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Human papillomavirus-associated immune profile of the tumor microenvironment in laryngeal squamous cell carcinoma: a Tunisian cohort study

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Abstract

Background

Human papillomavirus (HPV) infection plays a key role in the pathogenesis of several squamous cell carcinomas and may influence tumor immune responses. Although HPV-associated oropharyngeal squamous cell carcinoma has been extensively studied, the relationship between high-risk HPV (HR-HPV) status and the tumor immune microenvironment (TME) in laryngeal squamous cell carcinoma (LSCC) remains poorly understood. This study aimed to investigate the association between HR-HPV status and immune cell infiltration in LSCC.

Methods

A retrospective cohort of 70 patients with histologically confirmed LSCC was analyzed. HR-HPV DNA detection was performed on tumor specimens. Immunohistochemical analysis was used to evaluate $CD8^+$ cytotoxic T cells, $FOXP3^+$ regulatory T cells, and $CD68^+/CD163^+$ tumor-associated macrophages. Immune infiltration was assessed using a semi-quantitative immunoreactivity score. Associations between immune markers, HR-HPV status, clinicopathological parameters, and survival outcomes were evaluated.

Results

HR-HPV DNA was detected in 39/70 (55.7%) LSCC cases. HR-HPV-positive tumors showed significantly increased infiltration of $CD8^+$ T cells, $FOXP3^+$ Tregs, and $CD68^+/CD163^+$ macrophages compared with HR-HPV-negative tumors ($p < 0.0001$). High $CD8^+$ T-cell infiltration was associated with improved overall and disease-free survival, although without statistical significance. $FOXP3^+$ Treg infiltration was significantly associated with tumor grade.

Conclusions

HR-HPV-positive LSCC exhibits a distinct immune landscape characterized by increased infiltration of T cells and tumor-associated macrophages, suggesting HPV-driven immune modulation within the TME. These findings highlight the importance of immune profiling in HPV-related LSCC and support further studies exploring its prognostic and therapeutic implications.

Introduction

Head and neck squamous cell carcinomas (HNSCCs) represent a heterogeneous group of malignancies with distinct etiological, molecular, and clinical characteristics. Among these cancers, laryngeal squamous cell carcinoma (LSCC) remains a major health burden worldwide and is strongly associated with classical risk factors, including tobacco and alcohol consumption. In Tunisia, laryngeal cancer represents an important oncological concern. According to the Global Cancer Observatory (GLOBOCAN 2022), the 5-year prevalence of laryngeal cancer (all ages) was estimated at 12.4 cases per 100,000 inhabitants, with a marked male predominance. Histologically, squamous cell carcinoma (SCC) accounts for approximately 95% of laryngeal tumors, with most cases displaying low- to intermediate-grade differentiation [1].

In addition to conventional risk factors, high-risk human papillomavirus (HR-HPV) infection has emerged as an important etiological factor in a subset of HNSCCs, particularly oropharyngeal squamous cell carcinoma (OPSCC) [2–4]. HPV-driven tumors exhibit distinct biological and immunological characteristics resulting from viral antigen expression and the interaction between infected tumor cells and the host immune system. HPV-derived antigens may influence immune cell recruitment, antigen presentation, and the balance between effective antitumor immune responses and immune escape mechanisms [7–10]. Consequently, the composition and functional status of the tumor immune microenvironment (TME)

have gained increasing attention as potential determinants of tumor progression and therapeutic response in HPV-associated malignancies [11–12].

Unlike HPV-associated OPSCC, the role of HPV infection in LSCC remains less clearly established and is still a matter of debate [3–6]. Furthermore, the impact of HR-HPV infection on immune cell organization within the LSCC microenvironment remains poorly characterized, particularly in populations underrepresented in molecular and immunological studies.

Tumor-infiltrating immune cells play a crucial role in shaping tumor behavior. $CD8^+$ cytotoxic T lymphocytes are major effectors of antitumor immunity through their ability to recognize and eliminate malignant cells, whereas $FOXP3^+$ regulatory T cells (Tregs) contribute to immune tolerance and may facilitate tumor immune evasion. The prognostic relevance of these lymphocyte populations has been increasingly investigated in HNSCC [14–16]. In addition, tumor-associated macrophages (TAMs) are key components of the TME and regulate tumor progression, inflammation, immune suppression, and therapeutic resistance. CD68 is widely used as a pan-macrophage marker, while CD163 is commonly associated with an immunoregulatory M2-like macrophage phenotype [13, 17].

Despite the growing recognition of HPV-associated immune modulation, data regarding the immune landscape of HR-HPV-positive LSCC remain limited, especially in North African populations. In a previous Tunisian study, HR-HPV DNA was detected in 55.71% of LSCC tumors using in situ hybridization targeting high-risk HPV genotypes [6]. Therefore, the present study aimed to characterize HR-HPV-associated immune remodeling in LSCC by evaluating the intratumoral expression of $CD8^+$ T cells, $FOXP3^+$ Tregs, $CD68^+$ and $CD163^+$ macrophages, and by investigating their associations with clinicopathological parameters and patient outcomes in a Tunisian cohort.

Material and methods

Patients

A retrospective study was conducted using archived formalin-fixed, paraffin-embedded surgical specimens from 70 patients diagnosed with laryngeal squamous cell carcinoma (LSCC) who were treated between 2002 and 2011. The treatment consisted of surgery followed by radiotherapy, with or without concomitant chemotherapy. Clinical data collected included gender, age at diagnosis, smoking status, alcohol consumption, tumor location, tumor stage (T), nodal stage (N), overall disease stage, HPV status, treatment details, and the date of death or last follow-up (Table 1). Patients were classified into 39 HPV-positive (HPV+) and 31 HPV-negative (HPV−) groups, with the prevalence and prognostic significance of HPV previously established in this cohort.

Table 1: Clinicopathological characteristics of LSCC patients (N=70)

	Number	Percent (%)
Age		
<60 years	29	41.4
≥ 60 years	41	58.6
Gender		
Male	67	95.7
Female	3	4.3
pT stage		
T1	0	0
T2	2	2.86
T3	12	17.14
T4	56	80
pTNM stage		
I	0	0
II	2	2.86
III	7	10
IV	61	87.14
Lymph nodes		
N-	54	77.14
N+	15	21.43
Missed	1	1.43
Histological grade		
G1 (well differentiated)	66	94.3
G2 (moderatelydifferentiated)	3	4.3
G3 (poorly differentiated)	1	1.4
Tumor site		
Glottis+sus+subglottic	30	42.86
Glottis+sus glottic	15	21.43
Glottic+subglottic	17	24.28
Glottic	8	11.43
Smoking consumption		
Yes	62	88.57
No	3	4.28
Missed	5	7.15
Alcohol consumption		

Yes	30	42.9
No	19	27.1
Missed	21	30
Tumor recurrence		
Yes	22	31.25
No	68	68.75
Survival data (n=64)		
Alive	43	67.19
Death	21	32.81
HPV status		
Positive	39	55.71
Negative	31	44.29

Immunohistochemical staining of tumor

The expression of TILs (CD8, FoxP3) and TAM (CD68, CD163) was evaluated using an immunohistochemical assay. Tissue sections (4 μm thick) were cut from each paraffin block, deparaffinized in xylene, and rehydrated through a graded series of ethanol solutions. Antigen retrieval was performed by heating the sections at 98°C for 30 minutes in 10 mM citrate buffer, pH 6.0 (for CD163 and FoxP3) or pH 9.0 (for CD8 and CD68). The slides were then washed in phosphate-buffered saline (PBS), and endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Next, the sections were incubated at room temperature with specific primary antibodies: anti-CD8 (diluted 1:50, 30 minutes), anti-CD68 (NCL-L-CD68; diluted1:100, 30 minutes), anti-CD163 (NCL-L-CD163; diluted1:200, 30 minutes) (mouse monoclonal antibodies from Novocastra, Leica), and FoxP3 (ab 20034; diluted 1:100, 1 hour; Abcam, Cambridge, United Kingdom). After washing with PBS, the specimens were treated with the Novolink Max Polymer Detection System according to the manufacturer's instructions. Staining was visualized using 3,3-diaminobenzidine (DAB), and the slides were counterstained with Mayer's hematoxylin. Finally, the sections were dehydrated, cleared, and mounted.

Evaluation of immunohistochemical staining

Immunohistochemical staining for CD8, FoxP3, CD68 and CD163 was evaluated independently by two pathologists (SK, YH), co-authors of this paper. They compared the stained sections with the corresponding hematoxylin and eosin (H&E) stained slides to accurately identify tumor regions. Staining results were quantified by calculating the percentage of stained area to total tumor-infiltrated area, expressed as (stained area/total area) × 100 for each biopsy sample. The percentage of positively stained cells was classified as 0 (negative), 1 (< 25%), 2 (25–50%) or 3 (> 50%). Staining of at least 25% of cells was considered positive for FoxP3 expression. Staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). A composite score was obtained by multiplying the intensity score by the percentage score. On the basis of this composite score, cases were classified into two groups: low expression (composite score ≤ 2) and high expression (composite score > 2).

Statistical Analysis

Analyses were performed using SPSS 26. The chi-square test or Fisher's exact test was used to assess the association of CD8, FoxP3, CD68 and CD163 with clinicopathological parameters. Overall survival (OS) was calculated from the date of

anatomopathological diagnosis to the date of death or the date of the last follow-up. Disease -free survival (DFS) was defined as the time elapsed between diagnosis and the onset of progression (either imaging or clinical) or death. Survival curves were plotted using the Kaplan-Meier method, and median differences in disease -free survival (DFS) between groups were tested using the log-rank test. For the multivariate analysis of DFS, a Cox proportional hazards regression model was employed, including only variables with significant effects on survival identified in the univariate Cox regression. All statistical tests were two-tailed, with a p-value < 0.05 considered significant.

Results

Patient characteristics

The clinicopathological characteristics of the 70 patients with laryngeal squamous cell carcinoma (LSCC) are summarized in Table 1. The cohort included 67 males (95.7%) and 3 females (4.3%). Regarding tumor classification, no patient was diagnosed with T1 disease, whereas 2 patients (2.86%) were classified as T2, 12 (17.14%) as T3, and 56 (80%) as T4. Concerning nodal status, 54 patients (77.14%) were N-negative and 15 (21.43%) were N-positive. Most patients presented with advanced disease, with 2 cases (2.86%) classified as stage II, 7 (10%) as stage III, and 61 (87.14%) as stage IV.

Histologically, 66 tumors (94.3%) were classified as grade G1. Tumor location was glottic, supraglottic, or subglottic in 30 patients (42.86%). A history of tobacco smoking was reported in 62 patients (88.57%), while 30 patients (42.86%) reported alcohol consumption.

Survival data were available for 64 patients. Among them, 43 patients (67.19%) were alive at the last follow-up, whereas 21 (32.81%) had died. The mean overall survival (OS) was 88.22 months (95% CI: 72.55–103.90 months). Disease recurrence occurred in 20 patients (31.25%), with a mean disease-free survival (DFS) of 89.53 months (95% CI: 73.76–105.31 months).

Based on HR-HPV DNA status, patients were classified into two groups: 39 HR-HPV-positive (55.7%) and 31 HR-HPV-negative (44.3%) cases, as previously reported [6].

Association between immune cell infiltration and clinicopathological characteristics

The immunohistochemical evaluation of $CD8^+$ T cells, $FOXP3^+$ regulatory T cells (Tregs), $CD68^+$ and $CD163^+$ macrophages is shown in Fig. 1. The associations between immune cell infiltration levels and clinicopathological characteristics are summarized in Table 2. $CD8^+$ T-cell infiltration was significantly lower in smokers compared with non-smokers ($p = 0.034$). $FOXP3^+$ Treg infiltration also showed a lower frequency among smokers, with a borderline statistical significance ($p = 0.051$).

Table 2
Correlation of CD8, CD68, CD163 and FoxP3 expression with clinicopathological parameters of LSCC patients

Age	**CD8**			**CD68**			**CD163**			**FoxP3**		
	Low	**High**	**P**	**Low**	**High**	**P**	**Low**	**High**	**P**	**Low**	**High**	**P**
< 60 years	17	12	0.619	13	16	0.810	12	17	1	16	13	0.810
≥ 60 years	27	14		17	24		16	25		24	17	
Gender												
Male	42	25	1	28	39	0.573	26	41	0.560	38	29	1
Female	2	1		2	1		2	1		2	1	
pT stage												
T1	0	0	0.774	0	0	0.224	0	0	0.284	0	0	0.671
T2	2	0		2	0		2	0		2	0	
T3	7	5		6	6		5	7		7	5	
T4	35	21		22	34		21	35		31	25	
pTNM stage												
I	0	0	0.716	0	0	0.365	0	0	0.281	0	0	0.439
II	2	0		2	0		2	0		2	0	
III	4	3		3	4		3	4		5	2	
IV	38	23		25	36		23	38		33	28	
Lymph nodes												
N-	32	22	0.604	20	34	0.103	19	35	0.177	29	25	0.642
N+	11	4		9	6		8	7		10	5	
Missed	1	0		1	0		1	0		1	0	
Histological grade												
G1	43	23	0.140	28	38	0.760	28	38	0.389	40	26	**0.030**
G2	1	2		1	2		0	3		0	3	
G3	0	1		1	0		0	1		0	1	
Tumor site												
Glottis + sus+subglottic	21	9	0.653	11	19	0.846	11	19	0.915	16	14	0.839
Glottis + sus glottic	9	6		7	8		6	9		10	5	
Glottic+subglottic	9	8		8	9		7	10		9	8	
Glottic	5	3		4	4		4	4		5	3	

High expression: composite score > 2, low expression: composite score ≤ 2 ; Chi square test or Fisher exact test, statistical significance defines as $p < 0.05$

Age	CD8			CD68			CD163			FoxP3		
	Low	High	P	Low	High	P	Low	High	P	Low	High	P
Tabac consumption												
Yes	42	20	**0.034**	28	34	0.627	27	35	0.251	38	24	**0.051**
No	1	2		1	2		0	3		0	3	
Missed	1	4		1	4		1	4		2	3	
Alcohol consumption												
Yes	17	13	0.496	15	15	0.484	11	19	0.450	16	14	0.789
No	14	5		8	11		10	9		12	7	
Missed	13	8		7	14		7	14		12	9	
High expression: composite score > 2, low expression: composite score ≤ 2 ; Chi square test or Fisher exact test, statistical significance defines as $p < 0.05$												

FOXP3⁺ Treg infiltration was significantly associated with histological grade ($p = 0.030$), with lower infiltration observed in grade G1 tumors. No significant association was observed between CD68⁺ or CD163⁺ macrophage infiltration and clinicopathological parameters

Association between immune cell infiltration and HR-HPV status

The distribution of CD8⁺ T cells, FOXP3⁺ Tregs, CD68⁺ macrophages, and CD163⁺ macrophages according to HR-HPV status is summarized in Table 3.

Table 3
Correlation between CD8, CD163, FoxP3 expression (Low or High) and HPV status (number and percentage)

Biomarkers Immunostaining	HPV+	HPV-	P value
CD8			
Low	17 (24.28%)	27(38.57%)	**< 0.0001**
High	22 (31.42%)	4(5.73%)	
CD163			
Low	6(8.57%)	22(31.42%)	**< 0.0001**
High	33(47.14%)	9(12.87%)	
CD68			
Low	8(11.46%)	22(31.42%)	**< 0.0001**
High	31(44.27%)	9(12.87%)	
FoxP3			
Low	14(20%)	26(37.14%)	**< 0.0001**
High	25(35.71%)	5(7.15%)	
High expression: composite score > 2, low expression: composite score ≤ 2; Chi square test or Fisher exact test, statistical significance defines as $p < 0.05$			

All evaluated immune cell populations showed higher infiltration levels in HR-HPV-positive tumors compared with HR-HPV-negative tumors. This association between HR-HPV status and immune cell infiltration was statistically significant ($p < 0.0001$).

Survival analysis according to immune cell infiltration and HR-HPV status

Overall survival (OS) (Fig. 2A). and disease-free survival (DFS) (Fig. 2B). analyses were performed in 64 LSCC patients.

Among the evaluated immune markers, $CD8^+$ T-cell infiltration showed a trend toward improved survival outcomes. Patients with high $CD8^+$ infiltration had a longer OS compared with patients with low $CD8^+$ infiltration (mean OS: 101.12 ± 9.66 months, 95% CI: 82.20–120.05 vs 76.63 ± 10.04 months, 95% CI: 56.95–96.32), although this difference did not reach statistical significance ($p = 0.055$) (Fig. 2A).

Similarly, patients with high $CD8^+$ infiltration had a longer DFS compared with those with low $CD8^+$ infiltration (mean DFS: 100.68 ± 9.80 months, 95% CI: 81.48–119.89 vs 78.74 ± 10.18 months, 95% CI: 58.79–98.70), with a borderline statistical significance ($p = 0.067$) (Fig. 2B).

No significant association was observed between $CD68^+$, $CD163^+$ macrophage infiltration, or $FOXP3^+$ Treg infiltration and survival outcomes.

In this cohort, HR-HPV status was associated with DFS ($p = 0.016$), whereas the association with OS showed a trend toward significance ($p = 0.081$).

Multivariate Cox regression analysis for LSCC prognosis

Multivariate Cox regression analysis was performed to evaluate the independent prognostic contribution of HR-HPV status and $CD8^+$ T-cell infiltration (Tables 4a and 4b). HR-HPV positivity showed a potential association with improved DFS

independently of CD8⁺ T-cell infiltration; however, this association did not reach statistical significance (p = 0.062).

Table 4
a. Multivariate Cox regression analysis for prognosis of LSCC patients (CD8, HPV and OS)

HPV	B	SE	Wald	ddl	Sig.	Exp(B)
	− .561	**.464**	**1.466**	**1**	**.226**	**.570**
CD8	− .427	.287	2.216	1	.137	.652

Table 4
b. Multivariate Cox regression analysis for prognosis of LSCC patients (CD8, HPV and DFS)

HPV	B	SE	Wald	ddl	Sig.	Exp(B)
	− .940	**.505**	**3.471**	**1**	**.062**	**.391**
CD8	− .353	.290	1.480	1	.224	.703

Discussion

In the present study, we investigated the relationship between HR-HPV status and immune cell infiltration in LSCC. Our findings indicate that HPV-positive LSCC exhibits a distinct immune profile characterized by increased infiltration of CD8⁺ T cells, FOXP3⁺ regulatory T cells, and macrophage populations.

The association between HPV positivity and increased immune infiltration supports the concept that HPV-driven tumors may generate a different immune contexture compared with HPV-negative tumors. Viral antigens can enhance immune recognition and promote recruitment of immune cells into the tumor microenvironment. However, the presence of immune cells does not necessarily reflect effective antitumor activity, as regulatory mechanisms within the TME may limit immune responses [7–10, 11].

CD8⁺ T lymphocytes represent major effectors of tumor-directed immunity. In our cohort, high CD8⁺ infiltration showed a trend toward improved overall survival and disease-free survival. Although statistical significance was not reached, these observations suggest a potential role of cytotoxic immune infiltration in LSCC outcome that requires confirmation in larger cohorts. Previous studies have demonstrated that high densities of CD8⁺ T cells are associated with favorable outcomes in head and neck cancers and LSCC [15, 18–20].

The increased FOXP3⁺ regulatory T-cell infiltration observed in HPV-positive tumors highlights the complexity of HPV-associated immune responses. Tregs may coexist with activated immune populations and contribute to immune equilibrium within the tumor microenvironment. Their prognostic significance appears to vary according to tumor type, anatomical site, and viral status [21–23]. In HPV-associated head and neck cancers, Tregs have been reported to be associated with improved outcomes in some settings, suggesting that HPV-related immune modulation may influence their functional role [24–26].

Regarding macrophages, CD68⁺ and CD163⁺ cells were more frequently observed in HPV-positive tumors, suggesting HPV-related modulation of macrophage recruitment or polarization. However, no significant survival association was identified in our cohort, emphasizing the need for further characterization of macrophage subsets in LSCC. TAMs are major regulators of the tumor microenvironment and can display either antitumoral M1-like or immunosuppressive M2-like functions. CD68 and CD163 are commonly used markers to evaluate macrophage infiltration and polarization [13, 17, 27–28].

Compared with HPV-associated oropharyngeal carcinoma, where HPV positivity is strongly linked to prognosis and therapeutic response, the biological role of HPV in LSCC remains uncertain [3–6, 29–30]. Our findings suggest that HPV-

positive LSCC represents a biologically distinct subgroup with specific immune features, consistent with previous observations showing HPV-associated modulation of immune cell recruitment in head and neck cancers [7–10, 31–32].

This study has several limitations, including its retrospective design, relatively limited sample size, and single-center recruitment. In addition, immunohistochemistry provides information on immune cell distribution but does not fully reflect immune cell function. Future studies integrating molecular profiling and multiplex immune analyses will be required to better define HPV-driven immune mechanisms in LSCC.

Conclusion

Our study shows that HR-HPV status is associated with changes in the tumor immune microenvironment of laryngeal squamous cell carcinoma. HR-HPV-positive LSCC displayed higher infiltration of $CD8^+$ T cells, $FOXP3^+$ regulatory T cells, and tumor-associated macrophages compared with HR-HPV-negative tumors, suggesting the presence of distinct immune profiles according to HPV status.

The increased $CD8^+$ T-cell infiltration observed in HR-HPV-positive tumors and its association with a favorable survival trend highlight the potential relevance of immune profiling in LSCC. These findings support the consideration of HR-HPV status as an important biological parameter when evaluating immune biomarkers in laryngeal cancer.

Further multicenter studies with larger cohorts and advanced immune profiling approaches are required to validate these findings and determine their potential contribution to patient stratification and personalized therapeutic strategies.

Declarations

Declaration of generative AI in scientific writing

Generative AI was used to improve the readability and language of the manuscript.

Declaration of Competing Interest

We declare that there have no competing interests.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Salah Azaiez Cancer Institute in Tunis (Reference No. ISA/2020/14). All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

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Author Contribution

NMB : conceived and designed the study, supervised the project, contributed to data analysis and interpretation, and wrote the original manuscriptSK and YH : (pathologists) performed independent blinded assessment of immunohistochemical staining, including evaluation of marker expression and tumor immune cell infiltration.YZ and AM and RA : contributed to clinical data collection and patient information management.MD: reviewed and critically revised the manuscript and approved the final version.

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Figures

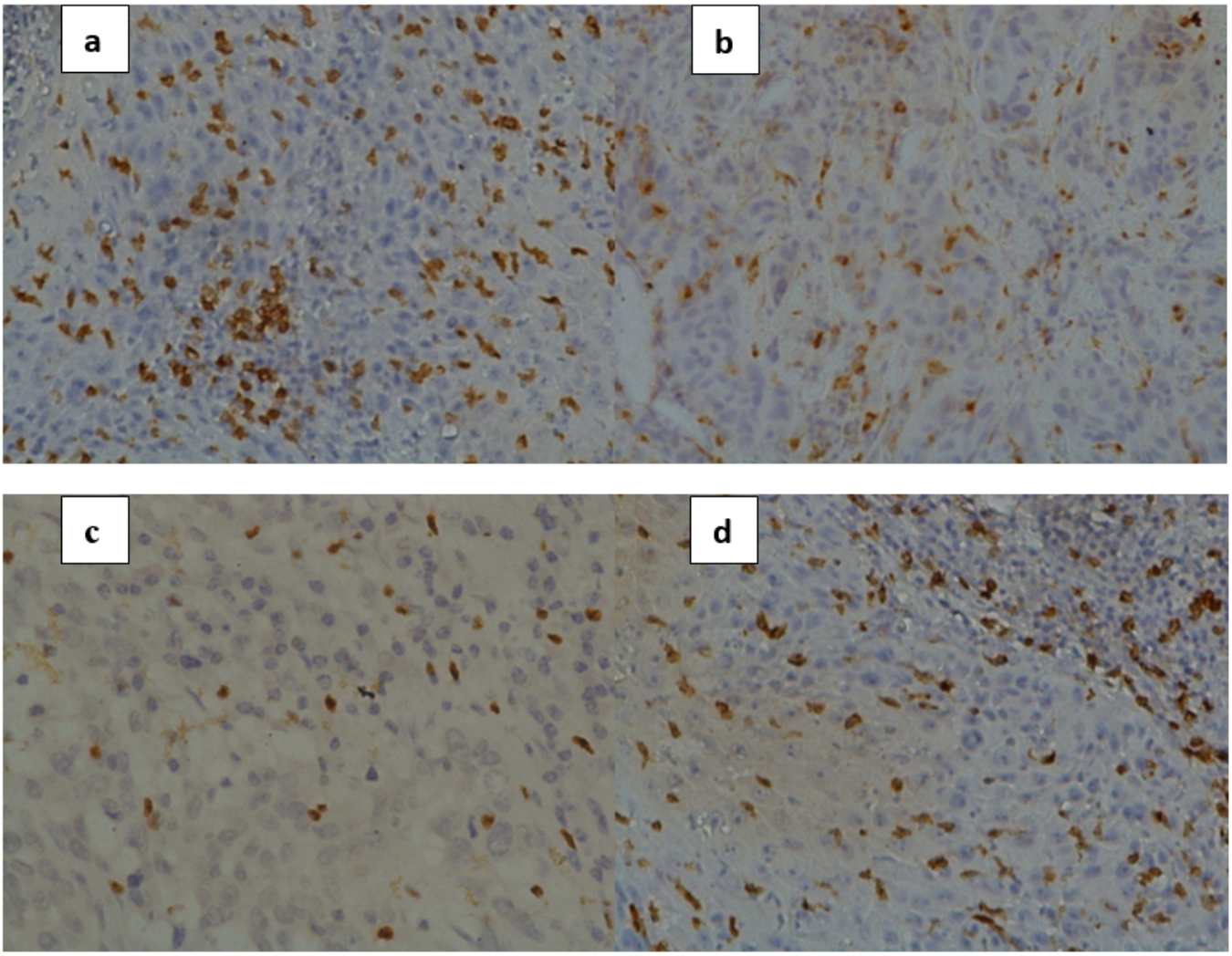


Figure 1

Immunohistochemical staining for CD8 (a), FoxP3 (b), CD68 (c), and CD163 (d) in Laryngeal Squamous Cell Carcinoma (LSCC). Original magnification x 40

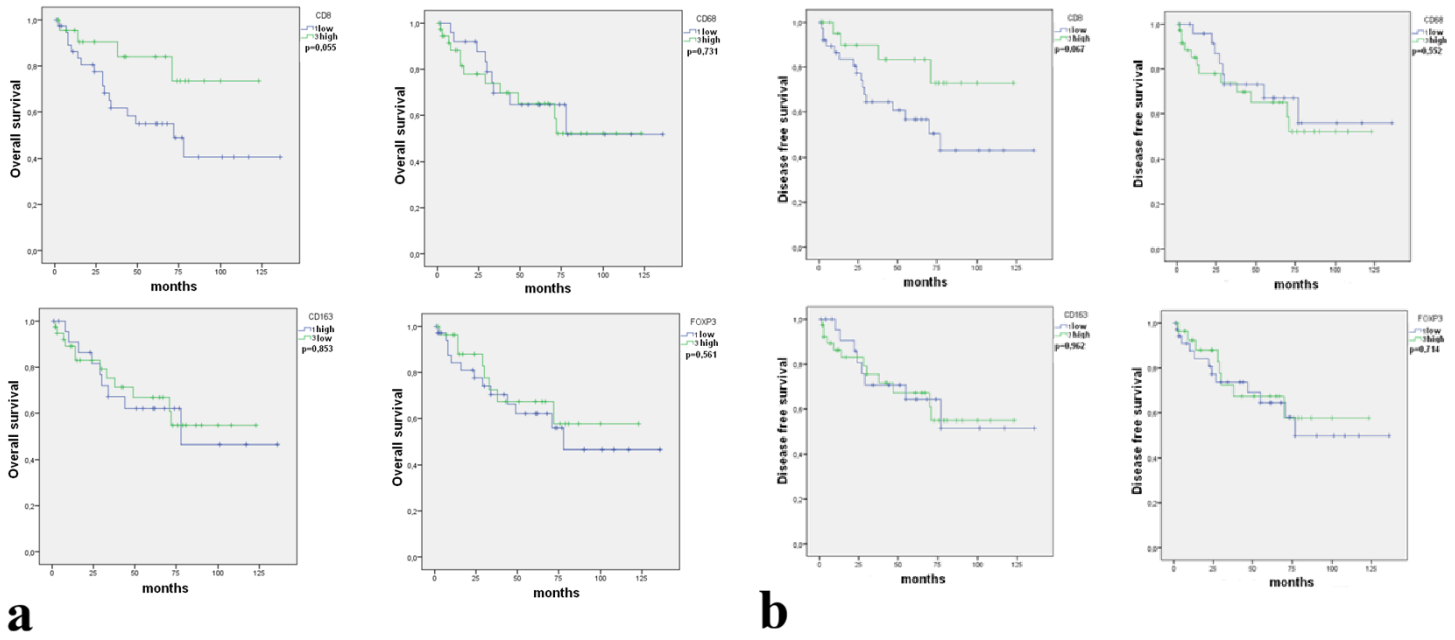


Figure 2

2A: Kaplan–Meier curves for overall survival (OS) in CD8, CD68, CD163 and FOXP3 expression of patients with Laryngeal Squamous Cell Carcinoma (p=0.055; p=0.731; p=0.853 and p=0.561 respectively)

2B: Kaplan–Meier curves for Disease Free Survival (DFS) in CD8, CD68, CD163 and FOXP3 expression of patients with Laryngeal Squamous Cell Carcinoma (p=0.067; p=0.552; p=0.962 and p=0.714 respectively)