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LETTER TO THE EDITOR

Immune cell score, PD-L1 expression and prognosis in esophageal cancer

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Introduction

Esophageal cancer (EC) is the ninth most common cancer worldwide and the sixth leading cause of cancer death [1]. Despite improvements in the treatment of EC, the general outcome remains poor [2–4].

Immunoscore measures densities of CD3+ and CD8+ lymphocytes at the tumor center and at the invasive margin [5,6]. This method has been internationally validated in colorectal cancer and its inclusion as a part of TNM-staging (TNMi) has been proposed [6]. The value of immune cell scoring in esophageal cancer is not clear. PD-L1 inhibitors are among the most well-known immune checkpoint modulators [7]. The PD-L1 molecule, produced by some tumor cells and inflammatory cells binds to PD-1 receptor on T cells, thereby reducing their activity, leading to the inhibition of anti-tumor immunity and immune escape [7,8].

The aim of the present study was to assess the prognostic significance of the host immunity elements in EC by measuring immune cell score (CD3+ and CD8+ cells) and PD-L1 tumor immune escape pathway in cohort of patients operated with mini-invasive techniques. The secondary aim was to investigate the potential impacts of esophageal stent, histology, stage and neoadjuvant therapy on immune cell densities.

Material and methods

Patients

All patients with histologically confirmed EC ($n = 97$) undergoing mini-invasive (laparoscopy + thoracoscopy $n = 87$) or hybrid (laparotomy + thoracoscopy $n = 4$ and laparoscopy + thoracotomy $n = 6$) esophagectomy in Central Finland Central Hospital from 1 January 2012 to 20 June 2018 were included. Primary outcome was the 3-year overall survival. Clinical data and survival data were obtained from the prospective surgical database and from patient records and confirmed from the Cause of Death Registry maintained by Statistics Finland. The follow-up ended on 31 December 2019. The study was duly approved by the hospital district. Use of the samples and patient data were approved by the Ethics Committee and by the National Authority for Medicolegal Affairs (VALVIRA). Treatment protocol has been previously published [9]. Tumor stage was determined according to the 8th edition of the UICC/AJCC TNM categories [10].

Immunohistochemical analyses

Immunohistochemical methods have been previously reported in detail [11]. For CD3 and CD8, strong immunoreaction was observed in a subset of lymphocytes (positive control), while no immunoreaction was observed in other cells, including epithelial cells (negative control). For PD-L1, strong immunoreaction was observed in the majority of epithelial crypt cells (positive control), while no immunoreaction was observed in superficial epithelial cells and in the majority of lymphocytes, including mantle zone B cells (negative control).

Scoring

IHC slides were scanned with a NanoZoomer-XR (Hamamatsu Photonics) at ×20 magnification. Cell detection method has been previously reported [11,12]. CD3+ and CD8+ immune cell densities were measured from the tumor center and the invasive margin (Supplementary Figure 1), defined as a 0.5 mm-wide region on each side of the tumor border. In tumors with complete response to neoadjuvant therapy, the central areas of the fibrotic scar at the site of the original tumor were evaluated as the tumor center and the margins of the fibrotic scar were evaluated as the invasive margin. Cases with complete histologic response to neoadjuvant therapy were excluded from survival analyses, considering that the factors influencing the immune infiltrates in these tumors may differ from other tumors due to the absence of

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tumor cells, and it also is more difficult to accurately define the regions of tumor center and invasive margin in these cases.

To calculate ICS based on dichotomized CD3 and CD8 densities at the invasive margin and tumor center, medians of all densities were chosen *a priori* as cutoff values. The cut-off values (cells/mm^2) were 600.2 for CD3+ at the tumor core, 662.7 for CD3+ at the invasive margin, 342.1 for CD8+ at the tumor core and 434.8 for CD8+ at the invasive margin. The sum of the dichotomized density variables was calculated, and, according to ICS protocol, three groups were formed: low ICS 0, moderate ICS 1–3 and high ICS 4.

For PD-L1 analysis Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100, was used. The samples were defined to be PD-L1 negative or positive if the PD-L1 CPS was $<5\%$ or $\geq 5\%$ respectively.

Statistical analysis

Survival times were calculated from the date of surgery until the time of death or the end of follow-up (31 December 2019). The Kaplan-Meier method with log-rank test was used to calculate overall survival (OS) stratified by the immune cell variables. The relationships between ICS groups and clinicopathological variables were evaluated by Chi-square test. Univariable and multivariable Cox proportional hazards regression models were used to calculate hazard ratios for survival with the following pre-determined confounders: age (continuous), sex, Charlson Comorbidity Index (0, 1, ≥ 2) [13], histology (EAC, ESCC), neoadjuvant treatment (no/yes), stage (I, II, III–IV). *P*-value less than 0.05 was considered significant. The statistical analyses were performed with IBM SPSS statistics 24 for Windows (IBM Corporation, Armonk, NY, USA).

Results

Patient demographics

A total of 97 EC specimens (EAC $n = 76$, ESCC $n = 21$) were included in the study. The mean age of the cohort was 66.3 years (SD 9.8) and 75.3% were men ($n = 73$). Charlson Comorbidity Index was 0 in 49 patients (50.5%), 1 in 27 patients (27.8%) and ≥ 2 in 21 patients (21.7%). Of 97 patients, 28 patients received neoadjuvant chemotherapy and 50 patients neoadjuvant radiochemotherapy. Nineteen patients did not receive any neoadjuvant treatment. Of 78 patients, 23 (29.5%) showed complete response, 34 (43.6%) partial response and 21 (26.9%) minor or no response to neoadjuvant therapy. Of 97 patients, 42 (43.3%) had ypTNM/pTNM stage I disease, 28 (28.9%) stage II and 27 (27.8%) stage III–IV disease.

Median follow-up time for the whole cohort ($n = 97$) was 35.5 months (IQR 19.5–51.4) and estimated mean overall survival 55.2 [95% CI: (49.1–61.3)] months. The 1-year, 3-year and 5-year overall survival rates were 91.8%, 68.4% and 58.5%.

Immune cell score

Neoadjuvant therapy was associated with a reduced number of CD3+ and CD8+ cells. Therefore, those 23 patients with a complete pathological response were excluded from further analysis (Supplementary Table 1). The 3-year overall survival in complete response subgroup ($n = 23$) was 82.6%.

The final cohort for ICS analysis consisted of 74 patients (EAC $n = 63$, ESCC $n = 11$). The 3-year overall survival rates were 42.4% for low, 63.4% for moderate and 82.4% for high ICS ($p = 0.018$, Figure 1). Hazard ratios (unadjusted and adjusted) of the 3-year overall mortality in EC patients with the low (0), moderate (1–3) and high (4) immune reactions are shown in Table 1(A,B) and association with clinicopathological variables are shown in Supplementary Table 1.

PD-L1

In CPS, $\geq 5\%$ was used as cutoff value for positive CPS. We observed PD-L1 positivity in 27 (27.8%) patients of whom 16 (59.3%) had high ICS, 9 (33.3%) had moderate ICS and 2 (7.4%) had low ICS. The 3-year overall survival in cases with CPS negative vs. positive PD-L1 was 62.3% vs. 61.6% ($p = 0.835$). The 3-year overall survival in analysis excluding low ICS patients CPS negative vs. positive PD-L1 76.0% vs. 62.3% ($p = 0.528$).

Esophageal stent, histology, stage and neoadjuvant–association with immune cell score

Fourteen patients (14.4%) had a stent at the time of the operation. Stent was not associated with ICS ($p = 0.675$) and there were no major differences in immune cell spread as highlighted in Box plot (Supplementary Table 1; Supplementary Figure 2(a)). The tumor histological type or stage were not significantly associated with ICS (Supplementary Table 1; Supplementary Figure 2(b,c)). Patients who received neoadjuvant therapy had a reduced number of CD3+ and CD8+ cells and low ICS, although without statistical significance ($p = 0.062$, Supplementary Table 1; Supplementary Figure 2(d)). In complete response group, median was 0. In partial or minor/no response groups median was 2 ($p = 0.327$, Supplementary Table 1, Supplementary Figure 2(d)).

Discussion

The main finding of this study indicates that high ICS in EC patients is associated with improved 3-year overall survival, although a statistically significant difference was not seen after adjustment for confounding factors. PD-L1 positivity was not significantly associated with survival.

The strength of this study is a consecutive series of esophageal resections during 2012–2019 from a single geographical area of Central Finland without apparent selection bias. All patients were operated by one operating specialized surgeon (ES) using minimally invasive techniques [9]. Single operating surgeon with a completed learning curve makes

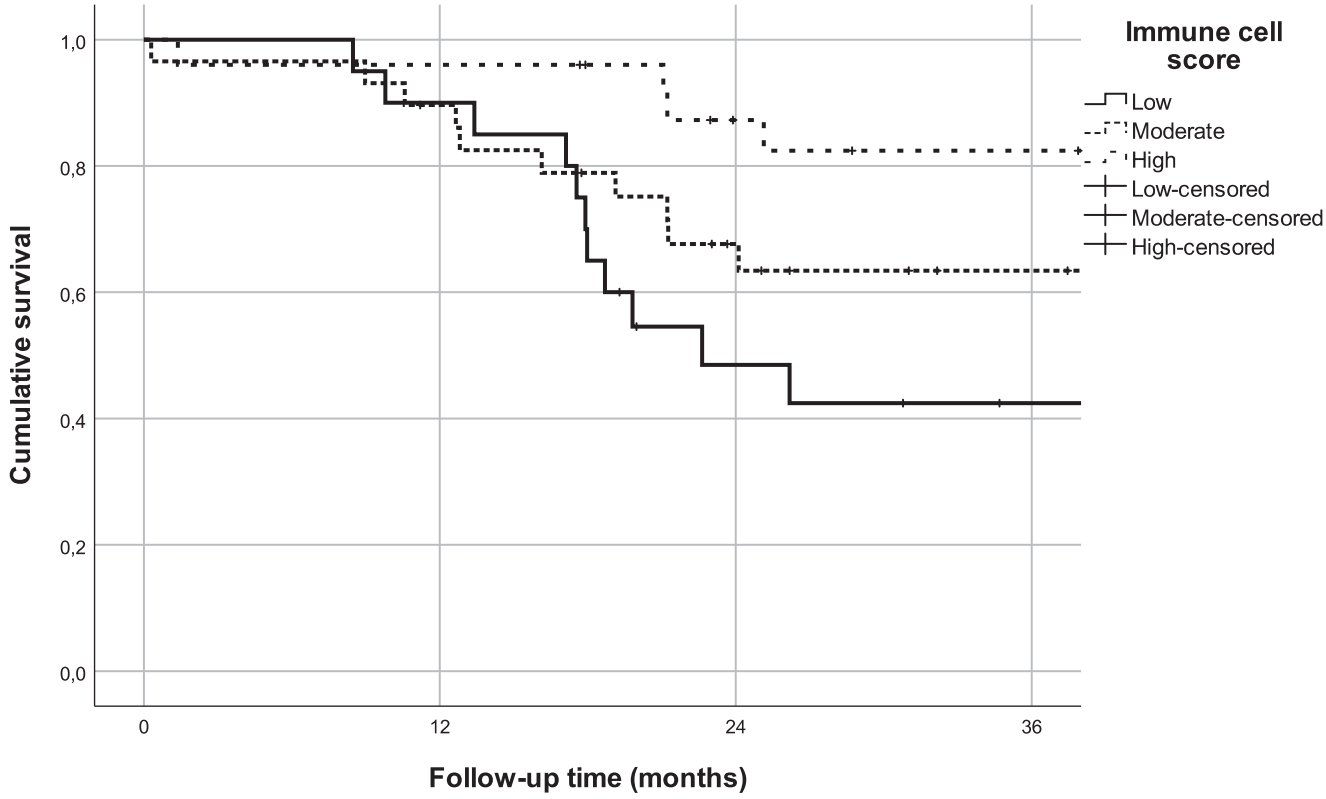

Figure 1. The 3-year overall survival in esophageal cancer patients stratified by immune cell score. Patients with a complete pathological response were excluded.

Table 1. (A) Hazard ratios (HRs) with 95% confidence intervals (CI) of the 3-year overall mortality in esophageal cancer patients with low (0), moderate (1–3) and high (4) immune reaction based on immune cell score (ICS). (B) HRs with 95% CIs of the 3-year overall mortality in esophageal cancer patients separately in variables used in ICS.

A	Number of patients	Immune cell score 0 HR (95% CI)	Immune cell score 1–3 HR (95% CI)	Immune cell score 4 HR (95% CI)
Overall mortality (3 years)				
All patients (crude)	74	1.00 (Reference)	0.58 (0.26–1.32)	0.24 (0.09–0.69)
All patients (adjusted)[a]	74	1.00 (Reference)	0.71 (0.30–1.72)	0.41 (0.11–1.49)
B	Number of patients	Low, HR (95% CI)		High, HR (95% CI)
Overall mortality (3 years)				
CD3 core (crude)	74	1.00 (Reference)		0.27 (0.12–0.61)
CD3 core (adjusted)[a]	74	1.00 (Reference)		0.36 (0.13–1.01)
CD3 front (crude)	74	1.00 (Reference)		0.72 (0.34–1.51)
CD3 front (adjusted)[a]	74	1.00 (Reference)		0.93 (0.42–2.05)
CD8 core (crude)	74	1.00 (Reference)		0.23 (0.10–0.55)
CD8 core (adjusted)[a]	74	1.00 (Reference)		0.36 (0.11–1.20)
CD8 front (crude)	74	1.00 (Reference)		0.58 (0.28–1.23)
CD8 front (adjusted)[a]	74	1.00 (Reference)		0.97 (0.42–2.28)

[a]Adjusted for age, sex, Charlson Comorbidity Index, histology, neoadjuvant treatment (no/yes), stage (I, II, III–IV).

the comparison of tumor biology related survival truly possible. All patients were followed up to five years after surgery and nationwide compulsory databases enabled us to obtain complete long-term mortality data. A major limitation of our study is a small sample size. This limits any subgroup analyses and possible positive associations may be missed due to a low statistical power both in ICS and PD-L1 analyses. Confidence intervals for reported hazard ratios are wide and replication studies are needed to confirm the findings.

So far, only few studies have evaluated the significance of ICS in esophageal cancer. Previously, CD3+ and CD8+ lymphocytes have been separately assessed associating mainly with improved prognosis [14–17]. In our study, ICS, established by a detailed computer-assisted quantification of CD3+ and CD8+ T cell densities, was significantly associated with improved overall 3-year survival. The method we used resembles the Immunoscore assay developed by Galon and colleagues [5,6] combining whole slide imaging with image analysis and has been introduced as a reproducible prognostic parameter for colorectal-cancer [5,18].

In our study, we observed PD-L1 positive CPS (≥5%) in 27 (27.8%) patients. Previous studies have reported variation of 9–71% in tumor cells and 25–87% in immune cells in PD-L1 expression [8]. PD-L1 expression has been associated with an unfavorable clinical outcome and survival in resected esophageal cancer, supporting its role as a prognostic biomarker [8,14,17].

There are some studies of EC patients combining CD3+ and CD8+ densities and PD-L1 expression. A classification of tumors into four groups based on PD-L1 expression and TIL

status has been suggested: type I (PD-L1 positive and TIL positive, driving adaptive immune resistance), type II (PD-L1 negative with no TIL, indicating immune ignorance), type III (PD-L1 positive, TIL negative, indicating intrinsic induction), and type IV (PD-L1 negative with TIL, indicating a role for other suppressor(s) in promoting immune tolerance) [17,19]. Type I (PD-L1-positive and TIL-positive) tumors with a rich immune infiltrate are considered more responsive to checkpoint [17,20].

There are few studies about immune cell densities in primary resected ECs compared to neoadjuvant treated ECs. In a small series, a significant decrease of T cell activity measured by CD3+ and CD8+ expression after neoadjuvant radio-chemotherapy was shown in both EAC ($n = 17$) and ESCC ($n = 21$) patients [21,22]. Our study supports these earlier findings of the impact of neoadjuvant radio-chemotherapy on the tumor immune microenvironment and immune cell densities, especially in patients with complete neoadjuvant response.

Immune checkpoint inhibitors are designed to release or enhance preexisting anti-cancer immune responses. FDA has given approval for PD-1 inhibitor Pembrolizumab for the treatment of patients with recurrent locally advanced or metastatic gastric or gastro-esophageal-junction adenocarcinoma with PD-L1 expression [23], and the effects of various immune modulators are increasingly studied in ongoing clinical trials [24]. Other than PD-L1 expression, tumor mutation burden (TMB) associating with high immune response could possibly identify patients benefiting from PD-1 inhibitors [25]. The first promising results in disease-specific survival of adjuvant immunomodulatory nivolumab with operated esophageal cancer patients have been published [26]. The expression level cutoffs, associations with clinicopathological features, and prognostic impact of many checkpoint molecules have, however, not been well-established in EC. Therefore, the optimal subgroups for a single or a combination of immunotherapy are not clear.

Conclusions

Our study describes the patterns of CD3+ and CD8+ T cell infiltration and PD-L1 expression in EC. ICS could serve as a marker for favorable prognosis in EC. The applicability of ICS and PD-L1 to guide immunotherapy are relevant subjects for further clinical research.

Authors' contributions

O.H. conceived and designed the study; A.J, J.P.V, M.A, T.K, J.M, E.S. and O.H acquired the data; O.H., J.V. J.M and E.S., performed the experiments; A.J., M.A, J.P.V, T.K and O.H. analyzed the data; A.J. and O.H. drafted the manuscript; all authors critically reviewed, edited and approved the manuscript; O.H. provided funding; O.H. supervised the study and is the guarantor of the study.

Ethics approval and consent to participate

The Central Finland Central hospital ethics committee approved the study and the need to obtain informed consent from the study patients was waived by the National Authority for Medicolegal Affairs (VALVIRA). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. The study was not registered due to retrospective nature.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Anonymized data are available upon request from the corresponding author.

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