Epithelial PD-L2 Expression Marks Barrett’s Esophagus and Esophageal Adenocarcinoma

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Abstract

Esophageal adenocarcinoma is an increasingly common disease with a dismal 5-year survival rate of 10% to 15%. In the first systematic evaluation of the PD-1 pathway in esophageal adenocarcinoma, we identify expression of PD-L2 in cancer cells in 51.7% of esophageal adenocarcinomas. Epithelial PD-L1 was expressed on only 2% of cases, although PD-L1 cancer cells in 51.7% of esophageal adenocarcinomas. We also evaluated expression in the precursor lesion of esophageal adenocarcinoma, Barrett’s esophagus, which emerges following gastric reflux–induced esophageal inflammation, and found PD-L2 expression in Barrett’s esophagus but not in non–Barrett’s esophagus epithelias. Because the progression from squamous esophagitis to Barrett’s esophagus is accompanied by a transition from a Th1 to Th2 immune response, we hypothesized that the Th1/Th2 cytokines IL4/IL13 could contribute to PD-L2 induction. We confirmed that these cytokines can augment PD-L2 expression in esophageal adenocarcinoma cell lines. These results suggest that the inflammatory environment in Barrett’s esophagus and esophageal adenocarcinoma may contribute to the expression of PD-L2. Furthermore, the potential for PD-1 receptor blockade to be effective in esophageal adenocarcinomas with epithelial PD-L2 or immune cell PD-L1 expression should be evaluated in clinical trials.

Introduction

The incidence of esophageal adenocarcinoma has increased dramatically in the Western world in the past decades (1). Five-year survival rates are 10% to 15%, and treatment is largely reliant upon minimally effective cytotoxic chemotherapy (2). Multiple attempts have been made to use molecularly targeted agents, but to date only the monoclonal antibody trastuzumab has proved effective, when used in the approximately 15% of patients with HER2 overexpression (3). There is a pressing need for new therapies.

Because esophageal adenocarcinomas harbor a high somatic mutation burden (4) and develop in a background of chronic inflammation caused by gastric reflux, esophageal adenocarcinomas are potentially immunogenic tumors and therefore promising candidates for immunotherapy. In response to gastric reflux, the lower esophagus often undergoes metaplasia to an intestinalized epithelium. Barrett’s esophagus. Barrett’s metaplasia is associated with a change from an acute (Th1 type) immune response accompanied with IFNγ expression (5, 6) to a Th2-type chronic inflammation with production of IL4/IL13, a transition that potentially induces an immunosuppressive, tumor-promoting environment.

Among the most promising targets in cancer immunology is the programmed cell death protein 1 (PD-1) pathway. PD-1 is a negative costimulatory receptor expressed primarily on activated T cells. The interaction of PD-1 with its ligands, programmed cell death ligand 1 or 2 (PD-L1 or PD-L2), inhibits T-cell activation (7). Expression of PD-L1 on cancer cells and immune cells can inhibit T-cell antitumor response and permit neoplastic growth. Expression of these ligands thus serves as a tool exploited by cancers to avoid immune clearance. PD-1 and PD-L1 inhibitors in melanoma, lung cancer, and renal cancer have shown marked response rates with durable clinical responses (8). However, the PD-1 pathway has not been systematically evaluated in esophageal adenocarcinoma.

The characterization of the inflammatory state is a critical initial step toward a rational development of immunotherapy for esophageal adenocarcinoma. Here, we evaluated PD-1, PD-L1, and PD-L2 expression in a series of 354 esophageal adenocarcinomas. These studies revealed expression of PD-L2 or
PD-L1 in a majority of tumors, raising immediate hypotheses regarding the role of PD-1 pathway activation in esophageal adenocarcinoma and the potential of PD-1–blocking drugs in this deadly disease.

Materials and Methods

Patient series

Tumor tissue microarrays (TMA) were constructed from 354 esophageal adenocarcinomas from the University of Pittsburgh (Pittsburgh, PA). Detailed clinical and pathologic data are summarized in Table 1.

Immunohistochemistry

PD-L1, PD-L2, and PD-L1/CD68 double staining was performed as previously described (refs. 9, 10; described in detail in Supplementary Methods). PD-L1/CD163 double staining was performed with anti-CD163 (Neomarkers). PD-L1 was considered positive if ≥5% of tumor cells had membranous staining or any positive immune cell. PD-L2 expression was considered positive when ≥50% of tumor cells on TMAs or ≥10% of tumor cells on whole-tissue slides had moderate–strong PD-L2 staining in the cytoplasm and/or membrane.

Cell lines

Esophageal adenocarcinoma cell lines OE19, OACM5.1C, ES026, KYAE-1, and FLO-1 were purchased from the European Collection of Cell cultures and authenticated by SNP arrays (2012). OE33 cells were purchased from Sigma and MKN7 cells from the Broad Institute (Cambridge, MA) and characterized within the Cancer Cell Line Encyclopedia project. Only low-passage cell aliquots of original stocks were used for experiments.

IL4/IL13 treatment. Cells were stimulated with human recombinant IL4 (20 ng/mL; BD Pharmingen) and IL13 (20 ng/mL; Peprotech) or medium for 4, 8, and 24 hours. STAT6 expression and phosphorylation were determined by Western blotting (anti-pSTAT6, BD Biosciences; anti-STAT6; Santa Cruz Biotechnology).

Flow cytometry

Cell lines. Adherent cells were harvested with EDTA and stained with anti-human PD-L2 antibody or isotype control (PE; BioLegend).

Statistical analyses

Associations with clinical and pathologic characteristics were analyzed using the Student t test, Pearson χ², or Fisher exact test. Associations with overall survival were analyzed using the Kaplan–Meier method, the log-rank test, and the multivariate Cox proportional hazards model. All P values are two sided. P < 0.05 was considered statistically significant.

Results

Esophageal adenocarcinomas commonly express PD-L2 in cancer epithelial cells

We assayed PD-1, PD-L1, and PD-L2 expression in TMAs containing cores from 354 esophageal adenocarcinomas using antibodies optimized for formalin-fixed, paraffin-embedded (FFPE) tissues (9, 10). The PD-L2 assay was recently validated on FFPE human lymphoid tissues (9). Clinical and pathologic characteristics of the study population are listed in Table 1. A strong majority (289 of 354, 81.6%) of esophageal adenocarcinomas showed evidence of at least weak epithelial PD-L2 expression in one of the cores. In 51.7% (183 of 354) of esophageal adenocarcinomas, we observed moderate–strong PD-L2 epithelial expression in at least one core. In 19.8% (70 of 354) of esophageal adenocarcinomas, all evaluated cores had moderate–strong PD-L2 staining (Fig. 1). Immune cells were negative for PD-L2. Because of the high frequency of weak epithelial PD-L2 expression, only moderate–strong PD-L2 expression was considered positive for further analyses.

PD-L1 and PD-L1 expression in stromal inflammatory cells

PD-L1+ tumor cells were observed in 1.7% (6 of 344) of esophageal adenocarcinomas. PD-L1+ inflammatory cells, however, were observed in 18% (62 of 344) of esophageal adenocarcinomas (Table 1). Morphologically, PD-L1+ inflammatory cells appeared to be macrophages, which was confirmed by PD-L1 double staining with macrophage markers CD68 and CD163 on whole-tumor sections of 16 PD-L1+ esophageal adenocarcinomas (data not shown). PD-L1+ tumor-infiltrating lymphocytes (TIL) were identified in 59.8% (215 of 349) of esophageal adenocarcinomas with one to 181 PD-L1+ TILs identified per core when present.

Coexpression of PD-L2, PD-L1, and PD-1

Although PD-L2 and PD-L1 expression was not mutually exclusive, tumors with PD-L2 expression in all evaluated cores were less likely to possess PD-L1+ immune cells (P = 0.045). Both PD-L2+ and PD-L1+ tumors had a higher average number of PD-L1+ TILs than tumors without PD-L2 or PD-L1 expression (7.2 vs. 3.7, P = 0.052; and 12.6 vs. 2.5, P < 0.001, respectively). In 15.5% (53 of 343) of esophageal adenocarcinomas, no PD-L2+, PD-L1+, or PD-1+ cells were observed.

TMA results were validated using immunohistochemistry (IHC) on whole-tissue slides from 45 tumors. We confirmed expression of PD-L2 in 27 of 30 cases that expressed PD-L2 on the TMA. Tumors with PD-L2 expression in all evaluated cores had a larger percentage of PD-L2+ cells (70%–100% of tumor cells positive) compared with tumors with one or two positive cores (30%–60% of tumor cells positive). Moreover, 6 of 14 tumors lacking evident PD-L2 expression on TMAs showed focal regions with PD-L2 expression in whole-tissue slides, suggesting that PD-L2 expression may be more frequent than suggested by the TMA results. Similarly, an evaluation of whole-tumor slices showed PD-L1+ immune cells in 16 of 45 (35.6%) tumors, of which 7 of 16 were not identified on the TMAs. In 5 of 16 esophageal adenocarcinomas, PD-L1+ immune cells were observed only at the tumor border, whereas in the remaining 11 patients, PD-L1+ cells infiltrated the tumor.
Validation of IHC results

Because this is the first study to report predominant PD-L2 expression in epithelial cancer cells, we next sought to validate our IHC results. We queried PD-L2 epithelial expression in gastroesophageal cell lines OE19, ESO26, OE33, OACM5.1C, FLO-1, KYAE-1, and MKN7 by IHC and identified PD-L2 positivity in OE33 and MKN7 lines (Fig. 2A). These IHC results were concordant with flow cytometry using a distinct PD-L2 antibody and with mRNA expression (Fig. 2A and B). As an additional validation, we identified two esophageal adenocarcinoma patient–derived xenografts with evidence of epithelial PD-L2 expression in the primary tissue and xenograft by IHC. Using fresh xenograft tissue, we confirmed PD-L2 expression by immunoblotting (Supplementary Fig. S1) and flow cytometry, which identified EpCAM/PD-L2 double-positive cells, further validating the IHC findings (Fig. 2C).

PD-L2 expression is detected at the transition of reflux esophagitis to Barrett’s esophagus and can be induced by IL4/IL13

We hypothesized that PD-L2 epithelial expression may also occur in Barrett’s esophagus, the precursor to esophageal adenocarcinoma patient–derived xenografts with evidence of epithelial PD-L2 expression in the primary tissue and xenograft by IHC. Using fresh xenograft tissue, we confirmed PD-L2 expression by immunoblotting (Supplementary Fig. S1) and flow cytometry, which identified EpCAM/PD-L2 double-positive cells, further validating the IHC findings (Fig. 2C).
adenocarcinoma. We evaluated PD-L2 expression via IHC in samples with Barrett’s esophagus (n = 21) and reflux esophagitis without Barrett’s esophagus (n = 14; Fig. 3A). Although no esophagitis samples exhibited PD-L2 expression, 42.8% (9 of 21) of Barrett’s esophagus cases (complete and incomplete intestinal metaplasia, high-grade and low-grade dysplasia) showed

Figure 1.
IHC staining of FFPE esophageal tissues in tissue microarrays (×20). Staining with anti–PD-1 antibody showing PD-1⁺ TILs (A), anti–PD-L1 antibody showing PD-L1⁺ immune cells (B), negative PD-L2 staining (C), weak PD-L2 staining in tumor epithelium scored as 1⁺ (D), moderate PD-L2 staining scored as 2⁺ (E), and strong PD-L2 staining scored as 3⁺ (F). High magnification images to show cytologic details for PD-1 and PD-L1.

Figure 2.
Validation of IHC results with flow cytometry. A, IHC and flow-cytometric results of PD-L2-expressing OE33 and MKN7 cells and PD-L2 nonexpressing ESO26 cells (black, isotype; white, PD-L2). B, PD-L2 mRNA expression in 7 gastroesophageal cell lines. Data are depicted as mean ± SD. C, IHC staining of 2 esophageal adenocarcinoma biopsies with anti–PD-L2 antibody (×20). Staining was scored strong-positive for both tumors. Tumor biopsies were implanted in the flanks of nude mice. At time of passage, tumors were disaggregated into single-cell suspensions and analyzed. Flow cytometry shows coexpression of EpCAM and PD-L2 in 11.9% and 10.2% of EpCAM⁺ cells. PE isotype was used as a control. A representative experiment of two independent experiments is shown.
PD-L2 expression was not observed. PD-L2 epithelial expression. PD-L1 expression in epithelial or immune cells was not observed.

Acquisition of PD-L2 expression with the development of Barrett's esophagus raised the hypothesis that IL4/IL13 expression, which accompanies the transition to Barrett's esophagus, can contribute to PD-L2 induction. We tested this hypothesis in vitro. Treatment of the PD-L2− cell lines OE33 and MKN7 with exogenous IL4 and IL13 induced STAT6 phosphorylation and an increase in PD-L2 mRNA (Fig. 3B). For OE33, an increase in protein expression was observed by flow cytometry (Fig. 3C).

Clinical and pathologic associations of PD-1 pathway member expression in esophageal adenocarcinoma

We next evaluated clinical correlates of PD-1, PD-L1, and PD-L2 expression. In tumors where all cores showed PD-L2 expression, PD-L2 expression was associated with early stage (P = 0.003), smaller tumor size (P = 0.014), and a well-differentiated grade (P < 0.001; Table 1). Moreover, cancers with consistent PD-L2 expression were more likely to have histologic evidence of Barrett's esophagus with intestinal metaplasia (P = 0.030). In contrast, PD-L1 expression was significantly enriched in those tumors lacking evidence of Barrett's esophagus (P = 0.034) or lacking a clinical history of gastroesophageal reflux disease (P = 0.017). Tumors with PD-1+ TILs had higher tumor stage (P < 0.001), were more frequently poorly differentiated (P < 0.001), and a trend toward absence of histologically confirmed Barrett's esophagus (P = 0.056). PD-1 positivity correlated with an increased mortality [univariate Cox regression: HR, 1.89; 95% confidence interval (CI), 1.38–2.6; P < 0.001; Supplementary Fig. S2]. However, the negative association between PD-1 and mortality was lost after adjustment for other prognostic factors. PD-L2 expression showed a trend toward an improved outcome (univariate Cox regression: HR, 0.75; 95% CI, 0.54–1.03; P = 0.078). For PD-L1 expression, no association with survival was observed.

Discussion

Esophageal adenocarcinoma is a highly lethal disease that lacks effective systemic treatment, making exploration of immunotherapy targets of clear importance. This study represents the first systematic effort to characterize expression and clinical correlations of the PD-1 pathway in esophageal adenocarcinoma. Our results show that the vast majority of esophageal adenocarcinomas harbor expression of at least one member of this pathway: expression of PD-1 on lymphoid cells or of ligands PD-L2 on tumor cells or PD-L1 on immune cells. A limitation of our data was the use of TMAs. Given the heterogeneous expression of these markers, our results may underestimate the frequency of expression of these markers in esophageal adenocarcinoma. Furthermore, we evaluated only tumors that have not been treated with chemotherapy or chemoradiation. Nonetheless, our data support the testing of PD-1 pathway inhibitors in esophageal adenocarcinoma. The presence of PD-L2 expression may mark a scenario in which inhibition of PD-1, a receptor for both PD-L1 and PD-L2, may have more efficacy than targeting PD-L1.

A surprising finding in our study was our observation of common PD-L2 expression in esophageal epithelial cells. Although PD-L1 expression has been described in epithelial cancer cells of various lineages (8, 11), predominant PD-L2
expression has been recorded only for primary mediastinal large B-cell lymphoma (9). Although PD-L2 expression has been described in esophageal squamous cell carcinomas (12), cervical carcinomas (13), and hepatocellular carcinomas (14), this was in co-occurrence with PD-L1 epithelial expression. Epstein–Barr virus–positive gastric cancers (15) show mRNA expression of both PD-L1 and PD-L2, which occurs in the setting of 9p24.1 amplification, the locus containing both of these genes. However, these amplifications have been seen only rarely in esophageal adenocarcinoma (4).

These data raise the question of what induces epithelial PD-L2 expression in the absence of PD-L1 coexpression in esophageal adenocarcinoma. In macrophages and dendritic cells, PD-L2 transcription is regulated by IL4/IL13/STAT6 signaling (16, 17). Notably, esophageal adenocarcinomas develop in a background of chronic inflammation and typically emerge from Barrett’s esophagus, a tissue with a documented type 2 inflammatory state with increased IL4/II13 expression (5, 6). PD-L2 epithelial expression in esophageal adenocarcinoma and Barrett’s esophagus may indeed be a consequence of IL4/II13 expression in the immune microenvironment (18).

The finding of PD-L2 expression in esophageal adenocarcinomas and Barrett’s esophagus suggests that PD-L2 may be a component of a larger chronic inflammatory microenvironment that facilitates tumor survival. Recent studies have shown that Th2 polarization in esophageal cancer is associated with the infiltration of myeloid-derived suppressor cells (MDSC) and M2-polarized macrophages (18, 19), suggesting the presence of other immune-evasion mechanisms beyond the PD-1 pathway. Future studies will need to evaluate the implications of strong inflammatory PD-L2 expression in many esophageal adenocarcinoma samples and to determine whether the PD-L2 expression we have observed in esophageal adenocarcinoma functions to mediate immune evasion and specifically predicts response to PD-1 blockade. The role of PD-L2 in inhibiting T-cell responses is controversial. Although PD-L1 and PD-L2 show structural similarity, each ligand has alternative secondary receptors, RGmB for PD-L2 (20) and CD80 for PD-L1 (21). Although a number of studies show an inhibitory role for PD-L2 (22, 23), others suggest that PD-L2 can stimulate T-cell proliferation (24) via a PD-1 receptor–independent mechanism, potentially involving a distinct PD-L2 binding partner.

Beyond PD-L2 expression, we also identified PD-L1+ immune cells in 18% to 36% of esophageal adenocarcinomas. PD-L1 expression may predominate in tumors with a different inflammatory state than PD-L2–predominant tumors. PD-L1 expression is strongly induced by Th1 cytokine IFNγ, whereas PD-L2 is only weakly induced (17), raising the hypothesis that PD-L1+ tumors emerge in the setting of a Th1-type acute inflammation. These findings raise the question as to why esophageal adenocarcinomas harbor distinct checkpoint inhibitors. Although all the tumors in this dataset carry a clinical diagnosis of esophageal adenocarcinoma, there is likely heterogeneity within our collection. Indeed, these tumors may comprise a combination of true esophageal tumors emerging from a Barrett’s esophagus precursor and other tumors that emerged directly from proximal gastric mucosa at the gastro-esophageal junction. The predilection for PD-L1 expression in resection samples without evidence of Barrett’s esophagus in our dataset suggests the hypothesis that tumors that do not emerge from Barrett’s esophagus may have a distinct inflammatory environment. However, because PD-L1+ tumors were also larger and more advanced compared with the PD-L2–expressing tumors in our dataset, we cannot exclude the possibility that the absence of Barrett’s esophagus in these samples may simply reflect overgrowth by the tumor.

Considerable additional work will be needed in the coming years to explore the nature of distinct inflammatory states in esophageal adenocarcinoma and their mechanisms for evading immune attack. Nonetheless, these data demonstrate for the first time that the majority of esophageal adenocarcinomas show evidence of PD-1 pathway activity. These data suggest that exploration of PD-1 inhibition is warranted in this disease and that studies will need to evaluate the potential capacity of PD-1 and both PD-L1 and PD-L2 to serve as biomarkers of response. Furthermore, in PD-L2+ tumors, the presence of secondary mechanisms of immune evasion, such as the presence of M2 macrophages, may also affect response to therapies, potentially leading to tumors in which combination immunotherapeutic approaches may ultimately be required.

**Disclosure of Potential Conflicts of Interest**

G.J. Freeman has ownership interest (including patents) from Amplimmune, Boehringer-Ingelheim, Bristol-Myers Squibb, EMD-Serono, Merck, Novartis, and Roche and is a consultant/advisory board member for Bristol-Myers Squibb, Eli Lilly, Novartis, and Roche. S.J. Rodig has received a commercial research grant from Bristol-Myers Squibb and has submitted a patent on PD-L1 IHC for diagnostics. No potential conflicts of interest were disclosed by the other authors.

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