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Exploring the combination of tumor-stroma ratio, tumor-infiltrating lymphocytes, and tumor budding with WHO histopathological grading on early-stage oral squamous cell carcinoma prognosis

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Abstract

Background: While the relevance of the World Health Organization histopathological grading system as a prognostic tool for oral squamous cell carcinoma has received many critics, other histopathological features such as tumor-stroma ratio, tumor-infiltrating lymphocytes, and tumor budding are displaying promising results. Here, we evaluated the prognostic impact of the incorporation of tumor-stroma ratio, tumor-infiltrating lymphocytes, and tumor budding into World Health Organization histopathological grading for patients with oral squamous cell carcinoma.

Methods: A total of 95 patients with early-stage oral squamous cell carcinoma were enrolled in the study, and World Health Organization tumor grading, tumor-stroma ratio, tumor-infiltrating lymphocytes, and tumor budding were evaluated in surgical slides stained with hematoxylin and eosin. Survival analyses for cancer-specific survival and disease-free survival were performed using Cox regression models, and receiver operating characteristic curves were applied for assessment of the performance of the combinations.

Results: Tumor-stroma ratio (stroma-rich) was significantly and independently associated with both shortened cancer-specific survival and poor disease-free survival, individually and in combination with World Health Organization histopathological grading. The combination of tumor-stroma ratio with World Health Organization grading did not improve the discriminatory ability compared to tumor-stroma ratio alone. Although low tumor-infiltrating lymphocytes were associated with shortened cancer-specific survival, the association did not withstand multivariate analysis. However, in combination with World Health Organization grading, low tumor-infiltrating lymphocytes were independently associated with poor cancer-specific survival. The combination of tumor-infiltrating lymphocytes and World Health Organization

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histopathological grading displayed a better discrimination of poor cancer-specific survival than tumor-infiltrating lymphocytes alone, but not at a significant level.

Conclusion: Our findings support tumor-stroma ratio as a potential prognostic marker for patients with oral squamous cell carcinoma, and the incorporation of tumor-infiltrating lymphocytes into the World Health Organization grading system improves the prognostic ability of the tumor grading alone.

KEYWORDS
histopathological features, oral cancer, tumor budding, tumor-stroma ratio, WHO grading system

1 | INTRODUCTION

Oral cancer, represented in at least 90% of the cases by oral squamous cell carcinoma (OSCC), shows an annual incidence of approximately 380 000 new cases, with close to 180 000 deaths every year.[1] In contrast to many other cancers, the 5-year survival of OSCC remains stagnant at 50% for many decades,[2] and this has become one of the main challenges for clinicians, oncologists, and researchers in the field. Indeed, the lack of biomarkers for clinical applications, such as early diagnosis, therapeutic targets, responsiveness to treatment, prognosis, and post-therapeutic monitoring, is one of the many factors contributing to these low survival rates.[3]

Investigations aiming to characterize OSCC biomarkers are frequent, but recent systematic reviews and meta-analyses have concluded that the evidences available for those markers are still insufficient for their recommendations for clinical applications.[4,5] It has empathized that the paucity of validation studies, particularly as multicenter, prospective, with large cohorts, applying complementary assays to capture the impact of biomarkers in different scenarios, precludes the clinical applications.[3] Among many prognostic markers, histopathological features, individually or combined in a grading system, are one of the oldest potential markers to be studied for OSCC. The 8th edition of the UICC Tumour, Node, Metastasis (TNM) classification has recognized the importance of histopathological features for OSCC staging, after the incorporation depth of invasion into the T category and extranodal extension into the N stage.[6]

The World Health Organization (WHO) histopathological grading system, which takes into account the degree of keratinization of the tumor, levels of cellular and nuclear pleomorphism, and presence of mitotic activity, has been universally criticized due to its subjectiveness, absence of important features related to tumorigenesis and, most importantly, its poor association with outcomes and responses to treatment.[7,8] On the other hand, many other histological features, some very traditional ones and examined in several studies such as perineural invasion, lymphovascular infiltration and involvement of the surgical margins, and others more recently described, only examined in a limited manner, including tumor-stroma ratio (TSR), tumor-infiltrating lymphocytes (TILs) and tumor budding (TB), demonstrated a very strong association with OSCC outcome.[9]

This study was conducted to evaluate the impact of incorporation of TSR, TILs, and TB into the WHO histopathological grading system on the outcome of 95 patients with early-stage OSCC.

2 | MATERIAL AND METHODS

2.1 | Patients and clinicopathological data

A total of 95 patients with early-stage OSCC, who underwent treatment at two referral hospitals in Brazil (the UOPECCAN Cancer Hospital in Cascavel-Parana and Hospital Bom Pastor in Varginha-Minas Gerais) between 1998 and 2014, were enrolled in this study. The clinicopathological features were obtained from medical records and included age, sex, smoking habit, alcohol consumption, location, clinical stage, treatment, margin status (≥5 mm or <5 mm), and survival (cancer-specific survival [CSS] and disease-free survival [DFS]). Using both clinical and pathological features, the patients, originally staged based on the 7th edition of the UICC TNM classification, were re-staged according to the 8th edition. All patients were treated by curative surgery, but 25 patients received postoperative radiotherapy and 24 patients were subject to chemoradiotherapy. Chemotherapy was based on cisplatin monotherapy. Postoperative follow-up ranged from 6 to 178 months, and cases of recurrence were histologically confirmed. The clinicopathological features of the patients are depicted in Table S1. Although this cohort was collected in two hospitals, there were no differences in the overall survival rates of patients (data not shown). This study was approved by the Human Research Ethics Committee of the School of Dentistry, University of Campinas (CAAE: 55927322.0.0000.5418).

2.2 | Histopathological grading

Histological slides stained with hematoxylin and eosin (HE) from the surgical resections were retrieved, and the WHO

TABLE 1 Univariate analysis for cancer-specific survival and disease-free survival of 95 patients with early-stage oral squamous cell carcinoma

	Cancer-specific survival			Disease-free survival		
	% in 5 years	HR (95% CI)	*p* value	% in 5 years	HR (95% CI)	*p* value
Age (years)						
≤62 years	70.7	1		60.5	1	
>62 years	62.6	1.46 (0.67–3.17)	0.33	65.5	0.75 (0.36–1,57)	0.45
Sex						
Male	71.0	1		65.6	1	
Female	62.1	1,67 (0.68–4.09)	0.26	61.1	1.02 (0.44–2.31)	0.96
Clinical stage (8th ed.)						
I	89.3	1		84.4	1	
II	56.2	2.59 (1.16–5.79)	0.02	59.5	2.26 (1.05–4.87)	0.04
Tumor site						
Tongue	61.8	1		59.9	1	
Others	68.3	0.84 (0.35–1.99)	0.69	79.2	0.64 (0.27–1.51)	0.32
Treatment						
Surgery	71.0	1		68.8	1	
Surgery + radiotherapy	71.3	0.96 (0.39–2.34)	0.94	76.2	0.78 (0.33–1,80)	0.71
Surgery + radiotherapy + chemotherapy	48.9	2.22 (0.79–6.23)	0.07	38.9	1.95 (0.76–4.94)	0.12
Margin status						
≥5 mm	69.5	1		65.9	1	
<5 mm	41.0	2.04 (0.61–6.78)	0.24	46.7	1.18 (0.42–3.29)	0.75
WHO histopathological grading						
Well-differentiated	64.7	1		58.0	1	
Moderately- and poorly-differentiated	67.7	0.88 (0.39–1.95)	0.75	66.9	0.97 (0.44–2.11)	0.94
Tumor-stroma ratio (TSR)						
<50% (stroma-poor)	85.2	1		81.8	1	
≥50% (stroma-rich)	48.3	4.10 (1.87–8.97)	0.0004	44.3	3.32 (1.51–7.29)	0.003
Tumor-infiltrating lymphocytes (TILs)						
≥20% (high density)	72.9	1		68.1	1	
<20% (low density)	27.8	6.58 (1.81–23.8)	0.004	42.6	1.86 (0.63–5.44)	0.25
Tumor budding (TB)						
<5 buds	76.1	1		74.5	1	
≥5 buds	52.7	1.96 (0.88–4.36)	0.09	50.9	1.44 (0.69–3.03)	0.33

histopathological grading system was used to classify the tumors.[10] The number of available slides of the primary tumor for each case ranged from 1 to 8.

2.3 | Tumor-stroma ratio

TSR was assessed in HE-stained slides at the invasive front according to van Pelt et al.[11] and based on the percentage of stroma and tumor cells, the tumors were classified as stroma-poor (<50%) or stroma-rich (≥50%). Representative cases classified as stroma-poor and stroma-rich are illustrated in Figure S1.

2.4 | Tumor-infiltrating lymphocytes

TILs, defined as the percentage of stromal tumor area occupied by lymphocytes, were evaluated in HE-stained sections following recommendations of the International Immuno-Oncology Biomarker Working Group.[12] The cut-off of 20% was used to separate tumors with high and low TILs (Figure S2).[13]

2.5 | Tumor budding

TB was identified in the invasive front on the basis of the recommendations previously published,[14] and the cut-off point of five buds per

field was applied to separate tumors with low (<5 buds) and high (≥5 buds) activity (Figure S3).

2.6 | Statistical analysis

For statistical purposes, we combined moderately- and poorly-differentiated tumors in a single group. Univariate and multivariate Cox regression analyses were performed to assess the impact of the clinicopathological parameters on the patient's outcome. In the multivariate analysis, several models, avoiding counting the same parameter twice, in a stepwise approach taking into consideration both clinical and pathological features of tumors were constructed. A receiver operating characteristic (ROC) curve with area under the curve (AUC) was applied to compare the discriminatory ability of the parameters alone and in combination with WHO histopathological grading. A p value of ≤0.05 was considered to be statistically significant.

3 | RESULTS

The patients' survival based on univariate analysis of the clinicopathological parameters is shown in Table 1. Significant differences in prognosis of cases classified at stages I and II, after re-staging according to the criteria of the 8th TNM classification, were observed. The stage II cases were associated with worse CSS (HR: 2.59, 95% CI: 1.16–5.79, $p = 0.02$) and DFS (HR: 2.26, 95% CI: 1.05–4.87, $p = 0.04$) compared with stage I cases. There was no statistically significant difference between stage I and stage II based on the previous 7th edition (data

TABLE 2 Impact of the combinations between WHO histopathological grading and tumor-stroma ratio (TSR), tumor-infiltrating lymphocytes (TILs), and tumor budding (TB) on cancer-specific survival and disease-free survival of 95 early-stage patients with oral squamous cell carcinoma

	Cancer-specific survival			Disease-free survival		
	% in 5 years	HR (95% CI)	p value	% in 5 years	HR (95% CI)	p value
WHO histopathological grading and tumor-stroma ratio						
WD and <50% (stroma-poor)	90.0	-		80.2	-	
MD/PD and <50% (stroma-poor)	82.8	1.04 (0.36–3.05)	0.97	73.5	1.30 (0.49–3.44)	0.75
WD and ≥50% TSR (stroma-rich)	55.3	3.17 (0.85–11.8)	0.08	52.1	2.89 (0.91–9.20)	0.07
MD/PD and ≥50% TSR (stroma-rich)	51.4	3.58 (1.28–10.1)	0.01	49.6	3.27 (1.09–9.79)	0.05
WHO histopathological grading and tumor-infiltrating lymphocytes						
WD and ≥20% (high density)	78.6	-		71.0	-	
MD/PD and ≥20% (high density)	69.8	1.74 (0.75–4.00)	0.28	66.1	1.20 (0.53–2.75)	0.67
WD and <20% (low density)	53.3	2.33 (0.91–15.1)	0.08	40.0	3.25 (0.46–22.8)	0.10
MD/PD and <20% (low density)	50.0	2.96 (0.60–14.6)	0.06	60.2	1.33 (0.35–5.15)	0.68
WHO histopathological grading and tumor budding						
WD and <5 buds	74.0	-		63.6	-	
MD/PD <5 buds	78.1	0.85 (0.31–2.33)	0.81	80.5	0.68 (0.25–1.89)	0.45
WD and ≥5 buds	64.3	1.31 (0.36–4.78)	0.58	72.2	0.89 (0.25–3.15)	0.69
MD/PD and ≥5 buds	53.6	1.95 (0.64–5.97)	0.25	57.0	1.25 (0.43–3.66)	0.62

Abbreviations: MD, moderately-differentiated; PD, poorly-differentiated; WD, well-differentiated.

TABLE 3 Mutivariate models for cancer-specific survival and disease-free survival of 95 early-stage patients with oral squamous cell carcinoma, considering WHO histopathological grading, tumor-stroma ratio (TSR), tumor-infiltrating lymphocytes (TILs), and tumor budding (TB), individually or in combination

	Cancer-specific survival		Disease-free survival	
	HR (95% CI)	p value	HR (95% CI)	p value
Individual parameters				
Tumor-stroma ratio	4.97 (1.97–12.5)	0.0007	3.22 (1.43–7.27)	0.005
Combinations				
WHO histopathological grading and tumor-stroma ratio	1.93 (1.28–2.90)	0.002	1.59 (1.11–2.27)	0.01
WHO histopathological grading and tumor-infiltrating lymphocytes	1.70 (1.05–2.74)	0.03	-	-

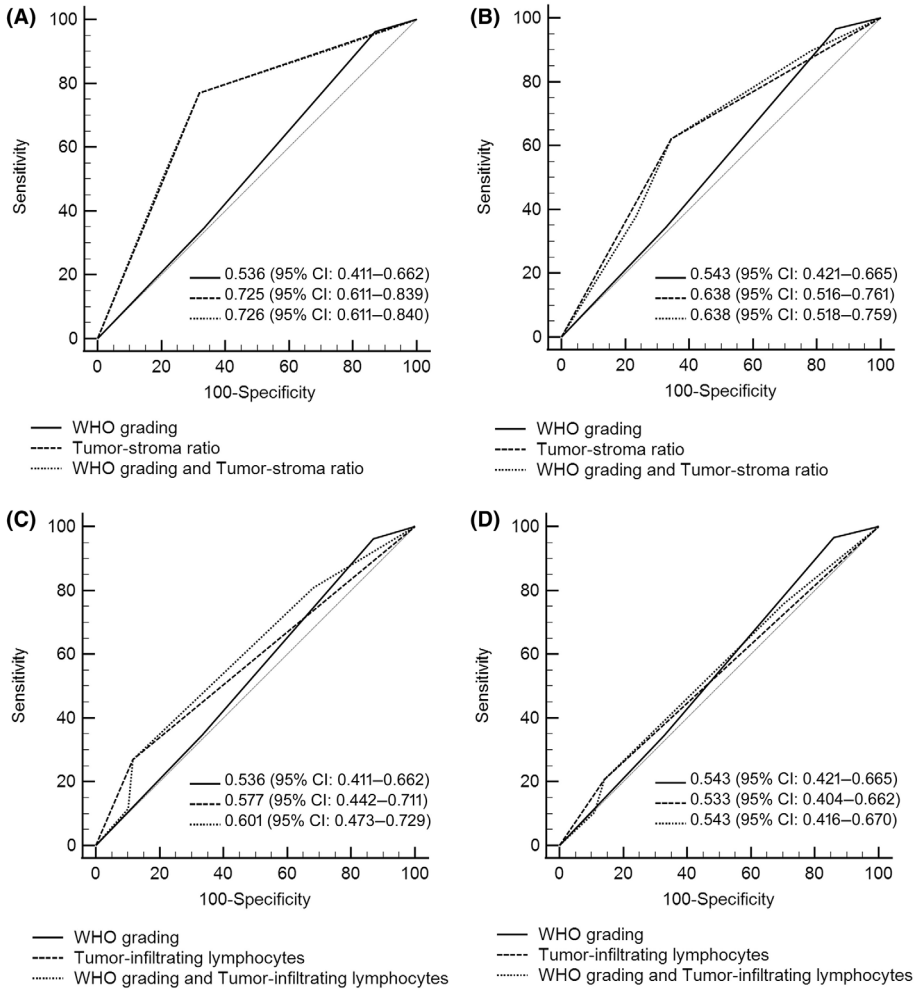

FIGURE 1 Receiver operating characteristic (ROC) curve with area under the curve (AUC) comparing the parameters individually and combined with the WHO histopathological grading system. The tumor-stroma ratio (TSR) alone or in combination with WHO grading system showed a significantly superior discrimination compared to WHO grading system alone, for both cancer-specific survival (A) and disease-free survival (B). For both outcomes, no differences between TSR alone and combined with WHO grading system were observed. Although not at a significant level, the combination of WHO grading system and tumor-infiltrating lymphocytes (TILs) showed a superior discrimination for cancer-specific survival than TILs alone or WHO grading system alone (C). No differences in disease-free survival were observed (D).

not shown). TSR (stroma-rich, ≥50%) was significantly associated with poor CSS (HR: 4.10, 95% CI: 1.87–8.97, $p = 0.0004$) and DFS (HR: 3.32, 95% CI: 1.51–7.29, $p = 0.003$). Patients with low TILs (<20%) had worse CCS (HR: 6.58, 95% CI: 1.81–23.8, $p = 0.004$) than those with high TILs (≥20%), but no impact on DFS was observed. The presence of TB was not associated with CCS or DFS in our cohort, showing only a tendency towards worse CSS ($p = 0.09$) (Table 1).

Next, we combined TSR, TILs, and TB with WHO grading system and performed univariate and multivariate survival analyses. As revealed in Table 2, the combination of WHO grading with TSR produced statistically significant results. Compared to the well-differentiated tumor with stroma-poor (<50% TSR), the patients with a well-differentiated tumor displaying a stroma-rich (TSR ≥50%) had shortened CSS (HR: 3.17, 95% CI: 0.85–11.8, $p = 0.08$), which was even worse for patients with moderately- or poor-differentiated tumor, yielding an HR of 3.58 (95% CI: 1.28–10.1, $p = 0.01$). For DFS, the combination of moderately- or poor-differentiated tumor with stroma-rich (≥50% TSR) revealed that patients with those features had significantly more relapses than patients with well-differentiated tumor and stroma-poor (TSR <50%) (HR 3.27, 95% CI: 1.09–9.79, $p = 0.05$) (Table 2). The combination involving TB did not show any significant association, whereas the combinations with low density of TILs (<20%) showed tendencies to have shortened CSS (Table 2).

Individually, TSR was the only parameter independently associated with the outcomes of patients (Table 3). Multivariate analysis revealed that stroma-rich (≥50%) is a risk factor for shortened CSS (HR: 4.97, 95% CI: 1.97–12.5, $p = 0.0007$) and poor DFS (HR: 3.22, 95% CI: 1.43–7.27, $p = 0.005$) in patients with OSCC. In terms of combination, the multivariate analyses showed that the combination of WHO grading system and TSR is an independent risk factor for both CSS (HR: 1.93, 95% CI: 1.28–2.90, $p = 0.002$) and DFS (HR: 1.59, 95% CI: 1.11–2.27, $p = 0.01$), whereas the combination between WHO grading system and TILs is an independent marker for poor CSS (HR: 1.70, 95% CI: 1.05–2.74, $p = 0.03$).

To determine the impact of incorporation of TSR and TILs into the WHO histopathological grading system, the AUC of ROC curves of the parameters alone and in combination were compared. Compared to WHO histopathological grading alone, both TSR alone or in combination showed a significantly superior discrimination for CSS with an AUC of 0.725 (95% CI: 0.611–0.839, $p = 0.03$) for TSR and of 0.726 (95% CI: 0.611–0.840, $p = 0.03$) for the combination compared to 0.536 (95% CI: 0.41–0.662) for WHO grading alone (Figure 1A). The discrimination for DFS showed similar pattern (Figure 1B), with

both TSR alone (AUC: 0.638, 95% CI: 0.516–0.761) and combination TSR and WHO histopathological grading (AUC: 0.638, 95% CI: 0.518–0.759) displaying better discrimination than WHO grading system alone (AUC: 0.543, 95% CI: 0.421–0.665). For both outcomes, no differences between TSR alone and combined with WHO grading system were observed. Although not at a significant level, the combination of WHO grading system and TILs (AUC: 0.601, 95% CI: 0.473–0.729) showed a superior discrimination for CSS than TILs alone (AUC: 0.577, 95% CI: 0.442–0.711) or WHO grading system alone (AUC: 0.536, 95% CI: 0.411–0.662) (Figure 1C). No differences in DFS were observed (Figure 1D).

4 | DISCUSSION

OSCC treatment and prognosis largely depend on the clinical stage at diagnosis. Surgery remains the mainstay of treatment, including neck dissection for involved cervical lymph nodes as well as for staging the disease and access during reconstructive surgery.[15] An important concern in early-stage OSCC is the presence of lymph node metastasis (clinically detected or occult), representing a major source of recurrence and cancer-related death.[16] Although occult lymph node metastasis is found in up to 30% of the OSCCs, the fear of its devastating effects often leads to elective dissection of the neck in early-stage OSCCs, mainly in those located in the tongue, causing overtreatment in many cases, with more adverse effects and higher financial burden than what is required.[17,18] In this context, it is essential to identify reliable biomarkers to accurately identify patients with a more aggressive tumor, in whom a complex therapy is necessary. Several studies have sustained the prognostic importance of histopathological features in OSCC, such as TSR, TILs, and TB, and highlighted that they might represent simple, universal, and inexpensive methods to drive the best treatment context for the patient.[19–21] However, there are limited studies investigating the prognostic significance of these new histopathological factors together with WHO grading system in OSCC. We undertook this retrospective study to determine the outcomes of 95 patients with early-stage OSCC in an attempt to verify the prognostic significance of TSR, TILs, and TB, individually and in combination with WHO histopathological tumor grade.

The tumor microenvironment promotes essential aspects of cancer cell proliferation, invasion, and metastasis, and recent studies have demonstrated that multiple parameters in the tumor microenvironment display prognostic potential, such as TSR. In OSCC, TSR, representing the proportion of tumor cells to stroma at the invasive tumor front, has been consistently associated with patient outcomes, as demonstrated by the recent meta-analysis of Almangush et al.,[22] who demonstrated a higher likelihood of recurrence and death for a stroma-rich tumor. Our data confirm that a TSR ≥50% (stroma-rich) might be prognostically significant and relevant in influencing decisions regarding the need for additional adjuvant therapy and optimal management of early-stage OSCC. However, there is no evidence that the combination with WHO tumor grading improves its discriminant ability. The underlying mechanisms connecting TSR and poor outcomes are not still fully understood, but among the many postulated theories, the one involving cancer-associated fibroblasts (CAFs) is getting attention. As previously highlighted, the presence of CAFs, as the main component of the OSCC microenvironment, is significantly associated with worse prognosis, and the ability of those cells in producing a repertoire of molecules, particularly collagen, promotes a complex cross-talk resulting in tumor cell proliferation, survival, migration, and invasion.[23,24]

The immune/inflammatory cells represent another important component of the OSCC microenvironment. Although the inflammatory response is considered as an attempt of an organism to eradicate tumor cells, and evasion of the immune destruction is considered one of the hallmarks of cancer, the prognostic role of the intensity of the inflammatory response, in the dependency of complex cell populations, in OSCC has generated inconsistent results. However, after the 2017 standardized protocol for assessing infiltrating lymphocytes in solid tumors based on hematoxylin and eosin-stained slides,[12] a consistent association of TILs with poor outcomes has been verified in many studies.[13,25,26] Furthermore, studies characterizing TILs in OSCCs showed the existent of an immunosuppressive milieu containing regulatory T cells, an immunosuppressive subset of $CD4^+$ T cells.[27] In this study, TILs set at a 20% cut-off (also when set at 30%, data not shown) were associated with poor CSS, but not as an independent factor. In addition, the combination of TILs and WHO grading system revealed *p* values in the univariate analysis that were interpreted as a *tendency* toward *significance*. *However*, when multivariate analysis was used to adjust the influence of confounders in the cohort, a significant relationship between the combinations of WHO histopathological grading and TILs with CSS appeared. Therefore, the addition of TILs to WHO tumor grading can serve as a potential prognostic marker for OSCC, impacting treatment decisions, but studies validating this finding is required.

The main limitations of this study are its retrospective approach since we could not eliminate selection bias because we excluded cases due to missing tumor slides/blocks, and the moderate sample size, which could explain the lack of association between TB and survival that has been detected in previous studies.[28,29] Moreover, due to the sample size, we grouped together moderate- and poorly-differentiated tumors. There were also some important strengths to this study. We have updated all cases with the 8th edition of the UICC TNM classification, and the inclusion criteria were stringent, allowing a statistical analysis based on correcting for multiple known confounders.

5 | CONCLUSION

In this study, we found that TSR in early-stage OSCC has significant implications for patient outcomes. The combination of TSR and WHO tumor grading does not improve its potential to indicate patients at high risk for recurrence or death due to tumor. Conversely, low density of TILs was only independently associated with a poor CSS when in combination with WHO grading system. As those features are low

cost, easy to assess, feasible, and reproducible, validation should be the target of future studies.

AUTHOR CONTRIBUTIONS

Gabriela Vivili Domingues Silva: Conceptualization, data curation, formal analysis, investigation, methodology, validation, writing—original draft, writing—review, and editing; **Eder da Silva Dolens**: Conceptualization; data curation; formal analysis; investigation, methodology, writing—original draft, writing— review, and editing; **Lívia Máris Ribeiro Paranaíba**: Data curation, formal analysis, writing—review, and editing; **Ana Lúcia Carrinho Ayroza**: Data curation, formal analysis, writing—review, and editing; **Clarissa Araujo Gurgel Rocha**: Conceptualization, formal analysis, writing—review, and editing; **Alhadi Amlangush**: Conceptualization, formal analysis, writing—review, and editing; **Tuula Salo**: Conceptualization, formal analysis, writing—review, and editing; **Peter A. Brennan**: Formal analysis, writing—review, and editing; **Ricardo D. Coletta**: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, writing—original draft, and writing—review and editing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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