

## PD-L1 Expression in Esophageal and Gastroesophageal Junction Carcinoma, Correlation with the Immune Infiltrate - Preliminary Study

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### Rezumat

*Expresia PD-L1 în carcinomul esofagian și de joncțiune esogastrică, corelată cu infiltratul imun - studiu preliminar*

**Scop:** Deși managementul tradițional pentru cancerul esofagian și esogastric a fost îmbunătățit, supraviețuirea la 5 ani este încă redusă, iar imunoterapia ar putea fi o cale de ameliorare a sa. Pe lângă valoarea predictivă a răspunsului la imunoterapie, PD-L1 are și valoare prognostică cunoscută. Scopul studiului este de a evalua expresia imunohistochimică a PD-L1, CD8+ T-cell, și CD4/CD25+ T-cell (Tregs) în infiltratul limfatic intratumoral în cancerul de esofag și joncțiune esogastrică.

**Material și Metode:** Au fost analizate biopsiile endoscopice la 14 pacienți cu cancer de esofag sau joncțiune esogastrică, înainte de începerea tratamentului neoadjuvant, internați în perioada 2019-2021 în Spitalul Clinic Sf. Maria București. S-au efectuat teste imunohistochimice pentru cercetarea expresiilor markerilor infiltratului limfocitar intratumoral.

**Rezultate:** Din cele 14 cazuri, 13 (93%) au fost bărbați, și o femeie (7%). Histologic, 4 cazuri au fost adenocarcinoame, iar 10 cazuri au fost carcinoame cu celule scuamoase. 10 cazuri au prezentat expresie imunohistochimică pozitivă pentru PD-L1 (78%). Folosind

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o evaluare cantitativă a PD-L1 am obținut o corelație statistică între valoarea mediană a acestui marker și expresia CD8. S-a obținut o corelație statistică între pozitivitatea PD-L1 și expresia scăzută a celulelor T CD4+/CD25+.

**Concluzii:** PD-L1 este exprimat în tumori cu densități mai mari de celule T CD8+ și mai mici de celule pozitive CD4/CD25 (Tregs), indicând că prognosticul bun al tumorilor PD-L1 pozitive s-ar datora mai degrabă inhibiției celulelor pozitive CD4/CD25 (Tregs) decât stimulării de celule T CD8 pozitive, printr-un mecanism de rezistență imună adaptivă.

**Cuvinte cheie:** PD-L1, cancer esofagian, CD8+T cells, Tregs, micromediul tumoral

## Abstract

**Background:** Although traditional management for esophageal and esogastric cancer has been improved, survival at 5 years is still low, and immunotherapy could be a way to improve it. In addition to the predictive value of the response to immunotherapy, PD-L1 also has a known prognostic value. We aimed to evaluate the immunohistochemical expression of PD-L1, CD8+ T-cell, and CD4/CD25+ T-cell (Tregs) infiltration and their relationship in esophageal and gastro-esophageal junction carcinoma.

**Material and Methods:** Endoscopic biopsies were analyzed in 14 patients with esophageal cancer or esogastric junction, before starting the neoadjuvant treatment, hospitalized from the period 2019- to 2021 in the St. Mary's Clinical Hospital, Bucharest. Immunohistochemical tests were performed to investigate the expression of lymphocyte intratumoral infiltrate markers.

**Results:** Of the 14 cases, 13 (93%) were male, and 1 (7%) were female. Histological, 4 cases were adenocarcinomas, and 10 cases were squamous cell carcinomas. 10 cases showed epithelial PD-L1 positivity (78%). Using a quantitative evaluation of PD-L1 we obtained a statistical correlation between the median values of this marker with the expression of CD8. There was obtained a statistical correlation between PD-L1 positivity and low expression of CD4 or CD4+/CD25 T cells.

**Conclusions:** PD-L1 is expressed in tumors with higher CD8+ T cell densities and lower CD4/CD25 positive cells (Tregs), indicating that the good prognosis of PD-L1-positive tumors could be due to the inhibition of CD4 / CD25-positive cells (Tregs) rather than the stimulation of CD8-positive T cells, by an adaptive immune resistance mechanism.

**Key words:** PD-L1, Esophageal cancer, CD8+T cells, TREGS, tumor microenvironment

## Introduction

Esophageal cancer, which includes squamous cell carcinoma and adenocarcinoma, is considered a serious malignancy in terms of prognosis and leads to death in the vast majority of cases (1). Currently, esophageal cancer is the eighth most common cancer worldwide due to its extremely aggressive nature and poor survival rate and affects more than 450,000 people worldwide and the incidence is increasing rapidly (2).

Cancer evades host immune surveillance via the utilization of immune checkpoints (3). Blocking of the PD-1/PD-L1 interaction has been recently shown to be a promising treatment in numerous tumor types, including esophageal cancer, in which it significantly improves survival in combination with chemotherapy, in comparison with chemotherapy alone (4). Nowadays, the level of PD-L1 expression, assessed by immunohistochemistry (IHC), is the biomarker used for indicating eligibility for treatment with immune checkpoint

inhibitors in patients with esophageal cancer (5). PD-L1 is implicated in tumor immune escape by impairing cytokine production and diminishing the cytotoxicity of tumor-infiltrating lymphocytes (TILs), notably activated T cells (6). Marked infiltration of CD8+ cells into a PD-L1 positive tumor has been reported for several cancers and is associated with better overall survival in other cancers (7,8). Regulatory T cells (Tregs), characterized by the co-expression of CD4 and CD25, suppress anticancer immunity (9) and can selectively inhibit the host's immune response, thus contributing to the progression of the disease (10). Besides PD-L1's use as an indicator for eligibility for immune checkpoint inhibitors treatment in cancer, there are a few studies that show that PD-L1 positivity has a positive impact on the prognosis of esophageal carcinoma (11), and other cancers, notably small cell carcinoma of the lung and colorectal carcinoma (12,13). As stated before, it has been suggested that the presence of particular TIL subsets, such as CD8-positive cytotoxic T-cells, correlates with a better prognosis in various malignancies (14). On the other hand, it has been shown that tumor infiltration by CD4/CD25+ T cells harms survival (15). There are no studies that correlate the expression of PD-L1, CD8+ infiltrating lymphocytes, and CD4/CD25+ T-cell Tregs.

Here, we evaluated the characteristics of PD-L1 expression, CD8+ T-cell, and CD4/CD25+ T-cell (Tregs) infiltration and their relationship in esophageal carcinoma, in a cohort of 14 therapy-naive ESCCs.

## Materials and Methods

### *Patients and Study Protocol*

Our tumor series comprised 14 esophageal carcinoma cancer patients, who underwent clinical evaluation and biopsy, before neo-adjuvant therapy between 2019 and 2021 at Saint Mary Clinical Hospital in Bucharest, Romania.

Additional clinical parameters (patient age

and sex, tumor site, tumor stage, tumor grade, histological type, C reactive protein (CRP), lymphocyte count, hemoglobin, and platelet count) were collected for all patients

All tumors were chemo-/radiation-naive, while no patient has received any of the PD-1/PD-L1 checkpoint inhibitors or other form of immune therapy. Tissue from esophageal primary tumors, obtained by endoscopic biopsy, was histologically assessed. The Hematoxylin-Eosin (HE) stained sections were analyzed by two pathologists (CI, CB), who diagnosed and graded the cases of esophageal carcinoma.

### *Immunohistochemistry*

IHC was done on 2 µm thick sections from each tumor using the DAKO clone 22C3, of the PD-L1 antibody for which a positive tissue control was used (tonsil); and primary antibodies against CD4, CD8, and CD25, using an automated immunostainer with its specific protocols (Ventana Medical Systems, Roche).

### *Scoring of PD-L1+, CD4+/CD25+ and CD8+ tumor infiltrating lymphocytes*

The assessment of TILs was performed by two pathologists, in a blinded fashion in regard to clinical information. The TILs were assessed separately by the two pathologists and the two scores were averaged and assigned as the final TIL score for the each case. The extent of membranous PD-L1 expression in tumor cells was assigned in each tumor. Spots showing at least 5% expression were considered positive. The average PD-L1 expression was calculated in each case and a 5% positivity cutoff was also used. Tumor-infiltrating CD4+/CD25+ and CD8+ were assessed in 4 high magnification/ power fields (HPF), in areas with the most elevated TIL density (hot-spots), and the values were transformed into averages. In regard to statistical analysis, the expression patterns were classified into low and high, the cutoff being 50 lymphocytes/HPF.



### Statistical Methods

The data were processed using SPSS version 23.0. For descriptive statistics, the mean and standard deviation were calculated, respectively the medians and quartiles for the quantitative variables, and the frequency and percentage for qualitative variables.

Compared to the quantitative data, depending on the normality of the data, the Student t-test (Independent Sample T-test) (for two groups with normally distributed data) and Mann-Whitney (for data that do not have a normal distribution) were used, respectively.

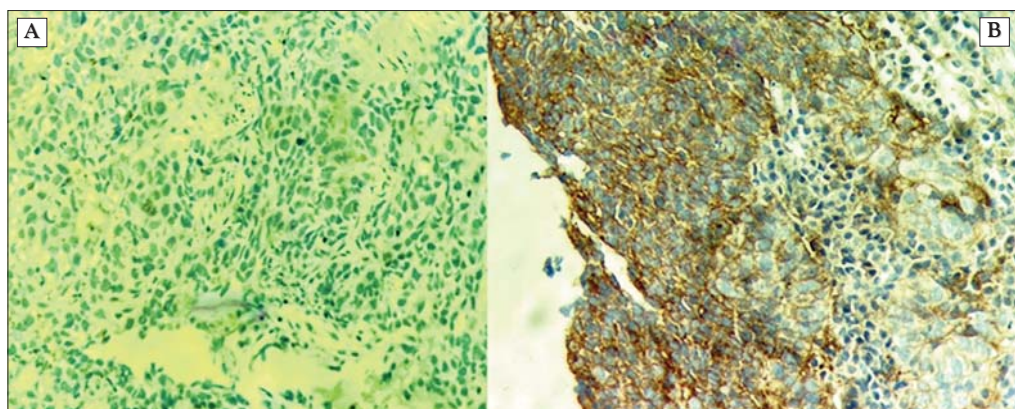
Quantitative data were tested for normality (Shapiro-Wilk test) and variant homogeneity using the Levene test. Fisher's Exact Test was used for the data. The probability of error less than 5% ( $p < 0.05$ ) was considered the threshold of significance.

### Results

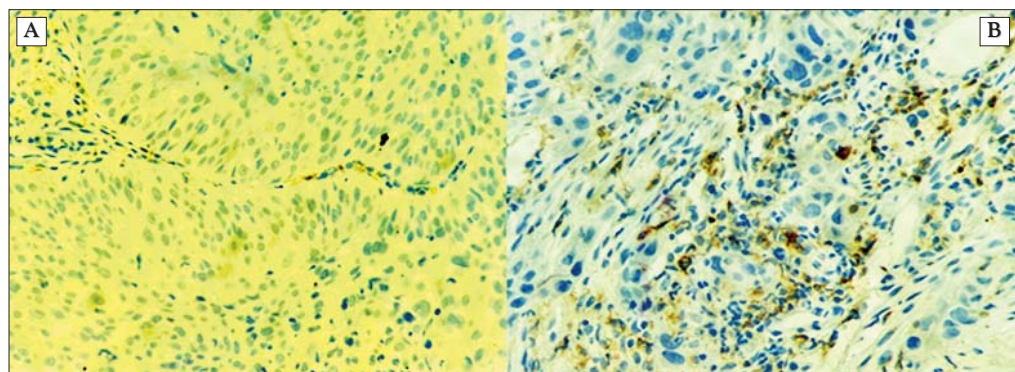
A total of 14 cases of esophageal cancers were analyzed. Of the 14 cases, 13 (93%) were male, and 1 female (7%). The age range was between 42 and 91 years, with a median of 65 years. Histological, 4 cases were adenocarcinomas, and 10 cases were squamous cell carcinomas, 28,5%, and 71,5% respectively. 7 tumors were moderately differentiated, and 7 were poorly differentiated (50% and 50% respectively). Regarding stage, all tumors were an advanced stage, 12 being TNM stage 3 and the remaining two being stage 4 (78,9% and 21,1% respectively).

Of the 14 cases, 10 showed epithelial PD-L1 positivity (78%), of which 3 were adenocarcinomas and 7 were squamous cell carcinomas (*Fig. 1*). CD4 expression was high in 4 cases and low in 10 cases (*Fig. 2*). 11 cases showed high expression of CD8 and 3 cases were appreciated with low

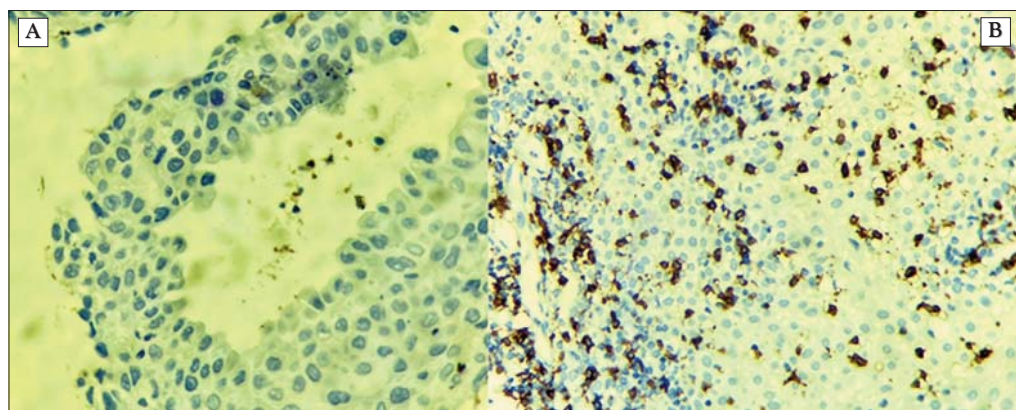
**Figure 1.** PD-L1 IHC expression, 40X: (A) low expression, (B) high expression



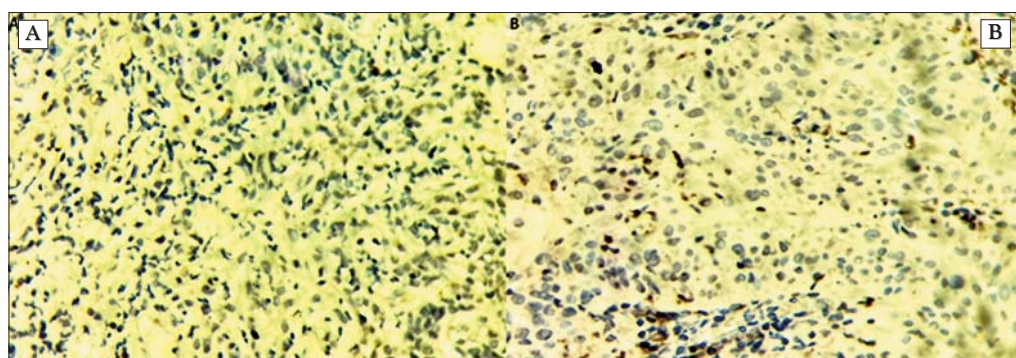
**Figure 2.** CD4 IHC expression, 40X: (A) low expression, (B) high expression



**Figure 3.** CD8 IHC expression, 40X: (A) low expression, (B) high expression



**Figure 4.** CD4/CD25 IHC expression, 40X: (A) low expression, (B) high expression



**Table 1.** Demographic and clinical – paraclinical characteristic of patients

Sex		CD8 IHC	
M	13	High	11
F	1	Low	3
Age		CD4/CD25 IHC	
>70	5	High	4
≤70	9	Low	10
Location		PD-L1	
E	12	40%	2
GEJ	2	30%	1
Pathological exam		25%	1
Squamous cell	10	20%	2
Adenocarcinoma	4	10%	1
Grading		8%	1
2	7	7%	1
3	7	6%	1
TNM		5%	1
3	12	2%	1
4	2	0%	2
CD4 IHC		PD-L1	
High	4	>5%	10
Low	10	≤5%	4

expression (Fig. 3). Cumulative Expressions of CD4 and CD25 were high in 4 cases and low in 10 cases (Fig. 4, Table 1).

### Statistical analysis

We did not obtain statistical correlations of PD-L1 immunohistochemical expression with age, sex, location, TNM stage, pathological type, and tumor grading or laboratory data. Using a qualitative evaluation of PD-L1 (high >5%, and low ≤5%) there was not a statistical correlation between PD-L1 expression and high CD8+ TILs, although 9 cases of PD-L1 positive showed increased CD8+ T cells (90%). Using a quantitative evaluation of PD-L1 we obtained a statistical correlation between the median value of this marker with an expression of CD8. There were obtained statistical correlations between PD-L1 positivity and low expression of CD4 or CD4+/CD25 T cells, of the 10 PD-L1 positive cases, only 1 showed an increase of CD4 or CD4/CD25 T cells (10%) (Table 2).



**Table 2.** Statistical analysis

	PD-L1=neg	PD-L1=poz	P_value (test)
SEX			1.000000
F	0/4 (0%)	1/10 (10%)	(Fisher's Exact Test)
M	4/4 (100%)	9/10 (90%)	
AGE	65.75±4.8562	65.60±12.4561	0.982083 (Independent Sample T test)
LOCATION			0.505495
E	3/4 (75%)	9/10 (90%)	(Fisher's Exact Test)
GEJ	1/4 (25%)	1/10 (10%)	
CD4			0.040959
high	3/4 (75%)	1/10 (10%)	(Fisher's Exact Test)
low	1/4 (25%)	9/10 (90%)	
CD8			0.175824
high	2/4 (50%)	9/10 (90%)	(Fisher's Exact Test)
low	2/4 (50%)	1/10 (10%)	
CD4/CD25			0.040959
high	3/4 (75%)	1/10 (10%)	(Fisher's Exact Test)
low	1/4 (25%)	9/10 (90%)	
AP			1.000000
Adenocarcinoma	1/4 (25%)	3/10 (30%)	(Fisher's Exact Test)
Squamous cell	3/4 (75%)	7/10 (70%)	
G			1.000000
2	2/4 (50%)	5/10 (50%)	(Fisher's Exact Test)
3	2/4 (50%)	5/10 (50%)	
TNM stage			1.000000
3	4/4 (100%)	8/10 (80%)	(Fisher's Exact Test)
4	0/4 (0%)	2/10 (20%)	
Hb			0.580420
N	3/4 (75%)	5/10 (50%)	(Fisher's Exact Test)
P	1/4 (25%)	5/10 (50%)	
Leukocyte			1.000000
N	2/4 (50%)	6/10 (60%)	(Fisher's Exact Test)
P	2/4 (50%)	4/10 (40%)	
Neutrophil cell	4/4 (100%)	10/10 (100%)	-
Lymphocyte	4/4 (100%)	10/10 (100%)	-
Trombocyte	4/4 (100%)	10/10 (100%)	-
CRP			1.000000
N	2/4 (50%)	4/10 (40%)	(Fisher's Exact Test)
P	2/4 (50%)	6/10 (60%)	
<b>Median (25%, 75%)</b>	<b>CD8=high (N=11)</b>	<b>CD8=Low (N=3)</b>	<b>P_value (test)</b>
PD-L1	20.00 [6.00, 30.00]	0.00 [0.00, -]	0.034952 (Mann-Whitney U)
Values	2,5,6,8,10, 20,20, 25,30,40,40	0,0,7	CD8=high - 50% of values are > 20

## Discussion

Immune checkpoint inhibitor treatments targeting the PD-L1–PD-1 axis are currently under investigation and have shown evidence of antitumor activity (16,17). Furthermore, PD-L1 expression has been shown repeatedly that it has a solid prognostic value in various cancers (18,19,20). However, the tumor microenvironment (TME) is very complex and it dictates the abnormal growth of cancer cells (21). The tumor microenvironment consists of mesenchymal stromal cells, blood vessels, and

lymphatic and immune cells (macrophages, T-cells, B-cells, and NK-cells) (22) and it is involved in all the aspects of cancer progression (23). Much of the cancer treatment wisdom is centered on multimodal treatments including surgery, chemotherapy, and radiation therapy, with variable results (24), and targets directly the tumor cells. Newer treatments, such as monoclonal antibodies, although effective, are sometimes plagued by treatment resistance and serious side effects (25). Better understanding the interplay of the tumor cells with the associated inflammatory

cells of the TME might yield other treatment targets (26), increasing the therapeutic arsenal against esophageal cancer.

### Conclusion of Preliminary Results

While we acknowledge the weakness of this study because of the low sample size, we tried to demonstrate that PD-L1 is expressed in tumors with higher CD8+ T cell densities and lower CD4/CD25 positive cells (Tregs), suggesting that the better prognosis associated with PD-L1 positivity might be due to suppression of CD4/CD25+ Tregs, rather than activation of CD8+ T-cells. Also, other adaptive immune resistance mechanisms may be occurring. Further characterization of the esophageal cancer tumor microenvironment regarding PD-L1 expression may highlight other mechanisms and targets for immune-based therapy.

### Conflict of Interests

The authors declare no conflicts of interests.

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