DR ALHADI ALMANGUSH (Orcid ID : 0000-0003-4106-314X)

PROFESSOR RICARDO COLETTA (Orcid ID : 0000-0001-5285-3046)

Article type : Original Article

Prognostication of oral squamous cell carcinoma patients based on tumor-stroma ratio and tumor budding

Maurício Rocha Dourado,[1] Karen Yumie Mendonça Miwa,[1] Guilherme Baptista Hamada,[1] Lívia Máris Ribeiro Paranaíba,[2] Íris Sawazaki-Calone,[3] Catherine Bueno Domingueti,[2,4] Carine Ervolino de Oliveira,[2] Emylle Caroline Barquez Furlan,[3] Bruna Cristina Longo,[3] Alhadi Almangush,[5] Tuula Salo,[6] & Ricardo D. Coletta[1]

[1]Department of Oral Diagnosis, Piracicaba Dental School, University of Campinas, Piracicaba, São Paulo, Brazil, [2]Department of Pathology and Parasitology, Institute of Biomedical Sciences, Federal University of Alfenas (UNIFAL-MG), Alfenas, Minas Gerais, Brazil, [3]Oral Pathology and Oral Medicine, Dentistry School, Western Paraná State University, Cascavel, Paraná, Brazil, [4]University José the Rosário Vellano (UNIFENAS), Biomedicine, Varginha, Minas Gerais, Brazil, [5]Department of Pathology, University of Helsinki, Helsinki, Finland; Institute of Biomedicine, Pathology, University of Turku, Turku, Finland; [6]Cancer and Translational Medicine Research Unit, Faculty of Medicine and Medical Research Center Oulu, Oulu University Hospital, University of Oulu, Oulu, Finland; Institute of Oral and Maxillofacial Disease, University of Helsinki, and HUSLAB, Department of Pathology, Helsinki University Hospital, Helsinki, Finland

Running title: Tumor-stroma ratio and tumor budding in oral cancer.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/HIS.14070

Address for correspondence: Ricardo D. Coletta, Department of Oral Diagnosis, Piracicaba Dental School, University of Campinas, 13414-018 Piracicaba, São Paulo, Brazil, Email: coletta@fop.unicamp.br

ABSTRACT

Aims: Previous studies have demonstrated that tumor-stroma ratio (TSR) and tumor budding are of prognostic value for oral squamous cell carcinomas (OSCC). Herein we evaluated the prognostic significance of those histological parameters, individually and in combination, for OSCC.

Methods: TSR and tumor budding (the presence of ≥ 5 buds at the invasive front) were estimated in 254 patients with OSCC. The clinicopathological association was investigated using a chi-square test, and the prognostic significance (cancer-specific survival and disease-free survival) was verified by Kaplan-Meier analysis and the Cox proportional hazard model.

Results: TSR (≥ 50%, stroma-rich) was significantly and independently associated with both shortened cancer-specific survival and poor disease-free survival, whereas tumor budding significantly reduced cancer-specific survival. The TSR/tumor budding model was independently associated with a high-risk of cancer-mortality and recurrence (disease-free survival). In patients with early-stage tumors (clinical stage I and II, n=103), TSR, tumor budding and the TSR/tumor budding model were significantly associated with both cancer-related death and recurrence, while in advanced-stage tumors (clinical stage III and IV, n=144), only TSR and the TSR/tumor budding model were significantly associated with cancer-specific survival.

Conclusions: TSR, tumor budding and their combination provide significant information on OSCC outcome, suggesting that their incorporation into the routine evaluation of histopathological specimens might be useful in the prognostication of OSCC patients.

Keywords: oral cancer, prognosis, tumor-stroma ratio, tumor budding.

Introduction

Oral squamous cell carcinoma (OSCC), the most common tumor in the head and neck region, affects more than 300,000 new individuals and is responsible for 177,000 deaths every year.[1] OSCC is considered a very aggressive tumor, and even for those receiving maximum treatment with curative intent, only half survive for more than 5 years.[2] Its management and prognosis are mainly based on clinical criteria, especially TNM classification, however, the behavior of some OSCCs is unpredictable.[3] Several pathological features, individually or combined in scoring systems, have shown an important role in the prognostication of patients with OSCCs.[4,5] Two of them, depth of invasion and extranodal extension in a metastatic lymph node, were incorporated into T and N stages, respectively, in the new edition of the clinical staging manual of the American Joint Cancer Committee.[6]

The histological analysis of the proportion of tumor cells to fibrotic stroma (tumor-stroma ratio-TSR) in hematoxylin and eosin (HE) stained slides has been shown to be of prognostic value for solid tumors,[7] including OSCCs.[8,9] The study by Niranjan and Sarathy,[8] which was composed of a small sample size and a short follow up, did not find a significant association between TSR and outcome. On the other hand, the study performed by Almangush and colleagues[9] revealed that TSR is a powerful marker for both cancer-related mortality and disease-free survival. Another potential histological prognostic marker for OSCC, which can also be assessed in HE stained slides, is tumor budding, single cells or clusters of up to 5 cancer cells at the invasive front, as revealed by recent systematic reviews with meta-analysis.[10,11] Moreover, the incorporation of tumor budding to the WHO histological tumor grade of OSCC showed a superior prognostic value compared to WHO histological tumor grade alone.[12]

The aim of the present study was to examine the prognostic value of TSR, tumor budding and a model combining those 2 histological parameters in a cohort of 254 patients with OSCC. Furthermore, the ability of those markers to determine clinical outcome was verified separating OSCCs at early-stage from advanced-stage.

Material and Methods

SAMPLE

This study included 254 patients with OSCC treated at reference hospitals in Brazil (the UOPECCAN and CEONC Cancer Hospitals in Cascavel-Parana, and the Hospital Bom Pastor in Varginha-Minas Gerais) between 1998 and 2014. Complete demographic and clinical data were collected from

patient's records, including age, gender, habits such as smoking and alcohol consumption, TNM clinical stage (7th edition), tumor site, type of treatment post-surgery, status of the surgical margins, recurrence and survival. Treatments were based on radical surgery with or without postoperative radiotherapy and/or chemotherapy, and no patient had ever received any therapy before surgery. Surgical margin, identified as the closest distance between the tumor and the surgical resection edge (both in deep muscle and laterally on the mucosa), was categorized into 2 groups based on a cut-off value of 5 mm (≥ 5 mm or < 5 mm). Histological grade of tumors was classified according to the World Health Organization (WHO) grading system.[13] After treatment, patients were followed up for at least 5 years or until death (mean of 47 months, ranging from 1 to 178 months after treatment), and recurrences were histologically confirmed. The outcomes were categorized as cancer-specific survival (time from treatment initiation until death due to disease or last known date alive) and disease-free survival (time from treatment initiation until diagnosis of the first recurrence (local, regional or distant) or last follow up information for those without recurrence). The study was approved by the ethics review board of each of the hospitals affiliated with the collaborative study, and revised by the Human Research Ethics Committee of the School of Dentistry, University of Campinas (protocol number: 090/2011).

ASSESSMENT OF TSR AND TUMOR BUDDING

The HE slides were retrieved from the pathology archives, and TSR and tumor budding were estimated. The number of available slides of the primary tumor for each case ranged from 2 to 16. TSR was assessed according to van Pelt et al.[14] After identification of the invasive front, the field with the highest amount of stroma was scored, ensuring that tumor cells were present on all four sides. The area was scored at x100 magnification with regard to the percentage of stroma and tumor cells, classifying the tumors as stroma-poor (< 50%) or stroma-rich (≥ 50%). Tumor budding was scored as described elsewhere.[15] In essence, the invasive front of the tumor was scanned with low magnification, and the field with the highest number of tumor buds was counted using high magnification (x200). The cut-off point was set at 5 buds/field (< 5 buds or ≥ 5 buds). A single calibrated evaluator scored the parameters. Twenty-five cases were evaluated twice, with 4 weeks between each evaluation, to test the intra-examiner reproducibility using Cohen's Kappa coefficient, which was 0.96 for TSR and 0.84 for tumor budding.

The 2 parameters were combined and grouped as follows: Low risk, tumors with < 50% of TSR and < 5 buds, Intermediate risk, tumors with ≥ 50% of TSR and < 5 buds or tumors with < 50% of TSR and ≥ 5 buds, and High risk, tumors with ≥ 50% of TSR and ≥ 5 buds.

STATISTICAL ANALYSIS

Associations between clinicopathological parameters and TSR, tumor budding and the TSR/tumor budding model were performed using a chi-square test. Survival curves were constructed based on the Kaplan-Meier method and compared with the Log-rank test. For multivariate survival analysis, the Cox proportional hazard model (stepwise approach) was employed. A p value of ≤0.05 was considered statistically significant.

Results

The clinicopathological characteristics of patients included in this study are depicted in Supplementary Table 1. Although this cohort was collected in different Brazilian cancer treatment centers, there were no differences in the overall survival rates of patients (data not shown). On TSR assessment, 55.9% (n=142) of tumors were stroma-poor (< 50%, Figure 1A) and 44.1% (n=112) were stroma-rich (≥ 50%, Figure 1B). TSR was significantly associated with smoking habit (p=0.04), location of primary tumor (p=0.002), local recurrence (p=0.0002) and recurrence in the cervical lymph nodes (p=0.05) (Table 1). Patients classified as stroma-rich (≥ 50% TSR) developed significantly more local and regional relapse than patients classified as stroma-poor (< 50% TSR). Regarding tumor budding, 148 (58.5%) were classified as < 5 buds/field (Figure 2A), and 105 (41.5%) with ≥ 5 buds/field (Figure 2B). Tumor budding was significantly associated with treatment (p=0.03) and involvement (< 5 mm) of surgical margins (p=0.004) (Table 1). Patients with ≥ 5 buds/field were treated significantly with more complex treatment and had more involvement of the surgical margins than patients with < 5 buds/field. Applying the TSR/budding model, 35.4% (n=90) of tumors were classified as low risk, 43.3% (n=110) as intermediate risk and 21.3% (n=54) as high risk (Figure 3). Table 2 displays the results of association between the clinicopathological features and the TSR/budding model. Local recurrence was significantly more frequent in patients with a tumor classified as high risk (27.8%) compared to intermediate risk (23.1%) and low risk (11.1%) (p=0.03).

On univariate survival analysis based on log-rank test, clinical stage (p=0.01), TSR (p<0.0001) and tumor budding (p=0.04) were significantly associated with cancer-specific survival (Table 3). The TSR/tumor budding model was also significantly associated with cancer-specific survival (Figure 4A). In comparison with low risk, patients classified at intermediate risk had a shortened survival (HR: 1.75, 95% CI: 0.99-3.07, p=0.05), which was even worse for patients at high risk, yielding a HR of

4.29 (95% CI: 2.36-7.79, p<0.0001) (Table 3). Individually TSR was the only parameter associated significantly with disease-free survival (p=0.001, Table 3). After the 5-year follow up, 79% of patients classified as stroma-poor (< 50% of TSR) remained without recurrence compared with 49.1% of those classified as stroma-rich (≥ 50% of TSR) (Table 3). For disease-free survival (Figure 4B), the proposed model showed that patients at high risk had significantly more relapse than patients at low risk (HR: 2.95, 95% CI: 1.45-5.99, p=0.003), whereas patients at intermediate risk showed only a tendency towards association with shortened disease-free survival (p=0.06) (Table 3). On multivariate survival analysis, TSR, tumor budding and the TSR/tumor budding model were all independently associated with cancer-specific survival (Table 4). For disease-free survival, TSR (p=0.006) and high risk TSR/tumor budding model (p=0.007) were significantly associated (Table 4).

We were also interested in determining whether TSR and tumor budding show differential prognostic significance in OSCCs at early-stage (clinical stages I and II) and advanced-stage (clinical stages III and IV). When only patients at early-stage (n=103) were analyzed, TSR (p=0.0002), tumor budding (p=0.001) and the TSR/tumor budding model (p<0.0001) were significantly associated with cancer-specific survival on univariate analysis (Figure 5). On multivariate analysis, TSR (HR: 4.73, 95% CI: 1.75-12.77, p=0.002), tumor budding (HR: 3.03, 95% CI: 1.30-7.07, p=0.01) and the TSR/tumor budding model (HR: 3.70, 95% CI: 1.99-6.88, p<0.0001) were independently associated with cancer-specific survival of patients diagnosed with tumors at early-stage. For disease-free survival, TSR (p=0.008) and the TSR/tumor budding model (p=0.02), but not tumor budding (p=0.07), showed a significant association in early-stage tumors (Figure 5). Cox multivariate analysis confirmed that both TSR (HR: 2.56, 95% CI: 1.18-5.55, p=0.017) and the TSR/tumor budding model (HR: 1.89, 95% CI: 1.16-3.10, p=0.01) are independent prognostic markers of disease-free survival.

One hundred and forty-four patients were diagnosed with tumors at advanced-stage, and in this group, the univariate survival analysis showed that TSR (p=0.002) and the TSR/tumor budding model (p=0.005) were significantly associated with cancer-specific survival, and none variable was associated with disease-free survival (Figure 6). TSR (HR: 2.50, 95% CI: 1.47-4.16, p=0.003) and the TSR/tumor budding model (HR: 1.77, 95% CI: 1.15-2.73, p=0.009) in advanced-stage tumors were independent prognostic factors for 5-year cancer-specific survival in Cox multivariate analysis.

Discussion

In this study we investigated the prognostic significance of TSR and tumor budding in OSCC. We found a significant association between high TSR and locoregional recurrence and between the

presence of tumor budding and involvement (< 5 mm) of surgical margins, both well-known adverse features of OSCC outcome. We also performed unadjusted and adjusted survival analysis to evaluate TSR and tumor budding effects on patients' survival. TSR was associated with cancer-specific and disease-free survival in both univariate and multivariate analysis, confirming it as an independent prognostic factor in OSCC. On the other hand, tumor budding was only significantly and independently associated with cancer-specific survival. Moreover, within the scoring system combining TSR and tumor budding, we found that the higher the score, the worse the outcome in 5-year cancer-specific and disease-free survival.

Currently it is known that high TSR is associated with increased cancer-mortality and high relapse in different solid tumors,[7] which is in line with our findings. In oral cancers, one early investigation reported reduced 3-year overall survival and disease-free survival rates in stroma-rich patients, but the differences were not statistically significant, mainly due to the small sample size and short follow up.[8] Another multicenter study with 311 early-stage oral tongue carcinomas showed the stroma-rich group (≥ 50% TSR) to have a HR of 1.71 with 95% CI of 1.02-2.86 for 5-year cancer-related mortality and a HR of 1.81 with 95% CI of 1.17-2.79 for 5-year disease-free survival.[9] Compared to this previous study, the current study found greater hazard ratios for mortality and recurrence when comparing stroma-rich and stroma-poor groups in the multivariate analysis. These differences may be explained by a number of factors. Whilst we have included tumor at different clinical stages (with a predominance of advanced-stage), from different sites of the oral cavity and mainly classified as moderately-differentiated tumors, the previous study included only early-stage tumors from oral tongue, mainly with well or poor cellular differentiation.

The reason for the worse outcome in tumors with a higher proportion of stroma is still unclear, but it is probably related to the interactions between tumor cells and cancer-associated fibroblasts (CAFs). The biological properties of CAFs in OSCC progression and metastasis have been extensively reported,[16-18] and several of those functions are related to the ability of CAFs to secrete large amounts of extracellular matrix, including collagen.[19,20] Collagen and its derived-peptides formed during collagen-fiber maturation or degradation by proteases such as the matrix metalloproteinases (MMPs) are directly associated with tumor cell proliferation, survival, migration and invasion, as well as affecting angiogenesis and immune function in the tumor microenvironment.[21] The fibrotic stroma also prevents drug delivery into the tumor mass, facilitating chemoresistance.[22] Together these features can explain why a tumor with higher stromal content is prone to having a highly aggressive phenotype, influencing patient's outcome.

Tumor budding is a histologic finding in which cells at the tumor front detach from the tumor mass, as single cell or as clusters with up to 5 tumor cells, and invade into the adjacent normal tissue. It has been indicated as a confident and reproducible predictor of clinical outcome for colorectal tumors.[23] In oral cancer, 2 recent systematic reviews with meta-analysis verified the value of tumor budding in OSCC, and demonstrated that high bud activity is frequently associated with parameters that worsen the prognosis, including lymph node metastasis, and more importantly, ≥ 5 buds/field significantly predicted shortened time to disease relapse and an overall decrease in survival.[10,11] In our analysis, previous data that revealed tumor budding as a prognostic factor for OSCC were confirmed. Indeed, patients with < 5 buds had a better cancer-specific survival compared with those with ≥ 5 buds in both univariate and multivariate analysis. The number of buds was not associated with disease-free survival, however, in early-stage tumors, a clear tendency of association was detected (p=0.07), yielding a HR of 1.98 with a 95% CI of 0.92-4.27. Interestingly, tumor budding was combined with depth of invasion to form the tumor budding and depth of invasion (BD) risk model for OSCC, which has been associated with high risk for locoregional recurrence and shortened survival for OSCCs in different studies.[15,24-26]

Evidence accumulated over the years reveals a connection between buds and epithelial-mesenchymal transition (EMT).[27] Low expression of E-cadherin and up-regulation of vimentin, both key features of cells in EMT, were frequently observed in tumor buds of OSCCs.[28-31] Moreover, cells in the buds exhibited a specific gene expression signature, with activation of the TGFβ pathway and overexpression of EMT transcription factors, including ZEB1 and PRRX1.[30] A high expression of other EMT transcription factors, such as SNAIL and TWIST, was also reported in OSCC buds.[31] Another important avenue to be explored is the association of tumor budding and cell stemness. A recent study revealed a significant correlation between CD44 overexpression and high tumor budding activity at the invasive margin of OSCCs.[32] Indeed, the connection among budding, EMT and cancer stem cells would be interesting to define, warranting further studies of stem cell markers in tumor buds.

The combination of TSR and tumor budding resulted in a risk model with a clear discriminatory ability to indicate prognosis of OSCC patients, especially for cancer-specific survival. This might be due to the combination of independent prognostic parameters, which significantly increase the prognostic power, thus leading to an even higher prognostic impact than the individual parameters alone. Of note, this combination model includes both a cancer-related feature (tumor budding) and a stromal-related feature (TSR). The prognostic significance was not observed in some of our

subgroup analysis, which could be due to the limited sample size. Further studies, especially conducted on larger cohorts, are necessary to confirm our findings and to improve understanding of the biological behavior of OSCC.

In summary, we show, based on a representative sample of 254 primary OSCCs, that TSR and tumor budding, assessed on regular HE-stained slides, are reliable markers of OSCC outcome. The combination of these features improved the discrimination of patients with low- and high-risk for poor prognosis, mainly in patients with early-stage tumors. Those included parameters have the advantage that they can be determined routinely in daily clinical practice, and their inclusion in histopathology reports may be of help in the more accurate prognostic classification of OSCC patients. The fact that the model was tested in only a cohort makes further validation warranted.

Funding

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (2018/16077-6 for RDC). LMRP is supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais - FAPEMIG (APQ 00205.16). MRD (2017/26764-8), KYMM (2018/16754-8) and GBH (2018/17689-5) are research fellows supported by FAPESP.

Conflict of interest

The authors declare no conflicts of interest related to this study.

Author's contributions

MRD, KYMM, GBH and RDC carried out data acquisition and analysis. LMRP and IS-C have contributed with clinical samples. CBD, CEO, ECBF and BCL and collected clinical information. AA and TS have participated in the design of the study. RDC drafted the manuscript and all authors have critically revised the manuscript.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018; **68;** 394-424.

2. Chi AC, Day TA, Neville BW. Oral cavity and oropharyngeal squamous cell carcinoma-an update. *CA Cancer J Clin*. 2015; **65;** 401-421.
3. Rodrigues PC, Miguel MC, Bagordakis E *et al*. Clinicopathological prognostic factors of oral tongue squamous cell carcinoma: a retrospective study of 202 cases. *Int J Oral Maxillofac Surg*. 2014; **43;** 795-801.
4. Woolgar JA. Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. *Oral Oncol*. 2006; **42;** 229-239.
5. Speight PM, Farthing PM. The pathology of oral cancer. *Br Dent J*. 2018; **225;** 841-847.
6. Ridge JA, Lydiatt WM, Patel SG *et al*. Head and Neck. In: Amin MB, Edge S, Greene FL, Byrd DR, Brookland RK, Washington MK, Gershenwald JE, Compton CC, Hess KR, Sullivan DC, Jessup JM, Brierley JD, Gaspar LE, Schilsky RL, Balch CM, Winchester DP, Asare EA, Madera M, Gress DM, Meyer LR, eds. AJCC Cancer Staging Manual, (8th ed). New York, NY: Springer; 2017: 79-94.
7. Wu J, Liang C, Chen M, and Su W. Association between tumor-stroma ratio and prognosis in solid tumor patients: a systematic review and meta-analysis. *Oncotarget*. 2016; **7;** 68954-68965.
8. Niranjan KC, Sarathy NA. Prognostic impact of tumor-stroma ratio in oral squamous cell carcinoma - A pilot study. *Ann Diagn Pathol*. 2018; **35;** 56-61.
9. Almangush A, Heikkinen I, Bakhti N *et al*. Prognostic impact of tumour-stroma ratio in early-stage oral tongue cancers. *Histopathology*. 2018; **72;** 1128-1135.
10. Almangush A, Pirinen M, Heikkinen I, Mäkitie AA, Salo T, Leivo I. Tumour budding in oral squamous cell carcinoma: a meta-analysis. *Br J Cancer*. 2018; **118;** 577-586.
11. Zhu Y, Liu H, Xie N *et al*. Impact of tumor budding in head and neck squamous cell carcinoma: A meta-analysis. *Head Neck*. 2019; **41;** 542-550.
12. Elseragy A, Salo T, Coletta RD *et al*. A proposal to revise the histopathologic grading system of early oral tongue cancer incorporating tumor budding. *Am J Surg Pathol*. 2019; **43;** 703-709.
13. Sloan P, Gale N, Hunter K *et al*. Malignant surface epithelial tumours. In El-Naggar AK, Chan JKC, Grandis JR, Takata T, Sootweg P (editors). WHO classification of head and neck tumours. 4th edition, 2017; 109-111. IARC: Lyon.
14. van Pelt GW, Kjaer-Frifeldt S, van Krieken *et al*. Scoring the tumor-stroma ratio in colon cancer: procedure and recommendations. *Virchows Arch*. 2018; **473;** 405-412.
15. Almangush A, Coletta RD, Bello IO *et al*. A simple novel prognostic model for early stage oral tongue cancer. *Int J Oral Maxillofac Surg*. 2015; **44;** 143-150.

16. Li YY, Tao YW, Gao S *et al*. Cancer-associated fibroblasts contribute to oral cancer cells proliferation and metastasis via exosome-mediated paracrine miR-34a-5p. *EBioMedicine*. 2018; **36;** 209-220.
17. Dourado MR, Korvala J, Åström P *et al*. Extracellular vesicles derived from cancer-associated fibroblasts induce the migration and invasion of oral squamous cell carcinoma. *J Extracell Vesicles*. 2019; **8;** 1578525.
18. Dourado MR, Guerra ENS, Salo T, Lambert DW, Coletta RD. Prognostic value of the immunohistochemical detection of cancer-associated fibroblasts in oral cancer: A systematic review and meta-analysis. *J Oral Pathol Med*. 2018; **47;** 443-453.8.
19. Bagordakis E, Sawazaki-Calone I, Macedo CC *et al*. Secretome profiling of oral squamous cell carcinoma-associated fibroblasts reveals organization and disassembly of extracellular matrix and collagen metabolic process signatures. *Tumour Biol*. 2016; **37;** 9045-9057.
20. Mellone M, Hanley CJ, Thirdborough S *et al*. Induction of fibroblast senescence generates a non-fibrogenic myofibroblast phenotype that differentially impacts on cancer prognosis. *Aging* (Albany NY). 2016; **9;** 114-132.
21. Pickup MW, Mouw JK, Weaver VM. The extracellular matrix modulates the hallmarks of cancer. *EMBO Rep*. 2014; **15;** 1243-1253.
22. Mezawa Y, Orimo A. The roles of tumor- and metastasis-promoting carcinoma-associated fibroblasts in human carcinomas. *Cell Tissue Res*. 2016; **365;** 675-689.
23. Lugli A, Kirsch R, Ajioka Y *et al*. Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. *Mod Pathol*. 2017; **30;** 1299-1311.
24. Sawazaki-Calone I, Rangel A, Bueno AG *et al*. The prognostic value of histopathological grading systems in oral squamous cell carcinomas. *Oral Dis*. 2015; **21;** 755-761.
25. Hori Y, Kubota A, Yokose T *et al*. Predictive significance of tumor depth and budding for late lymph node metastases in patients with clinical N0 early oral tongue carcinoma. *Head Neck Pathol*. 2017; **11;** 477-486.
26. Yu P, Wang W, Zhuang Z *et al*. A novel prognostic model for tongue squamous cell carcinoma based on the characteristics of tumour and its microenvironment: iBD score. *Histopathology*. 2019; **74;** 766-779.
27. Grigore AD, Jolly MK, Jia D, Farach-Carson MC, Levine H. Tumor budding: The name is EMT. Partial EMT. *J Clin Med*. 2016; **5**. pii: E51. doi: 10.3390/jcm5050051.

28. Wang C, Huang H, Huang Z *et al*. Tumor budding correlates with poor prognosis and epithelial-mesenchymal transition in tongue squamous cell carcinoma. *J Oral Pathol Med*. 2011; **40;** 545-551.
29. Attramadal CG, Kumar S, Boysen ME *et al*. Tumor budding, EMT and cancer stem cells in T1-2/N0 oral squamous cell carcinomas. *Anticancer Res*. 2015; **35;** 6111-6120.
30. Jensen DH, Dabelsteen E, Specht L *et al*. Molecular profiling of tumour budding implicates TGFβ-mediated epithelial-mesenchymal transition as a therapeutic target in oral squamous cell carcinoma. *J Pathol*. 2015; **236;** 505-516.
31. Hong KO, Oh KY, Shin WJ, Yoon HJ, Lee JI, Hong SD. Tumor budding is associated with poor prognosis of oral squamous cell carcinoma and histologically represents an epithelial-mesenchymal transition process. *Hum Pathol*. 2018; **80;** 123-129.
32. Boxberg M, Götz C, Haidari S et al. Immunohistochemical expression of CD44 in oral squamous cell carcinoma in relation to histomorphological parameters and clinicopathological factors. *Histopathology*. 2018; **73;** 559-572.

Figure legends

Figure 1 Representative samples of tumor-stroma ratio (TSR). (A) Tumor classified as stroma-poor (< 50% TSR) and (B) tumor with stroma-rich (≥ 50% TSR).

Figure 2 Tumor budding in oral squamous cell carcinomas, with arrows to indicate budding foci. (A) Tumor with low budding activity (< 5 tumor buds/field) and (B) tumor with more than 5 tumor buds/field (high activity).

Figure 3 Model associating tumor-stroma ratio (TSR) and tumor budding. (A) Low risk: < 50% TSR and no tumor buds, (B) Intermediate risk: ≥ 50% TSR but no tumor buds, (C) High risk: ≥ 50% TSR and ≥ 5 tumor buds/field. Arrows indicate examples of tumor buds.

Figure 4 Kaplan-Meier curves for cancer-specific survival (A) and disease-free survival (B) of patients with OSCC based in the tumor-stroma ratio (TSR)/tumor budding model.

Figure 5 Kaplan-Meier survival curves for cancer-specific survival (A-C) and disease-free survival (D-F) based on tumor-stroma ratio (TSR) (A and D), tumor budding (B and E) and the TSR/tumor budding model (C and F) in patients with tumors at early-stage.

Figure 6 Kaplan-Meier survival curves for cancer-specific survival (A-C) and disease-free survival (D-F) based on tumor-stroma ratio (TSR) (A and D), tumor budding (B and E) and the TSR/tumor budding model (C and F) in patients with tumors at advanced-stage.

Table 1. Association of tumor-stroma ratio (TSR) and tumor budding with clinicopathological parameters of the tumors.

	Tumor-stroma ratio (TSR)			Tumor budding		
	< 50%	≥ 50%	p value	< 5 buds	≥ 5 buds	p value
Age (years)						
≤ 61 years	68 (47.9%)	65 (58%)		62 (43.1%)	41 (40.2%)	
> 61 years	74 (52.1%)	47 (42%)	0.11	82 (56.9%)	61 (59.8%)	0.65
Gender						
Male	107 (75.4%)	81 (72.3%)		109 (73.6%)	78 (74.3%)	
Female	35 (24.6%)	31 (27.7%)	0.58	39 (26.4%)	27 (25.7%)	0.91
Smoking habit						
No	14 (12.6%)	23 (23.5%)		25 (20.7%)	12 (13.8%)	
Yes	97 (87.4%)	75 (76.5%)	0.04	96 (79.3%)	75 (86.2%)	0.20
Drinking habit						
No	39 (37.5%)	42 (46.6%)		50 (45%)	31 (37.8%)	
Yes	65 (62.5%)	48 (53.4%)	0.19	61 (55%)	51 (62.2%)	0.31
Clinical stage						
I + II	58 (42%)	45 (41.3%)		62 (43.1%)	41 (40.2%)	
III + IV	80 (58%)	64 (58.7%)	0.90	82 (56.9%)	61 (59.8%)	0.65
Location						
Tongue	104 (73.2%)	66 (58.9%)		91 (61.5%)	78 (74.3%)	
Floor of mouth	35 (24.6%)	32 (28.6%)		46 (31.1%)	21 (20%)	
Other	3 (2.2%)	14 (12.5%)	0.002	11 (64.7%)	6 (5.7%)	0.09
Histological grade						

Well-differentiated	40 (28.2%)	32 (28.6%)		44 (29.7%)	28 (26.7%)	
Moderately-differentiated	88 62%)	66 (58.9%)		84 (56.8%)	69 (65.7%)	
Poorly-differentiated	14 (9.8%)	14 (12.5%)	0.78	20 (13.5%)	8 (7.6%)	0.23
Treatment						
Surgery	44 (31.7%)	31 (28.4%)		52 (35.9%)	23 (22.5%)	
Surgery + Radiotherapy	49 (35.3%)	44 (40.4%)		54 (37.2%)	38 (37.3%)	
Surgery + Radiotherapy + Chemotherapy	46 (33.1%)	34 (31.2%)	0.70	39 (26.9%)	41 (40.2%)	0.03
Margin status						
≥ 5 mm	89 (74.8%)	84 (80%)		110 (84%)	62 (67.4%)	
< 5 mm	30 (25.2%)	21 (20%)	0.35	21 (16%)	30 (32.6%)	0.004
Local recurrence						
No	124 (88.6%)	78 (69.6%)		119 (80.4%)	82 (79.6%)	
Yes	16 (11.4%)	34 (30.4%)	0.0002	29 (19.6%)	21 (20.4%)	0.87
Regional (cervical) recurrence						
No	138 (97.2%)	101 (91.8%)		139 (95.2%)	99 (94.3%)	
Yes	4 (2.8%)	9 (8.2%)	0.05	7 (4.8%)	6 (5.7%)	0.74
Distant recurrence						
No	136 (95.8%)	106 (96.4%)		141 (96.6%)	100 (96.2%)	
Yes	6 (4.2%)	4 (3.6%)	0.81	5 (3.4%)	5 (4.8%)	0.59

Table 2. Association of tumor-stroma ratio (TSR)/tumor budding model with clinicopathological parameters of the tumors.

	TSR/tumor budding model			
	Low risk	Intermediate risk	High risk	p value
Age (years)				
≤ 61 years	41 (45.6%)	61 (55.5%)	31 (57.4%)	
> 61 years	49 (54.4%)	49 (44.5%)	23 (42.6%)	0.37
Gender				
Male	65 (72.2%)	86 (78.2%)	37 (68.5%)	
Female	25 (27.8%)	24 (21.8%)	17 (31.5%)	0.37
Smoking habit				
No	10 (14.5%)	19 (20.2%)	8 (17.4%)	
Yes	59 (85.5%)	75 (79.8%)	38 (82.6%)	0.64
Drinking habit				
No	25 (37.9%)	36 (43.4%)	20 (44.4%)	
Yes	41 (62.1%)	47 (56.6%)	25 (55.6%)	0.73
Clinical stage				
I + II	39 (44.8%)	42 (38.8%)	22 (42.3%)	
III + IV	48 (55.2%)	66 (61.1%)	30 (57.7%)	0.70
Location				
Tongue	60 (66.7%)	75 (68.2%)	35 (64.8%)	
Floor of mouth	28 (31.1%)	25 (22.7%)	14 (25.9%)	
Other	2 (2.2%)	10 (9.1%)	5 (9.3%)	0.23
Histological grade				
Well-differentiated	27 (30%)	30 (27.3%)	15 (27.8%)	
Moderately-differentiated	51 (56.7%)	70 (63.6%)	33 (61.1%)	
Poorly-differentiated	12 (13.3%)	10 (9.1%)	6 (11.1%)	0.85
Treatment				
Surgery	31 (35.2%)	34 (31.5%)	10 (19.2%)	
Surgery + Radiotherapy	32 (36.4%)	39 (36.1%)	22 (42.3%)	
Surgery + Radiotherapy + Chemotherapy	25 (28.4%)	35 (32.4%)	20 (38.5%)	0.37
Margin status				
≥ 5 mm	58 (78.4%)	83 (81.4%)	32 (66.7%)	
< 5 mm	16 (21.6%)	19 (18.6%)	16 (33.3%)	0.13
Local recurrence				
No	80 (88.9%)	83 (76.9%)	39 (72.2%)	

Yes	10 (11.1%)	25 (23.1%)	15 (27.8%)	0.03
Regional (cervical) recurrence				
No	87 (96.7%)	103 (95.4%)	49 (90.7%)	
Yes	3 (3.3%)	5 (4.6%)	5 (9.3%)	0.28
Distant recurrence				
No	87 (96.7%)	103 (95.4)	52 (96.3%)	
Yes	3 (3.3%)	5 (4.6%)	2 (3.7%)	0.89

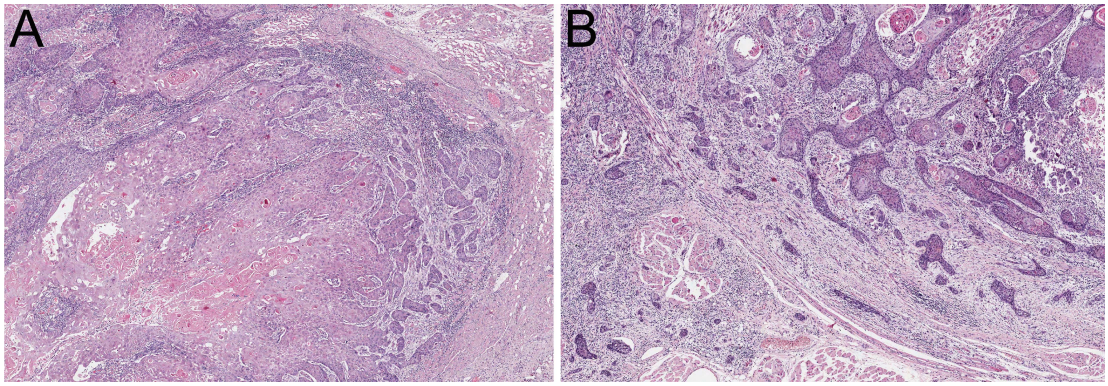
Table 3. Univariate analysis for cancer-specific survival and disease-free survival of patients with the oral squamous cell carcinoma.

	Cancer-specific survival			Disease-free survival		
	% in 5 years	HR (95% CI)	p value	% in 5 years	HR (95% CI)	p value
Age (years)						
≤ 61 years	60.7	1		64.1	1	
> 61 years	56.4	1.35 (0.87-2.08)	0.18	64.2	1.03 (0.55-1.22)	0.79
Gender						
Male	57.7	1		64.5	1	
Female	61.5	0.86 (0.53-1.41)	0.54	68.0	0.78 (0.45-1.35)	0.38
Clinical stage						
I + II	68.2	1		64.1	1	
III + IV	53.2	1.72 (1.11-2.67)	0.01	64.7	0.96 (0.58-1.58)	0.87
Location						
Tongue	63.1	1		66.4	1	
Floor of mouth	53.0	1.40 (0.83-2.37)	0.20	64.7	1.09 (0.60-1.97)	0.76
Other	55.7	1.28 (0.75-2.93)	0.41	44.1	2.53 (0.87-7.28)	0.08
Histological grade						
Well-differentiated	62.8	1		69.0	1	
Moderately-differentiated	57.5	1.08 (0.66-1.76)	0.75	54.7	1.44 (0.82-2.51)	0.19
Poorly-differentiated	55.7	1.34 (0.60-2.99)	0.46	66.4	1.19 (0.53-2.69)	0.67
Treatment						
Surgery	64.7	1		64.5	1	
Surgery + Radiotherapy	50.9	1.24 (0.73-2.09)	0.42	69.0	0.78 (0.41-1.50)	0.47
Surgery + Radiotherapy + Chemotherapy	61.8	1.13 (0.64-1.99)	0.67	61.9	1.09 (0.60-1.98)	0.76

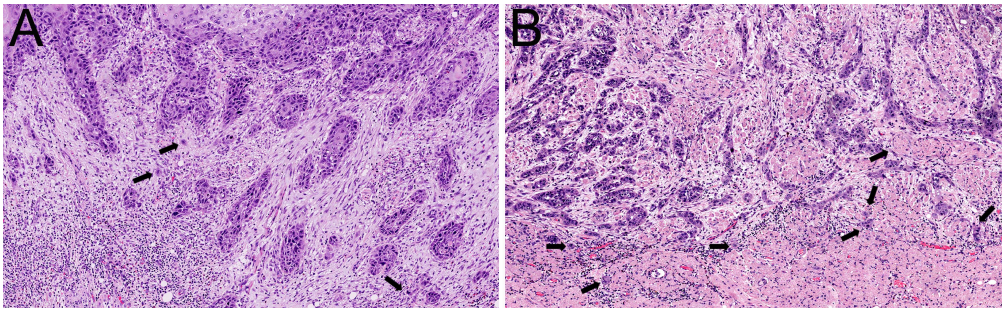
Margin status						
≥ 5 mm	62.9	1		65.1	1	
< 5 mm	47.5	1.40 (074-2.65)	0.29	44.5	1.29 (0.69-2.42)	0.42
Tumor-stroma ratio (TSR)						
< 50% (stroma-poor)	75.2	1		79.0	1	
≥ 50% (stroma-rich)	43.2	2.93 (1.89-4.52)	<0.0001	49.1	2.29 (1.40-3.76)	0.001
Tumor budding						
≥ 5 buds	67.1	1		69.0	1	
< 5 buds	44.8	1.89 (1.01-2.49)	0.04	55.1	1.29 (0.78-2.14)	0.31
TSR/tumor budding model						
Low risk	76.6	1		79.5		
Intermediate risk	62.0	1.75 (0.99-3.07)	0.05	63.8	1.77 (0.98-3.19)	0.06
High risk	29.0	4.29 (2.36-7.79)	<0.0001	40.1	2.95 (1.45-5.99)	0.003

Table 4. Multivariate analysis of cancer-specific survival and disease-free survival for the 254 patients with oral squamous cell carcinoma.

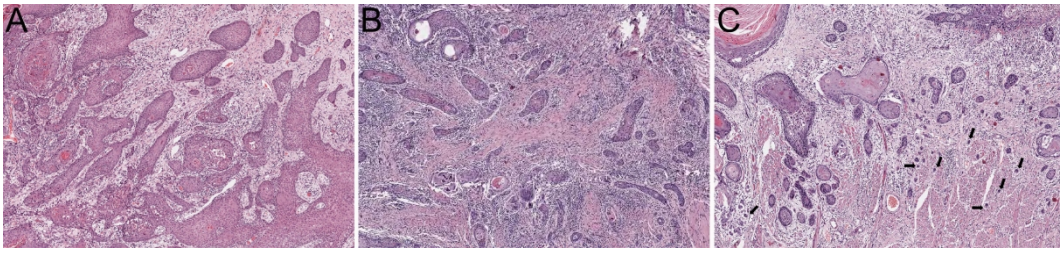
	Cancer-specific survival		Disease-free survival	
	HR (95% CI)	p value	HR (95% CI)	p value
Model 1				
Tumor-stroma ratio (TSR)				
< 50% (stroma-poor)	1		1	
≥ 50% (stroma-rich)	3.58 (2.05-6.27)	<0.0001	2.05 (1.23-3.44)	0.006
Tumor budding				
< 5 buds	1			
≥ 5 buds	1.47 (1.05-2.05)	0.02		
Model 2				
TSR/tumor budding model				
Low risk	1		1	
Intermediate risk	2.15 (1.05-4.39)	0.03		
High risk	2.62 (1.76-3.91)	<0.0001	1.61 (1.14-2.28)	0.007



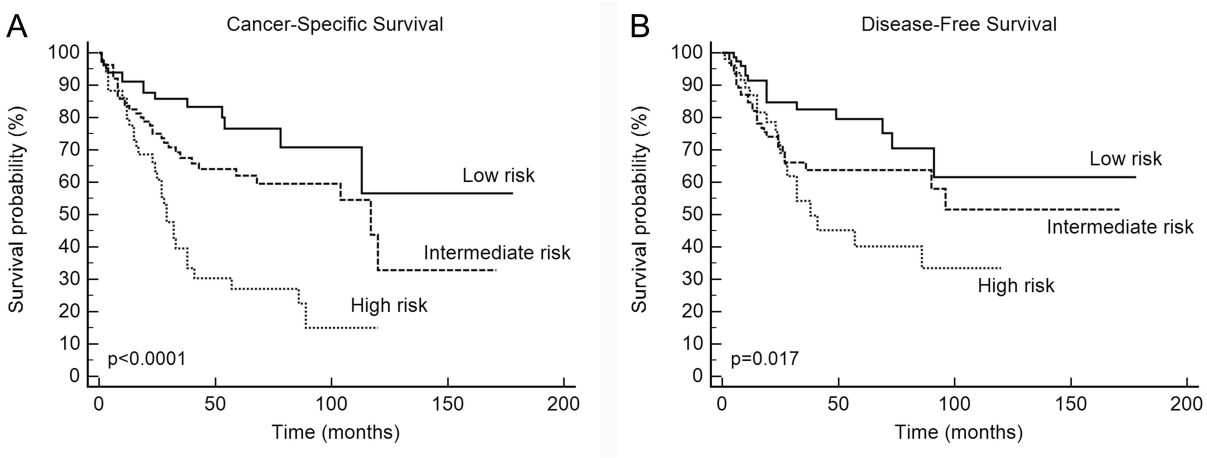
his_14070_f1.jpg



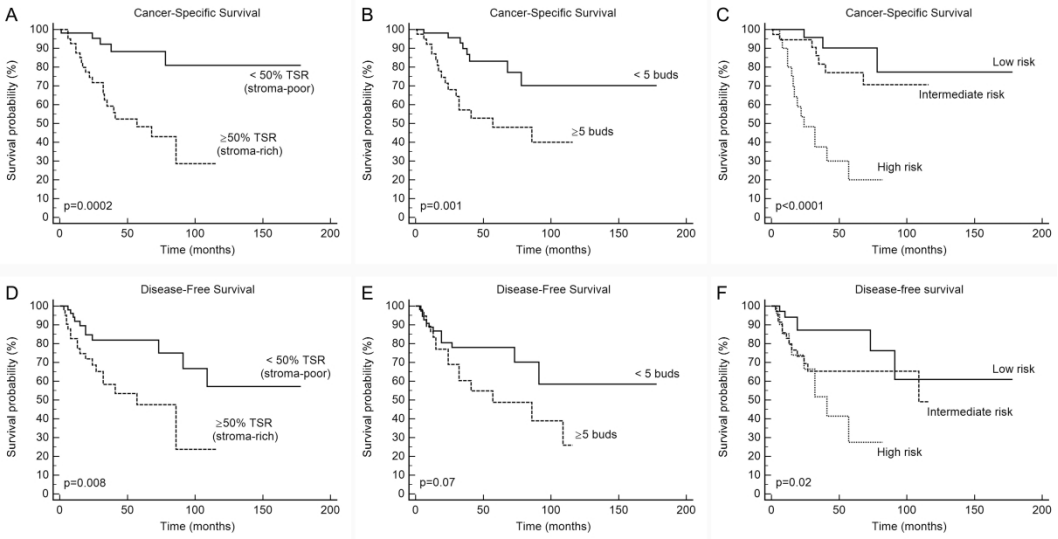
his_14070_f2.jpg



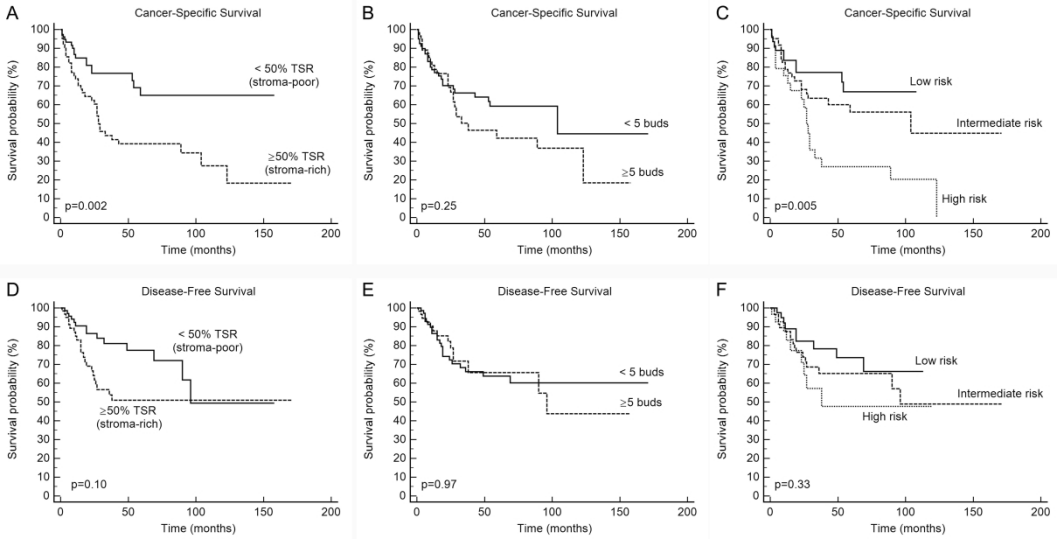
his_14070_f3.jpg



his_14070_f4.jpg



his_14070_f5.jpg



his_14070_f6.jpg