Prognostication for oral squamous cell carcinoma patients based on the tumour–stroma ratio and tumour budding

Running title: *Tumour–stroma ratio and tumour budding in oral cancer*

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Aims: Previous studies have demonstrated that the tumour–stroma ratio (TSR) and tumour budding are of prognostic value for oral squamous cell carcinomas (OSCCs). The aim of this study was to evaluate the prognostic significance of those histological parameters, individually and in combination, for OSCC.

Methods and results: The TSR and tumour budding (the presence of five or more buds at the invasive front) were estimated in 254 patients with OSCC. The clinicopathological association was investigated with a chi-square test, and the prognostic significance (cancer-specific survival and disease-free survival) was verified with Kaplan–Meier analysis and the Cox proportional hazard model. The TSR (≥50%, stroma-rich) was significantly and independently associated with both shortened cancer-specific survival and poor disease-free survival, whereas tumour budding was significantly associated with reduced cancer-specific survival. The TSR/tumour budding model was independently associated with a high risk of cancer mortality and recurrence (disease-free survival). In patients with early-stage tumours (clinical stage I and II, $n = 103$), the TSR, tumour budding and the TSR/tumour budding model were significantly associated with both cancer-related death and recurrence, whereas, in advanced-stage tumours (clinical stage III and IV, $n = 144$), only the TSR and the TSR/tumour budding model were significantly associated with cancer-specific survival.

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

Introduction

Oral squamous cell carcinoma (OSCC), which is the most common tumour in the head and neck region, affects >300 000 new individuals and is responsible for 177 000 deaths globally every year.[1] OSCC is considered to be a very aggressive tumour, and, of those receiving maximum treatment with curative intent, only half survive for >5 years.[2] Its management and prognosis are mainly based on clinical criteria, especially TNM classification; however, the behaviour of some OSCCs is unpredictable.[3] Several pathological features, individually or combined in scoring systems, have been shown to have important roles in prognostication for patients with OSCC.[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]

Histological analysis of the proportion of tumour cells relative to fibrotic stroma [tumour–stroma ratio (TSR)] on haematoxylin and eosin (H&E)-stained slides has been shown to be of prognostic value for solid tumours,[7] including OSCCs.[8,9] A study by Niranjan and Sarathy,[8] which had a small sample size and a short follow-up, did not find a significant association between the TSR and outcome. On the other hand, a study by Almangush *et al*.[9] revealed that the 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 on H&E-stained slides, is tumour budding, i.e. single cells or clusters of up to five cancer cells at the invasive front, as revealed by recent systematic reviews with meta-analyses.[10,11] Moreover, the incorporation of tumour budding in the World Health Organization (WHO) histological tumour grade of OSCC resulted in superior prognostic value to that of the WHO histological tumour grade alone.[12]

The aim of the present study was to examine the prognostic value of the TSR, tumour budding and a model combining these two histological parameters in a cohort of 254 patients

with OSCC. Furthermore, the ability of those markers to indicate clinical outcome was verified by separating early-stage OSCCs from advanced-stage OSCCs.

Materials and methods

SAMPLE

This study included 254 patients with OSCC treated at referral hospitals in Brazil (the UOPECCAN and CEONC Cancer Hospitals in Cascavel-Parana, and Hospital Bom Pastor in Varginha-Minas Gerais) between 1998 and 2014. Complete demographic and clinical data were collected from patients' records, including age, sex, habits such as smoking and alcohol consumption, TNM clinical stage (7th edition), tumour site, type of treatment post surgery, status of surgical margins, recurrence, and survival. Treatments were based on radical surgery with or without postoperative radiotherapy and/or chemotherapy, and no patient had received any therapy before surgery. The surgical margin, identified as the closest distance between the tumour and the surgical resection edge (both in deep muscle and laterally on the mucosa), was categorised into two groups on the basis of a cut-off value of 5 mm (≥5 mm or <5 mm). The histological grade of tumours was classified according to the 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 month to 178 months after treatment), and recurrences were histologically confirmed. The outcomes were categorised 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 THE TSR AND TUMOUR BUDDING

The H&E-stained slides were retrieved from the pathology archives, and the TSR and tumour budding were estimated. The number of available slides of the primary tumour for each case ranged from two to 16. The 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 tumour cells were present on all four sides. The area was scored at ×100 magnification with regard to the percentage of stroma and tumour cells, and the tumours were classified as stroma-poor (<50%) or stroma-rich (≥ 50%). Tumour budding was scored as described elsewhere.[15] In essence, the invasive front of the tumour was scanned at low magnification, and the field with the highest number of tumour buds was counted at high magnification (×200). The cut-off point was set at five buds per field (fewer than five buds, or five or more 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 by the use of Cohen's kappa coefficient, which was 0.96 for the TSR and 0.84 for tumour budding.

The two parameters were combined and grouped as follows: low risk—tumours with a TSR of <50% and fewer than five buds; intermediate risk—tumours with a TSR of ≥50% and fewer than five buds, or tumours with a TSR of <50% and five or more buds; and high risk—tumours with a TSR of ≥50% and five or more buds.

STATISTICAL ANALYSIS

Associations between clinicopathological parameters and the TSR, tumour budding and the TSR/tumour budding model were determined with a chi-square test. Survival curves were constructed according to the Kaplan–Meier method, and compared by use of the log-rank test. For multivariate survival analysis, the Cox proportional hazard model (stepwise approach) was used. A *P*-value of ≤ 0.05 was considered to be statistically significant.

Results

The clinicopathological characteristics of the patients included in this study are shown in Table S1. Although this cohort was collected in different Brazilian cancer treatment centres, there were no differences in the overall survival rates of patients (data not shown). On TSR assessment, 55.9% ($n = 142$) of tumours were stroma-poor (<50%; Figure 1A) and 44.1% ($n = 112$) were stroma-rich (≥50%; Figure 1B). The TSR was significantly associated with smoking ($P = 0.04$), location of the primary tumour ($P = 0.002$), local recurrence ($P = 0.0002$), and recurrence in the cervical lymph nodes ($P = 0.05$) (Table 1). Patients with tumours classified as stroma-rich (TSR of ≥50%) developed significantly more local and regional relapses than patients with tumours classified as stroma-poor (TSR of <50%). Regarding tumour budding, 148 (58.5%) tumours were classified as having fewer than five buds per field (Figure 2A), and 105 (41.5%) tumours were classified as having five or more buds per field (Figure 2B). Tumour budding was significantly associated with treatment ($P = 0.03$) and involvement (<5 mm) of surgical margins ($P = 0.004$) (Table 1). Patients with five or more buds per field received significantly more complex treatment and had more involvement of the surgical margins than patients with fewer than five buds per field. On application of the TSR/budding model, 35.4% ($n = 90$) of tumours were classified as low

risk, 43.3% (n = 110) as intermediate risk, and 21.3% (n = 54) as high risk (Figure 3). Table 2 shows the results regarding associations between the clinicopathological features and the TSR/budding model. Local recurrence was significantly more frequent in patients with tumours classified as high risk (27.8%) than in those with tumours classified as intermediate risk (23.1%) and low risk (11.1%) (P = 0.03).

On univariate survival analysis based on the log-rank test, clinical stage (P = 0.01), the TSR (P < 0.0001) and tumour budding (P = 0.04) were significantly associated with cancer-specific survival (Table 3). The TSR/tumour budding model was also significantly associated with cancer-specific survival (Figure 4A). In comparison with patients with tumours classified as low risk, patients with tumours classified as intermediate risk had shortened survival [hazard ratio (HR) 1.75, 95% confidence interval (CI) 0.99–3.07, P = 0.05], which was even worse for patients with tumours classified as high risk, yielding an HR of 4.29 (95% CI 2.36–7.79, P < 0.0001) (Table 3). Individually, the 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 with tumours classified as stroma-poor (TSR of <50%) remained without recurrence, as compared with 49.1% of those with tumours classified as stroma-rich (TSR of ≥50%) (Table 3). For disease-free survival (Figure 4B), the proposed model showed that patients with tumours classified as high risk had significantly more relapses than patients with tumours classified as low risk (HR 2.95, 95% CI 1.45–5.99, P = 0.003), whereas patients with tumours classified as intermediate risk showed only a tendency to have shortened disease-free survival (P = 0.06) (Table 3). On multivariate survival analysis, the TSR, tumour budding and the TSR/tumour budding model were all independently associated with cancer-specific survival (Table 4). Also, the TSR (P = 0.006) and high-risk TSR/tumour budding model (P = 0.007) were significantly associated with disease-free survival (Table 4).

We were also interested in determining whether the TSR and tumour budding showed differential prognostic significance for early-stage tumours (clinical stages I and II) and advanced-stage tumours (clinical stages III and IV). When only patients with early-stage tumours (n = 103) were analysed, the TSR (P = 0.0002), tumour budding (P = 0.001) and the TSR/tumour budding model (P < 0.0001) were significantly associated with cancer-specific survival on univariate analysis (Figure 5). On multivariate analysis, the TSR (HR 4.73, 95% CI 1.75–12.77, P = 0.002), tumour budding (HR 3.03, 95% CI 1.30–7.07, P = 0.01) and the TSR/tumour 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 tumours at an early stage. For disease-free survival, the TSR (P = 0.008) and the TSR/tumour budding model (P = 0.02), but not tumour budding (P = 0.07), showed significant associations in early-stage tumours (Figure 5). Cox multivariate analysis confirmed that both the TSR (HR 2.56, 95% CI 1.18–5.55, P = 0.017) and the TSR/tumour 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 tumours at an advanced stage, and, in this group, the univariate survival analysis showed that the TSR (P = 0.002) and the TSR/tumour budding model (P = 0.005) were significantly associated with cancer-specific survival, and that no variable was associated with disease-free survival (Figure 6). In advanced-stage tumours, the TSR (HR 2.50, 95% CI 1.47–4.16, P = 0.003) and the TSR/tumour budding model (HR 1.77, 95% CI 1.15–2.73, P = 0.009) were independent prognostic factors for 5-year cancer-specific survival on Cox multivariate analysis.

Discussion

In this study we investigated the prognostic significance of the TSR and tumour budding in OSCC. We found significant associations between a high TSR and locoregional recurrence, and between the presence of tumour budding and involvement (<5 mm) of surgical margins, both of which are well-known adverse features of OSCC outcome. We also performed unadjusted and adjusted survival analyses to evaluate the effects of the TSR and tumour budding on patients' survival. The TSR was associated with cancer-specific and disease-free survival in both univariate and multivariate analyses, confirming it as an independent prognostic factor in OSCC. On the other hand, tumour budding was only significantly and independently associated with cancer-specific survival. Moreover, within the scoring system combining the TSR and tumour budding, we found that the higher the score, the worse the outcome regarding 5-year cancer-specific and disease-free survival.

Currently, it is known that a high TSR is associated with increased cancer mortality and a high rate of relapse in patients with different solid tumours,[7] which is in line with our findings. For 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 because of the small sample size and short follow-up.[8] Another multicentre study with 311 early-stage oral tongue carcinomas showed the stroma-rich group (TSR of ≥50%) to have an HR of 1.71 with a 95% CI of 1.02–2.86 for 5-year cancer-related mortality, and an HR of 1.81 with a 95% CI of 1.17–2.79 for 5-year disease-free survival.[9] The current study found greater HRs than these for mortality and recurrence when the stroma-rich and stroma-poor groups were compared in multivariate analysis. These differences may be explained by a number of factors. Whereas we included tumours at different clinical stages (with a predominance of advanced-stage tumours), from different sites of the oral cavity and mainly classified as moderately differentiated tumours, the

previous study included only early-stage tumours from the oral tongue, mainly with good or poor cellular differentiation.

The reason for the worse outcome in patients with tumours with a higher proportion of stroma is still unclear, but it is probably related to the interactions between tumour cells and cancer-associated fibroblasts (CAFs). The biological effects 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 fibre maturation or degradation by proteases such as matrix metalloproteinases are directly associated with tumour cell proliferation, survival, migration, and invasion, and also affect angiogenesis and immune function in the tumour microenvironment.[21] The fibrotic stroma also prevents drug delivery into the tumour mass, facilitating chemoresistance.[22] Together, these features could explain why a tumour with higher stromal content is prone to having a highly aggressive phenotype, influencing patient outcome.

Tumour budding is a histological process whereby cells at the tumour front detach from the tumour mass, as single cells or as clusters of up to five tumour cells, and invade the adjacent normal tissue. It has been indicated to be a reliable and reproducible predictor of clinical outcome for colorectal tumours.[23] For oral cancer, two recent systematic reviews with meta-analyses verified the value of tumour budding in OSCC, and demonstrated that high bud activity is frequently associated with parameters that worsen the prognosis, including lymph node metastasis; more importantly, five or more buds per field significantly predicted a shortened time to disease relapse and an overall decrease in survival.[10,11] In our analysis, previous data that revealed tumour budding as a prognostic factor for OSCC were confirmed. Indeed, patients with fewer than five buds had better cancer-specific survival

than those with five or more buds in both univariate and multivariate analyses. The number of buds was not associated with disease-free survival; however, in early-stage tumours, a clear tendency for association was detected ($P = 0.07$), yielding an HR of 1.98 with a 95% CI of 0.92–4.27. Interestingly, tumour budding was combined with depth of invasion to form the tumour budding and depth of invasion risk model for OSCC, which has been associated with a high risk of locoregional recurrence and shortened survival for patients with 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 of which are key features of cells in EMT, were frequently observed in tumour buds of OSCCs.[28–31] Moreover, cells in the buds showed a specific gene expression signature, with activation of the transforming growth factor-β pathway and overexpression of EMT transcription factors, including ZEB1 and PRRX1.[30] High expression levels of other EMT transcription factors, such as SNAIL and TWIST, have also been reported in OSCC buds.[31] Another important avenue to be explored is the association of tumour budding and cell stemness. A recent study revealed a significant correlation between CD44 overexpression and high tumour budding activity at the invasive margin of OSCCs.[32] Indeed, the connections among budding, EMT and cancer stem cells would be interesting to define, warranting further studies of stem cell markers in tumour buds.

The combination of TSR and tumour budding resulted in a risk model with a clear discriminatory ability to indicate the 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. It is of note that this

combination model includes both a cancer-related feature (tumour budding) and a stroma-related feature (TSR). The prognostic significance was not observed in some of our subgroup analyses, 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 our understanding of the biological behaviour of OSCC.

In summary, we show, on the basis of a representative sample of 254 primary OSCCs, that the TSR and tumour budding, assessed on regular H&E-stained slides, are reliable markers of OSCC outcome. The combination of these features improved the discrimination between patients with a low risk and a high risk of having a poor prognosis, mainly in those with early-stage tumours. These 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.

Conflicts of interest

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

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Author contributions

M. R. Dourado, K. Y. M. Miwa, G. B. Hamada and R. D. Coletta carried out data acquisition and analysis. L. M. R. Paranaíba and I. Sawazaki-Calone contributed clinical samples. C. B. Domingueti, C. E. de Oliveira, E. C. B. Furlan and B. C. Longo collected clinical information. A. Almangush and T. Salo participated in the design of the study. R. D. Coletta drafted the manuscript. All authors 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 *et al*. eds. *AJCC cancer staging manual*, 8th edn. New York, NY: Springer, 2017; 79–94.
7. Wu J, Liang C, Chen M, 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 eds. *World Health Organization classification of head and neck tumours*, 4th edn. Lyon: IARC Press, 2017; 109–111.
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.
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.

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 1. Representative examples of the tumour–stroma ratio (TSR). **A,** A tumour classified as stroma-poor (TSR of <50%). **B,** A tumour classified as stroma-rich (TSR of ≥50%).

Figure 2. Tumour budding in oral squamous cell carcinomas, with arrows to indicate budding foci. **A,** A tumour with low budding activity (fewer than five tumour buds per field). **B,** A tumour with high budding activity (more than five tumour buds per field.

Figure 3. A model associating the tumour–stroma ratio (TSR) and tumour budding. **A,** Low risk: a TSR of <50% and no tumour buds. **B,** Intermediate risk: a TSR of ≥50% but no tumour buds. **C,** High risk: a TSR of ≥50% and five or more tumour buds per field. Arrows indicate examples of tumour buds.

Figure 4. Kaplan–Meier curves for cancer-specific survival (**A**) and disease-free survival (**B**) of patients with oral squamous cell carcinoma based on the tumour–stroma ratio/tumour budding model.

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

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

Supporting information

Table S1. Clinicopathological features of patients with oral squamous cell carcinoma included in this study.

Supplementary Table 1. Clinicopathological features of patients with oral squamous cell carcinoma included in this study.

	N	%
Age (years)		
Mean ± SD: 60.5 ± 12.1		
Range: 17-88		
Gender		
Male	188	74.0
Female	66	26.0
Smoking habit		
No	37	14.6
Yes	172	67.7
Missing data	45	17.7
Drinking habit		
No	81	31.9
Yes	113	44.5
Missing data	60	23.6
Clinical stage		
I	37	14.6
II	66	26.0
III	55	21.7
IV	89	35.0
Missing data	7	2.7
Location		
Tongue	170	66.9
Floor of month	67	26.4
Retromolar area	9	3.5
Palate	5	2.0
Gingiva	3	1.2
Histological grade		
Well-differentiated	72	28.3
Moderately-differentiated	154	60.6
Poorly-differentiated	28	11.1
Treatment		
Surgery	75	29.5
Surgery + Radiotherapy	93	36.6
Surgery + Radiotherapy + Chemotherapy	80	31.5
Missing data	6	2.4
Margen status		
≥ 5 mm	173	68.1
< 5 mm	51	20.1
Missing data	30	11.8
Local recurrence		
No	202	79.5
Yes	50	19.7
Missing data	2	0.8
Regional (cervical) recurrence		
No	239	94.1
Yes	13	5.1
Missing data	2	0.8
Distant recurrence		

No	242	95.3
Yes	10	3.9
Missing data	2	0.8
Status		
Alive	171	67.3
Dead	83	32.7

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

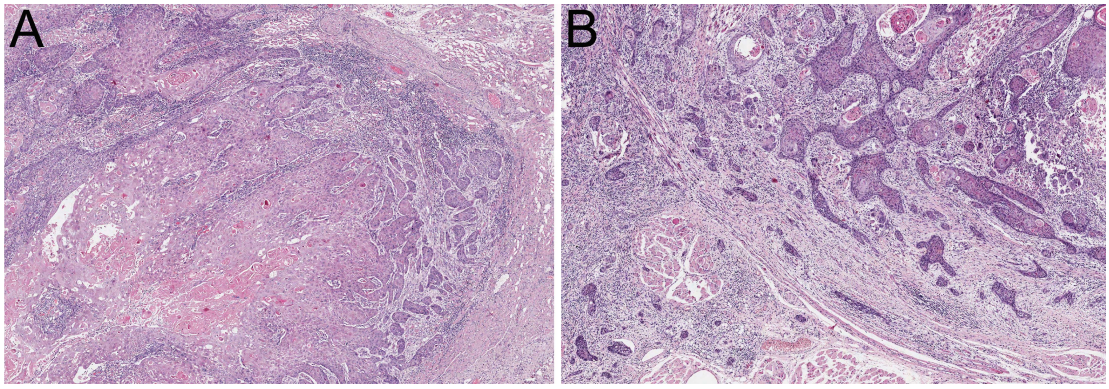
Table 3. Univariate analysis for cancer-specific survival and disease-free survival of patients with the oral squamous cell carcinoma.

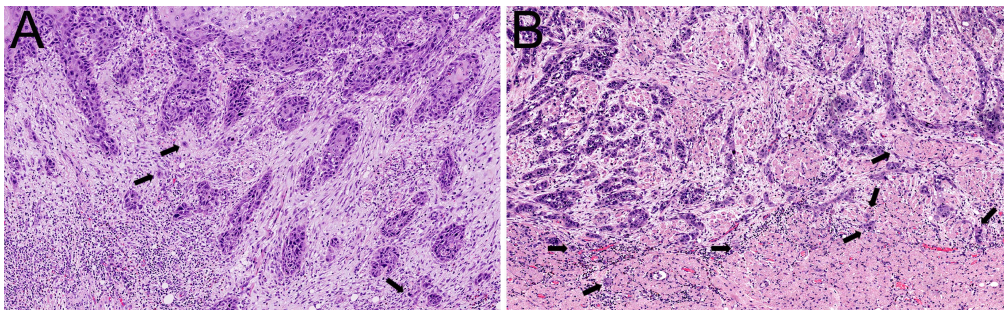
	Cancer-specific survival			Disease-free survival		
	% in 5 years	HR (95% CI)	p value	% in 5 years	HR (95% CI)	p value
Age (years)						
≤ 61 years	60.7	1		64.1	1	
> 61 years	56.4	1.35 (0.87-2.08)	0.18	64.2	1.03 (0.55-1.22)	0.79
Gender						
Male	57.7	1		64.5	1	
Female	61.5	0.86 (0.53-1.41)	0.54	68.0	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

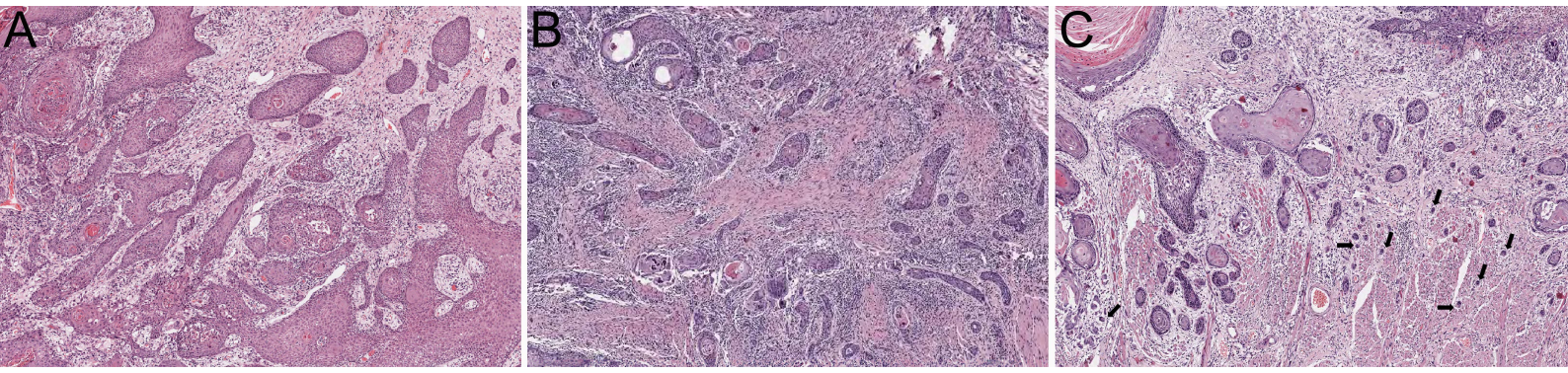
TSR/tumor budding model						
Low risk	76.6	1		79.5		
Intermediate risk	62.0	1.75 (0.99-3.07)	0.05	63.8	1.77 (0.98-3.19)	0.06
High risk	29.0	4.29 (2.36-7.79)	<0.0001	40.1	2.95 (1.45-5.99)	0.003

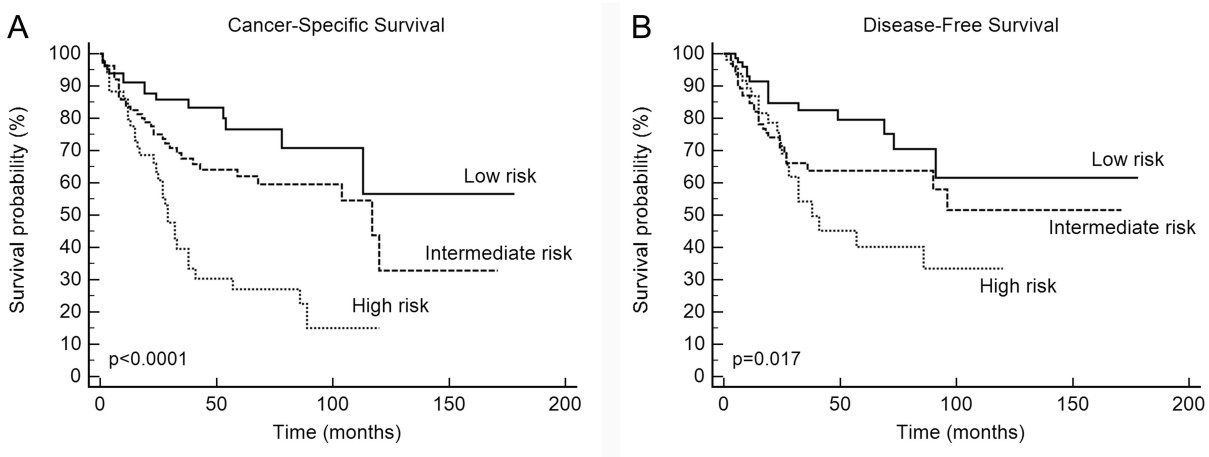
Table 4. Multivariate analysis of cancer-specific survival and disease-free survival for the 254 patients with oral squamous cell carcinoma.

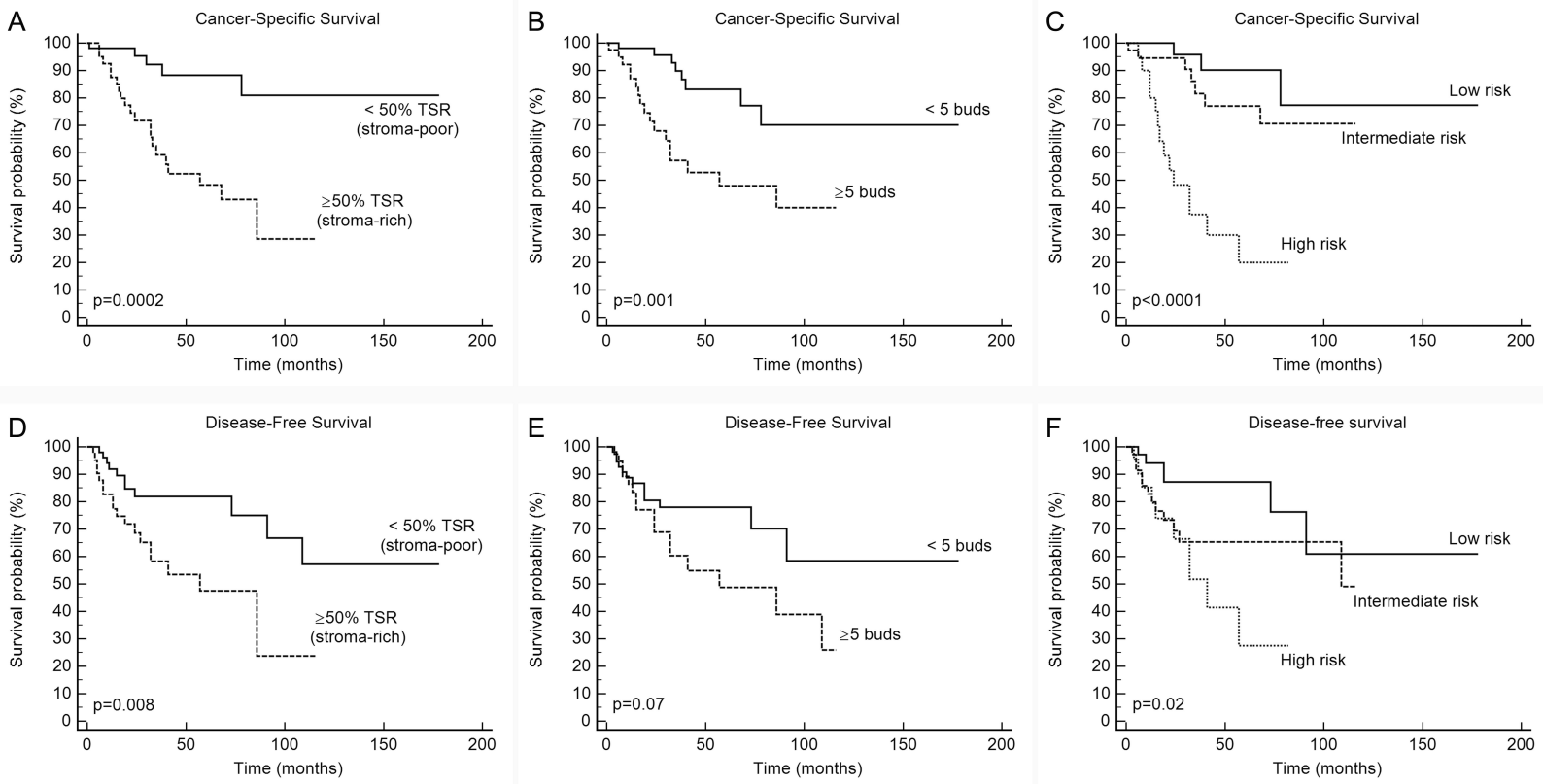
	Cancer-specific survival		Disease-free survival	
	HR (95% CI)	p value	HR (95% CI)	p value
Model 1				
Tumor-stroma ratio (TSR)				
< 50% (stroma-poor)	1		1	
≥ 50% (stroma-rich)	3.58 (2.05-6.27)	<0.0001	2.05 (1.23-3.44)	0.006
Tumor budding				
< 5 buds	1			
≥ 5 buds	1.47 (1.05-2.05)	0.02		
Model 2				
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

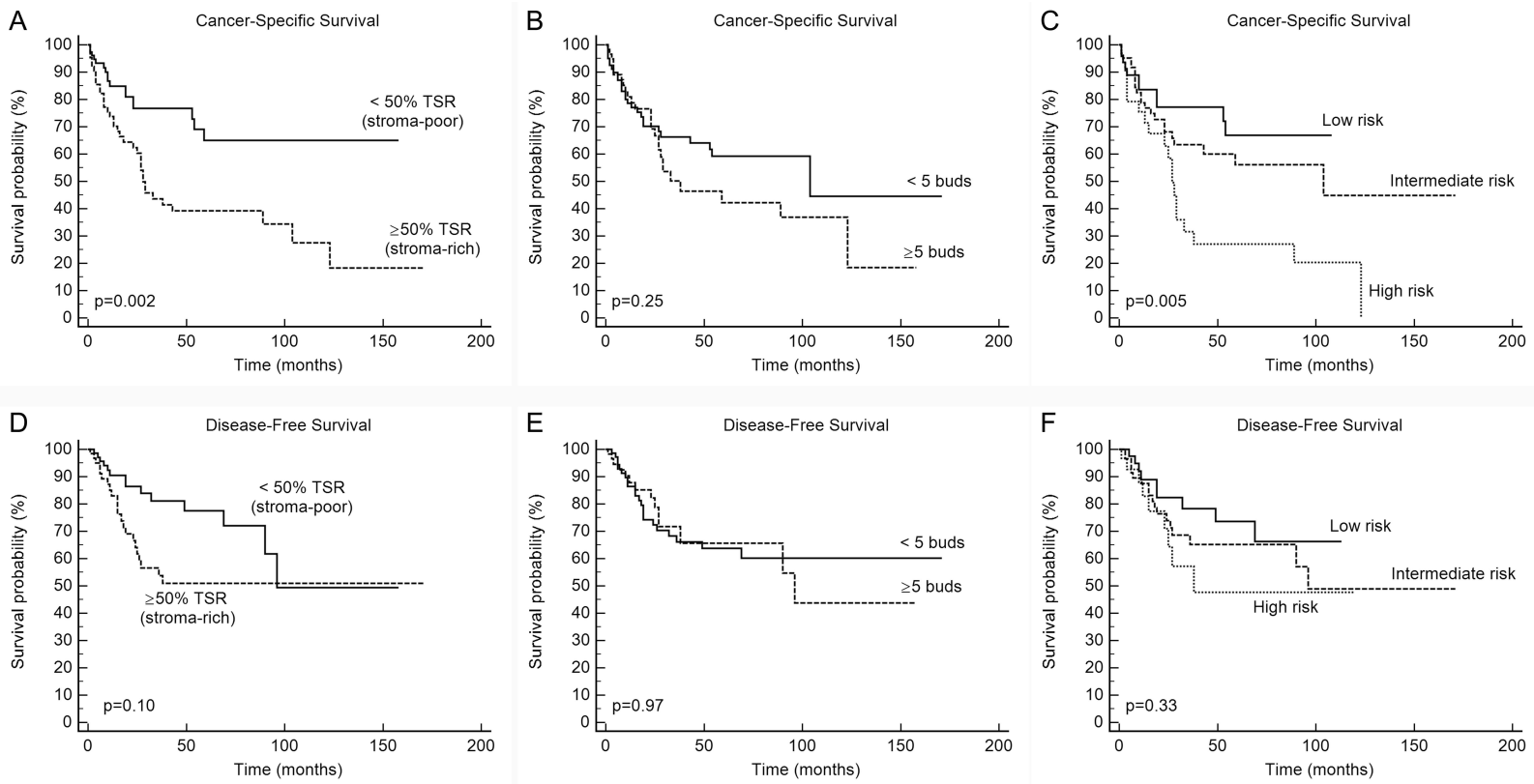

A
B


A
B


A
B
C


A
Cancer-Specific Survival
Survival probability (%)
100
90
80
70
60
50
40
30
20
10
0
Low risk
Intermediate risk
High risk
p<0.0001
0
50
100
150
200
Time (months)
B
Disease-Free Survival
Survival probability (%)
Low risk
Intermediate risk
High risk
p=0.017
Time (months)


A
Cancer-Specific Survival
Survival probability (%)
Time (months)
< 50% TSR
(stroma-poor)
≥50% TSR
(stroma-rich)
p=0.0002
B
Cancer-Specific Survival
Survival probability (%)
Time (months)
< 5 buds
≥5 buds
p=0.001
C
Cancer-Specific Survival
Survival probability (%)
Time (months)
Low risk
Intermediate risk
High risk
p<0.0001
D
Disease-Free Survival
Survival probability (%)
Time (months)
< 50% TSR
(stroma-poor)
≥50% TSR
(stroma-rich)
p=0.008
E
Disease-Free Survival
Survival probability (%)
Time (months)
< 5 buds
≥5 buds
p=0.07
F
Disease-free survival
Survival probability (%)
Time (months)
Low risk
Intermediate risk
High risk
p=0.02


A
Cancer-Specific Survival
Survival probability (%)
< 50% TSR
(stroma-poor)
≥50% TSR
(stroma-rich)
p=0.002
Time (months)
B
Cancer-Specific Survival
Survival probability (%)
< 5 buds
≥5 buds
p=0.25
Time (months)
C
Cancer-Specific Survival
Survival probability (%)
Low risk
Intermediate risk
High risk
p=0.005
Time (months)
D
Disease-Free Survival
Survival probability (%)
< 50% TSR
(stroma-poor)
≥50% TSR
(stroma-rich)
p=0.10
Time (months)
E
Disease-Free Survival
Survival probability (%)
< 5 buds
≥5 buds
p=0.97
Time (months)
F
Disease-Free Survival
Survival probability (%)
Low risk
Intermediate risk
High risk
p=0.33
Time (months)