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High CD3$^+$ lymphocytes, low CD66b$^+$ neutrophils, and scarce tumor budding in the invasive front of lip squamous cell carcinomas*

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ABSTRACT

Objective: This study aimed to evaluate tumor budding (TB) and quantify the neutrophilic and lymphocytic infiltration in the invasive front of lip squamous cell carcinomas. In addition, the associations between these features and the histopathological grading in the invasive front were analyzed.

Design: A total of 43 lip squamous cell carcinoma surgical specimens were included and classified in accordance with a histological invasive front grading system. Immunohistochemistry was performed for CD66b and CD3 for the evaluation of neutrophils and T lymphocytes, respectively, in the invasive front of the tumors. Tumor budding was evidenced by AE1/AE3 staining.

Results: Thirty-six (83.7%) of the tumors were well-differentiated tumors. Eleven (25.6%) of the cases exhibited high-intensity tumor budding. There were low neutrophil and high T lymphocyte infiltrations in the invasive front, leading to a low neutrophil/T lymphocyte ratio in the same region. Moreover, we found an association between tumor budding and the pattern of invasion, and between the CD3$^+$ cell count and the inflammatory infiltrate ($p < 0.05$).

Conclusions: The low neutrophil and high T lymphocyte infiltration in the invasive front, and the few high-intensity tumor budding cases are in accordance with the histopathological features of well-differentiated lip tumors. If these characteristics remain in lip squamous cell carcinomas with more aggressive histopathological features, it deserves to be investigated.

1. Introduction

Lip cancer is mostly represented by squamous cells carcinoma (Silveira et al., 2010; Zini, Czerninski, & Sgan-Cohen, 2010). Like the basal cell carcinoma of the skin, lip squamous cell carcinoma (LSCC) is mostly associated with chronic, long-lasting sun exposure (Dawn & Lawrence, 2013; Domínguez-Gordillo et al., 2015; Osterne et al., 2011). Lip cancer has remarkable prevalence in parts of Australia, Europe, the USA, and Brazil, mainly among fair-skinned people who work outdoors and are chronically exposed to UV radiation (Domínguez-Gordillo et al., 2015; Osterne et al., 2011). The labial site for squamous cell carcinoma frequently provides early diagnosis of the lesions and facilitates broad surgical treatment (Rena et al., 2014). Additionally, lip squamous cell carcinoma (LSCC) presents more favorable characteristics than intra-oral squamous cell carcinoma, such as cellular differentiation, a low metastasis index, and 5-year disease-free survival rates that can reach 100% in initial cases (Bhandari et al., 2015; Silveira et al., 2010).

Histopathologic grading is an adjunct to the tumor-node-metastasis system (El-Naggar, Chan, Grandis, Takata, & Slootweg, 2017) and can assist the surgeon in therapeutic decisions. Most recently, tumor

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Table 1
Reagents and procedures for immunohistochemistry.

Primary antibody	Clone/fabricator	Antigen retrieval	Dilution	Detection system
CD66b (neutrophils)	Clone G10F5; BD Biosciences, San Diego, California, USA, code 555723	TRIS–EDTA solution (pH 9.0) in a 96 °C water bath for 20 min	1:600	Kit Spring BioScience, SPB-999, Pleasanton, California, USA
CD3 (T-lymphocytes)	Clone F7.2.38; Dako Cytomation, Glostrup, Denmark, code M7254	TRIS–EDTA solution (pH 9.0) in a 96 °C water bath for 20 min	1:200	Kit Spring BioScience, SPB-999, Pleasanton, California, USA
PAN cytokeratins (tumor buds)	Clone AE1/AE1, Dako Cytomation, Glostrup, Denmark, code M3515	Citric acid solution (pH 6.0) in a 96 °C water bath for 30 min	1:100	Kit Spring BioScience, SPB-999, Pleasanton, California, USA

budding (TB) has been suggested as a simple and effective tool for the prognostic determination of oral cancer (Almangush et al., 2014, 2018; Marangon Junior et al., 2018; Strieder et al., 2016; Wang et al., 2011). Tumor budding is a histopathological feature referring to clusters ("buds") of malignant cells localized mainly in the invasive front of the tumor, the most invasive neoplasm area (Almangush et al., 2014; Wang et al., 2011). Also, TB has been related to an increased potential for tumor spreading and metastasis, including in LSCC, since it represents a loss of adhesion and local invasion of the epithelial cells (Almangush et al., 2014, 2018; Marangon Junior et al., 2018; Strieder et al., 2016). Further, the histological invasive front grading (IFG) system, proposed by Bryne, Koppang, Lilleng, and Kjaerheim (1992)), powered the predictive value malignancy grading of oral squamous cell carcinomas, considering the invasive front the most indicative site for evaluating the aggressiveness of the tumor. According to this system, a greater intensity of the inflammatory infiltrate (specially composed of lymphocytes) is considered to be beneficial to the patient and associated with a low grade of malignancy. However, it has been shown that the inflammatory infiltrates may have both anti- and pro-tumor functions (Galdiero et al., 2013; Houghton, 2010), with different roles according to cell secretion.

Recent studies highlight that neutrophils should be further investigated in cancer (Fridlender et al., 2009). Some tumor-associated neutrophils may be involved in the initiation, invasion, and progression of the disease, as well as in its dissemination and angiogenesis (Dumitru, Lang, & Brandau, 2013; Fridlender et al., 2009). The degranulation and release of arginase-1, by tumor-associated neutrophils, inhibits T-cell function (Dumitru et al., 2013; Tecchio, Scapini, Pizzolo, & Cassatella, 2013). Additionally, they may influence the recruitment and function of $CD8^+$ and $CD4^+$ T lymphocytes. In turn, the function of neutrophils can be modulated by T lymphocytes, revealing an interaction between these immune cells in the tumor microenvironment (Millrud et al., 2012; Trellakis et al., 2011). Studies on many types of cancer, including intra-oral squamous cell carcinoma, have demonstrated an association of unfavorable clinical outcomes, unsatisfactory results in treatment, and poor survival with a high neutrophil/lymphocyte ratio (NLR) in the blood and/or tumor (Millrud et al., 2012; Trellakis et al., 2011).

Therefore, the objective of this study was to evaluate TB and quantify the neutrophilic and lymphocytic infiltration in the invasive front of a series of LSCCs. Additionally, we investigated the associations among these features and the specific criteria of histopathological grading in the invasive front.

2. Material and methods

2.1. Samples and eligibility criteria

This study was approved by the Institutional Research Ethics Committee (protocol #1143507). Forty-three paraffin blocks from the surgical resection (excisional biopsy) of lower lip squamous cell carcinomas were recovered from a reference center for oral diagnosis, comprising a convenience sample, as used by other authors in similar research (Caldeira, Sousa, & Aguiar, 2015; Strieder et al., 2016). Cases in which the biopsy record accompanying the specimen mentioned the use of immunosuppressive medicines were excluded. Clinical and demographic data such as sex and age were obtained from the biopsy records.

2.2. Histopathological grading of LSCC samples

The histopathological grading was carried out by two oral and maxillofacial pathologists (M.C.F.A. and K.D.S.), who were blinded in relation to the clinical characteristics of the lesions. They evaluated hematoxylin and eosin stained slides in the invasive front area under a two-headed optical microscope (Zeiss Axiostar Plus, Ser. 3109007734,

Table 2
Descriptive analysis of the clinical and pathological characteristics of the sample (n = 43).

Characteristics	n (%)
Sex (n = 43)	
Female	11 (25.6)
Male	32 (74.4)
Age (n = 41)	
< 40 years	2 (4.9)
40-60 years	16 (39.0)
> 60 years	23 (56.1)
Size (n = 38)	
T1 (up to 2 cm)	30 (78.9)
T2 (> 2-4)	6 (15.8)
T3 (> 4 cm)	2 (5.3)
Clinical aspect (n = 39)	
Erythroplakia	2 (5.1)
Ulcer	33 (84.6)
Associated aspects*	4 (10.3)
Evolution time (n = 30)	
0-6 months	13 (43.3)
6-12 months	6 (20)
> 12 months	11 (36.7)

* Associated aspects mean more than one clinical aspect, such as erythroplakia + ulcer or ulcer + leukoplakia.

Oberkochen, Germany). The criteria proposed by Bryne et al. (1992) was followed, and there were no disagreements in the final classification.

2.3. Immunohistochemistry

Immunohistochemical staining was used to identify neutrophils (CD66b), T lymphocytes (CD3), and tumor buds (AE1/AE3). Tissue sections (3 μm thick) were dewaxed in xylol and hydrated in an ethanol solution. After antigen retrieval, we performed hydrogen peroxide blocking, protein blocking, and detection steps with ready-to-use solutions (Table 1). The reaction was revealed with 3,3′-diaminobenzidine (Spring BioScience, code DAB-999), and hematoxylin was used for the counterstaining. The negative controls were included by omitting the antibody. The positive controls were the epithelium for cytokeratins, lymphoid hyperplasia for CD3, and one inflammatory process for CD66b.

2.4. Evaluation of immunohistochemistry

2.4.1. Neutrophils and T lymphocytes (CD66 and CD3)

All the cases were evaluated by one observer (K.D.S.) using an eyepiece grid coupled to an optical microscope (Zeiss Axiostar, Ser. 48824, Oberkochen, Germany). Intravascular neutrophils and T lymphocytes were not counted, and necrotic areas or artifacts were ignored. We performed a semiquantitative analysis according to the recommendations by Caldeira et al. (2015). Infiltrates were counted in five consecutive, non-overlapping, high-power fields (400x magnification) in the invasive front, starting from the left border of it. The positivity was graded as scores 1 (no staining of neutrophils or lymphocytes in relation to the total infiltrate), 2 (1–25% of positive neutrophils or lymphocytes), 3 (> 25–50% positive cells), or 4 (> 50%). The mean scores from the five fields were set as the CD66b and CD3 index of staining for each case. We calculated the neutrophil/T lymphocyte ratio (NLR). Concordance with an experienced oral pathologist (P.C.C.) was done for a representative sample (ten cases), as in the study by Jensen et al. (2009), with intraclass correlation coefficient values of 0.9 for both neutrophil and T lymphocyte counting.

2.4.2. Tumor budding (AE1/AE3)

The analysis of TB was performed by two observers (M.C.F.A. and K.D.S.), who were blind to the clinical characteristics of the lesions, under a two-headed optical microscope (Zeiss Axiostar Plus, Ser. 3109007734, Oberkochen, Germany). Buds are represented by isolated cells or small clusters of a maximum of four cells (Almangush et al., 2014; Wang et al., 2011). The hot spot area of the slide was first identified under 100x magnification and corresponded to the area in which a greater number of AE1/AE3 isolated or clustered positive cells were seen. Afterward, in a 200x power field, the case was classified as “high-intensity tumor budding” if it presented five or more tumor buds, and “low-intensity or no tumor budding” if less than five tumor buds or no tumor bud was detectable (Almangush et al., 2014; Wang et al., 2011). Disagreements in the classification were discussed until a consensus was reached.

2.5. Statistical analysis

Statistical tests were performed using SPSS 19.0 software (IBM®, Armonk, New York, USA). The Shapiro-Wilk normality test revealed that the data distribution was not normal. The absolute and relative frequencies were estimated with descriptive analysis. We compared Bryne’s parameters with TB (high vs. low) and tumor size with TB using Pearson’s $\chi 2$ tests. We used Kruskal-Wallis and Mann-Whitney tests to compare $CD66b^+$ and $CD3^+$ indexes and the neutrophil/T lymphocyte ratio with TB and the parameters described by Bryne et al. (1992). The level of significance was set at 5% ($p < 0.05$) for all tests.

3. Results

The clinical and pathological features of 43 patients are presented in Table 2. In relation to the clinical stage, only the tumor size could be recovered, as in most cases there was no information regarding the N (lymph node metastasis) and M (distant metastasis) parameters in the files. Regarding the histological IFG system, most of the cases were patients with well-differentiated tumors (Table 3).

Tumor budding was high in 11 (25.6%) and low in 32 (74.4%) of the cases. There was no statistically significant association between the total malignancy score of the system proposed by Bryne et al. (1992) and TB, but there was a statistically significant association (p = 0.03) between the pattern of invasion and TB. The most common pattern of

Table 3
Descriptive analysis of the histological grading (Bryne et al., 1992) (n = 43).

Parameters	n (%)
Total malignancy score[1]	
Grade I	36 (83.7)
Grade II	7 (16.3)
Degree of keratinization	
[1] Highly keratinized (> 50% of the cells)	20 (46.5)
[2] Moderately keratinized (20–50% of the cells)	18 (41.9)
[3] Minimal keratinization (5–20% of the cells)	3 (7)
[4] No keratinization (0–5% of the cells)	2 (4.6)
Nuclear pleomorphism[2]	
[1] Little pleomorphism (> 75% mature cells)	24 (55.8)
[2] Moderately abundant pleomorphism (50–75% mature cells)	18 (41.9)
[3] Abundant pleomorphism (25–50% mature cells)	1 (2.3)
Pattern of invasion[2]	
[1] Pushing, well delineated infiltrating borders	12 (27.9)
[2] Infiltrating, solid cords, bands and/or strands	21 (48.8)
[3] Small groups or cords of infiltrating cells (n > 15)	10 (23.3)
Inflammatory cell infiltration[2]	
[1] Marked	26 (60.5)
[2] Moderate	16 (37.2)
[3] Slight	1 (2.3)

[1] There were no cases with Grade III.
[2] The degree 4 did not occur for these features.

Table 4
Frequencies of cases in relation to scores determined according to the total inflammatory infiltrate in the invasive front (n = 43).

	CD66b$^+$ n (%)	CD3$^+$ n (%)
Score 1 (0%)	6 (14)	2 (4.6)
Score 2 (1-25%)	28 (65.1)	13 (30.2)
Score 3 (> 25-50%)	8 (18.6)	10 (23.3)
Score 4 (> 50%)	1 (2.3)	18 (41.9)

invasion was infiltrative with bundles, strips, or solid cords (Table 3). There were no statistically significant associations between TB and CD66b$^+$ neutrophil or CD3$^+$ T lymphocyte counts, or between TB and the NLR ($p \geq 0.05$).

The immunohistochemical analysis of CD66+ neutrophils and CD3$^+$ T lymphocytes is given in Table 4. Regarding CD66b$^+$ and CD3$^+$ cells, the neutrophils presented a median index of 0.8 (min 0.0; max 2.4) and T lymphocytes had a median value of 2.0 (min 0.0; max 3.0). Also, it is important to highlight that when the entire tumor was examined, the region that exhibited a greater infiltration of these cells (the hot spot) coincided with the invasive front.

Considering the NLR, the mean value of CD66b$^+$ neutrophils was lower than that of CD3$^+$ T lymphocytes in 37 of the cases (90.2%). The median index for the NLR was 0.4 (min 0.0; max 2.4). In two of the cases, the NLR could not be calculated because the CD3$^+$ cell count was 0.0.

There was no association between CD66b$^+$ or CD3$^+$ cell counts or the NLR and the total malignancy score of Bryne's system ($p \geq 0.05$). However, there was a statistically significant association between the CD3$^+$ cell count and the lymphoplasmacytic infiltration parameter of this system ($p = 0.03$). For the CD66b$^+$ cell count, there was no association with the inflammatory infiltration of Bryne's system ($p > 0.05$). The clinical parameter tumor size was not associated with TB or the NLR ($p > 0.05$). The aspects of the invasive fronts of the tumors, TB, and immunostaining of inflammatory infiltrate can be seen in Fig. 1.

4. Discussion

The aim of this study was to investigate neutrophils, T lymphocytes, and TB in the invasive front of LSCC, and to verify their association with the morphological features of the tumors. Our main findings were: (1) The majority of LSCCs were well-differentiated tumors; (2) LSCCs have low CD66b$^+$ neutrophils and high CD3+ lymphocytes in the invasive front, leading to a low NLR in the same region; (3) Few LSCCs expressed high-intensity TB, however, in these cases TB was associated with the tumor pattern of invasion. To the best of our knowledge, this is the first study to analyze the NLR in the invasive front of LSCCs and the second to evaluate TB specifically in these tumors (Strieder et al., 2016).

The clinical and demographic data of the sample are similar to those reported in the literature, with a higher prevalence of LSCCs in males, individuals aged over 40 years, white-skinned people, and workers chronically exposed to sunlight (Domínguez-Gordillo et al., 2015; Osterne et al., 2011). Regarding the histological IFG system (Bryne et al., 1992) applied to the sample, most cases were well-differentiated tumors, displaying cells with a moderate to high keratinization, little nuclear pleomorphism, a less invasive pattern of invasion (degree 1 and 2), and marked inflammatory infiltrate, findings that are similar to those presented by Santos et al. (2014) for T1 LSCCs.

There was a low percentage of polymorphonuclear neutrophil cells (0 and 25% of the total inflammatory infiltrate) in most of the cases analyzed (Table 4). Neutrophils may increase the invasiveness of tumor cells in a neutrophil/tumor cell ratio-dependent manner, reinforcing the participation of neutrophils within the tumor microenvironment (Glogauer, Sun, Bradley, & Magalhães, 2015). Caldeira et al. (2015) reported that most T4 head and neck squamous cell carcinomas displayed a medium or strong infiltration of CD66$^+$ neutrophils, whereas smaller and less-invasive tumors exhibited a lower degree of neutrophil infiltration. Our findings were similar to the last, taking into account that most of the LSCCs were small and less-invasive tumors, although the evaluation of neutrophilic infiltration in more aggressive LSSCs, even though the tumors are uncommon, needs further investigation.

Bryne's system aggregated the additional parameters that were not present in the World Health Organization system (El-Naggar et al., 2017), including the pattern of invasion and inflammatory infiltration. Dense inflammatory infiltrates have been associated with a good host response, giving a probability of grading the tumor as well-differentiated. The present study showed high CD3$^+$ T lymphocyte counts and a statistically significant association with the inflammatory infiltrate of lymphocytes and plasma cells of Bryne's system. Although we have not evaluated the functionality of T lymphocytes, we speculate

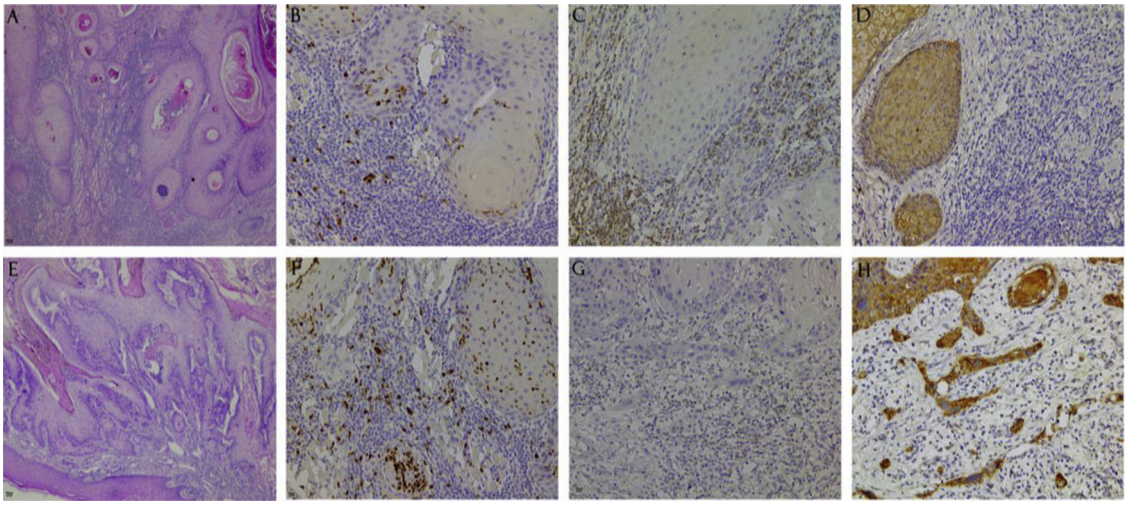

Fig. 1. Photomicrographs of lip squamous cells carcinoma cases in the invasive front. At the top of the figure can be seen tumor with blander histopathological features than in the lower part. A- Infiltrative pattern of invasion with solid cords, bands and/or strands (H&E, x40). B- Low CD66b$^+$ neutrophilic infiltration (IHC, x200). C- High CD3$^+$ lymphocytic infiltration (IHC, x200). D- Low-intensity tumor budding case (IHC, PAN cytokeratins, x200). E- Pattern of invasion revealing small groups or cords of infiltrating tumor cells (H&E, x40). F- High CD66b$^+$ neutrophilic infiltration (IHC, x200). G- Low CD3$^+$ lymphocytic infiltration (IHC, x200). H- High-intensity tumor budding case (IHC, PAN cytokeratins, x200).

that lymphocytic infiltration may have contributed to a more favorable profile of our sample, as already observed in a similar study (Silveira et al., 2010).

The median NLR value in our study of 0.4 (min 0.0; max 2.40) was similar to the reported mean ratio in the invasive front of T1-T2 squamous cell carcinomas from the tongue and floor of the mouth (0.36 ± 0.28), but lower than the value for T3-T4 tumors (0.85 ± 0.79) (Caldeira et al., 2015). Research on head and neck squamous cell carcinoma has shown that a high baseline neutrophil count and a high NLR in the blood and/or tumor were associated with unfavorable clinical outcomes, such as tumor size, poor prognosis, and lower survival rate (Caldeira et al., 2015; Trellakis et al., 2011). It is known that UV-induced LSCCs have a more favorable course than intraoral squamous cell carcinomas (Rena et al., 2014; Silveira et al., 2010) and the low NLR value in our study can play an important role in this regard. Meanwhile, since the function of neutrophils varies according to the phenotype (Houghton, 2010; Tecchio et al., 2013), the role of these cells needs to be further elucidated in cases of LSCC.

High-intensity TB was present in only a small percentage of cases in our study (25.6%), similar to a previous report on LSCCs (Marangon Junior et al., 2018). TB has been shown to have a prognostic significance for LSCC, with high-intensity TB lesions showing a decreased 5-year overall survival (Strieder et al., 2016). Additionally, associations between TB and tumor size, clinical stage, histological differentiation grade, lymph node metastasis, and low survival rates were previously reported for tongue squamous cell carcinomas (Wang et al., 2011).

Decision making for treatment planning and targets for future personalized oncology therapy are interesting clinical applications of histopathological parameters such as TB and NLR. Accordingly, previous studies on oral squamous cell carcinoma have suggested TB could guide treatment, in relation to the indication of prophylactic neck dissection (Seki, Sano, Yokoo, & Oyama, 2016; Seki, Sano, Yokoo, & Oyama, 2017). Moreover, the NLR can be an important marker for stratifying patients who could benefit from adjuvant therapy for head and neck cancer (Tham, Bardash, Herman, & Costantino, 2018). In the current research, we failed to find an association between TB and the NLR with the clinical parameters, but as this was the first study to address this issue in LSCCs, further research should confirm these results.

In the assessment of the histopathological parameters, we found an association between TB and the pattern of invasion ($p = 0.03$), but not for the tumor size and total malignancy score ($p > 0.05$). The most common pattern of invasion was infiltrative with bundles, strips, or solid cords. Almangush et al. (2014) found that the depth of invasion, TB and a high-risk worst pattern of invasion (represented by small tumor islands < 15 cells and satellite tumor(s) located at least 1 mm away from the main tumor) were prognostic indicators in early-stage tongue squamous cell carcinoma. Similar findings were displayed by Shimizu et al. (2018), in which TB and a high-risk worst pattern of invasion were associated with regional metastasis as well as with lymphovascular and perineural invasion. Therefore, TB and the pattern of invasion may appropriately reflect the aggressiveness of the tumor in the invasive front. The pattern of invasion is an easy parameter to evaluate which does not depend on equipment for the measurements. TB has been considered a promising morphological marker of cancer invasion, with also easy identification, especially after immunohistochemistry for cytokeratins (Marangon Junior et al., 2018).

The extrapolation of our results should be taken with caution. The main limitation of this study is the absence of follow-up information, also the N and M parameters of the tumor-node-metastasis staging. The samples used in the present study were from an oral diagnosis center, not a therapeutic center for oral cancer. Despite many efforts, no information on the follow-up could be retrieved. Missing data due to the incomplete filling of the biopsy records is also a challenge often found in retrospective studies. All assumptions were made taking into account these limitations. Nevertheless, the literature points out that LSCCs have an overall good prognosis with a low recurrence, metastasis, or death (Rena et al., 2014; Silveira et al., 2010).

In summary, we show that LSCCs present low neutrophil and high lymphocytic infiltration in the invasive front. Also, most lesions did not show high-intensity TB. High-intensity TB was associated with an infiltrative tumor pattern of invasion, and the $CD3^+$ cell count was associated with the lymphoplasmacytic infiltration parameter of Bryne´s system. Altogether, these findings corroborate with those of previous studies and are in accordance with the histopathological features of well-differentiated tumors. We encourage further studies with larger samples of LSCCs, including more aggressive cases, in an attempt to best identify the characteristics of inflammatory infiltrate and TB in squamous cell carcinomas of this site.

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Declaration of interest

None.

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