Prognostic significance of tumor budding thresholds in oral tongue squamous cell carcinoma

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**Title**

Prognostic significance of thresholds for tumor budding categorization in oral tongue squamous cell carcinoma

**Running title (75 caratteri)**

Prognostic role of tumor budding categorization in oral tongue cancer

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*Conflict of interest statement*

The Authors declare no conflict of interest.

**Abstract**

Purpose**:** Oral tongue squamous cell carcinoma (OTSCC) represents the most common malignancy of the oral cavity. Despite the improvements in prognostic stratification of the current staging system, personalized treatments are still not used in the clinical practice. Accumulating evidence suggested tumor budding (TB) is a reliable prognostic factor in OTSCC. However, a standardized scoring system is still necessary to improve assessment replicability and interobserver agreement. The study aims to evaluate the prognostic role of TB in 211 OTSCC patients treated between 1997-2018.

Procedures: TB was evaluated on haematoxylin and eosin-stained sections in the hotspot area of the tumoral infiltrative front under ×200 magnification. TB was scored using a two-tier and a three-tier system and according to BD model and revised Grading system. Univariate and multivariate Cox regression analyses of disease-specific survival (DSS) and disease-free survival (DFS) were performed. A p-values $< 0.05$ was considered as statistically significant.

Findings: On multivariate analysis, the two-tier and three-tier system resulted independent prognostic factors of worst DSS. High-risk patients had a 2.21 and 3.08 times-increased probability of poor DSS compared to low-risk group ($p \leq 0.001$). It is significantly increased even for intermediate-risk group patients (HR = 1.83; $p = 0.0021$). No statistically differences emerged classifying patients according to BD model and revised Grading.

Conclusion: These data confirm the prognostic value of TB in predicting DSS in OTSCC. Classifying patients in two groups using the 5-buds cut-off significantly discriminates their survival outcomes. Since the established role of DOI in pTNM-staging and the poor prognostic value of WHO grading, TB could be considered an independent prognostic marker.

**Keywords**. Tongue Neoplasms; Squamous Cell Carcinoma of Head and Neck; prognosis.

## Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck region, with over 400.000 new cases diagnosed annually worldwide, accounting for over the 90% of all oral cavity cancers [1, 2]. OSCC can develop from any oral region and the tongue is the most involved oral subsite, accounting for 40% of all oral cancer cases. In particular, the National Cancer Institute estimated about 17.960 new cases and 2.870 new deaths in the 2021. Due to the high density of muscle and nerve bundles and the complexity of the lymphovascular system, the oral tongue squamous cell carcinoma (OTSCC) is characterized by highly aggressive clinical behaviour and high rate of locoregional recurrences. Despite the therapeutic and prognostication improvements, a short-term survival rate equal to 68.1% is still reported [3].

Recently, literature data suggested OTSCC exhibits distinctive molecular and clinical behaviours compared to oral cancer of other subsites, leading to an "anatomical bias" both for research and for clinical decision making [2, 4-9]. Despite the advancements in improving prognostic stratification and accuracy, the 8$^{th}$ edition of American Joint Committee on Cancer (AJCC) staging system failed to identify OTSCC characterized by poor prognosis [10]. Moreover, no prognostic biomarkers have been validated to stratify the OTSCC-related risk groups. Therefore, there is the critical need for comprehensive predictive model leading the therapeutic management, based on the individual clinicopathological features.

Recently, research has been focused on the morphological features of tumor microenvironment and accumulating evidence suggested tumor budding (TB) as an independent prognostic factor for locoregional recurrences in many solid cancers, such as colorectal [11-13], nasopharyngeal, esophageal [14], pancreatic [15], and lung [16] cancer. Based on these findings, Wang et al. firstly proposed the evaluation of TB in OTSCC [17]. TB is defined as a single tumor cell or a small cluster of less than five neoplastic cells at the infiltrative tumor front [13, 18]. TB resulted an independent prognostic factor of overall survival (OS) in oral cancer, regardless the pathological Stage and subsite, [19-32] and an independent predictor of lymph node metastasis (LNM) in OTSCC [17, 27, 29, 32-44]. TB represents a snapshot of a dynamic process undertaken by an aggressive tumor with metastatic potential. Indeed, it is characterized by the loss of cellular adhesion, resistance to apoptosis, degradation of peritumoral connective tissues, and active tumoral invasion ability. Therefore, TB could represent an epithelial phenotype undergoing to epithelial-mesenchymal transition (EMT) or to dedifferentiation [19-21, 23, 30, 34, 37, 45-48].

To reach an agreement on an international evidence-based standardized scoring system, the International Tumor Budding Consensus Conference (ITBCC) for colorectal cancer, proposed a

method to TB assessment, validated in OTSCC [13, 34]. However, the lack of a standardized stratification and a cut-off point, and a validate staining and detection methods prevents its evaluation in a multidisciplinary setting. Therefore, the study aims to assess the prognostic role of TB in OTSCC, comparing several scoring systems proposed by the literature.

## Methods

### *Study population*

This retrospective study included randomly selected patients with histological diagnosis of OTSCC. All patients were treated with a curative intent at the Department of Maxillofacial Surgery, "Ospedali Riuniti" General Hospital (Ancona, Italy), between 1997 and 2018. The clinicopathological data were collected from the archive of the Institute of Pathology, Marche Polytechnic University, Italy, and from clinical records, by a single examiner to ensure their uniformity. The analyzed cohort was partially composed by cases already analyzed in another study from our group [49].

The inclusion criteria were: (a) primary OTSCC; (b) age more than 18 years; (c) no preoperative chemo- or radiation therapy; (d) no human papilloma virus (HPV) infection, assessed by HPV 16-specific fluorescence in situ hybridization and $p16^{Ink4a}$-specific immunohistochemistry; (e) follow-up data of at least 3 years for living patients. The exclusion criteria were: (a) OTSCC cases that also involve other anatomical sites, and where the exact site from which the tumor originated could not be identified; (b) relapsed or secondary primary OTSCC; (c) OTSCC patients with immediate postoperative death.

To confirm the original diagnosis, each sample was histologically revaluated and reclassified by 2 expert pathologists (C.R. and M.M.) according to the $8^{th}$ Edition of the AJCC, Cancer Staging Manual [50] and to the $4^{th}$ Edition of the World Health Organization (WHO) classification of Head and Neck tumors [51].

The surgical samples were further stratified to allocate the same number of patients in each pathological Stage group. Recall for the updating of follow-up data was made by phone call by a single operator (L.T.), blinded to clinical and pathological data and group allocation.

All patients had postoperative follow-up every 2 months for the first year, every 3 months during the second year, and every 6 months thereafter. If patient had symptoms or signs of suspected recurrence, an immediate postoperative visit was scheduled. A maximum follow-up period of 10 years was settled.

Clinical endpoints were the disease-specific survival (DSS) and disease-free survival (DFS). Follow-up time was defined as the time from the date of surgical treatment to the date of recurrence (for DFS), to the date of death due to cancer (for DSS), or the date of the last follow-up. Informed consent was obtained from all included patients, and the study was conducted in accordance with the "Ethical Principles for Medical Research Involving Human Subjects" statement of the Helsinki Declaration. This study received ethical approval from the institutional review board of Marche Polytechnic University, Italy (CERM 2019-308). The study was conducted according to REMARK checklist and TRIPOD statement [52, 53].

***Histopathologic evaluation***

Haematoxylin and eosin (HE) stained sections obtained from formalin-fixed, paraffin-embedded blocks of the primary tumor specimens, were considered in this study. Two pathologists (C.R. and M.M.) independently performed the histological evaluation, blinded to the clinical and pathological data. The block which included the most invasive part of the primary tumor was selected for each case. From each selected block, a 4 μm-HE slide was carried out (i.e., the same routinely used to assess the depth of invasion status).

TB was visually assessed as previously reported [13]. Briefly, the slide was initially scanned at ×40 magnification (×4 objective lens and ×10 ocular) using an Olympus BM50 light microscope (Olympus, Tokyo, Japan), to select the area with the greatest number of budding foci. Subsequently, number of budding foci was counted in one field along the tumoral infiltrative front at ×200 magnification.

Patients were subsequently divided into two or three groups, according to the most used cut-off values reported in literature. In particular, we used: (a) a two-tier system with a single cut-off of 3 buds/field (low TB < 3/field, high TB ≥ 3/field) [13]; (b) a two-tier system with a single cut-off of 5 buds/field (low TB < 5/field, high TB ≥ 5/field) [17]; and (c) a three-tier system with two cut-offs of 5 and 10 buds/field (low TB < 4/field, intermediate TB = 5-9/field, high TB ≥ 10/field) [24]. Furthermore, two other combined models using TB were investigated: (d) the BD model, using TB and DOI values (BD0: DOI < 4 mm and TB < 5 buds, BD1: DOI ≥ 4 mm and TB < 5 buds/field or DOI < 4 mm and TB ≥ 5 buds/field, BD2: DOI ≥ 4 mm and TB ≥ 5 buds/field) [43]; and (e) revised Grading (RG) system, using TB and pathological grading (RG1: G1 tumor without TB, RG2: G2 tumor with TB < 5 buds/field, RG3: G3 tumor with TB ≥ 5 buds/field) [54]. Therefore, in addition to considering TB as a continuous variable, 5 different methods to count TB were evaluated.

### *Statistical analysis*

All the statistical analyses were performed by using the following tools: SPSS 21.0 (IBM Corporation, Chicago, IL, USA) and Stata 16.0 (StataCorp LLC, USA).

Normal distribution of variables was explored through Shapiro-Wilk normality test and the Kolgomorov-Smirnoff test. To evaluate the correlation between TB and the clinicopathological variables, Spearman rank correlation analysis was performed entering TB either as continuous or categorical variable. The non-parametric tests of Mann-Whitney and Kruskal-Wallis were used to investigate the differences of the continuous variables among different clinicopathological groups. Univariate Cox Regression analysis was used to estimate the association among variables and survival outcomes (DSS and DFS). In addition, stepwise multivariate Cox proportional hazard models were built to assess the association among predictive variables and their influences on the prognostic outcomes, the proportional hazard assumption was checked with the test of nonzero slope in a generalized linear regression of the scaled Schoenfeld residuals on time (*estat* command on Stata 16.0). Furthermore, the prognostic performance of multivariate models including each time a different method for TB categorization, in conjunction to well established prognostic factor resulting from the stepwise process, was assessed by calculating the Harrell c-index for both the univariate and the multivariate models. A p-values $< 0.05$ was considered as statistically significant.

## Results

### *Demographic and clinicopathological variables*

The study included 211 OTSCC surgical samples (Table 1), consisting of 135 males (64%) and 76 females (36%). The mean age at diagnosis was 64.7 ± 13.8 years (range: 23–93 years) and the mean tumoral diameter was equal to 2.5 ± 1.3 cm (range: 0.3–7 cm). All patients were tested for HPV infection and none of them resulted positive. According to the 8$^{th}$ Edition of AJCC staging system, 40 patients (19.0%) were Stage I, 60 (28.4%) were Stage II, while 51 (24.2%) and 60 (28.4%) patients belonged to Stage III and IV, respectively. According to 4$^{th}$ Edition of WHO Grading, 37 cases (17.5%) resulted well differentiate tumors (G1), 115 cases (54.5 %) were moderately differentiated (G2) and 59 cases (28%) showed a poor grade of differentiation (G3). Finally, perineural invasion (PNI), lymph vascular invasion (LVI), and worst pattern of invasion (WPOI-5) were observed in 39.3%, 14.2%, and 4.7% of OTSCCs, respectively.

### *Assessment of different TB models*

The mean count of TB was equal to 4.5 ± 5.0 (range: 0–26), and 38 patients (18%) did not exhibited TB (Table 2). According to the two-tier system proposed by Seki et al., a low TB and high TB were observed in 96 (45.5%) and 115 (54.5%) of cases, respectively [37]. In agreement with Wang et al., 141 (66.8%) and 70 (33.2%) OTSCCs were scored as low and high TB, respectively [17]. Using the three-tier system of Lugli et al., 141 cases (66.8%) were classified as low TB, 42 (19.9%) as intermediate TB, and 28 (13.3%) as high TB [11]. According to BD model, 48 (22.9%), 103 (48.8%) and 60 (28.4%) patients were scored as BD0, BD1, and BD2, respectively. Regarding the RG model, 11 (5.2%), 92 (43.6%), and 108 (51.2%) cases were classified as RG1, RG2, and RG3, respectively. Locoregional recurrences occurred in 31.3% of cases after 24.0 ± 25.0 months from the initial surgical treatment (range: 3–108 months), while the 45.0% of patients died due to OTSCC, whit a DSS equal to 31.8 ± 25.9 months (range: 3–116 months).

### *Correlation among TB and clinicopathological variables*

Shapiro-Wilk test showed a non-normal distribution of TB (p-value < 0.001). Clinicopathological Spearman's rank correlations are collected in Table 3. In particular, a raw number of buds correlated with tumoral size, higher grade, advanced Stage and DOI. The mean number of buds did not differ between male and female (Mann-Whitney p-value = 0.338), positive or negative LVI (Mann-Whitney p-value = 0.104), node positive or negative patients (Mann-Whitney p-value = 0.090) and the presence or absence of extra-nodal extension (ENE) (Mann-Whitney p-value = 0.105). Higher number of buds was found in higher G grading system (Kruskal-Wallis p-value = 0.033, mean value G3 = 5.86 ± 5.76 vs G1 = 3.59 ± 4.64). Noteworthy, multifocal PNI tumors reported highest mean number of buds compared to patients without PNI (Kruskal-Wallis p-value < 0.001, mean value multifocal PNI = 7.07 ± 5.52) vs negative PNI = 3.41 ± 4.50); at last, advanced Stage tumors showed higher mean number of buds (Kruskal-Wallis p-value < 0.001, mean value Stage IV = 5.25 ± 4.98 vs Stage I = 2.65 ± 4.52).

### *Univariate and multivariate analysis of different TB models for DSS and DFS*

As a continuous variable, a significant association was found between TB and DSS in the analysed cohort (HR = 1.07, 95%CI: 1.04–1.11, p-value < 0.001). Similar results were obtained when TB was included in a multivariate model cohort (HR = 1.07, 95%CI: 1.03–1.11, p-value < 0.001) with other significant factors (Stage, Age, Sex, PNI and LVI). Significant results were obtained also for the risk groups categorized according to Wang et al. both at univariate (HR = 2.38, 95%CI: 1.57–3.55, p-value < 0.001) and multivariate (HR = 2.21, 95%CI: 1.41–3.45, p-value < 0.001) analysis. Conversely, the model by Seki et al. was not able to predict DSS both at univariate (HR = 1.38, 95%CI: 0.91–2.07, p-value = 0.124) and multivariate (HR = 1.03, 95%CI: 0.66–1.60, p-value = 0.888) analysis. The model by Lugli et al. significantly predicted poor survival in OTSCC both at univariate and multivariate analysis (Table 4 and 5). At univariate analysis, only the high-risk group (BD2) of BD model was associated with poor DSS compared to the BD0 patients, while no significant differences were detected between BD0 and BD1 groups. Focusing on the revised Grading system, no significant differences were detected between the risk groups (Table 3 and 4). Regarding the DFS, only the two-tier system proposed by Wang et al., correlated with poor DFS (p = 0.021) at the univariate analysis. All the analysis performed for DFS are summarized in the Supplemental Table 1.

### *Comparison of the prognostic performance of TB models*

To assess the prognostic stratification of the models and to detect at baseline a future aggressive behaviour of the disease, the prognostic performance of the included models was analysed by the Harrell's c-index. Results of the univariate analysis showed as the model by Lugli et al. had the better prognostic stratification capability (c-index = 0. 616), followed by the revised Grading system (0.615) and the inclusion of the normal TB evaluation as a continuous variable (0.614). All the other models showed a lower prognostic performance (Table 3). In addition, the prognostic performance was also assessed in multivariate models with other known predictive variables resulting from a stepwise process (Table 4). Likewise, the model by Lugli et al. showed a better prognostic stratification capability (c-index = 0.739) compared to the others.

## Discussion

TB represents a single tumor cell or a small cluster of less than five neoplastic cells at the infiltrative tumor front [13]. TB emerged as an independent prognostic factor in many solid tumors, included

OSCC and OTSCC. Several Authors demonstrated a significantly correlation between high TB and LNM, either in OSCC [19, 23, 25, 28, 30, 31, 47] and in OTSCC [17, 27, 32, 35-39, 41]. In OSCC, TB resulted an independent prognostic factor of OS and DFS, regardless the pathological Stage and oral subsite [23, 26, 30, 31, 47]. Furthermore, TB showed an association with younger patients, ENE, tongue and mouth floor subsite [23], and positive surgical margins [22]. The correlation with younger patients could be attribute to their higher immune response, which in turn promotes the epithelial-mesenchymal transition (EMT) and the formation of tumor buds. TB is promoted even from the dense muscle bundles and from the lymphovascular network of the tongue and the mouth floor. Moreover, TB was associated with other prognostic markers, like DOI, POI, LVI, and PNI [19, 24, 28, 30, 32-34, 55, 56]. A possible explanation for the formation of tumor buds is that hypoxia could influence the behaviour of cancer cells by triggering the neoplastic cell migration and invasion [30]. In early OSCC, TB resulted an independent prognostic factor of local recurrences [20], occulted LNM [24, 30, 55], worst OS and DFS [24, 55]. In early OTSCC, occulted LNM was found in all cases with ≥ 3 buds/field. In particular, the combination of TB with a DOI ≥ 3 mm better predicted the occulted LNM [29, 42].

Our results confirm the prognostic value of TB in OTSCC, and the two-tier system with a cut-off of 5 buds/field is highly predictive of LNM and DSS, regardless the pathological Stage. This cut-off is the most commonly reported in literature [17, 20, 32, 33, 35, 43, 44, 57-63], compared to 3 buds/field [36, 37, 47] and to 10 buds/field [25, 33, 55, 64, 65]. Although the three-tier system further improve the risk stratification, our results do not show significant differences between high and intermediate risk group. However, due to the uncertain prognostic value of these cut-off values, it is recommended to report the total bud count for each case, to avoid the loss of information.

The integration of TB into a predictive model does not improve the patient's stratification. However, BD model resulted a prognostic factor of LNM, local recurrences, and OS [20, 29, 32-34, 41, 43, 62, 66], and the revised Grading showed a significant worsened DSS and DFS in OTSCC patients with RG3 respect to G3 cases [54]. Since the poor prognostic value of the WHO grading and the inclusion of DOI as a staging parameter of pTNM, the BD model and the revised Grading do not improve the stratification of risk group compared to the only evaluation of TB. Moreover, Boxberg et al. criticised the inclusion of DOI into the BD model, because it is already a staging parameter [31, 67]. Therefore, they proposed a model based on bud activity and intratumoral cell nest size (CNS), showing its prognostic value in predicting OSCC LNM [31].

The iBD model, based on Glasgow Microenvironment Score and BD score, significantly predicted the DFS and OS of OTSCC patients, suggesting the critical role of local inflammation and stroma reaction in the spreading of TB. Tumor cell dedifferentiation and dissemination at invasive front could

be related to a decreased local anti-tumor activity, and the increased tumor stroma ratio (TSR) might provide cell buds energy substrates. Contrary, Domingueti et al. did not confirmed the prognostic value of inflammatory response of iBD model; indeed, they demonstrated the higher prognostic value of BD model compared to iBD model in predicting DSS, especially in early OSCC. [66]. Also, the integration of TB and TSR and/or tumor infiltrating lymphocytes (TIL), resulted an independent prognostic factor of DSS and DFS, regardless the OSCC Stage [22, 26]. Finally, the evaluation of TIL and TB, resulted in a better prediction of occulted LNM in early OTSCC [41].

It has been suggested the prognostic value of TB does not differ between immunohistochemical (IHC) and HE evaluation. Therefore, the ITBCC group recommended its evaluation on HE sections. However, TB could be obscured by the peritumoral infiltrate, the reactive stromal cells, and the glandular fragmentation. Moreover, its assessment could be difficult in poorly differentiate OSCC, because of bud cells are surrounded by many lymphocytes, cancer associated fibroblasts and other stromal cells [17, 33, 34, 63]. Furthermore, single bud cells could be confused with the adjacent tumoral stroma [11, 45]. So, a cytokeratin immunostaining would allow a better visualization, showing the limits of neoplastic epithelial cells [17, 33, 34, 63]; although, the IHC may also stain apoptotic bodies and cellular debris, which should not be counted [17]. Finally, the IHC showed a better reproducibility compared to the HE, resulting more easier, regardless the examiners experience [45, 57, 63, 68]. However, the ITBCC recommendations have been validated as a reliable and simple scoring method in early and / or advanced OTSCC [33]. To ensure the field standardization, they recommended the TB evaluation by area (0.785 $mm^2$) and by the "hotspot" method, scanning 10 separate fields (×10 objective) along the invasive front and counting TB in the "hotspot" area (×20 objective) [13].

A quantitative and semi-automatic digital image analysis algorithm was proposed by Pedersen et al. This method showed a better accuracy and reproducibility compared to the conventional ones, overcoming some limitations of TB detection, such as the different scoring system and the inter- and intraobserver variability [60].

Only few studies evaluated the TB on incisional biopsy [24, 35, 37, 44, 58, 59, 61, 69]. The small sample size and fragmentation, the possible lack of infiltrative front, and the presence of artifacts or extensive necrosis could prevent its accurate assessment. However, it has been demonstrated a good correlation between pre- and post-operative TB score, and it has been confirmed the value of preoperative TB as a good predictor of DFS and LNM in OSCC [55, 69, 70]. Therefore, it has been recommended to perform a biopsy or several biopsies from different tumor sites, including its deepest infiltrative part [37, 69].

Recently, Seki-Soda et al. suggested to perform a biopsy (8 mm x 5 mm) including the clinically health tissue [61]. Alternatively, the intratumoral TB of fresh frozen sections could be evaluated. However, only one study demonstrated the prognostic role of CNS in OSCC patients [31].

TB seems to reflect the biological aggressiveness of neoplastic epithelial cells, representing a phenotype undergoing to partial EMT and promoting the tumoral invasion and metastasis. Indeed, bud cells are involved in the degradation of the peritumoral connective tissue and in the invasion of lymphatic and blood vessels leading to lymph node and distant metastasis [17, 20, 37, 44, 55, 57, 58, 60, 69, 71-73]. The overexpression of EMT-transcriptional factors (EMT-TFs) and the downregulation of MET-TFs [17, 20], the lower expression of EMT-TFs of epithelial cells of the invasive front rather than to the adjacent stroma, the keratins expression, and the up-regulation of N-cadherin, suggest a partial EMT [20, 23].

Despite the advancements in improving prognostic stratification and accuracy, the AJCC staging system fails to identify OTSCC characterized by poor prognosis. In early Stage OTSCC, an elective neck dissection (END) is strongly recommended for tumor with DOI $\geq$ 4 mm, whereas, if DOI < 4 mm, it is performed based on clinical judgment, potentially resulting in over- or undertreatment [74]. Therefore, the integration of TB could be relevant to select patients undergoing to END and / or adjuvant radiotherapy. During surgical treatment of early OTSCC, it has been recommend performing a cytokeratin staining on frozen sections to carry out a definitive surgical treatment without interrupting the anaesthesia [43], while other Authors suggested to assess TB and POI to select patients undergoing to closer follow-up or radical surgical treatment [55].

These data suggest TB should be included in daily practice to improve the pathological staging and better predict the patient's outcomes. Therefore, a standardized score system should be validate based on tumor site and Stage, and further studies and accuracy analysis should be conducted. Finally, TB could be a prognostic marker in oral cavity subsites in which the DOI is not suitable to predict the tumoral behaviour.

*Code availability statement*

The study was approved by the Regional Ethics Committee of Marche, University-Hospital Company, Ancona General Hospital (Protocol No. 2020-365).

*Data availability*

Data available on request from the Authors.

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*Patient consent statement*

Informed consent was obtained from all included patients, and the study was conducted in accordance with the "Ethical Principles for Medical Research Involving Human Subjects" statement of the Helsinki Declaration.

*Statement of Author contribution*

Conceptualization,; methodology, ; formal analysis,.; investigation, ; data curation,.; writing—original draft preparation,.; writing—review and editing,.; supervision, .
All authors have read and agreed to the published version of the manuscript.

*Supplemental materials*

- Supplemental Table 1.

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Table 1.

| **Parameter** | **n (%)** | **Mean ± SD (range)** |
|---|---|---|
| Age | | 64.7 ± 13.8 (23 - 93) |
| Sex | | |
| *M* | 135 (64) | |
| *F* | 76 (36) | |
| Size (cm) | | 2.5 ± 1.3 (0.3 - 7) |
| Tumor Budding | | 4.5 ± 5.0 (0 - 26) |
| Positive margins | 100 (48.1) | |
| Grading (WHO) | | |
| *G1* | 37 (17.5) | |
| *G2* | 115 (54.5) | |
| *G3* | 59 (27.0) | |
| Staging ($8^{th}$ ed. AJCC) | | |
| *I* | 40 (19.0) | |
| *II* | 60 (28.4) | |
| *III* | 51 (24.2) | |
| *IV* | 60 (28.4) | |
| PNI | | |
| *Positive* | 82 (39.3) | |
| *Negative* | 128 (60.7) | |
| LVI | | |
| *Positive* | 30 (14.2) | |
| *Negative* | 181 (85.8) | |
| WPOI | | |
| *1* | 31 (14.7) | |
| *2* | 32 (15.2) | |
| *3* | 72 (34.1) | |
| *4* | 66 (31.3) | |
| *5* | 10 (4.7) | |

Table 2.

| **Scoring system** | **n (%)** |
|---|---|
| TB1 | |
| *< 5 buds/field* | 141 (66.8) |
| *≥ 5 buds/field* | 70 (33.2) |
| TB2 | |
| *< 3 buds/field* | 96 (45.5) |
| *≥ 3 buds/field* | 115 (54.5) |
| TB3 | |
| *0 - 4 buds/field* | 141 (66.8) |
| *5 - 9 buds/field* | 42 (19.9) |
| *≥ 10 buds/field* | 28 (13.3) |
| BD model | |
| *BD0* | 48 (22.8) |
| *BD1* | 103 (48.8) |
| *BD2* | 60 (28.4) |
| RG model | |
| *RG1* | 11 (5.2) |
| *RG2* | 92 (43.6) |
| *RG3* | 108 (51.2) |

Table 3.

| Variable | Age | Size | n. of buds | Grading (WHO) | RG model | TB1 | TB2 | TB3 | BD model | WPOI | pT | Staging | DOI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Age** | ρ = 1 | 0.110 | -0.104 | **-0.222** | **-0.215** | -0.101 | -0.041 | -0.100 | -0.101 | **-0.136** | -0.025 | -0.046 | -0.048 |
| | p-value=1 | 0. 877 | 0.131 | **0.001** | **0.002** | 0.144 | 0.556 | 0.149 | 0.144 | **0.048** | 0.720 | 0.503 | 0.490 |
| **Size** | | ρ = 1 | **0.148** | 0.135 | **0.258** | 0.123 | 0.130 | 0.117 | **0.376** | 0.081 | **0.696** | **0.505** | **0.543** |
| | | p-value=1 | **0.035** | 0.054 | **<0.001** | 0.079 | 0.065 | 0.096 | **<0.001** | 0.247 | **<0.001** | **<0.001** | **<0.001** |
| **n. of buds** | | | ρ = 1 | **0.179** | **0.636** | **0.823** | **0.870** | **0.839** | **0.696** | **0.519** | **0.215** | **0.276** | **0.175** |
| | | | p-value=1 | **0.009** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **0.002** | **<0.001** | **0.011** |
| **Grading (WHO)** | | | | ρ = 1 | **0.600** | 0.130 | 0.088 | **0.142** | 0.109 | 0.063 | 0.109 | **0.250** | 0.050 |
| | | | | p-value=1 | **<0.001** | 0.059 | 0.200 | **0.040** | 0.113 | 0.361 | 0.116 | **<0.001** | 0.466 |
| **RG model** | | | | | ρ = 1 | **0.673** | **0.498** | **0.660** | **0.564** | **0.274** | **0.252** | **0.334** | **0.154** |
| | | | | | p-value=1 | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **0.025** |
| **TB1** | | | | | | ρ = 1 | **0.644** | **0.981** | **0.802** | **0.371** | **0.178** | **0.178** | 0.128 |
| | | | | | | p-value=1 | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **0.010** | **0.010** | 0.063 |
| **TB2** | | | | | | | ρ = 1 | **0.631** | **0.597** | **0.410** | **0.220** | **0.274** | **0.201** |
| | | | | | | | p-value=1 | **<0.001** | **<0.001** | **<0.001** | **0.001** | **<0.001** | **0,003** |
| **TB3** | | | | | | | | ρ = 1 | **0.777** | **0.402** | **0.166** | **0.171** | 0,121 |
| | | | | | | | | p-value=1 | **<0.001** | **<0.001** | **0.016** | **0.013** | 0,079 |
| **BD model** | | | | | | | | | ρ = 1 | **0.372** | **0.472** | **0.340** | **0.539** |

| | | | | | |
|---|---|---|---|---|---|
| | p-value=1 | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| **WPOI** | | ρ = 1 | 0.131 | **0.192** | **0.230** |
| | | p-value=1 | 0.057 | **0.005** | **0.001** |
| **pT** | | | ρ = 1 | **0.497** | **0.752** |
| | | | p-value=1 | **<0.001** | **<0.001** |
| **Staging** | | | | ρ = 1 | **0.447** |
| | | | | p-value=1 | **0.001** |
| **DOI** | | | | | ρ = 1 |
| | | | | | p-value=1 |

Table 4.

| | **H.R.** | **S.E.** | **p-value\|** | **95% CI** | **c-index** |
|---|---|---|---|---|---|
| TB | 1.0726 | 0.0175 | 0.000 | 1.0387 – 1.1076 | 0.614 |
| RG model | | | | | |
| *RG2* | 0.9470 | 0.5783 | 0.929 | 0.2861 – 3.1344 | 0.615 |
| *RG3* | 2.7704 | 1.6374 | 0.085 | 0.8698 – 8.8237 | |
| TB1 | 2.3668 | 0.4920 | 0.000 | 1.5747 – 3.5574 | 0.606 |
| TB2 | 1.3782 | 0.2877 | 0.124 | 0.9154 – 2.0750 | 0.545 |
| TB3 | | | | | |
| *Bd1* | 2.0661 | 0.5058 | 0.003 | 1.2786 – 3.3385 | 0.616 |
| *Bd2* | 2.9704 | 0.8184 | 0.000 | 1.7309 – 5.0975 | |
| BD model | | | | | |
| *BD1* | 1.0522 | 0.2921 | 0.855 | 0.6106 – 1.8131 | 0.593 |
| *BD2* | 2.1821 | 0.6244 | 0.006 | 1.2453 – 3.8236 | |

Table 5.

| | **H.R.** | **S.E.** | **p-value** | **95%CI** | **c-index** |
|---|---|---|---|---|---|
| TB | 1.0700 | 0.0201 | 0.000 | 1.0312 – 1.1103 | 0.728 |
| RG model | | | | | |
| *RG2* | 0.8614 | 0.5463 | 0.814 | 0.2485 – 2.9856 | 0.734 |
| *RG3* | 2.0320 | 1.2960 | 0.266 | 0.5821 – 7.0933 | |
| TB1 | 2.2142 | 0.5037 | 0.000 | 1.4176 – 3.4585 | 0.735 |
| TB2 | 1.0321 | 0.2321 | 0.888 | 0.6642 – 1.6039 | 0.715 |
| TB3 | | | | | |
| *Bd1* | 1.8318 | 0.4820 | 0.021 | 1.0936 – 3.0683 | 0.739 |
| *Bd2* | 3.0829 | 0.9047 | 0.000 | 1.7344 – 5.4797 | |
| BD model | | | | | |
| *BD1* | 0.4977 | 0.1629 | 0.033 | 0.2620 – 0.9455 | 0.735 |
| *BD2* | 1.2446 | 0.4478 | 0.543 | 0.6148 – 2.5194 | |

Figure and Table legends

Figure 1.

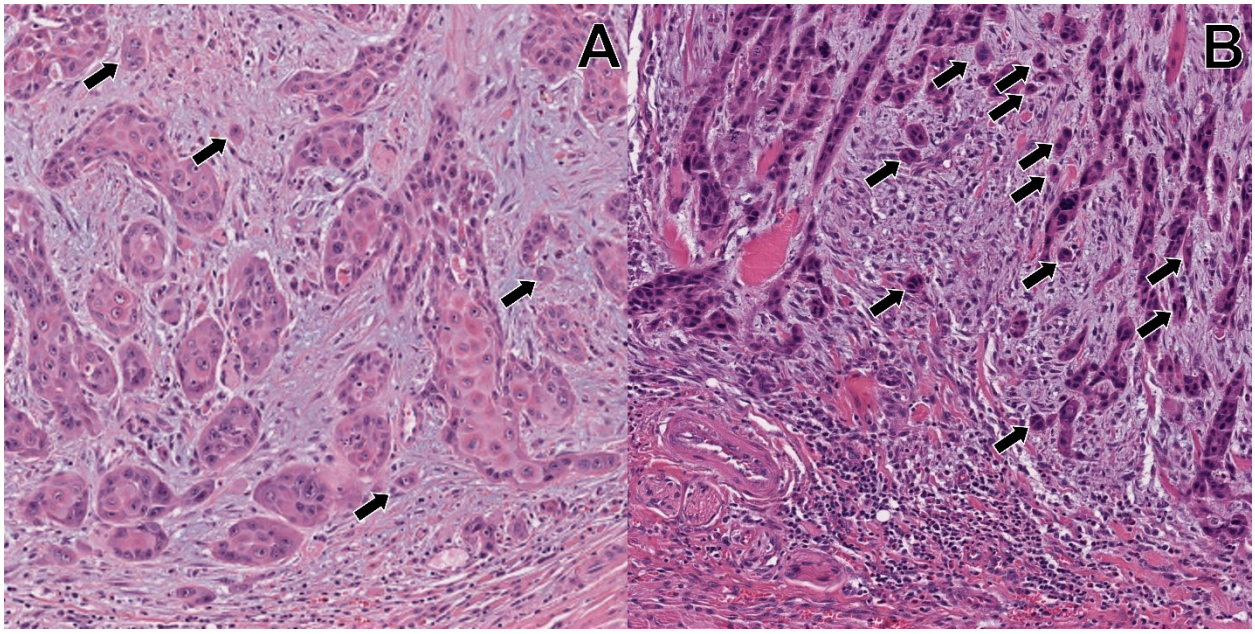


Figure 2.

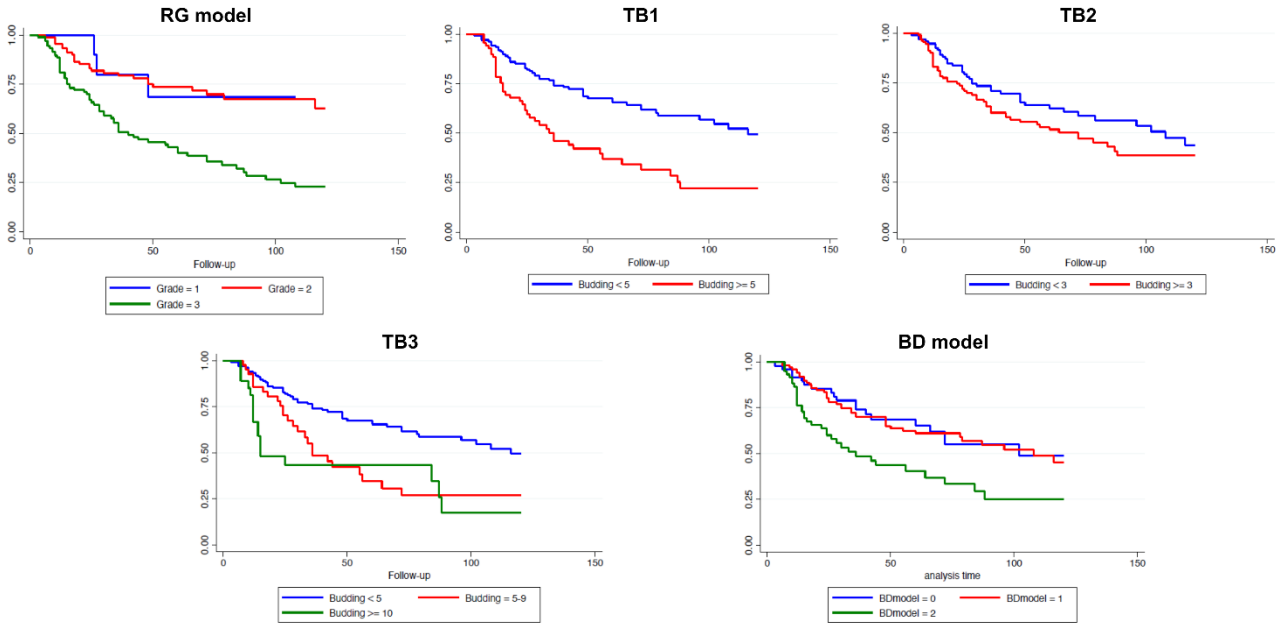


Table 1. Clinicopathological characteristics of patients included in the studied cohort. (M: male; F: female; G: grading; PNI: perineural invasion; LVI: lymphovascular invasion; WPOI: worst pattern of invasion; S.D.: standard deviation; WHO: world health organization; AJCC: American joint committee on cancer).

Table 2. Overall distribution of patients based on different tumor budding models. (TB: tumor budding; RG: revised Grading).

Table 3. Spearman's rank correlation analysis of tumor budding models and clinicopathological characteristics.

Table 4. Univariate analysis for the association of different tumor budding models and Disease-Specific Survival in oral tongue squamous cell carcinoma patients (TB: tumor budding; RG: revised Grading; H.R.: hazard ratio; S.E.: standard error; 95%CI: 95% confidence interval; c-index: Harrel's c-index).

Table 5. Multivariate analysis for the association of different tumor budding models and Disease-Specific Survival in oral tongue squamous cell carcinoma patients. (TB: tumor budding; RG: revised Grading; H.R.: hazard ratio; S.E.: standard error; 95%CI: 95% confidence interval; c-index: Harrel's c-index; AIC: Akaike information criterion; BIC: Bayesian information criterion).