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ORIGINAL ARTICLE – CANCER RESEARCH

Assessment of tumor-associated immune cells in laryngeal squamous cell carcinoma

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Abstract

Purpose This study investigated the characteristics of tumor-associated immune cells (TAICs) in laryngeal squamous cell carcinoma (LSCC) and their correlation with clinicopathological variables.

Methods The immune cell infiltrates of 71 specimens of stages I–IV LSCC were examined. The density of TAICs expressing CD3, CD4, CD8, CD68, and CD163 was assessed using immunohistochemical staining and image analysis in peritumoral and intratumoral regions.

Results Higher densities of CD3+ and CD8+ cell and lower densities of CD68+ and CD163+ cell infiltrations were found in early tumor stages than in late tumor stages. A higher percentage of patients with strong CD3+ and CD8+ immune cell infiltration and weak CD68+ cell infiltration in both tumor regions presented with T1 stage tumors compared with T4 stage tumors. Further, strong CD68+ cells infiltration in both regions was observed in a greater number of patients who had a relapse, while a weak CD3+ cells infiltration in both regions was found in a greater number of patients with nodal lymphatic metastasis. The univariate analysis showed that a high density of peritumoral CD3+ and CD8+ immune cells in both regions was significantly associated with a favorable overall survival (OS) ($P=0.004$; $P=0.006$; $P=0.042$). In contrast, a high density of intratumoral CD68+ cells and peritumoral CD163+ cells was significantly associated with poor OS durations ($P=0.026$; $P=0.030$). The multivariate analysis demonstrated that a high density of peritumoral CD163+ cells correlated with poor OS after adjusting for tumor stage, recurrence, and nodal lymphatic metastasis ($P=0.034$). This study found different patterns of TAIC infiltration in LSCC. The density and location of TAICs infiltration correlated with the clinicopathological characteristics of LSCC.

Conclusion A combined analysis of the density of TAICs and their location may help predict patient survival and response to checkpoint inhibitors.

Keywords Tumor-associated immune cells (TAICs) · Immunohistochemistry · Laryngeal squamous cell carcinoma (LSCC)

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Introduction

Laryngeal squamous cell carcinoma (LSCC) is the second common head and neck cancer worldwide (Jemal et al. 2011). Smoking and alcohol consumption are the main risks factors for LSCC patients (Galbiatti et al. 2013). A treatment regimen of a combination of appropriate surgery, radiation therapy, and chemotherapy did not improve the survival rate of LSCC patients in recent decades. Effective therapeutic strategies and increased understanding of the relevant mechanism are thus needed to improve the prognosis of LSCC (Leemans et al. 2011).

The immune system plays a vital role in the control of tumor growth and progression (Marini et al. 2016). However,

the role of immune cells in human neoplasia is less clear (Greten et al. 2004; Kauer and White 2003; Pollard 2004; Pikarsky et al. 2004; Pardoll 2001). Burnet (1971) stated that cancer develops when the host's immunity changes from an extrinsic tumor suppressor to a facilitator of tumor growth and progression through constant interactions between tumor cells and immune cells in the tumor microenvironment (Dunn et al. 2002). Lymphocytes are responsible for this process (Burnet 1971). Tumor-infiltrating lymphocytes (TILs) have been shown to inhibit tumor growth and are associated with improved prognosis in many cancers (Diederichsen et al. 2003; Sato et al. 2005; Zhang et al. 2003). A majority of studies have reported an association of Th1 cells with good clinical outcomes in many different tumor types, including melanoma, lung cancers, colorectal cancer, breast, bladder, urothelial, ovarian, renal, prostate, and head and neck (Marini et al. 2016; Angell and Galon 2013). In contrast, a low density of TILs was associated with a poor prognosis (Pagès et al. 2005; Galon et al. 2006, 2007). In general, high densities of myeloid cells, including macrophages and myeloid-derived suppressor cells, correlate with poor prognosis (Gabrilovich et al. 2012).

Infiltrating tumor immune cells are highly heterogeneous, not only among tumor types but also within different tumor regions and patients with the same cancer types. Some studies demonstrated TILs expressing CD3 or CD8 as an independent factor for favorable overall survival (OS) and distant metastasis-free survival in head and neck cancer (Balermpas et al. 2016). However, other studies found that the number of CD4+ or CD8+ TTLs was not associated with OS in a small cohort of patients with oral cavity carcinoma (Wolf et al. 2015). A previous study showed that the prognostic value of CD3+ and CD8+ TILs depended on the infiltration patterns. CD3+ TILs within the tumor cells or in proximity to tumor parenchyma bear a prognostic value. In contrast, CD3+ TILs in the tumor stroma or the periphery of tumor islands do not exhibit a prognostic value (Balermpas et al. 2014).

Galon et al. reported that major immune cell infiltration both in the center and in the invasive margin of human colorectal tumors varied with time and tumor progression. The evolution and spatial distribution of these subpopulations of immune cells might affect tumor recurrence. Therefore, Galon et al. believed that immune cell infiltrates varied considerably from tumor to tumor and evolved over time (Pagès et al. 2005; Galon et al. 2006, 2007). Some studies found that the T-cell immune infiltrate is the most important predictive criterion for patient survival (Atreya and Neurath 2008; Bindea et al. 2010; Mlecnik et al. 2011; Galon et al. 2006; Pagès et al. 2005). The local coordination of various immune compartments has not been comprehensively analyzed in patients with LSCC to date. In the present study, Deeper insights into tumor–immune system interaction during LSCC progression was gained by assessing the patterns of tumor-associated immune cells (TAICs) infiltrating in peritumoral and intratumoral compartments in LSCC specimens and investigating the relationship between the type, density, and location of infiltrating TAICs and the clinical outcomes of LSCC.

Materials and methods

Patient population

The retrospective study was conducted on a cohort of 71 patients with primary laryngeal squamous cell carcinoma (LSCC) who underwent surgery with curative intent between March 2002 and May 2008 at The First Hospital of Shanxi Medical University. Formalin-fixed, paraffin-embedded histological sections were surgically resected from primary LSCC tumors. Prior to treatment, the cancer stage was determined using electronic laryngoscopy, B-mode ultrasound, X-ray analysis, computed tomography, magnetic resonance imaging, and pathological examination and classified using the Union for International Cancer Control Tumor Node Metastasis Classification System for Laryngocarcinoma, Eighth Edition (Bertero et al. 2018). This study was approved by the Institutional Review Board of Center for Clinical and Translational Research of Shanxi Medical University.

Immunohistochemical staining

Four-micrometer-thick sequential histologic tumor sections were obtained from a representative formalin-fixed, paraffin-embedded tumor block and used for immunohistochemical (IHC) analysis. IHC was performed with primary mouse anti-human antibody against CD3 (T-cell lymphocytes; D7A6E, dilution 1:200; Cell Signaling Technology, MA, USA), CD8 (cytotoxic T cell; D8A8Y, dilution 1:200; Cell Signaling Technology), CD163 (macrophages; D6U1G, dilution 1:500; Cell Signaling Technology), and CD68 (macrophages; D4B9C, Cell Signaling Technology) and rabbit anti-human antibody against CD4 (helper T cell; 8–25 μg/mL; R&D System, MN, US). IHC staining was performed using a Super Vision IHC kit (cat. no. SV0002; Wuhan Boster Biological Technology, Ltd., Wuhan, China). The slides were deparaffinized with xylene and rehydrated through ethanol series with decreasing concentrations. Subsequently, the slides were washed three times with phosphate-buffered saline (PBS) for 5 min each, following which endogenous peroxidase was quenched using 3% hydrogen peroxide for 30 min and the slides were washed again with PBS three times for 5 min each. Antigen retrieval was performed by boiling the slides in a pressure-boiling container with pH 6.0 citrate buffer (0.01 M sodium citrate–citric acid

buffer) at 102 °C for 10 min, followed by washing three times and then incubation with 10% goat serum blocking solution (cat. no. AR0009; Wuhan Boster Biological Technology, Ltd.) for 20 min at room temperature. The slides were incubated with a previously described primary antibody overnight at 4 °C. After three washes with PBS, the slides were incubated with a secondary antibody (provided in the Super Vision IHC kit) at 37 °C for 30 min. Following 3,3′-diaminobenzidine staining, the sections were counterstained with hematoxylin for 2 min at room temperature, dehydrated, and attached to coverslips. Human tonsil formalin-fixed, paraffin-embedded tissues with and without primary antibody were used as positive and negative controls, respectively, with each run of IHC staining.

Image analysis

The IHC expression of different markers was evaluated and the immune cells were quantified by digitally scanning whole tumor sections at 200 × magnification using a Panoramic slide scanner II (3D Histech, Budapest, Hungary) and analyzed using CaseViewer2.2(3D Histech, Budapest, Hungary). Two pathologists with 10 years of experience who performed the image analysis were blinded to patients' clinical data. The densities of cells expressing CD3, CD4, CD8, CD68, and CD163 were evaluated by counting the positive cells in five random square areas (1 mm^2 each) in both intratumoral and peritumoral regions. Each 1 mm^2 area was histologically assessed to ensure that the tumor tissue (at least 75% malignant cells and tumor stroma) was included in the selected intratumoral region and only nonmalignant cells were included in the peritumoral region. For this analysis, each examined area was overlapped sequentially with the IHC-stained slides to quantify each marker in the same location of tumor specimens. The average number of cells positive for each marker in the five square areas was expressed as density per square millimeter. The samples were divided into low and high groups using median cutoff values for the density of immune cells positive for each marker (50% of patients with a high density and 50% of patients with a low density).

Statistical analysis

The Wilcoxon rank-sum test and Kruskal–Wallis test were used to identify markers with significantly different expression among patient groups. The χ^2 test or Fisher exact test was used to examine the differences in percentages of patients between different groups. OS was defined as the interval from surgery to death or last contact. OS data were censored at 10 years if a patient was alive or died after 5 years. The OS distributions for the patients were estimated using the Kaplan–Meier method. A log-rank test was performed to determine the difference in OS between the groups. Regression analysis of the OS data was performed using a Cox proportional hazards model. The statistical software SPSS (version 22) was used to perform the computations for all analysis.

Results

Baseline clinical and experimental data

The image analysis based on examination of whole tumor sections obtained from 71 patients with primary LSCC revealed that the mean patient age was 61.3 years. The majority were men (93%) and smokers (98.6%). Most of the patients were diagnosed as supraglottic or glottic (94.4%) and more often node negative on a clinical/radiologic examination (76.1%). Further, 15 (21.1%) patients had relapsed and 27 (38%) had died. The 10-year survival rate and 5-year survival rate were 60.4% and 71.7%, respectively. The clinicopathological data of patients are shown in Table 1.

TAIC density

The present study revealed different TAICs densities in tumor specimens, particularly while examining the intratumoral and peritumoral compartments separately. Representative images of immunohistochemically stained TAICs for these markers are shown in Fig. 1. The density of overall TAICs was found to be significantly higher in the peritumoral compartment than in the intratumoral compartment (CD3, $P = 0.002$; CD8, $P < 0.001$; CD4, $P < 0.001$; CD68, $P < 0.001$; and CD163, $P < 0.001$) (Table 2). In the peritumoral compartment, the density of cells expressing CD4 was significantly higher than that of cells expressing CD8 ($P < 0.001$). Similar to TILs, the density of cells expressing CD68 was significantly higher than that of cells expressing CD163 in the peritumoral compartment ($P < 0.001$) (Fig. 2). Unexpectedly, no significantly higher numbers of cells expressing CD4 were present in the intratumoral compartment compared with cells expressing CD8 ($P = 0.999$). The differences in densities between CD68+ and CD163+ cells were less noticeable in the intratumoral compartment ($P = 0.146$) (Fig. 2). These findings indicated that the densities of TILs and tumor-associated macrophages (TAMs) varied according to the tumor regions.

Relationship between the extent of TAIC infiltration and tumor stage, recurrence, and nodal lymphatic metastasis of LSCC

The data showed an inverse correlation between the density of TILs and tumor stage, nodal stage, and recurrence.

Table 1 Clinicopathological characteristics of patients with LSCC

Category	*N* (%)
Age (years)	
Median	61.3 (39–83)
Sex	
Male	66 (93.0%)
Female	5 (7.0%)
Smoking	
No	1 (1.4%)
Yes	70 (98.6%)
Alcohol abuse	
No	42 (59.1%)
Yes	29 (40.9%)
Tumor (T) stage	
T1	19 (26.8%)
T2	22 (30.9%)
T3	19 (26.8%)
T4	11 (15.5%)
Histologic grade	
Well	25 (35.2%)
Moderate	31 (43.7%)
Poor	15 (21.1%)
Nodal (N) stage	
N0	54 (76.1%)
N1	11 (15.5%)
N2	6 (8.4%)
Localization	
Glottic	34 (47.9%)
Subglottic	33 (46.5%)
Supraglottic	4 (5.6%)
Recurrence	
No	56 (78.9%)
Yes	15 (21.1%)
Vital status after 10 years	
Alive	44 (62.0%)
Dead	27 (38.0%)

Peritumoral densities of cells expressing CD3 and CD8 were high at an early stage (T1) and decreased gradually with tumor progression (T1–T4; $P<0.001$, $P=0.003$). A similar result was obtained when analyzing the intratumoral densities of cells expressing CD3 and CD8 among T1–T4 stages (T1–T4; $P<0.001$, $P<0.001$) (Fig. 3a, b). The peritumoral density of cells expressing CD3 was higher in patients without nodal lymphatic metastasis than in those with lymphatic metastasis ($P<0.001$) (Fig. 3c). Tumors of patients without recurrence had higher peritumoral CD3 immune cell densities than tumors of patients with a tumor recurrence ($P=0.036$) (Fig. 3d). No significant correlation was found between the density of cells expressing CD4 and the clinicopathological characteristics of LSCC (Fig. 3a–d). In contrast, the density of TAMs positively correlated with tumor stage and recurrence. Patients with T4 stage had higher numbers of cells expressing CD163 in the peritumoral compartment and cells expressing CD68 in the intratumoral compartment than those with T1 stage ($P=0.002$, $P=0.014$) (Fig. 3a, b). Higher numbers of TAMs (peritumoral cells expressing CD68 and cells expressing CD163 in both regions) were observed in patients who had a relapse compared with those with no relapse ($P<0.001$, $P=0.001$, $P=0.038$) (Fig. 3d). No notable correlation was found between the number of TAICs and histologic grade and tumor location.

Patients were divided into four groups on the basis of the densities of TAICs in both tumor regions using a median cutoff value of TAICs (HiHi, LoHi, HiLo, and LoLo). Patients with high densities of TAICs in both peritumoral and intratumoral compartments were classified into the HiHi group. Patients with low densities of TAICs in both peritumoral and intratumoral compartments were classified into the LoLo group. Patients with low peritumoral densities and high intratumoral densities of TAICs were classified into the LoHi group. Patients with high peritumoral densities and low intratumoral densities of TAICs were classified into the HiLo group. The study found that 58% of patients had a stage T1 tumor compared with only 9% of patients with a stage T4 tumor in the CD8HiHi group. In contrast, only 10% patients presented with a low density of CD8+ cells in both tumor regions (CD8LoLo) in early-stage T1 tumors, whereas 46% of patients with a weak CD8 immune cell infiltrate had stage T4 tumors. Similar results were obtained for immune cells expressing CD3. Heterogeneous CD8 cell infiltrates (HiLo and LoHi) in tumor regions were equally distributed among T stages (Fig. 4a, b). The Chi square test comparing four groups of patients on the basis of the densities of CD3 and CD8 (HiHi, LoHi, HiLo, and LoLo) revealed significant differences in the distribution of patients between tumor stages (T1/2 and T3/4) ($P=0.025$, $P=0.032$). Further, 11% of patients had a stage T1 tumor compared with 46% of patients with a stage T4 tumor in the CD68 HiHi group. In contrast, 42% of patients presented with a low density of CD68+ cells in both tumor regions (CD68LoLo) in T1 tumors, whereas 9% of patients with weak CD68 immune cell infiltration had stage T4 tumors. However, the difference was not statistically significant among the four groups (CD68HiHi, CD68LoHi, CD68HiLo, and CD68LoLo, $P=0.257$) or between the two groups of patients (CD68HiHi and CD68LoLo, $P=0.121$). Similar results were obtained for the density of cells expressing CD163. No differences were found between the T stage and heterogeneous TAM cell infiltration (HiLo and LoHi).

The analysis of TAICs and tumor recurrence demonstrated low CD3+ cell infiltration and high CD68+ cell infiltration in both regions on a tumor relapse. Patients with a tumor recurrence had a higher frequency of CD3LoLo cell

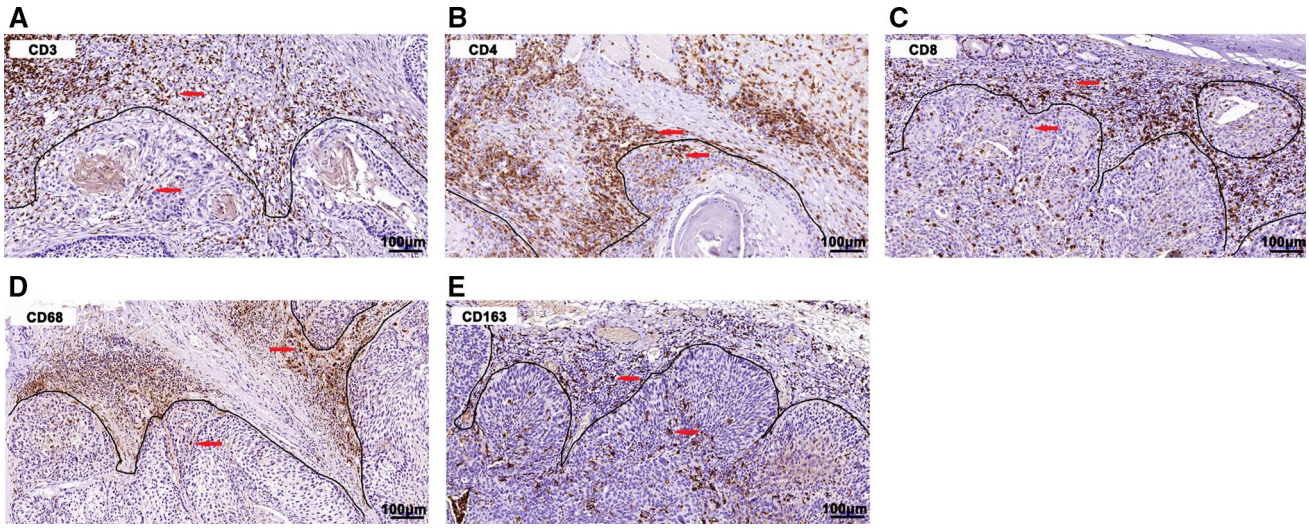


Fig. 1 Microphotographs of representative examples of the five immune markers in LSCC specimens (**a** CD3; **b** CD4; **c** CD8; **d** CD68 and **e** CD163). A black line divides LSCC tissue into two compartments: intratumoral (below the image) and peritumoral (above the image). Arrows indicate cells expressing each immune marker (red)

Table 2 Median density of TAIC in the intratumoral and peritumoral compartments in LSCC

Markers	Peritumoral	Intratumoral	*P*
CD3	1517	735	0.002
CD4	1071	415	< 0.001
CD8	603	330	< 0.001
CD68	550	132	< 0.001
CD163	315	123	< 0.001

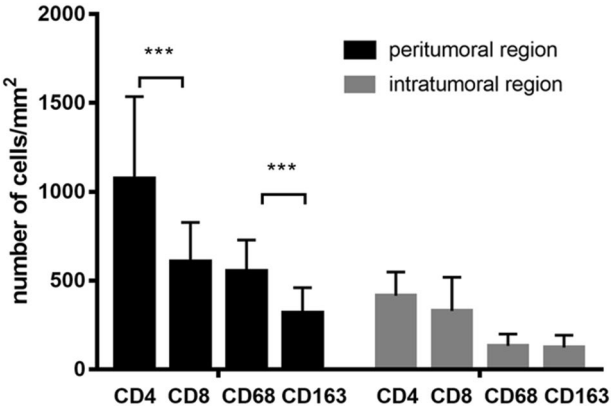


Fig. 2 A comparison of the mean of immune cell densities in the peritumoral region (black bars) and intratumoral region (gray bars). In the peritumoral region, the density of cells expressing CD4 was significantly higher than that of cells expressing CD8. The density of cells expressing CD68 was significantly higher than that of cells expressing CD163 in the peritumoral compartment. In the intratumoral compartment, the differences in densities were not significant. ***$P < 0.001$

infiltration compared with CD3HiHi cell infiltration (60% and 7%, $P = 0.016$). In contrast, 40% of patients who had a relapse had CD68HiHi cell infiltration compared with 7% of those with CD68LoLo cell infiltration ($P = 0.046$) (Fig. 4c, d). Smaller differences were found for homogeneous CD3+ and CD68+ cell infiltrations (HiLo and LoHi) in patients with no relapse (Fig. 4c, d). When analyzing TAIC infiltration and nodal lymphatic metastasis, 65% of patients were found to have nodal lymphatic metastasis compared with only 19% of patients without nodal lymphatic metastasis in the CD3LoLo group. In contrast, 37% of patients without nodal lymphatic metastasis presented CD3HiHi cell infiltration compared with 0% of patients with nodal lymphatic metastasis had CD3HiHi cell infiltration ($P < 0.001$) (Fig. 4e). No significant difference was observed between the percentage of patients in other TAIC infiltrate (CD4, CD8, CD163, and CD68) groups when presenting with nodal lymphatic metastasis.

Correlation between immune marker expression and LSCC prognosis

The present study evaluated the correlation between the extent of immune cell infiltration in situ in both tumor regions with patient outcomes. Kaplan–Meier curves highlighted the shorter OS of patients with low densities of peritumoral CD3+ cells and CD8+ cells in both compartments compared with high densities of them ($P = 0.002$, $P = 0.003$, $P = 0.036$). In contrast, patients with high densities of intratumoral CD68+ and peritumoral CD163+ cells had poor OS ($P = 0.021$, $P = 0.025$) (Fig. 5a–d). The peritumoral density

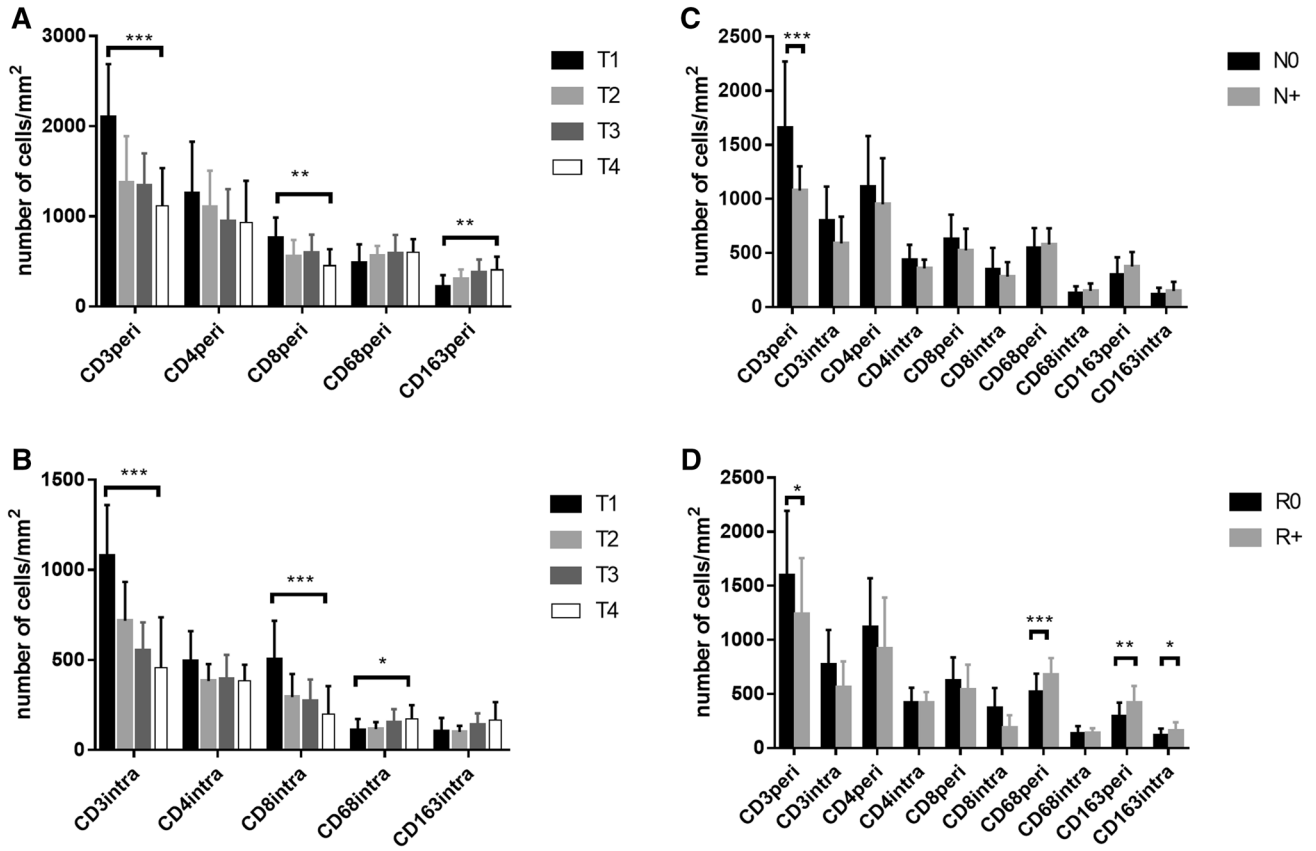


Fig. 3 A comparison of the mean of immune cell densities in the peritumoral and intratumoral regions among patients with different T (tumor stage), N (nodal lymphatic metastasis), and R (recurrence) stages. TAIC densities were recorded as cells per square millimeter of tissue. Histograms show the mean ± standard error of TAIC densities in the peritumoral (**a**) and intratumoral regions (**b**) in the different groups of patients according to the T stage. A comparison of the mean ± standard error of TAIC densities in the peritumoral and intratumoral regions according to nodal lymphatic metastasis (**c**) and recurrence (**d**). N0, patients without nodal lymphatic metastasis; N+, patients with nodal lymphatic metastases; R0, patients without recurrence; R+, patients with a recurrence. Data were statistically analyzed using the Wilcoxon–Mann–Whitney test. $*P<0.05$; $**P<0.01$; $***P<0.001$

of cells expressing CD3 greater than the median density significantly correlated with good OS duration in the univariate analysis [hazard ratio (HR), 0.293; 95% confidence interval (CI), 0.128–0.671; $P=0.004$]. Similar results were obtained for cells expressing CD8 in peritumoral and intratumoral compartments (HR 0.297; 95% CI 0.125–0.705; $P=0.006$; HR 0.435; 95% CI 0.195–0.969; $P=0.042$). However, the intratumoral density of cells expressing CD68 and the peritumoral density of cells expressing CD163 greater than the median density were significantly associated with poor OS durations in the univariate analysis (HR 2.489; 95% CI 1.117–5.548; $P=0.026$; HR 2.382; 95% CI 1.090–5.208; $P=0.030$). The multivariate analysis demonstrated that the peritumoral density of cells expressing CD163 greater than the median density correlated with poor OS durations after adjusting for tumor stage, recurrence, and nodal lymphatic metastasis (HR 2.530; 95% CI 1.074–5.959; $P=0.034$).

The study next determined whether TAIC infiltration could discriminate the patient outcome in different tumor regions. A strong CD8 immune reaction in both regions (CD8HiHi) correlated with a favorable prognosis. However, a weak CD8 in situ immune reaction in both tumor regions (CD8LoLo) correlated with a poor prognosis (HR 0.160; 95% CI 0.046–0.556; $P=0.004$) (Fig. 5a, b). In contrast, a high density of CD163+ cells in both tumor regions (CD163HiHi) was found to be associated with poor prognosis (HR 4.099; 95% CI 1.259–13.342; $P=0.019$) (Fig. 5c, d) in the univariate analysis. The multivariate analysis demonstrated that the density of CD163+ cells greater than the median density correlated with poor OS durations after adjusting for tumor stage, recurrence, and nodal lymphatic metastasis (HR 0.181; 95% CI 0.043–0.764; $P=0.020$).

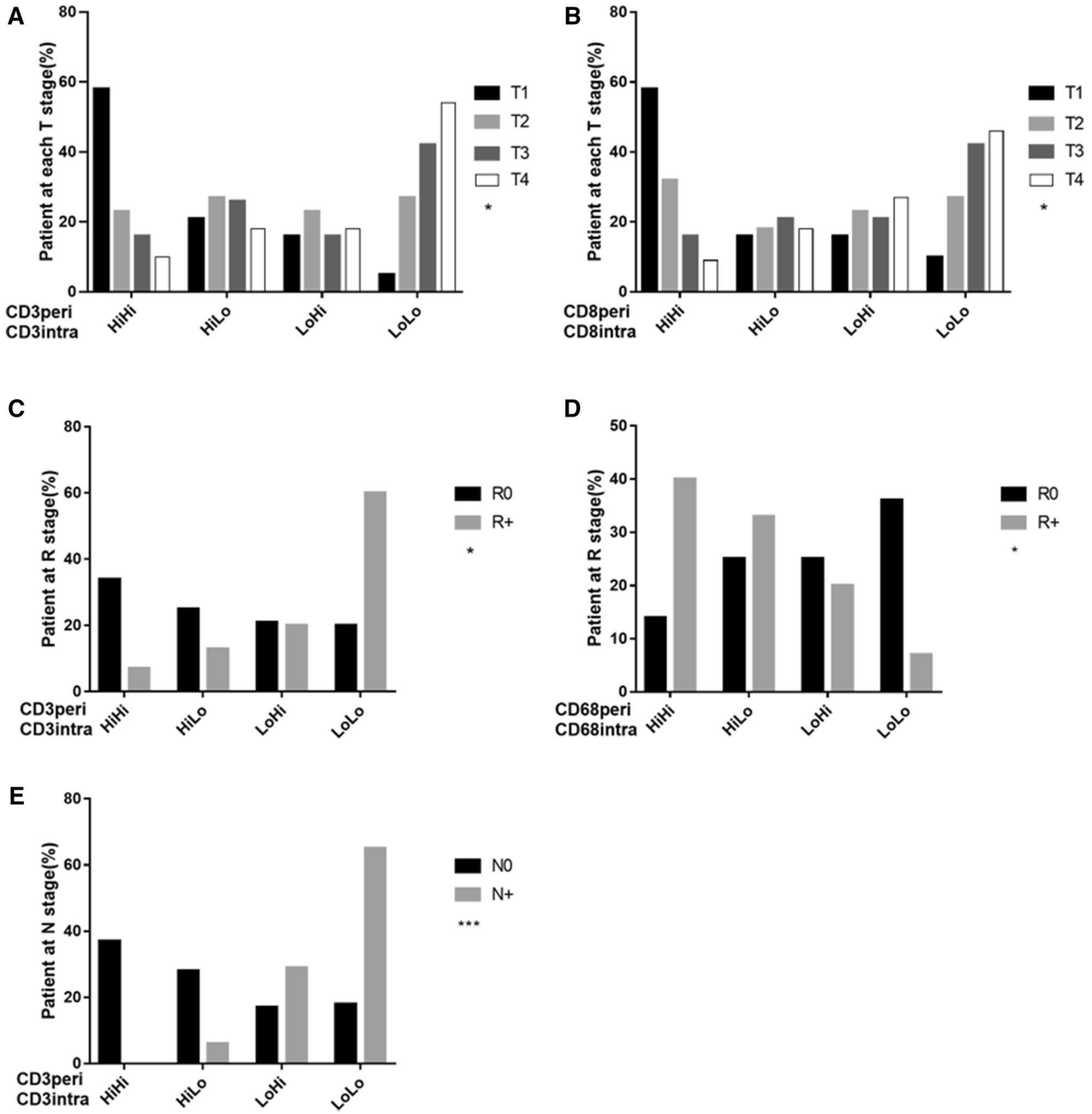
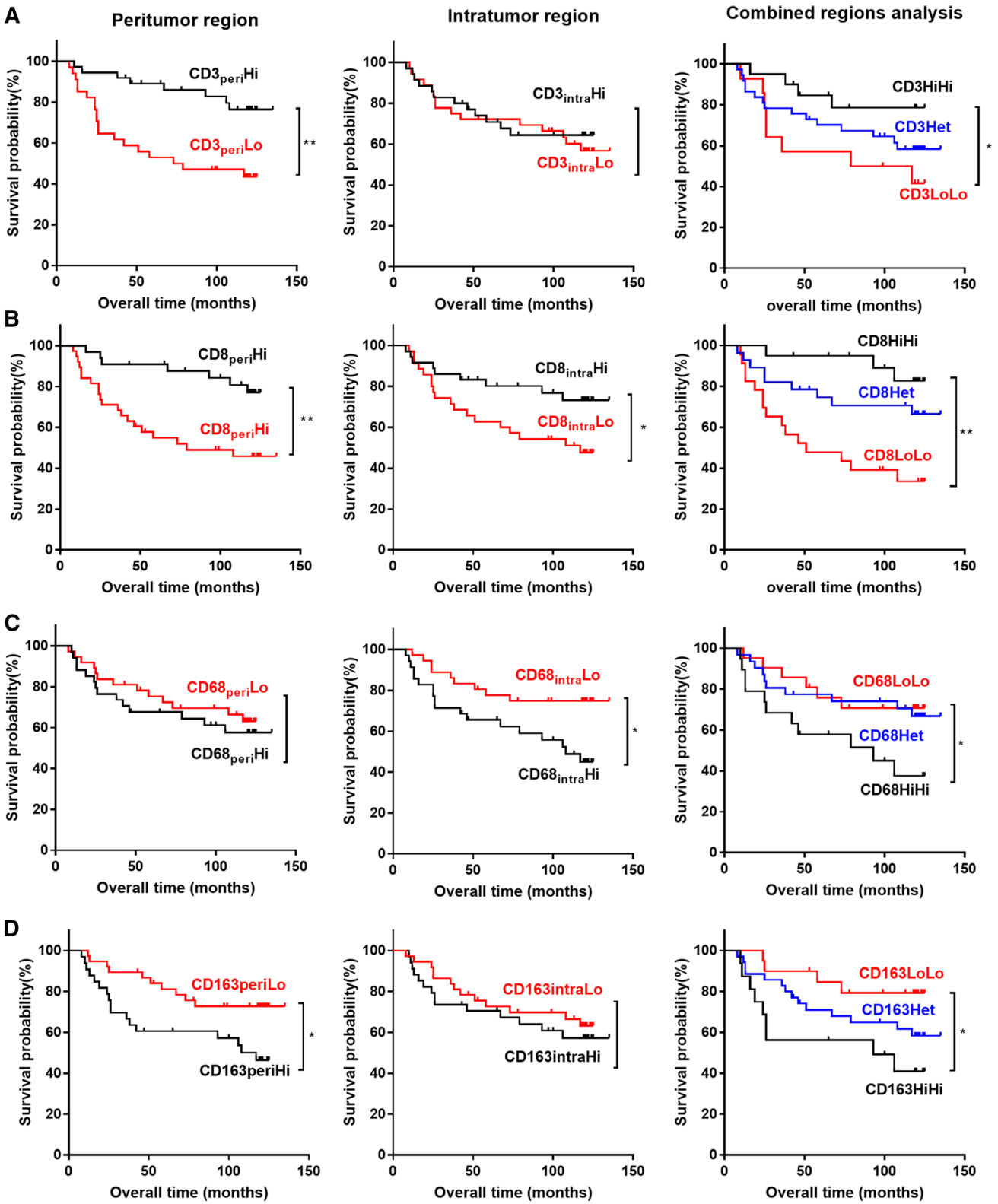


Fig. 4 Percentages of patients in the four groups of TAICs (HiHi, HiLo, LoHi, and LoLo) at each T (tumor stage), R (recurrence), and N (nodal lymphatic metastasis) stages of LSCC. Patients were divided into four groups on the basis of TAIC densities in the peritumoral and intratumoral regions using a median cutoff value of TAICs (HiHi, HiLo, LoHi, and LoLo). **a**, **b** Histograms show the percentages of patients in different CD3 and CD8 groups (CD3HiHi, CD3HiLo, CD3LoHi, and CD3LoLo; CD8HiHi, CD8HiLo, CD8LoHi, and CD8LoLo) at each tumor stage. Percentages of patients in different CD3 and CD68 groups are shown according to nodal lymphatic metastasis (**c**, **d**) and recurrence (**e**). Percentage of patients in all four groups (HiHi, HiLo, LoHi, and LoLo) at each stage adds up to 100%. N0, patients without nodal lymphatic metastasis; N+, patients with nodal lymphatic metastases; R0, patients without recurrence; R+, patients with recurrence. Statistical analysis was performed using the Chi square test or Fisher exact test. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$



Discussion

In this study, the patterns of TAICs infiltrating in primary LSCCs were assessed and complex interactions between tumors and their microenvironment were observed. The density of TAICs expressing most of the immune markers tested was found to be significantly higher in peritumoral than in intratumoral compartments. An inverse correlation

◂**Fig. 5** Kaplan–Meier curves illustrating the prognostic effect of the expression of immune markers on OS in LSCC. Kaplan–Meier curves illustrate the duration of OS according to the densities of CD3 (**a**), CD8 (b), CD68 (**c**), and CD163 (**d**) cells in a single tumor region (left panels, peritumoral region; middle panels, intratumoral region) and in both tumor regions (right panels). For each tumor region, high (Hi) and low (Lo) densities of TAICs were plotted according to the cutoff value of each cell density defined at the median of the cohort (50% of patients with high cell density and 50% of patients with low cell density). In a single-region analysis (left and middle panels), black lines indicate CD3Hi and red lines indicate CD3Lo. In a combined analysis (right panels), black lines indicate CD3periHiCD3intraHi(CD3HiHi), red lines indicate CD3periLoCD3intraLo(CD3LoLo), and blue lines indicate heterogeneous densities of CD3 with CD3periLo plus CD3intraHi or CD3periHi plus CD3intraLo (CD3Het). $*P<0.05$; $**P<0.01$; $***P<0.001$

was observed between the densities of CD3+ and CD8+ cells and tumor stage, nodal stage, and recurrence. However, a positive correlation was found between the densities of CD68+ and CD163+ cells and tumor stage and recurrence in both tumor regions. Kaplan–Meier curves highlighted that low peritumoral densities of CD3+ cells and CD8+ cells in both compartments correlated with poor outcomes. In contrast, a high density of intratumoral CD68 and peritumoral CD163 cells was related to poor OS duration. The multivariate analysis demonstrated that the density of CD163+ cells greater than the median density correlated with poor OS durations after adjusting for tumor stage, recurrence, and nodal lymphatic metastasis. The study concluded that the density of TAICs varied according to tumor compartment and progression in surgically resected specimens. Importantly, the present study identified several immune markers whose expression correlated with the outcome of LSCC.

The present study showed that the density of TAICs was higher in the peritumoral compartment than in the intratumoral compartment. However, the density of CD4+ cells was nearly equal to that of CD8+ cells in the intratumoral region in most specimens, while the density of CD4+ cells was higher than that of CD8+ cells in the peritumoral region. It was hypothesized that the nonhomogeneous or clustered secretion of cytokines and chemokines at the invasive tumor margin might enhance the recruitment of different TAIC subtypes to the tumor microenvironment (Marini et al. 2016). This study showed that the densities of CD3+ and CD8+ cells in both compartments gradually decreased with tumor progression. A significantly high percentage of patients with early tumor stage had strong CD3+ and CD8+ immune cell infiltration in both tumor regions (HiHi). These results were in agreement with previous findings indicating the association between a greater number of TILs with the earlier stages of HNSCC (Mlecnik et al. 2011; Wolf et al. 1986). It was concluded that the immune cell infiltrates varied considerably from tumor to tumor and evolved over time. The analysis of patients according to the presence of lymph nodes and recurrence revealed a higher density of peritumoral CD3+ cells in patients with lymph nodes metastasis than in those without lymph nodes metastasis. Tumors of patients without a recurrence had higher CD3+ immune cell densities within both tumor regions than those of patients with a recurrence. A high percentage of patients with strong CD3HiHi immune cell infiltration had no nodal lymphatic metastasis and recurrence. Mlecnik et al. (2011) demonstrated significant differences in immune cell densities between patient groups according to the extension of tumor (T stage) and the presence of lymph node or distant metastases. It is possible that TIL infiltration could reflect a level of antitumor immunity shaped by different mechanisms from the tumor and the microenvironment (Campoli and Ferrone 2008; Finn 2006; Galon et al. 2007). A strong immune reaction at the early tumor stage could be a major determinant for controlling the tumor evolution, and the progressive decrease in immune cell densities along with tumor invasion could indicate a progressive immune escape. It was hypothesized that a specific localized immune reaction to LSCC could influence the evolution and recurrence of tumor. The prognostic importance of tumor infiltration with CD3+ and CD8+ cells has been demonstrated in breast, esophageal, lung, ovarian, colon, and anal cancers (Gooden et al. 2011). In addition, Vassilakopoulou et al. (2016) reported that the intratumoral localization of TILs influenced their prognostic impact in LSCC. The present study found that patients with higher densities of peritumoral CD3+ and CD8+ TILs in both tumor regions had a favorable prognosis compared with those with lower densities of CD3+ and CD8+ TIL.

TAMs have a significant role in tumor progression and seem to affect responses to immunotherapy. TAMs include both M1 macrophages, which are involved in promoting antitumor immunity, and the M2 macrophages, which possess pro-tumorigenic properties (Chanmee et al. 2014). CD68 is the best established generic macrophage marker. CD163 is the most commonly used and best established marker for M2 polarized macrophages (Kawamura et al. 2009; Hasan et al. 2012). Macrophages are often found in the stromal compartment of solid tumors, including breast, ovarian, pancreatic, and hepatocellular carcinomas (McGettrick et al. 2012; Feig et al. 2012; Wu and Zheng 2012; Ruffell et al. 2012). TAMs help the tumor escape other immune responses by recruiting immunosuppressors such as regulatory T cells and encouraging angiogenesis (Morrison 2016). Clinical studies have shown an association between higher frequencies of TAMs and poor prognosis across a range of tumors (Kawamura et al. 2009; Hu et al. 2016; Kurahara et al. 2011). The present study demonstrated a significant association between strong infiltration of intratumoral CD68+ and CD163+ cells in both peritumoral and intratumoral compartments with tumor progression and recurrence in LSCC. High densities of intratumoral CD68+ cells and peritumoral CD163+

cells correlated with poor clinical outcomes. Similar to the findings of this study, Weber et al. and Wehrhan et al. also showed that increased numbers of CD163+ macrophages in the epithelial compartment of patients with oral squamous cell carcinoma (OSCC) correlated with the late T stage, recurrence of locoregional lymph node metastases, and poor outcomes. Furthermore, an increased malignant behavior of tumors was associated with increased numbers of CD163+ TAMs in the regional lymph nodes (Weber et al. 2014; Wehrhan et al. 2014). CD163+ subset macrophages can frequently recruit effector T cells not capable of mounting a protective antitumor immunity through secreting interleukin (IL-4, IL-13, IL-10) and other immunosuppressive cytokines (Qian and Pollard 2010; Noy and Pollard 2014). Reports suggest that macrophages can directly suppress T-cell responses through programmed cell death ligand 1(PD-L1) in hepatocellular carcinoma and B7-H4 in ovarian carcinoma (Kuang et al. 2009; Kryczek et al. 2006).

Cancer immunotherapy with immune checkpoint inhibitors in HNSCC has shown promising preliminary results. However, ongoing trials with anti-PD1/PD-L1 antibodies demonstrated only a fraction of patients benefiting from therapy (Segal et al. 2019). Immune cell infiltration and recognition of tumor cells by the immune system were required for the successful response of the tumor to immune checkpoint blockade (Mandal et al. 2016). Teng proposed four types of tumor microenvironments based on the presence or absence of TILs and PD-L1 expression. When TILs are present in sufficient numbers inside the tumor and these cells induce adaptive expression of PD-L1, the patients may respond to PD-1/L1 blockade. However, in patients with a low TIL infiltrate tumor microenvironment, single-agent checkpoint blockade is most likely not successful given the lack of preexisting T-cell infiltrates (Teng et al. 2015). Vassilakopoulou and Kluger suggested that TILs could be included as a predictive biomarker of patient response to checkpoint inhibitors (Vassilakopoulou et al. 2016; Kluger et al. 2015). Le and Zhu found that neither PD-1 nor CTLA-4 blockade could significantly reduce tumor growth in the murine model and patients with pancreatic cancer with high TAM infiltrates. However, the combination of CSF-1R blockade and PD-1 or CTLA-4 blockade inhibited tumor growth after CSF-1R blockade-induced reduction of TAMs (Le et al. 2013; Zhu et al. 2014). These findings showed that a quantitative assessment of TAICs could help predict patient response to immune checkpoint blockades.

The present study had a number of limitations. One limitation was that the study included retrospectively collected cases. Second, the number of cases was insufficient and we need more cases for further verification. The third was the cutoff value of the number of infiltrating TAICs. The clinically relevant threshold for the number of TAICs infiltrating in LSCC cells is yet to be defined. Various cutoffs of the number of infiltrating TAICs have been examined, including at least 5%, 30%, 25 cells/mm^2, and greater than the median value (Mlecnik et al. 2011; Vassilakopoulou et al. 2016; Mattox et al. 2017; Weber et al. 2016). Considering different patterns of TAICs in different tumors, the median value was used as the cutoff value in our research. This cutoff value was more frequently used to assess the number of TAICs in many tumors (Mlecnik et al. 2011; Weber et al. 2016).

Conclusion

In summary, the present study demonstrated that TILs infiltration positively correlated with a favorable tumor prognosis, while the density of TAMs was a poor prognostic indicator in LSCC. It concluded that different patterns of TAICs infiltrating in LSCCs correlated with tumor prognosis in LSCC. The evolution of tumor-associated immune cell subpopulations along with tumor progression could lead to different strategies for treating patients with LSCC. A combined analysis of the type, density, and location of TAIC infiltration can help scientists better understand the interaction between the tumor and microenvironment and the ways to therapeutically manipulate this system.

Author contributions LYZ, YJ L, and BQ W conceived and designed the study. LYZ and YJL performed the experiments. HHF, SXW, WG, CMZ, QLZ, and ZD collected the data. CXQ, GDL, LNW, and GY managed, analyzed, and interpreted the data. LYZ and YJL wrote the paper. LYZ and YJL reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Compliance with ethical standards

Conflict of interest All authors declare to have no COIs.

Ethical approval The Medical Research Ethics Committee of the first hospital, Shanxi Medical University, approved the sample acquisition (Taiyuan, China).

Informed consent Informed consent was obtained from all individual participants included in the study.

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