Annals of Diagnostic Pathology 50 (2021) 151657

Contents lists available at ScienceDirect

Annals of Diagnostic Pathology

journal homepage: www.elsevier.com/locate/anndiagpath

Original Contribution

Prognostic impact of neutrophils-to-lymphocytes ratio (NLR), PD-L1 expression, and tumor immune microenvironment in laryngeal cancer

Leonardo Franz [a], Lara Alessandrini [b], Elena Fasanaro [c], Piergiorgio Gaudioso [a], Alessandro Carli [a], Piero Nicolai [a], Gino Marioni [a,*]

[a] Department of Neuroscience DNS, Otolaryngology Section, University of Padova, Padova, Italy
[b] Department of Medicine DIMED, University of Padova, Padova, Italy
[c] Department of Neuroscience DNS, S.D.O.U. Otolaryngology at S. Antonio Hospital, University of Padova, Padova, Italy

ARTICLE INFO

Keywords:
Neutrophils-to-lymphocytes ratio
NLR
PD-L1
Tumor immune microenvironment
Laryngeal carcinoma prognosis
Immunotherapy

ABSTRACT

Purpose: In laryngeal carcinoma (LSCC), tumor immune microenvironment is attracting increasing interest, given the recent progresses in immunotherapy. Immune cells migrate to tumors as a result of a tumor antigen-induced immune reaction and cancer cells recruit immune regulatory cells to induce an immunosuppressive network, resulting in the escape from host immunity. This interaction reflects both on tumor microenvironment and systemic inflammatory status. Blood neutrophil-to-lymphocyte ratio (NLR), reflecting a highly pro-inflammatory status, has been related to worse oncological survival outcomes.

The aim of this study was to analyze in LSCC the relationship between circulating inflammatory cells (also in terms of NLR) and tumor immune microenvironment histopathological features (programmed cell death ligand 1 [PD-L1] expression, and tumor-infiltrating lymphocytes [TILs]), also investigating their clinical-pathological and prognostic significance.

Methods: Blood pre-operative NLR, and, at pathology, PD-L1 (in terms of combined positive score [CPS]) and TILs were assessed on 60 consecutive cases of LSCC.

Results: Blood NLR, neutrophils, and lymphocytes counts showed a significant value in predicting DFS and recurrence risk. Moreover, PD-L1 CPS $\geq$ 1 and TILs count rate $\geq$30% were associated with higher disease-free survival (DFS) and reduced recurrence risk. A logistic regression model found a significant positive association between increasing NLR values, and PD-L1 CPS $<$ 1 and TILs count rate $<$30%.

Conclusions: Further studies are needed to better characterize the role of pre-operative blood NLR in association with PD-L1 expression and tumor immune microenvironment features as prognostic factors and markers of anti-tumor immune response in LSCCs, also with regard to the effectiveness of immunotherapeutic protocols.

1. Introduction

During the last decade, research on laryngeal squamous cell carcinoma (LSCC) has shifted its focus from the traditional clinical-pathological factors to novel biological markers [1,2], aiming to more accurately profile tumor prognosis and identify targeted treatment strategies.

Tumor immune microenvironment is attracting increasing interest, also in the light of the recent progresses in immunotherapy, which has begun to be considered in association with platinum and 5-fluorouracil or alone as an appropriate first-line choice for recurrent or metastatic head and neck carcinomas (HNSCCs) [3]. As is well-known, a two-way interaction links inflammation and cancer: immune cells migrate to tumors as a result of a tumor antigen-induced immune reaction and cancer cells recruit immune regulatory cells to induce an immunosuppressive network, resulting in the escape from host immunity [4]. This interaction reflects both on tumor microenvironment and systemic inflammatory status, also resulting in changes in the circulating white blood cells counts. The latter have been recently regarded as promising markers for tumor-induced inflammation, with possible relationships with clinical features and prognosis in several tumor types, including HNSCC [5-10]. In particular, high neutrophils counts and neutrophil-to-lymphocyte ratio (NLR), reflecting a highly pro-inflammatory status, have been related to worse oncological survival outcomes [7-12], while high

* Corresponding author.
E-mail address: gino.marioni@unipd.it (G. Marioni).

https://doi.org/10.1016/j.anndiagpath.2020.151657

Available online 5 November 2020

lymphocyte counts have been considered as positive prognostic factors, as a result of an improved antigen-driven immune response [12]. NLR can synthetically express the balance between pro-tumor inflammatory status and antitumor immune response, with high values of the ratio representing a tumor-induced change of the immune system toward a pro-tumor pattern [13].

Similarly, the interaction between cancer and immune cells in the tumor microenvironment has relevant prognostic and therapeutic implications, in particular regarding regulation of the immune checkpoints. Programmed cell death ligand 1 (PD-L1) is a crucial molecule in the immune checkpoint biological pathway, which has been studied as both a prognostic marker and a target for therapy in a wide range of human malignancies [3,14-16]. When PD-L1 (expressed on tumor cells) binds to the programmed cell death protein 1 (PD-1) on lymphocytes, the latter undergo PD-1/PD-L1 checkpoint pathway activation, resulting in an attenuation of their viability and immune responsiveness. According to this biological model, PD-L1 has long been regarded as a factor determining tumor immune escape and its overexpression on cancer cells has therefore been considered as a negative prognostic factor [7,16]. However, according to a more articulated view of immune checkpoint biology, in some cancer types, PD-L1 expression seems to result from a complex interaction between tumor and immune cells in the tumor microenvironment, as a response to an effective antigen-induced antitumor immune pressure mediated by tumor-infiltrating lymphocytes (TILs) [14]. In line with this interpretation, PD-L1 expression has been reported to be positively related with increased TILs count and more favorable prognosis in LSCCs [17,18].

The aim of this exploratory study was to analyze the relationship between circulating inflammatory cells (in terms of NLR, blood neutrophils and lymphocytes counts) and some tumor immune microenvironment features (PD-L1 expression, and TILs) in LSCC, and to investigate their clinical-pathological and prognostic significance.

2. Materials and methods

2.1. Patients

This study was conducted in accordance with the principles of the Helsinki Declaration. Data were examined in compliance with Italian privacy and sensitive data laws and the in-house rules of Padova University's Otolaryngology Section. All patients preoperatively signed a consent form for disclosure of privacy in managing personal data for scientific purposes. Moreover, before undergoing surgery, all patients included in the study signed a detailed informed consent form.

The investigation involved 60 consecutive patients with LSCC treated with primary surgery. As previously described [19,20], all patients (51 males, 9 females; median age 64.5 years, IQR 60.0–69.0 years) underwent microlaryngoscopy with laryngeal biopsy, upper aerodigestive tract endoscopy, neck ultrasonography (with or without fine needle aspiration cytology), head and neck contrast-enhanced computerized tomography (CT), and/or magnetic resonance imaging, chest X-ray and liver ultrasonography. No distant metastases were found. LSCCs were staged according to the 8th edition of the TNM Classification of Malignant Tumors [21].

All patients underwent laryngeal surgery at our Institution, including unilateral or bilateral cervical lymph node dissection in 57 cases. Pathological findings warranted postoperative adjuvant RT (with or without concomitant chemotherapy) in 22 cases in accordance with current guidelines [22].

After treatment, the previously reported clinical follow-up schedule [23] (adjustable to patients' individual characteristics) was the following: once a month for the 1st year; every 2 months in the 2nd year; every 3 months in the 3rd year; every 4 months in the 4th year; every 6 months in the 5th year; every 12 months thereafter. Contrast-enhanced CT of the neck, total body positron emission tomography, chest CT, and neck and liver ultrasonography were repeated if clinically indicated.

The median follow-up time was 58.0 months (inter-quartile range [IQR] 29.0–90.5 months).

2.2. TILs evaluation

For each case of LSCC, all available hematoxylin and eosin (H&E)-stained slides were retrieved from the archives and reviewed by a dedicated pathologist (L.A.), selecting the most representative formalin-fixed, paraffin-embedded tissue blocks for immunohistochemical analysis.

As previously reported [18], to count TILs, the most representative H&E-stained slide was assessed as a whole, obtaining a mean infiltrate score based on all available tissue. Only stromal TILs were considered, recording them semi-quantitatively on a continuous scale as a percentage of stromal area, using a light microscope at 200×–400× magnification. All mononuclear cells (including lymphocytes and plasma cells, excluding granulocytes) in the stromal areas were included in the count; carcinomatous and necrotic areas were excluded [24].

2.3. Immunohistochemistry

Immunohistochemistry (IHC) was performed using the Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle upon Tyne, UK) in the BOND-MAX system (Leica Biosystems) with the primary antibody for PD-L1 (22C3, IHC PharmDx, Dako, Carpinteria, Ca, USA; 1:50 dilution). A positive section of tonsil was added for each sample of LSCC as an internal positive control. As a negative control, the primary antibody was replaced by phosphate buffer saline solution. At least 100 viable tumor cells on the slide from each case stained for PD-L1 were required for an adequate PD-L1 assessment, and LSCCs were considered PD-L1-positive if the combined positive score (CPS) was 1 or greater. The CPS was calculated as the number of PD-L1–positive cells (tumor cells, macrophages, and lymphocytes) divided by the total number of tumor cells, multiplied by 100 [25]. Any viable tumor cells with partial or complete linear membrane staining perceived as distinct from cytoplasmic staining were considered positive for the purpose of scoring PD-L1. Any mononuclear inflammatory cells with convincing membrane and/or cytoplasmic staining within tumor nests and/or adjacent supporting stroma were considered PD-L1-positive and included in the score. PD-L1 was scored by our head and neck pathologist, who was unaware of the related clinical data.

2.4. Peripheral blood cell counts and NLR

At the time of admission, immediately before surgery, routine analysis of all patients' blood samples, including a complete blood cell count, was performed at the laboratory of Padova General Hospital (EIA Unit, Laboratory Medicine Service), which is certified in compliance with ISO 15189. Each patient's preoperative blood cell counts were retrospectively retrieved. NLR was calculated by dividing neutrophil count (cells $\times 10^9$/L) by lymphocyte count (cells $\times 10^9$/L).

2.5. Statistical analysis

PD-L1 CPS, TILs count rates, neutrophils and lymphocytes blood counts, and NLR were considered both as continuous and dichotomous variables. For dichotomization purposes, as previously reported [18,26], the CPS for PD-L1 was binarized using a value of 1 as cut-off. Therefore, cases with a CPS $\geq$1 were considered as positive, and those with a CPS $<$1 as negative. Similarly, the cut-off to distinguish cases with higher versus lower TIL counts was set at a rate of 30%, based on the literature [17,27]. The chosen cut-offs coincided with the median values in our population for NLR, blood neutrophils and lymphocytes counts, as they were not subjective and achieved the best fit analytically.

The correlation between continuous variables was explored through linear regression models, while the correlation between blood counts or

NLR and dichotomized PD-L1 and TILs was approached through logistic regression models. Fisher's exact test and Mann-Whitney's *U* test were also applied, as appropriate. The accuracy with which each variable predicted time to LSCC recurrence was tested using a univariate Cox's regression analysis. The results of the Cox's regression are expressed as p-values, hazard ratios and 95% confidence intervals. The disease free-survival (DFS), measured as the time (in months) from completing treatment for primary LSCC to disease recurrence or to last follow up control (for patients experiencing no recurrence), predicted from the analyzed variables was represented graphically with Kaplan-Meier curves.

A p-value <0.05 was considered indicative of statistical significance, while values between 0.05 and 0.10 were assumed to indicate a statistical trend.

The STATA 16.0 IC statistical package (Stata Corp LP, College Station, TX, USA) was used for all analyses.

3. Results

3.1. Outcomes and conventional clinical-pathological variables

Forty-one patients (68.3%) experienced no disease recurrence after treatment, while 19 (31.7%) relapsed after a median 12.0 months (IQR 7.0–18.0 months). Cox's proportional hazards model for DFS found no significant predictive value for T classification (pT1–2 vs pT3–4), pathological grading (G1–2 vs G3), N status (cN0 + pN0 vs pN+), or pathological staging (stage I-II vs stage III-IV) (p = 0.311, p = 0.214, p = 0.212, and p = 0.468, respectively).

3.2. Circulating blood cells counts and prognosis

Table 1 summarizes the distribution of neutrophils and lymphocytes counts and NLR in the study population, vis-à-vis the other clinical-pathological variables and outcome.

Based on the Cox's regression model (see Table 2), NLR value, neutrophils, and lymphocytes counts were statistically significant in predicting DFS and recurrence risk. In particular, increasing NLR values and neutrophils counts were associated with higher recurrence risk and shorter DFS (p = 0.002, HR: 1.15 [95% C.I. 1.05; 1.26] per single-unit increase, and p = 0.030, HR: 1.16 [95% C.I. 1.01; 1.33] per single-unit increase, respectively) (see also Fig. 1A, B). Instead, increasing lymphocytes counts were associated with lower recurrence risk and longer DFS (p = 0.029, HR: 0.49 [95% C.I. 0.26; 0.93] per single-unit increase) (see also Fig. 1C).

Table 2
Hazard ratio of recurrence (with 95% C.I.) according to the Cox's regression model for each studied variable.

Variables	HR (95% C.I.)	p-Value
T1–2	1	–
T3–4	1.59 (0.65; 3.93)	0.311
G1–2	1	–
G3	1.77 (0.72; 4.37)	0.214
Stage I–II	1	–
Stage III–IV	1.41 (0.56; 3.59)	0.468
N0 status[a]	1	–
pN+ status	1.81 (0.71; 4.61)	0.212
NLR	1.15[b] (1.05; 1.26)	**0.002**
NLR <2.68	1	–
NLR ≥2.68	2.76 (1.04; 7.29)	**0.041**
Neutrophils count (cells × 10^9/L)	1.16[b] (1.01; 1.33)	**0.030**
Neutrophils count <5.11 cells × 10^9/L	1	–
Neutrophils count ≥5.11 cells × 10^9/L	2.15 (0.85; 5.48)	0.108
Lymphocytes count (cells × 10^9/L)	0.49[b] (0.26; 0.93)	**0.029**
Lymphocytes count <1.85 cells × 10^9/L	1	–
Lymphocytes count ≥1.85 cells × 10^9/L	0.53 (0.21; 1.34)	0.176
PD-L1 CPS < 1	1	–
PD-L1 CPS ≥ 1	0.20 (0.06; 0.70)	**0.012**
TILs count rate <30%	1	–
TILs count rate ≥30%	0.29 (0.10; 0.80)	**0.017**

Bold: significant p-Values.
NLR: neutrophil-to-lymphocyte ratio; HR: hazard ratio; 95% C.I.: 95% confidence interval.
[a] pN0: 40 cases, cN0: 3 cases.
[b] OR per each 1-unit increase.

Considering NLR and lymphocytes count as dichotomized, based on their median distribution values (2.68 [IQR: 1.95–4.57], and 1.85 cells × 10^9/L [IQR: 1.22–2.38 cells × 10^9/L], respectively), no significant associations were found between the two variables and the presence of neck lymph nodes metastases (Fisher's exact test, p = 0.126 and p = 0.284 for NLR and lymphocytes count, respectively). On the other hand, a neutrophil count higher than its median value (5.11 cells × 10^9/L [IQR 3.56–7.11 cells × 10^9/L]) was significantly associated with an increased nodal metastases risk (Fisher's exact test: p = 0.042).

3.3. Tumor immune microenvironment, PD-L1 expression, and prognosis

Table 2 reports each variable prognostic value in terms of recurrence hazard ratio. Based on Cox's regression model, PD-L1 CPS ≥ 1 and TILs count rate ≥30% were associated with longer DFS and reduced recurrence risk (p = 0.012, HR: 0.20 [95% C.I. 0.06; 0.70], and p = 0.017, HR:

Table 1
Distribution of NLR, neutrophils and lymphocytes counts in the considered cohort, vis-à-vis pT-stage, pathological grading, N-status, TNM stage grouping, PD-L1 expression, TILs count rate, and relapse occurrence.

Clinical-pathological variables	No. of cases	NLR Median (IQR)	p-Value[a]	Neutrophils count (cells × 10^9/L) Median (IQR)	p-Value[a]	Lymphocytes count (cells × 10^9/L) Median (IQR)	p-Value[a]
pT1–2	33	2.74 (1.71–4.60)	0.818	5.12 (3.45–6.59)	0.953	1.87 (1.2–2.34)	0.917
pT3–4	27	2.51 (1.98–4.40)		5.09 (3.68–7.46)		1.83 (1.23–2.49)	
G1–2	34	2.30 (1.71–4.46)	0.199	4.93 (3.42–6.33)	0.5607	1.92 (1.42–2.49)	0.161
G3	26	2.76 (2.07–8.029)		5.30 (3.68–5.80)		1.64 (1.02–2.34)	
Stage I–II	26	2.94 (1.94–5.04)	0.290	5.62 (3.45–5.46)	0.438	1.79 (1.02–2.10)	0.442
Stage III–IV	34	2.33 (1.97–3.86)		4.78 (3.66–6.76)		1.86 (1.36–2.50)	
N0 status[b]	43	2.79 (2.07–4.60)	0.166	5.60 (3.45–7.46)	0.512	1.72 (1.16–2.14)	0.341
pN+ status	17	2.06 (1.55–3.37)		4.70 (3.66–6.76)		1.93 (1.36–2.50)	
PD-L1 CPS < 1	36	3.35 (2.29–7.63)	**0.0008**	5.72 (4.10–8.23)	0.0563	1.50 (1.01–1.97)	**0.0017**
PD-L1 CPS ≥ 1	24	2.06 (1.53–2.72)		4.20 (3.44–5.70)		2.26 (1.63–2.75)	
TILs count rate <30%	32	2.82 (2.21–6.51)	**0.0225**	5.67 (3.56–8.01)	0.257	1.71 (1.00–2.02)	**0.0408**
TILs count rate ≥30%	28	2.15 (1.53–4.20)		4.88 (3.56–5.94)		2.03 (1.39–2.62)	
Without LSCC recurrence	41	2.30 (171–3.32)	**0.0103**	4.84 (3.30–6.17)	**0.0396**	2.00 (1.42–2.50)	**0.0149**
With LSCC recurrence	19	4.52 (2.37–8.08)		6.03 (4.62–8.55)		1.37 (0.92–1.09)	

NLR: neutrophil-to-lymphocyte ratio; IQR: inter-quartile range; bold: significant p-Values.
[a] Mann-Whitney U test.
[b] N0 status = pN0: 40 cases and cN0: 3 cases.

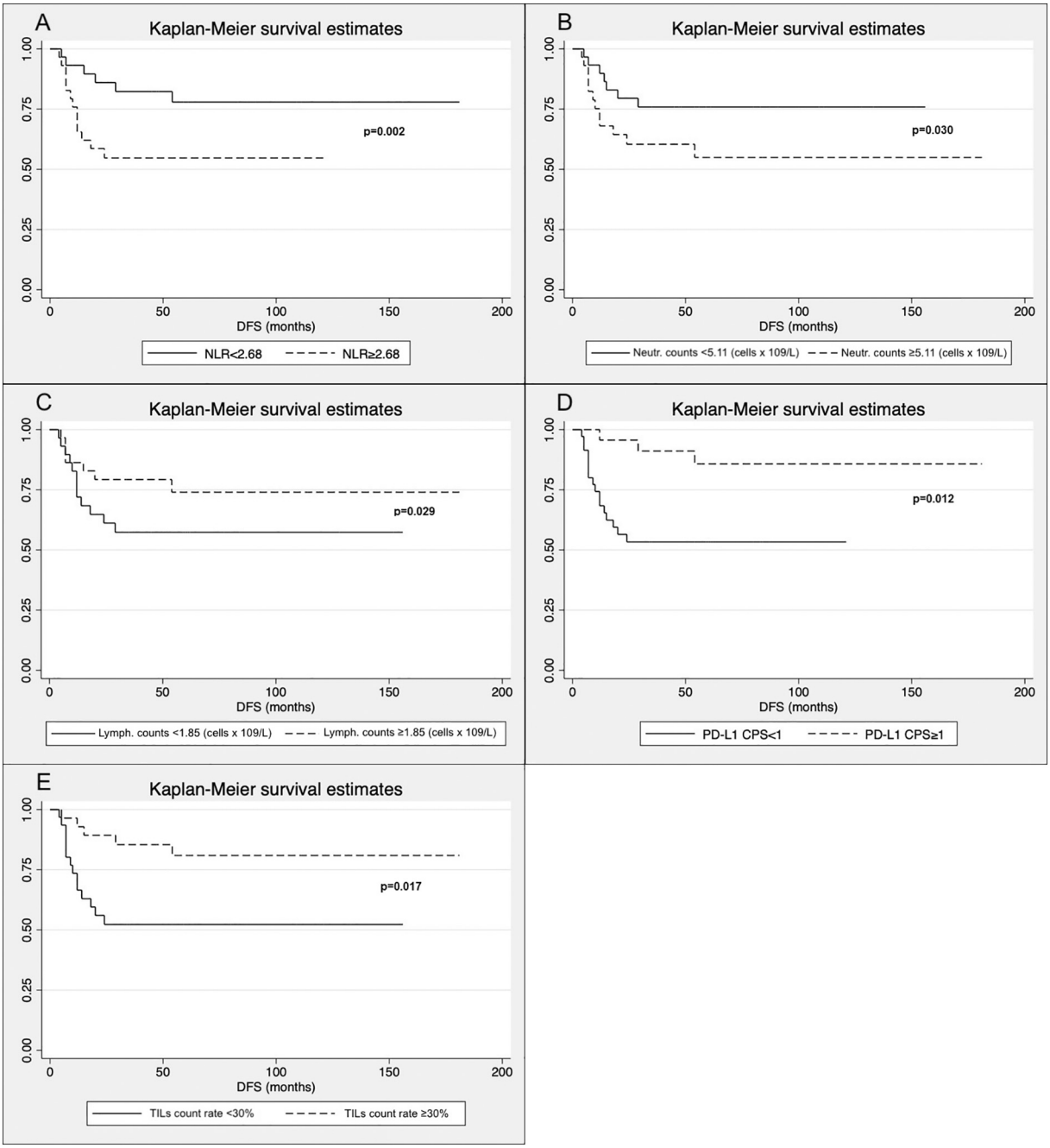

Fig. 1. Kaplan-Meier graphs showing differences in disease-free survival: (A) between cases with NLR <2.68 vs. ≥2.68; (B) between cases with neutrophils counts <5.11 vs. ≥5.11 cells × 10^9/L; (C) between cases with lymphocytes counts <1.85 vs. ≥1.85 cells × 10^9/L; (D) between cases with PD-L1 CPS <1 vs. ≥1; (E) between cases with TILs count rates <30% vs. ≥30%.

0.29 [95% C.I. 0.10; 0.80], respectively) (see also Figs. 1D, E, 2A–D). No significant associations were disclosed between N-status and tumor immune microenvironment markers (Fisher's exact test: p = 0.434 for PD-L1 CPS < 1 vs. ≥1 and p = 0.403 for TILs count rate <30% vs. ≥30%).

3.4. Relationships between circulating blood cell values, PD-L1 expression, and tumor microenvironment markers

Table 3 summarizes the correlations between circulating inflammatory cells (neutrophils, lymphocytes and NLR) and tumor immune microenvironment markers (PD-L1 CPS and TILs count rate), by linear regression models. A significant negative correlation emerged between NLR and TILs count rate (p = 0.019, R^2 = 0.09, c: −1.73 [95% C.I. −3.16; −0.29]) (Fig. 3).

Considering tumor immune microenvironment markers as dichotomous variables, based on the previously-described positivity cut-offs, the logistic regression model found that increasing NLR values were correlated with a decreased probability of PD-L1 CPS ≥1 and TILs count rate ≥30% (p = 0.014, OR: 0.59 [95% C.I. 0.39; 0.90], and p = 0.047, OR: 0.83 [95% C.I. 0.686; 0.997], respectively) (see also Fig. 4A, B).

Similarly, also increasing neutrophils counts correlated with a decreased probability of PD-L1 CPS ≥ 1 (p = 0.041, OR: 0.78 [95% C.I. 0.62; 0.99]), whereas increasing lymphocytes counts appeared to predict higher probability of PD-L1 CPS ≥ 1 (p = 0.004, OR: 3.27 [95% C.I. 1.47; 7.26]) (see also Fig. 4C, D).

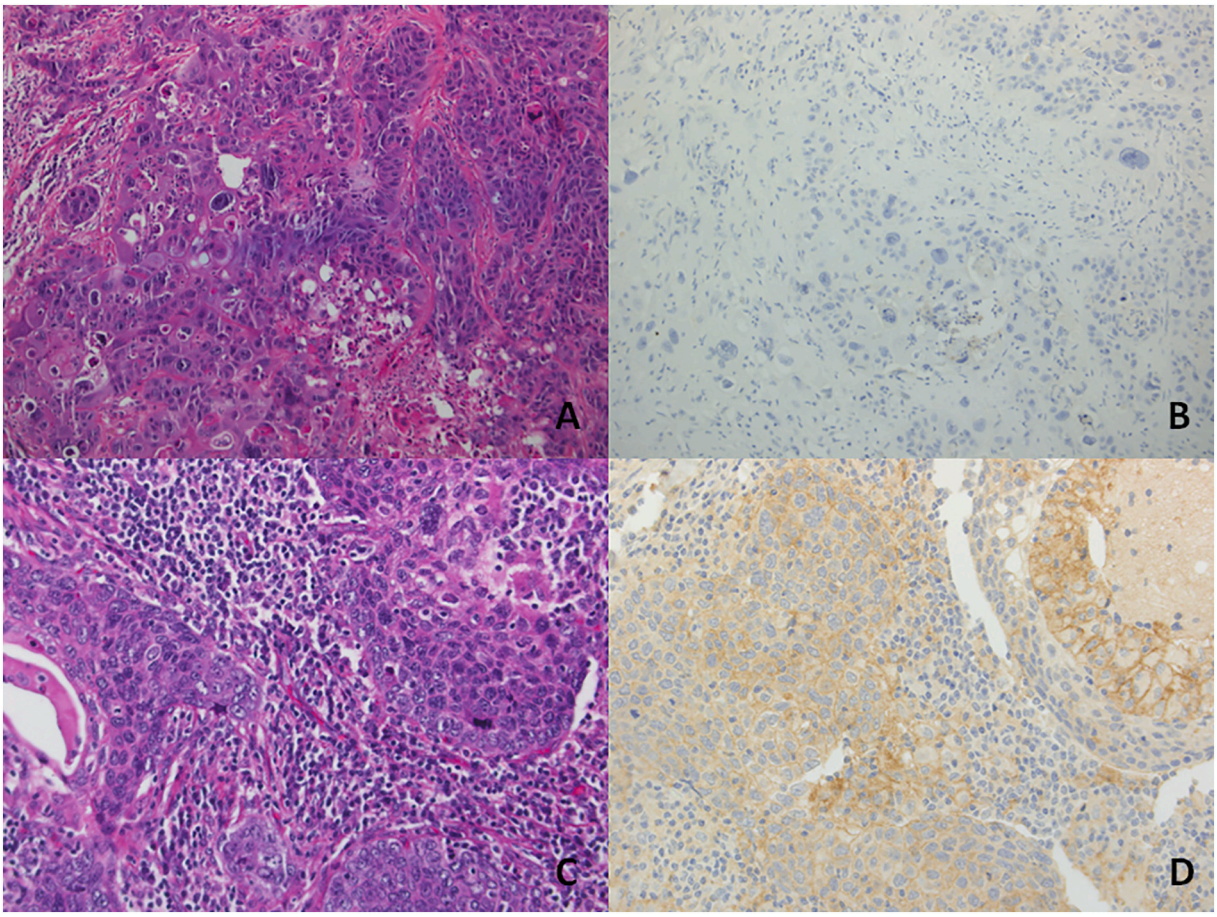

Fig. 2. A case of LSCC that developed local disease recurrence, showing necrosis and nuclear pleomorphism, with a low stromal TILs count rate (A, H&E, original magnification 100×) and paired negative immunostaining for PD-L1 (B, original magnification 100×). A case of LSCC who did not experience disease recurrence, displaying a high stromal TILs count rate (C, H&E, original magnification 200×) and PD-L1 CPS score >1 (D, original magnification 200×).

Table 3
Correlations between circulating inflammatory cells (neutrophils, lymphocytes and NLR) and LSCC immune microenvironment markers (PD-L1 CPS and TILs count rate), according to a linear regression model.

Circulating inflammatory cells	Tumor immune microenvironment markers	C (95% C. I.)	R^2	p-Value
NLR	PD-L1 CPS	−0.69 (−1.62; 0.24)	0.04	0.14
NLR	TILs count rate	−1.73 (−3.16;-0.29)	0.09	**0.019**
Neutrophils count	PD-L1 CPS	−0.94 (−2.17; 0.29)	0.04	0.13
Neutrophils count	TILs count rate	−1.64 (−3.58; 0.30)	0.05	0.096
Lymphocytes count	PD-L1 CPS	1.19 (−2.99; 5.37)	0.006	0.57
Lymphocytes count	TILs count rate	5.72 (−0.77; 12.20)	0.05	0.083

NLR: neutrophil-to-lymphocyte ratio; C: estimated coefficient; 95% C.I.: 95% confidence interval; bold: significant p-Values.

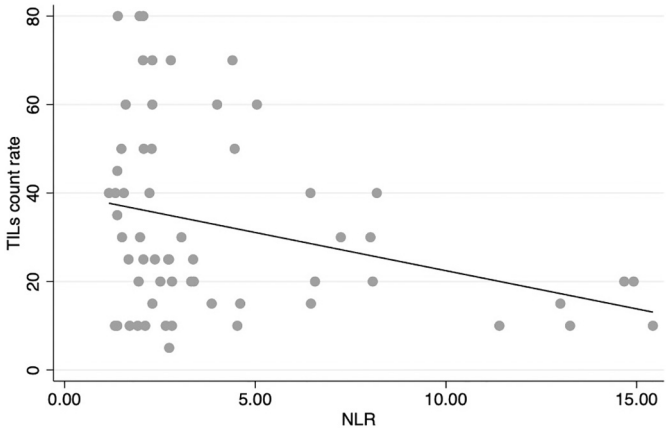

Fig. 3. Scatter plot depicting the correlation between NLR and TILs count rate according to the linear regression model (p = 0.019, R^2 = 0.09, c: −1.73 [95% C.I. −3.16; −0.29]).

A trend toward a positive correlation between increasing lymphocytes count values and higher probability of TILs count rate ≥30% emerged from the logistic regression model (p = 0.058, OR: 1.91 [95% I. C. 0.98; 3.72]), while no correlation emerged between neutrophils count values and TILs count rate (p = 0.130) (see also Fig. 4E, F).

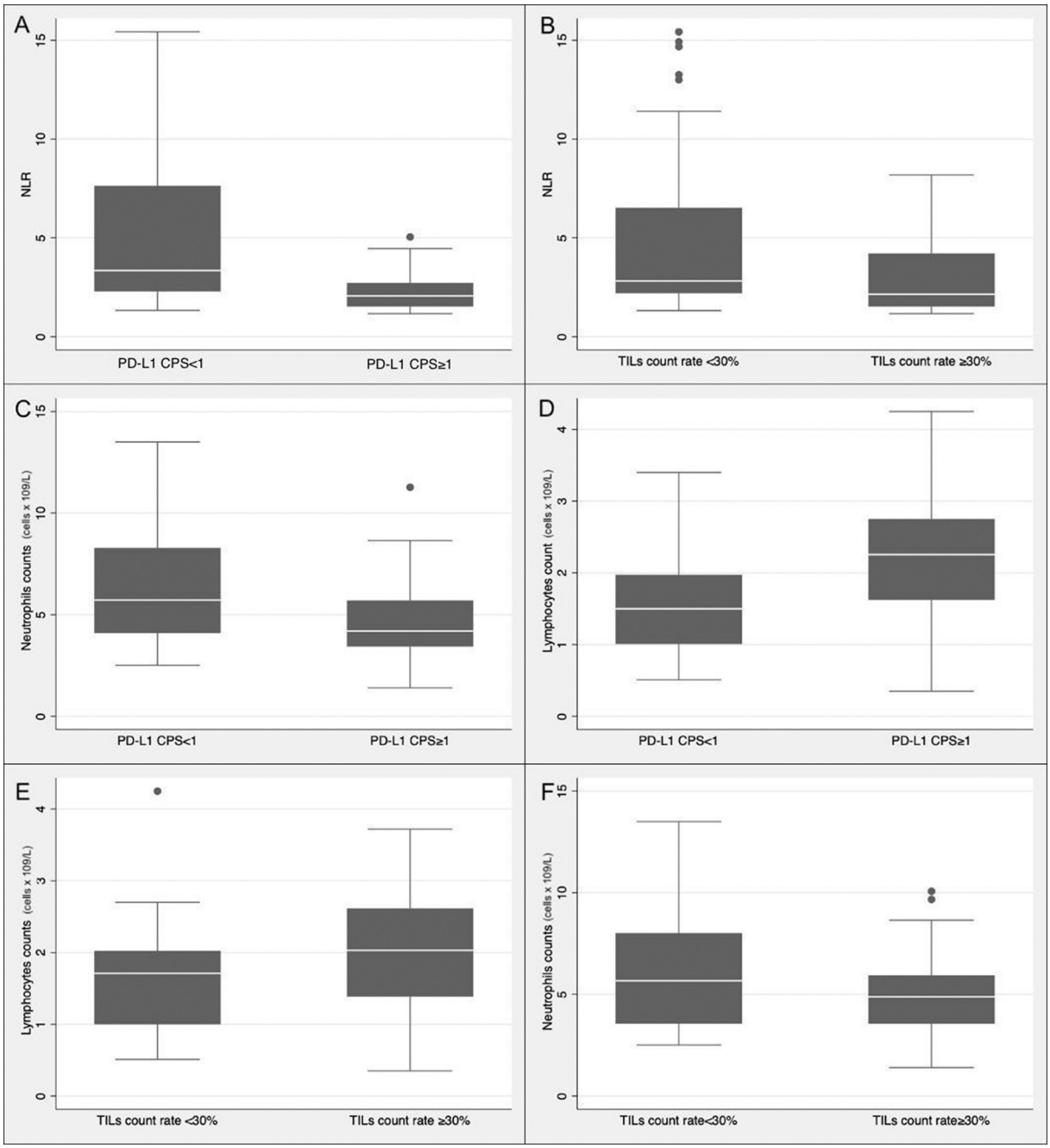

Fig. 4. Box plot showing differences in distribution of: (A) NLR vis-à-vis PD-L1 CPS <1 vs. ≥1; (B) NLR vis-à-vis TILs count rate <30% vs. ≥30%; (C) neutrophils counts vis-à-vis PD-L1 CPS <1 vs. ≥1; (D) lymphocytes counts vis-à-vis PD-L1 CPS <1 vs. ≥1; (E) neutrophils counts vis-à-vis TILs count rate <30% vs. ≥30%; (F) Lymphocytes counts vis-à-vis TILs count rate <30% vs. ≥30%.

4. Discussion

Tumors harness elements of both the innate and adaptive immune systems to facilitate their growth and metastasis. Recent reports have revealed that tumor-induced perturbations in the immune system can be the target of therapeutic strategies aiming to initiate or reinvigorate antitumor immune responses [28]. Circulating inflammatory cells have been explored as prognostic markers in several tumor types for about a decade [5-7]; in particular, NLR has been considered a promising clinical prognostic biomarker [29]. Recent studies found that blood neutrophils were directly linked with the number of intratumoral neutrophil populations, which may have the potential to compromise the antitumor immune response [29,30]. It has been established that neutrophils play a significant role in tumor development, progression and metastasis, either by exercising a direct effect on tumor cells or by indirectly affecting other components of the tumor microenvironment. This effect is achieved through the secretion and release of various chemokines and cytokines, including transforming growth factor-beta, vascular endothelial growth factor, IL-6, IL-8 and matrix metalloproteinase. Lower counts of lymphocytes usually reflect an impairment of cell-mediated immunity [31].

Local tumor immune microenvironment markers have been increasingly studied in head and neck oncology, with special reference in recent years to their predictive value in response to immunotherapy [3,26,32,33]. Among the available markers, tumor PD-L1 expression in terms of CPS is currently recommended to be routinely tested in HNSCCs

samples to select patients for immunotherapy [26]. In HNSCC there is a dearth of information regarding the prognostic significance of the association between PD-L1 and circulating inflammatory cells counts and ratios, including NLR. To evaluate the role of systemic and local inflammation in LSCC, Wang et al. [34] retrospectively analyzed NLR and TILs density in 120 patients who had undergone postoperative radiotherapy or chemo-radiotherapy. Statistical analysis revealed that high NLR (cut-off value 2.79) and low TILs density were both significantly correlated with inferior overall survival and DFS. In 2019, Kucuk et al. [35] investigated the possible association between local (TIL density in tumor area) and systemic (NLR with the same cut-off value of Wang et al. [34]) inflammation in 116 previously operated LSCCs. They found no statistically significant relationship between NLR, TIL density and overall survival.

To the best of our knowledge, this study is the first to investigate in LSCC the prognostic relationship between NLR and tumor immune microenvironment histopathological features in terms of both PD-L1 expression and TILs. In our series, NLR value, blood neutrophils, and lymphocytes counts were shown to be significant in predicting DFS and recurrence risk. Moreover, PD-L1 CPS $\geq$ 1 and TILs count rate $\geq$30% were associated with higher DFS and reduced recurrence risk. This was in line with previous studies on PD-L1 expression in LSCCs [17,18,20,36]. Our logistic regression model found a significant positive association between increasing NLR values, and PD-L1 CPS $<$ 1 and TILs count rate $<$30%. These results seem to highlight a close relationship between circulating inflammatory cells and LSCC immune microenvironment characteristics: in particular, the association between high blood neutrophils counts, NLR values vs tumor microenvironment features was suggestive of a weak antigen-driven local immune response. On the other hand, high lymphocytes counts were associated with tumor microenvironment features, such as high TILs count rates and PD-L1 expression, which might be indicative of a strong local anti-cancer immune pressure.

The main weakness of our study is the retrospective setting of the investigation and the limited number of cases included. Conversely, its main strength lies in the homogeneity of the series of patients considered because: (i) they all underwent primary laryngeal surgery; (ii) their surgical treatment was performed consecutively by the same team; (iii) only surgical specimens (not biopsies) of LSCC were assessed; (iv) only squamous cell carcinomas located in a single head and neck structure (the larynx) were considered; (v) the PD-L1 antibody used (22C3 IHC PharmDX) was a commercial clone, validated by a panel of experts for therapeutic purpose, as was the scoring system adopted (CPS) [26]; (vi) clinical-radiological follow-up criteria were defined; (vi) all pre-operative laboratory blood tests were performed at the same laboratory.

Given our promising results, further prospective studies on larger series are needed to better characterize the role of pre-operative blood NLR in association with PD-L1 expression and tumor immune microenvironment features as prognostic factors and markers for anti-tumor immune response in LSCCs, with special reference to the efficacy of immunotherapeutic protocols. In particular, the potential of simple, inexpensive, accessible clinical pre-treatment measures such as NLR should be further investigated as stratification factors to help identifying patients with advanced LSCC who could benefit from PD-1/PD-L1 inhibitors also in the adjuvant and neo-adjuvant setting.

Funding

This study was partly supported by grant No. DOR2090593/20 (G. Marioni) from the University of Padova, Italy.

Declaration of competing interest

The authors have no conflict of interest to disclose.

Acknowledgments

The authors thank Alison Garside for correcting the English version of this paper.

References

[1] Marioni G, Ottaviano G, Lovato A, et al. Expression of maspin tumor suppressor and mTOR in laryngeal carcinoma. Am J Otolaryngol 2020;41:102322.
[2] Lovato A, Franz L, Carraro V, et al. Maspin expression and anti-apoptotic pathway regulation by bcl2 in laryngeal cancer. Ann Diagn Pathol 2020;45:151471.
[3] Burtness B, Harrington KJ, Greil R, et al. Pembrolizumab alone or with chemotherapy versus cetuximab with chemotherapy for recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-048): a randomised, open-label, phase 3 study. Lancet 2019;394:1915–28.
[4] Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011;331:1565–70.
[5] Tsai YD, Wang CP, Chen CY, et al. Pretreatment circulating monocyte count associated with poor prognosis in patients with oral cavity cancer. Head Neck 2014;36:947–53.
[6] Wu G, Yao Y, Bai C, et al. Combination of platelet to lymphocyte ratio and neutrophil to lymphocyte ratio is a useful prognostic factor in advanced non-small cell lung cancer patients. Thorac Cancer 2015;6:275–87.
[7] Tashima Y, Kuwata T, Yoneda K, et al. Prognostic impact of PD-L1 expression in correlation with neutrophil-to-lymphocyte ratio in squamous cell carcinoma of the lung. Sci Rep 2020;10:1243.
[8] Takahashi H, Sakakura K, Tada H, Kaira K, Oyama T, Chikamatsu K. Prognostic significance and population dynamics of peripheral monocytes in patients with oropharyngeal squamous cell carcinoma. Head Neck 2019;41:1880–8.
[9] Formica V, Morelli C, Patrikidou A, et al. Gastric inflammatory prognostic index (GIPI) in patients with metastatic gastro-esophageal junction/gastric cancer treated with PD-1/PD-L1 immune checkpoint inhibitors. Target Oncol 2020;15:327–36.
[10] Yamada T, Hayashi T, Inokuchi Y, et al. Impact of the neutrophil-to-lymphocyte ratio on the survival of patients with gastric cancer treated with nivolumab monotherapy. Target Oncol 2020;15:317–25.
[11] Proctor MJ, McMillan DC, Morrison DS, Fletcher CD, Horgan PG, Clarke SJ. A derived neutrophil to lymphocyte ratio predicts survival in patients with cancer. Br J Cancer 2012;107:695–9.
[12] Valero C, Zanoni DK, Pillai A, et al. Host factors independently associated with prognosis in patients with oral cavity cancer. JAMA Otolaryngol Head Neck Surg 2020;146(8):1–9.
[13] Mattavelli D, Lombardi D, Missale F, et al. Prognostic nomograms in oral squamous cell carcinoma: the negative impact of low neutrophil to lymphocyte ratio. Front Oncol 2019;9:339.
[14] Taube JM, Anders RA, Young GD, et al. Colocalization of inflammatory response with B7-H1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med 2012;4:127ra37.
[15] Schalper KA, Velcheti V, Carvajal D, et al. In situ tumor PD-L1 mRNA expression is associated with increased TILs and better outcome in breast carcinomas. Clin Cancer Res 2014;20:2773–82.
[16] Zhuang Y, Liu C, Liu J, Li G. Resistance mechanism of PD-1/PD-L1 blockade in the cancer-immunity cycle. Onco Targets Ther 2020;13:83–94.
[17] Vassilakopoulou M, Avgeris M, Velcheti V, et al. Evaluation of PD-L1 expression and associated tumor-infiltrating lymphocytes in laryngeal squamous cell carcinoma. Clin Cancer Res 2016;22:704–13.
[18] Alessandrini L, Franz L, Ottaviano G, et al. Prognostic role of programmed death ligand 1 (PD-L1) and the immune microenvironment in laryngeal carcinoma. Oral Oncol 2020;108:104836.
[19] Franz L, Tealdo G, Contro G, et al. Biological tumor markers (maspin, CD105, nm23-H1) and disease relapse in laryngeal cancer: a cluster analysis. Head Neck 2020;42:2129–36.
[20] Franz L, Alessandrini L, Ottaviano G, et al. Postoperative radiotherapy for laryngeal cancer. The prognostic role of programmed death-ligand 1: an immune microenvironment-based cluster analysis. Pathol Res Pract 2020;216:153120.
[21] Amin MB. AJCC staging manual. 8th ed. Basel: Springer; 2017.
[22] National Comprehensive Cancer Network. NCCN guidelines for head and neck cancer care. www.nccn.org/professionals/physiciangls/PDF/head-and-neck.pdf. [Accessed 23 May 2020].
[23] Marioni G, Franz L, Ottaviano G, et al. Prognostic significance of CD105- and CD31-assessed microvessel density in paired biopsies and surgical samples of laryngeal carcinoma. Cancers 2020;12:2059.
[24] Salgado R, Denkert C, Demaria S, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol 2015;26:259–71.
[25] Fuchs CS, Doi T, Jang RW, et al. Safety and efficacy of pembrolizumab monotherapy in patients with previously treated advanced gastric and gastroesophageal junction cancer: phase 2 clinical KEYNOTE-059 trial. JAMA Oncol 2018;4:e180013.
[26] Cohen EEW, Bell RB, Bifulco CB, et al. The Society for Immunotherapy of Cancer consensus statement on immunotherapy for the treatment of squamous cell carcinoma of the head and neck (HNSCC). J Immunother Cancer 2019;7:184.
[27] Wotman M, Herman SW, Costantino P, et al. The prognostic role of programmed death ligand 1 in nasopharyngeal carcinoma. Laryngoscope 2020. https://doi.org/10.1002/lary.28523 (in press).

[28] Sio A, Chehal MK, Tsai K, et al. Dysregulated hematopoiesis caused by mammary cancer is associated with epigenetic changes and hox gene expression in hematopoietic cells. Cancer Res 2013;73:5892–904.
[29] Moses K, Brandau S. Human neutrophils: their role in cancer and relation to myeloid-derived suppressor cells. Semin Immunol 2016;28:187–96.
[30] Petrova MP, Eneva MI, Arabadjiev JI, et al. Neutrophil to lymphocyte ratio as a potential predictive marker for treatment with pembrolizumab as a second line treatment in patients with non-small cell lung cancer. Biosci Trends 2020;14:48–55.
[31] Jiang X, Xu J, Liu M, et al. Adoptive CD8+ T cell therapy against cancer: challenges and opportunities. Cancer Lett 2019;462:23–32.
[32] Ferris RL, Blumenschein Jr G, Fayette J, et al. Nivolumab for recurrent squamous-cell carcinoma of the head and neck. N Engl J Med 2016;375:1856–67.
[33] Ferris RL, Blumenschein Jr G, Fayette J, et al. Nivolumab vs investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck: 2-year long-term survival update of CheckMate 141 with analyses by tumor PD-L1 expression. Oral Oncol 2018;81:45–51.
[34] Wang J, Wang S, Song X, et al. The prognostic value of systemic and local inflammation in patients with laryngeal squamous cell carcinoma. Onco Targets Ther 2016;9:7177–85.
[35] Kucuk U, Ekmekci S, Bozkurt P, Bulgurcu S, Cukurova I. Relationship between local and systemic inflammatory response and prognosis in laryngeal squamous cell carcinoma. North Clin Istanb 2020;7:180–4.
[36] Birtalan E, Danos K, Gurbi B, et al. Expression of PD-L1 on immune cells shows better prognosis in laryngeal, oropharygeal, and hypopharyngeal cancer. Appl Immunohistochem Mol Morphol 2018;26:e79–85.