positive PD-L1 expression, which are both associated with higher survival rates. As shown in Figure 3, the differences in survival were greater between these two groups than between groups with low versus high EGFR-(18) or negative versus positive PD-L1 expression (Fig. 2). The results indicated that ESCC patients with low EGFR/positive PD-L1 expression had much better survival rates and durations no matter whether PD-L1 was localized on TCs or tumor-infiltrating ICs (ICs: OS, 53.3%, 62.7 months vs 22.8%, 19.5 months,  P = 0.001; DFS: 63.2%, 76.4 months vs 42.7%, 60.0 months,  P = 0.001) (Fig. 2b,d).

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groups; and patients with EGFR low/PD-L1 positive expression had the longest survival (Fig. S1).

**Multivariate analysis.** We performed a multivariate analysis to determine independent predictors of OS and DFS. As shown in Table 2, age, sex, T stage, N stage, histologic grade, treatment modality, PD-L1 expression on ICs, and EGFR expression were all independent factors that predicted OS. Age, sex, T stage, N stage, histologic grade, treatment modality, and PD-L1 expression on ICs were independent factors that predicted DFS, but EGFR expression was not an independent prognostic factor for DFS.

**Radiation induction of PD-L1 in ESCC cell lines with high EGFR expression.** With the goal of comparing radiation-induced PD-L1 expression in ESCC cell lines with different level of wild-type EGFR expression, we treated ESCC cell lines using six MV X-rays at varying doses. We selected three ESCC cell

![Fig. 2](image-url)

**Fig. 2.** Patients with PD-L1 positive expression showed better survival. Kaplan–Meier survival curves for PD-L1 expression and overall survival (a and b) and disease-free survival (c and d). PD-L1 was expressed on tumor-infiltrating immune cells (a and c) or tumor cells (b and d).

![Fig. 3](image-url)

**Fig. 3.** Kaplan–Meier survival curves according to PD-L1 and EGFR expression. Overall survival and disease free survival was compared between groups with high EGFR/negative PD-L1 and low EGFR/positive PD-L1 expression. PD-L1 was expressed on tumor-infiltrating immune cells (a and c) or tumor cells (b and d).
lines of kys30, TE1, and CaEs17, which have high, moderate, and low EGFR expression, respectively (Fig. 4a). After 24 h of radiation exposure, PD-L1 expression on the surface of the tumor cells increased significantly in a dose-dependent manner and corresponding with different levels of EGFR expression (Fig. 4a,b). Next, we assessed the changes of PD-L1 expression when EGFR activation was inhibited by the EGFR tyrosine kinase inhibitor AG1478 before radiation. AG1478 inhibited radiation-induced PD-L1 expression dramatically, to levels even lower than those found in the un-irradiated controls (Fig. 4c). Western blot results confirmed that radiation induced phosphorylation of EGFR in all three cell lines, and that AG1478 blocked EGFR phosphorylation. The level of phosphorylated STAT3 increased after cells were exposed to radiation and decreased after administration of AG1478 only in kys30 and TE1 cells, not CaEs17 cells (Fig. 4d).

Discussion

In the present study, we found that PD-L1 expression on tumor-infiltrating ICs is an independent prognostic factor in patients with ESCC; patients with IC PD-L1 expression had improved survival rates and durations. PD-L1 expression on both TCs and tumor-infiltrating ICs was negatively correlated

Table 2. Multivariate analysis of overall survival rate and disease-free survival rate

| Variable                              | Overall survival |      | Disease-free survival |      |
|---------------------------------------|-----------------|------|-----------------------|------|
|                                       | Hazard ratio (95% CI) | P-value | Hazard ratio (95% CI) | P-value |
| Age (ref. < 60 years)                 | 1.393 (1.099–1.766) | 0.006 | 1.370 (1.085–1.730) | 0.008 |
| Sex (ref. male)                       | 0.745 (0.563–0.986) | 0.040 | 0.791 (0.599–1.045) | 0.098 |
| T stage (ref. T1)                     | 1.420 (1.154–1.747) | 0.001 | 1.371 (1.120–1.679) | 0.002 |
| N stage (ref. N0)                     | 1.907 (1.491–2.440) | 0.000 | 1.980 (1.554–2.524) | 0.000 |
| Histologic grade (ref. high)          | 1.221 (1.014–1.471) | 0.035 | 1.268 (1.055–1.523) | 0.011 |
| EGFR expression (ref. low)            | 1.360 (1.033–1.790) | 0.029 | 1.251 (0.948–1.650) | 0.114 |
| ICs PD-L1 expression (ref. low)       | 0.720 (0.553–0.939) | 0.015 | 0.671 (0.516–0.874) | 0.003 |
| Treatment, S or S+RT (ref. S)         | 0.729 (0.577–0.921) | 0.008 | 0.684 (0.542–0.863) | 0.001 |

CI, confidence interval; EGFR, epidermal growth factor receptor; ICs, tumor-infiltrating immune cells; S, surgery; RT, radiotherapy. P ≤ 0.05 was considered statistically significant.

Fig. 4. Radiation induced PD-L1 in ESCC cells with high EGFR expression. (a,b) PD-L1 expression increased after radiation exposure. Complete culture media (10% fetal calf serum in RPMI 1640) was replaced by RPMI 1640 before exposure of the cells to radiation for 30 min. After 24 h, the cells were collected and tested using flow cytometry. Columns indicate mean values; bars indicate standard deviation. ▲, P < 0.05 compared with data of 0 Gy. (c). AG1478 inhibited the radiation-induced expression. AG1478 (10 lM) was added before cells were exposed to radiation for 30 min. PD-L1 expression was tested 24 hours later by flow cytometry. ▲, P < 0.05 compared with corresponding solvent-only control. (d) Changes in protein phosphorylation by radiation. Cells were treated as above and collected 30 min after exposure to radiation for Western blot assay. All experiments were repeated three times.
with tumor EGFR expression. EGFR/PD-L1 pairs could more greatly separate the difference of the survival between groups with high EGFR/negative PD-L1 and low EGFR/positive PD-L1 expression when compared with that between the negative/positive PD-L1 or high/low EGFR groups. PD-L1 expression in ESCC cell lines with EGFR high expression increased when EGFR signaling was activated by radiation exposure.

Our present results showed that tumor-infiltrating IC PD-L1 expression was associated with longer OS and was an independent predictor in patients with ESCC. Several studies have shown an association between PD-L1 protein expression and survival durations; interesting, the improved survival was also related with increased numbers of tumor-infiltrating lymphocytes (TILs). A recent study of a large and relatively homogeneous group of patients suggested that the presence of intense tumor IC infiltrates was a favorable prognostic marker for survival in patients with resected non-small cell lung cancer. In our study, we further pinpointed the predictive factor as PD-L1 expression on tumor-infiltrating ICs. The upregulation of PD-L1 in the tumor microenvironment not only presents immune suppression but is also associated with endogenous antitumor immune activity. The patient outcomes, therefore, are influenced by both immune suppression and activation. Since the tissue samples were all collected before the patients received postoperative radiotherapy, PD-L1 expression exhibited here presented naive immune response.

We found patients with IC PD-L1 expression, but not TC PD-L1 expression, had improved survival. Our result agreed with a recent study which showed that PD-L1 expression on tumor cells was not correlated with PFS or OS in patients with locally advanced non-small cell lung cancer, but CD8+ TIL density was. A study of multiple types of cancer using the same monoclonal antibody clone as ours (cloneSP142, Spring, USA) also indicated that patients with PD-L1 expression on tumor-infiltrating ICs, which stained as macrophages, dendritic cells, and T cells, had better responses to PD-L1 blockade therapy and improved survival. Tumor-infiltrating ICs in the tumor microenvironment are much closer to antitumor lymphocytes. They are probably more sensitive to PD-L1 induction by IFN-γ released from these cells. The presence of tumor-infiltrating ICs expressing PD-L1 may implicate the development of an ongoing immune response. In our study, we did not distinguish different subsets of immune cells. The effect of immune infiltrates on prognosis was mediated by both pro-tumor and anti-tumor immune populations, but no evidence suggested this balance could be used to predict outcomes in individual patients. It probably exists complicated interaction among different subsets of tumor-infiltrating ICs, which is a fascinating topic deserving to be explored in further study.

We found that PD-L1 expression in both TCs and tumor-infiltrating ICs was negatively related to EGFR expression. Much more sophisticated regulatory network links EGFR and PD-L1 in vivo than in vitro. Activation of the EGFR pathway might involve suppressing the immune response in murine melanoma models, either through activating regulatory T cells or reducing the levels of the T cell chemoattractant. It is possible that in patients with high EGFR expression, the endogenous antitumor immune response was inhibited, which could be partially reflected by decreased PD-L1 expression and devote to the high rates of recurrence and metastasis and shorter survival time for these patients. We found that EGFR/ PD-L1 pairs could further separate the survival between ESCC patients with high EGFR/negative PD-L1 and low EGFR/positive PD-L1 expression. As patients with low EGFR/positive PD-L1 tumors have their antitumor immune potential, their median OS time was much longer than that of patients with high EGFR/negative PD-L1 expression.

Two mechanisms are involved in regulation of PD-L1 expression: native and adaptive. In adaptive resistance, PD-L1 expression can be induced by IFN-γ which is released from activated tumor-specific T cells by chemotherapy and radiotherapy. The present study, we found low levels of PD-L1 expression in ESCC cell lines cultured in starved conditions, which was associated with EGFR expression and was sensitive to EGFR inhibitors (data not shown), indicating that in vitro condition, EGFR signaling pathways might participate in the development of intrinsic resistance of normal PD-L1 expression in TCs. More importantly, our results showed that radiation directly and significantly deregulated PD-L1 expression on TCs and the level of induced PD-L1 was associated specifically with the level of EGFR overexpression. It has been reported that mutant EGFR pathway activation induced PD-L1 expression in both bronchial epithelial cells and non-small cell lung cancer cells. We demonstrated here that PD-L1 expression was upregulated following radiation induced activation of wild-type EGFR signaling in ESCC cells and that an EGFR-specific inhibitor could block this activation and efficiently inhibit the radiation-induced PD-L1 expression. This finding is consistent with the notion that EGFR signaling activation deregulated PD-L1 in ESCC. The phosphorylation of STAT3 changed along with the phosphorylation of EGFR in EGFR-high and -moderate cells, but did not increase in EGFR-low cells after radiation, indicating that EGFR-STAT3 might be involved in the regulation of PD-L1 expression. Our study, therefore, has described an additional mechanism of radiation-induced adaptive immune resistance via direct modulation of PD-L1 expression in TCs. The detailed downstream signaling pathways were not explored in this study, which require further research in future.

We found that PD-L1 expression in ICs suggested better prognosis. This PD-L1 expression exhibited a balance between pro-tumor and anti-tumor capability in ESCC patients before they received any treatment (in our study, postoperative radiotherapy or not) and indicated ongoing immune response. While high EGFR expression in tumors devotes to immunological suppression. Accordingly, the negative association between EGFR and PD-L1 expression was found in the tissue samples; and patients with high EGFR/negative PD-L1 expression had much worse survival than that of patients with low EGFR/positive PD-L1 expression. Interestingly, we also found induced PD-L1 expression in radiated ESCC cells with EGFR high expression, which was directly regulated by activated EGFR signaling resulting from radiation exposure. The effect of radiotherapy on anti-tumor immune ability has two sides. Radiation leads to cell death and promotes anti-tumor immune response by increased release of tumor antigens, maturation of antigen processing cells and production of co-stimulus molecules. Simultaneously, inhibitory immune factors emerge soon after radiotherapy. At this time point, the upregulated PD-L1 expression represents negative feedback mechanism against activated anti-tumor immune response and might be disadvantageous to patient survival. In our previous study, we found patients with high EGFR expression would benefit from postoperative radiotherapy. However, in the present study, we did not find significant differences of survival among patients who had either positive or negative PD-L1 expression and received postoperative radiotherapy or not (Figs S2, S3). This result suggests that besides induced tumor cell death, negative
immune mechanisms such as expression of checkpoints both on tumor and stromal cells in tumor microenvironment complicates the outcome of the treatment and consequent survival.

In conclusion, our results show that expression of PD-L1 on tumor-infiltrating ICs is an independent prognostic factor for patients with ESCC and the association between EGFR and PD-L1 is vital to survival. A combination of radiotherapy and targeted therapy against PD-L1 or its associated signaling pathway may reveal new treatment strategies for patients with ESCC.

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Disclosure Statement

The authors declare that they have no competing interests.

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Supporting Information

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

**Table S1.** Association between tumor cell and tumor-infiltrating immune cell PD-L1 expression by χ² analysis.

**Fig. S1.** Kaplan–Meier survival curves according to different PD-L1 and EGFR expression.

**Fig. S2.** Kaplan–Meier survival curves according to tumor-infiltrating immune cell PD-L1 expression and postoperative radiotherapy.

**Fig. S3.** Kaplan–Meier survival curves according to tumor cell PD-L1 expression and postoperative radiotherapy.

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