Programmed Death Ligand 1: A Step Toward Immunoscore for Esophageal Cancer

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Background. **This study sought to evaluate the effect of tumor-infiltrating lymphocyte (TIL) density and programmed death ligand 1 (PD-L1) expression on the prognosis of esophageal cancer.**

Methods. **Banked tissue specimens from 53 patients who underwent esophagectomies for malignancy at a single institution over a 6-year period were stained for cluster of differentiation 3 (CD3), CD8, and PD-L1. Tumors were characterized as staining high or low density for CD3 and CD8, as well as positive or negative for PD-L1. TIL density and PD-L1 expression were analyzed in the context of survival, recurrence, and perioperative characteristics.**

Results. **Median follow-up was 823 days, with 92.5% survival and 26.8% recurrence rates. All tumors were adenocarcinomas. Neoadjuvant chemotherapy was given in 56.6% of cases, and neoadjuvant radiotherapy was given in 37.7%. High CD3 density was found in 83%, whereas high CD8 density was found in 56.6%. A total of 18.9% of the tumors stained positive for PD-L1. Survival was significantly shorter in Kaplan-Meier analysis for patients with primary tumors staining positive for PD-L1 (log rank: $p = 0.05$). Multivariable analysis controlling for neoadjuvant therapy, TIL markers, PD-L1, age, and sex found no significant difference in recurrence or survival.**

Conclusions. **Positive staining for PD-L1 may be a prognostic marker for decreased survival in esophageal adenocarcinoma. Additional TIL cell types should be investigated for creation of an esophageal cancer Immunoscore. PD-L1 has potential as a therapeutic target.**

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The current understanding of solid organ malignancies continues to evolve as we uncover the complex microenvironment of tumor cells, immune cells, and stroma. The characterization of immune cell types and signaling factors surrounding and infiltrating the tumor, an organization termed the immune contexture, has allowed for enhanced prognostication in colorectal, lung, and breast cancers [1–3]. This new framework, known as the Immunoscore, represents a progression in the field of oncology that extends the established tumor, node, metastasis (TNM) staging system to include the density of tumor-infiltrating lymphocytes (TILs), with promising results [4]. Essential to the formation of an Immunoscore for other malignancies is a clear delineation of the relevant immune cell types, their densities, and their infiltration sites, which have been found to vary considerably among organ sites [5].

Inextricable from the immune contexture are the immune signaling pathways used by TILs and exploited by the tumor cells to interrupt the antitumor response. The development of highly effective immunotherapies has harnessed our growing recognition of key checkpoints that can be targeted to counteract the malignancy defense tactics. Major successes in limiting cancer progression and prolonging patients' survival have been achieved using immunotherapies for treatment of several malignancy types, including melanoma, lung cancer, Hodgkin lymphoma, and others [6]. A well-described pathway of this type is the programmed death 1 (PD-1) and its ligand (PD-L1), for which several therapeutic agents are currently available and in use for multiple cancer types [7].

The immune contexture and optimal signaling targets remain largely undefined for esophageal cancer, although success in other cancer types suggests substantial

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potential for improving the management of this malignancy as well. Our group sought to explore the nature of TILs in esophageal cancer by staining surgically resected cancer specimens for immune cell markers cluster of differentiation 3 (CD3) and CD8, known for their prognostic value in lung and colorectal Immunoscoring systems [4], and subsequently stained for PD-L1. The histologic data were analyzed along with clinical data to evaluate the impact on survival and recurrence.

Material and Methods

Specimens were obtained from the tissue bank at a single institution from esophagectomies performed for esophageal adenocarcinomas by three thoracic surgeons over a 6-year period from 2009 to 2015. Clinical data were collected retrospectively for these patients. Cases with benign pathologic features, squamous cell carcinoma, or malignant disease with a pathologic complete response were excluded from the study. Institutional Review Board approval was obtained.

Tissue specimens were fixed in 10% phosphate buffered formalin, embedded in paraffin, with 5-μm-thick sections mounted on glass slides for hematoxylin and eosin staining. Immunohistochemistry stains (IHC) were performed with CD3, CD8, and PD-L1 on representative blocks for each specimen.

PD-L1 IHC staining was carried out with the Dako platform using 22C3 PharmDx kits (Agilent, Santa Clara, CA). The 22C3 clone was selected on the basis of Food and Drug Administration (FDA) approval for PD-L1 staining and prior validation at our institution [8]. Tissue sections mounted on glass slides were pretreated with heat-induced epitope retrieval using diluted EnVision FLEX Target Retrieval Solution at 65°C, then incubated for 20 min at 95°C, and subsequently cooled at 65°C. The treated slides were stained with PD-L1 22C3 pDx Blocking solution (SK006) for 5 min, then PD-L1 22C3 Ab pDx antibody with an incubation time of 30 min, then PD-L1 22C3 pDx Linker (SK006) for 30 min, PD-L1 22C3 pDx Visualization (SK006) for 30 min, PD-L1 22C3 pDx DAB with Substrate-Chromogen (SK006) for 10 min, PD-L1 22C3 DAB Enhancer (SK006) for 5 min, and finally Flex Hematoxylin (SM806) for 5 min. Wash buffer was applied before the addition of each reagent for 5 min to guarantee that no residual remains of any reagent were left on the slide.

CD3 and CD8 IHC staining was carried out with the Dako platform using CD3 polyclonal antibodies and monoclonal CD8 144B antibody. Tissue sections mounted on glass slides were pretreated with heat-induced epitope retrieval using diluted EnVision FLEX Target Retrieval Solution at 65°C, incubated for 20 min at 95°C, and cooled at 65°C. The pretreated slides were subsequently treated with Flex Peroxidase Blocking solution (SM901) CD8 antibody with an incubation time of 15 min, Flex Mouse Linker (SM804) for 15 min, Flex HRP (SM802) for 15 min, Flex DAB with Substrate-Chromogen (SM803) for 10 min, and then Flex Hematoxylin (SM806) for 5 min. Wash buffer was applied before the addition of each reagent for 5 min to guarantee that no residual remains of any reagent were left on the slide.

The IHC-stained slides were independently reviewed by two pathologists using an Olympus microscope (Olympus, Tokyo, Japan) and scored semiquantitatively. A positive PD-L1 stain was defined as, regardless of staining intensity, a membranous staining pattern in 1% or more of tumor cells and TILs. Low positivity was defined as PD-L1 expression in less than 50% of tumor cells. High positivity was defined as 50% or greater on the basis of the FDA-approved algorithm for PD-L1 Immunohistochemistry 22C3 pharmDx Interpretation Manual [9]. Given that only two samples showed high positivity, for the purpose of the statistical analysis, PD-L1 expression results were considered either negative or positive. TIL was semiquantitatively assessed on the basis of the approximate percentage of $CD3^+$ T cells and $CD8^+$ T cells in relation to all nonepithelial cells. CD3 and CD8 TIL density was categorized as low (10% or less) or high (greater than 10%).

Analyses were performed to compare patients whose samples stained positive versus negative for PD-L1 and high density versus low density for CD3 and CD8. The primary outcome evaluated was survival. The secondary outcome was recurrence. Univariate analyses of clinical data were performed using the *t* test for continuous variables, χ^2 test for categorical variables, and Wilcoxon rank-sum test for nonparametric data. Kaplan-Meier estimates were performed to evaluate survival and recurrence. Multivariable analyses were performed using Cox proportional hazard regression models, which included neoadjuvant chemotherapy, age, sex, and densities of CD3 and PD-L1 within tumor specimens. Statistical analysis was performed using STATA statistical software version 14 (StataCorp, College Station, TX).

Results

Fifty-three banked tissue specimens were collected from the designated time period. Clinical data were collected for a median follow-up of 823 days, ranging from 8 to 2,697 days. Overall survival during that time was 92.5%. Overall recurrence was 26.8%. All of the tumors were adenocarcinomas. Neoadjuvant chemotherapy had been administered to 56.6% of these patients and neoadjuvant radiation therapy to 37.7%. PD-L1 was positive in 18.9% of tumors. CD3 was found to be high density for 83% of specimens, whereas CD8 was found to be high density for 56.6%. The TILs stained positive for PD-L1 in 92.5% of specimens. The concordance rate of IHC interpretation between two pathologists was 100% on the basis of FDA-approved scoring algorithms.

At the time of diagnosis 28.6% of tumors were stage I, primarily T1b N0 lesions. Stage II tumors made up 44.6%, including T2 N0, T3 N0, and T2 N1 lesions. Stage III lesions disease comprised the remaining 26.8% of cases. At our institution over the 7-year period, the neoadjuvant treatment paradigm shifted to include more chemoradiotherapy for gastroesophageal junction tumors after the publication of the CROSS (Chemoradiotherapy for