Published in final edited form as:

J Oral Pathol Med. 2011 August ; 40(7): 545–551. doi:10.1111/j.1600-0714.2011.01041.x.

Tumor budding correlates with poor prognosis and epithelial-mesenchymal transition in tongue squamous cell carcinoma

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Abstract

BACKGROUND—Tumor budding is a readily detectable histopathological feature and has been recognized as an adverse prognostic factor in several human cancers. However, the prognostic value of tumor budding in tongue squamous cell carcinoma (TSCC) has not been reported. The purpose of this study is to assess the correlation of tumor budding with the clinicopathologic features, and the known molecular biomarkers (E-cadherin and Vimentin), as well as to evaluate its prognostic significance for TSCC.

METHODS—Archival clinical samples of 230 patients with TSCC were examined for tumor budding. Immunohistochemistry analyses were performed to examine the expression of E-cadherin and Vimentin. Statistical analyses were carried out to assess the correlation of tumor budding with clinicopathologic parameters and patient survival. The potential association between tumor budding and alterations of E-cadherin and Vimentin expression was also assessed.

RESULTS—Of the 230 TSCC cases examined, tumor budding was observed in 165 cases (71.7%), with a mean tumor bud count of 7.5 (range from 1 to 48 buds). High-intensity budding (≥ 5 tumor buds) was observed in 111 cases (48.3%). Statistical analysis revealed that tumor budding was associated with tumor size ($P < 0.05$), differentiation ($P < 0.05$), clinical stage

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Conflict of interest statement: None declared.

($P<0.05$), lymph node metastasis ($P<0.01$), and correlated with reduced overall survival. In addition, significant associations were observed among tumor budding and the deregulation of E-cadherin ($P<0.001$) and Vimentin ($P<0.001$).

CONCLUSIONS—Tumor budding, which associates with epithelial-mesenchymal transition, is a frequent event and appears to be an independent prognostic factor in TSCC.

Keywords

Tumor budding; tongue squamous cell carcinoma; prognostic factor; invasive tumor front; epithelial-mesenchymal transition

Introduction

Tongue squamous cell carcinoma (TSCC) is one of the most common cancers within the oral cavity. According to the American Cancer Society, an estimated 10,990 new cases of tongue cancer are expected each year, accounting for approximately 30% of all oral cavity and pharynx cancers [1]. TSCC is significantly more aggressive than other forms of oral cancer, with a propensity for rapid local invasion and spread [2], and a high recurrence rate [3]. The major causes of TSCC-related deaths are local/regional relapse and metastasis. It has been reported that 40% of all TSCC patients have neck metastasis at the time of diagnosis and 20–40% of patients with early-stage TSCC (T1/T2N0) showed occult nodal metastasis [4–7]. These facts point to the immediate need for new diagnostic/prognostic strategies and additional biomarkers will improve the clinical decision-making and the management of patients with TSCC.

It has been suggested that cancer cells located in the invasive tumor front (ITF) are more aggressive in terms of metastatic potential [8]. For oral cancer, a histopathological grading system of the ITF was firstly introduced by Bryne et al in 1992 [9, 10]. This approach have been utilized in a number of studies for evaluating invasive tumor margins, and its values as a prognostic marker for oral cancer patients were well recognized [11–15]. Based on this approach, Brandwein-Gensler et al [16, 17] developed a novel histologic risk assessment system to evaluate the aggressiveness of head and neck squamous cell carcinoma. Recently, a number of unique features of cancer cells located in ITF have been defined, including a cell morphological switch characterized by the epithelial-mesenchymal transition (EMT), which is associated with increased cell motility and invasiveness. Differential expression of several crucial EMT-related genes, including E-cadherin/ β -catenin [18], Vimentin [19], claudins [20], laminin 5 and γ 2 chains [21–22] in oral cancer cells located in the ITF have further confirmed the existence of EMT cells in ITF.

Tumor budding is defined as the presence of either isolated single cells or small cell clusters (up to four) scattered in the stroma ahead of the ITF [23]. Budding represents two malignant features: cellular discohesion and active invasion. The presence of tumor buds has been considered to be characteristic of aggressive cancer. Tumor budding has previously been demonstrated as a valuable prognostic marker for colorectal cancer patients [24–28]. More recently, it has also been reported as a significant prognostic marker for patients with esophageal cancers [29–31], lung and ampullary adenocarcinoma [32, 33] and laryngeal cancers [34]. An attractive feature of a tumor budding-based assessment is that, as it is based on the examination of the H&E-stained specimens, it can be performed easily as part of routine histopathological examination and the results are fairly reproducible [24]. However, to our knowledge, the effectiveness of this relatively straight-forward histopathological assessment and its prognostic value for TSCC have not been investigated so far. The aims of this study are to investigate the possible association of tumor budding and clinicopathologic

features in TSCC, as well as to determine its prognostic significance. In addition, the EMT status of the cancer cells in the tumor buds will also be investigated.

Material and methods

Patients and tissue specimens

Archived tissue samples from 230 cases of TSCC from the Department of Oral Pathology, Hospital of Stomatology and the Second Affiliated Hospital, Sun Yat-sen University were utilized in this study. Clinical characterization of the TSSC patients is summarized in supplemental Table 1. All patients received curative surgery (resection of the primary tumor and radical neck dissection) between May 1996 and June 2005. None of the patients received any form of adjuvant therapy prior to their surgery. The tumor extent was classified according to the TNM system by UICC, and the tumor grade was classified according to the WHO classification of histological differentiation. Survival was calculated based on the date of surgery and the date of the last follow-up (or death). Among 230 cases of TSCC that we examined, follow-up results were available on 133 cases (57.8%). Median duration of follow-up was 65 months (range 3–120). This study was approved by the ethical committee of Sun Yat-Sen University.

Histo- and Immunohistopathological analyses

Paraffin sections (4 μ m) were routinely prepared and stained with H&E. The immunohistochemical assay was performed as previously described [18, 19]. The slides were immuno-stained with anti-E-cadherin antibody (1:100, BD, USA), anti-Vimentin antibody (1:100, Cell signaling, USA), and pan-cytokeratin antibody (1:100, Cell signaling, USA), respectively. 3,3'-diaminobenzidine (DAB, Sigma-Aldrich) was used for visualization of E-cadherin and Vimentin staining. For pan-cytokeratin staining, AEC chromogen was used for visualization. Negative controls were included by substituting non-immune serum for primary antibodies. All sections were assessed by 2 independent observers who were unaware of the clinical data. Tumor budding was defined as the presence of isolated single tumor cells or small clusters (< 5 cancer cells) ahead of the invasive front as previously described (Figure 1A and B) [23]. Tumor specimens were initially scanned at the $\times 4$ objective lens (and $\times 10$ ocular) to select the areas with the highest density of budding. Tumor budding in the selected areas was then counted using the $\times 20$ objective lens, and the highest count per slide was used as the number of budding. The intensity of tumor budding (budding index) was arbitrary classified as low (< 5 buds/field) or high ($\geq$ 5 buds/field). To test the reproducibility of the tumor budding assessment (for both intra- and inter-observer), 50 randomly selected cases were subjected for a second review by the same investigators one month after the initial review. Excellent agreement was obtained between the initial and the second reviews by both observers ($\kappa=0.880$ and 0.838 , respectively, based on a hierarchical kappa test [35]). Similarly, agreement between two observers was also achieved ($\kappa=0.717$), demonstrating good inter-observer reproducibility.

The expression of E-cadherin in ITF, budding and central/superficial tumor parts was compared with that of adjacent non-cancerous epithelium, which served as an internal positive control in the same cases. When the positivity of E-cadherin was more than 90%, the case was designated as having preserved expression; otherwise the case was designated as having reduced expression, as described previously [36]. For the Vimentin expression, no detectable or < 10% positive staining of tumor cells was served as negative, while $\geq$ 10% positive staining of tumor cells was defined as positive [37].

Statistical analysis

Spearman Correlation Coefficient was used to assess correlations among the gene expression and clinical and histopathological parameters. Kaplan-Meier plots were constructed to present the survival outcomes. Cox regression was used for both univariate and multivariate analysis. For multivariate analysis, tumor size, pathological T-stage (pT), pathological N-stage (pN), clinical stages and budding index were considered as co-variables. For all statistical analyses, $P < 0.05$ was considered statistically significant.

Results

Intensity of tumor budding and its correlation with clinicopathological parameters

H&E staining was performed on 230 TSCC cases. As illustrated in Figure 1, tumor buds can be readily identified based on standard H&E staining. A second staining for pan-cytokeratin was also performed on 30 of the TSCC cases (13%) to assist the identification of tumor buds (Supplementary Figure 1). The tumor budding counts ranged from 0 to 48 buds (mean 7, median 4). Among 230 TSCC cases that we examined, 111 cases (48.36%) revealed high-intensity tumor budding (≥ 5 tumor buds) ahead of the invasive front; 119 cases (51.74%) revealed low-intensity budding (< 5 tumor buds). Of the 119 low-intensity budding cases, no tumor bud was observed in 65 cases.

Correlations were tested among tumor budding and clinical parameters of the TSCC cases (Table 1). As expected, correlations were observed among tumor size, pT, pN, and Clinical stage. Strong correlations were observed between the intensity of tumor budding and tumor size ($P < 0.05$), cell differentiation ($P < 0.05$), clinic stage ($P < 0.05$), and lymph node metastasis ($P < 0.01$). Interestingly, correlation between sex and tumor size (and pT) was also observed ($p < 0.05$).

The prognostic value of tumor budding for TSCC patients

Among 230 cases of TSCC that we examined, follow-up results were available for 133 cases (57.83%). Median duration of follow-up was 65 months (ranges from 3 to 120 months). Among these 133 cases, 74 cases (55.6%) were low-intensity or had no detectible tumor bud, and 59 cases (44.4%) had high-intensity tumor budding. As illustrated in Figure 2, a striking difference in 5-year survival rate was observed between the high-intensity budding group (79.7%) and the low-intensity or no budding group (49.2%). Both univariate and multivariate analysis demonstrated the adverse effect of high-density budding for prognosis (Table 2). Based on univariate analysis, the effects of tumor size, pT, pN and clinical stages on prognosis were also observed. Based on multivariate analysis pT was also identified as an independent prognostic factor.

High-intensity tumor budding is associated with reduced E-cadherin expression and enhanced Vimentin expression in TSCC

Distinct membrane staining of E-cadherin protein was detected in the adjacent non-cancerous epithelium. The expression pattern of E-cadherin in the center/superficial tumor parts was similar to that in the adjacent non-cancerous epithelium (Figure 3A, B). In contrast, an intensive reduction in membranous E-cadherin expression was observed in the ITF and tumor budding, when compared to that in the center/superficial tumor parts (64% vs 90%, $P < 0.05$). In particular, at the tumor budding site ahead of ITF, a loss of E-cadherin expression was frequently observed (Figure 3C). Cytoplasmic Vimentin expression was observed in the stromal cells of the adjacent non-cancerous tongue tissues, but not in the epithelium (Figure 3D). This is in agreement with the previous observation made in OSCC [19]. No staining or weak staining ($< 10\%$ of tumor cells) was found in the center/superficial tumor parts (Figure 3E). Increased expression of Vimentin was detected in the ITF and

tumor budding (40% vs 76%, $P<0.05$) (Figure 3F). Statistical analysis revealed that the high-intensity tumor budding is associated with reduced E-cadherin expression ($P<0.001$) and enhanced Vimentin expression ($P<0.001$) (Table 3).

Discussion

The molecular heterogeneities of tumor cells reside in different locations of solid tumors (e.g., center/superficial portions, ITF) are well recognized. Accumulating evidence now supports the idea that characteristics of the ITF provide the most useful prognostic information [8]. Accordingly, a malignancy grading of ITF (IFG), or the deep invasive margins of oral cancer, was firstly developed in 1992 [9, 10, 38]. This grading system is based on semiquantification of the following histological parameters of the ITF: 1) degree of keratinization; 2) nuclear polymorphism; 3) pattern of invasion; and 4) infiltration of lymphocytes. Based on this grading system, the prognostic value of ITF has been confirmed by multiple studies [12, 13, 18, 19]. Moreover, Brandwein-Gensler et al [16, 17] combined the pattern of invasion and lymphocytic host response with perineural invasion and developed a novel risk model to assess the aggressiveness of head and neck cancer. This model demonstrated a significant predictive performance. Intriguingly, tumor budding, which usually resides ahead the invasive front, has recently been suggested as a potential index of aggressiveness and poor prognosis for a number of cancer types [23–34]. However, little is known about the prognostic value of tumor budding in patients with TSCC. In this study, we found that the 5-year survival was significantly reduced in patients exhibiting high-intensity tumor budding compared to patients with low-intensity budding. Our observations suggested that high-intensity budding is an adverse prognostic factor in patients with TSCC. This is in agreement with studies on other solid tumors (e.g., larynx and esophageal cancer, colorectal cancer) showing strong associations of tumor budding with a poor prognosis [27, 28, 33,34].

An important advantage of tumor budding-based index as prognostic indicator is the simplicity and reproducible measurement of the budding. It is readily adaptable to routine H&E staining based histopathological examination without the need for additional cost-demanding techniques. This feature is clinically important and may have therapeutic benefits for the patients with TSCC. In this study, in accordance with previous reports in other cancers [23, 24, 39], a good reproducibility for tumor budding evaluation was achieved based on the intra- and inter-observer agreement studies ($\kappa=0.880$ or 0.818 and 0.717 , respectively). In contrast, the IFG system is more susceptible to intra- and inter-observer disagreement. Several independent groups reported that the inter-observer reproducibility of the IFG system is clinically unacceptable, with a value of κ ranging from 0.193 – 0.57 [40–42]. In Brandwein-Gensler's system, the reproducibility seems much better than the original IFG system ($\kappa=0.67$). However, it should be noted that a direct comparison between the budding index and the IFG-based grading system or Brandwein-Gensler's system may not be justifiable. Based on the IFG criteria proposed by Bryne et al [9,10], the tumor budding probably been classified into grade 4 (the pattern of invasion is defined as marked and wide-spread cellular dissociation in small groups and / or in single cells [$n<15$]). The tumor cells in these small groups showed a high tendency to metastasize to regional lymph nodes compared with those that invade in pushing fronts (IFG, score 1) or in solid cords, bands and / or strands (IFG, score 2 and 3). Same observations were also reported by Brandwein-Gensler's et al [16, 17] in two consecutive studies in which they found that the worst pattern of invasion 4 (tumor budding can be classified into this group) and 5 significantly associated with lymph node metastasis and overall survival. In agreement with these studies, positive associations of high intensity tumor budding with lymph node metastasis and poor prognosis were reported in these studies. In addition, we observed that it is difficult to identify the budding tumor cells in a subset of poorly differentiated TSCC

cases because the tumor budding at the ITF are surrounded by many lymphocytes, cancer associated fibroblasts and other stromal cells. To clearly define the budding status in such cases, we performed additional staining for pan-cytokeratin. This, together with the E-cadherin staining improved the identification of budding tumor cells (as shown in Figure 3C and Supplementary Figure 1 and Supplementary Figure 2). However, it is possible that there are still some unidentified tumor budding cells at the invasive front. For example, some isolated individual cells with multiple nuclei and spindle cells that can't be distinguished from fibroblasts and/or endothelial cells were not counted. Nevertheless, in agreement with previous reports on other cancer types [23, 24, 39], a good reproducibility for tumor budding evaluation was achieved based on the intra- and inter-observer agreement studies ($\kappa=0.880$ or 0.818 and 0.717, respectively).

Microscopically, tumor cells in ITF and tumor buds exhibit distinct morphological features, including de-differentiation and loss of cell-cell adhesion. This fibroblast-like morphological appearance is characteristic of cells undergoing epithelial to mesenchymal transition (EMT), characterized at the molecular level by loss of E-cadherin (a key component of the adherens junctions) and the increase in expression of Vimentin (a ubiquitous mesenchymal intermediate filament). Our results confirmed that the expression of E-cadherin is significantly reduced in cells located in ITF (64%) and cells located in tumor buds (90%), when compared with those located in the central/superficial portions of the tumor samples. The observed reduction in E-cadherin in ITF and budding cells is accompanied by an increase in Vimentin expression. Our observation is consistent with previous observations of reduced E-cadherin expression in ITF of OSCC [18, 19]. Taken together, these findings demonstrate that cancer cells located in the tumor buds underwent EMT, which is associated with enhanced metastatic potential. Additional studies may be warranted to further investigate the molecular events associated with tumor cells that reside in the ITF/budding areas, which will lead to a better understanding of TSCC invasion and metastasis, as well as potential targeted therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by National Natural Science Grant of China (81072223 and 30700952), and NIH PHS grants (CA135992, CA139596, DE014847). We also thank for Mrs. Weixin Cai and Huibin Ma for their assistance with data analysis and Ms. Katherine Long for her editorial assistance.

REFERENCES

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60:277–300. [PubMed: 20610543]
2. Lydiatt DD, Robbins KT, Byers RM, Wolf PF. Treatment of stage I and II oral tongue cancer. *Head Neck.* 1993; 15:308–312. [PubMed: 8360052]
3. Yuen AP, Lam KY, Chan AC, et al. Clinicopathological analysis of elective neck dissection for N0 neck of early oral tongue carcinoma. *Am J Surg.* 1999; 177:90–92. [PubMed: 10037317]
4. Layland MK, Sessions DG, Lenox J. The influence of lymph node metastasis in the treatment of squamous cell carcinoma of the oral cavity, oropharynx, larynx, and hypopharynx: N0 versus N+ Laryngoscope. 2005; 115:629–639. [PubMed: 15805872]
5. Teichgraber JF, Clairmont AA. The incidence of occult metastases for cancer of the oral tongue and floor of the mouth: treatment rationale. *Head Neck Surg.* 1984; 7:15–21. [PubMed: 6490380]

6. Cunningham MJ, Johnson JT, Myers EN, Schramm VL Jr, Thearle PB. Cervical lymph node metastasis after local excision of early squamous cell carcinoma of the oral cavity. *Am J Surg.* 1986; 152:361–366. [PubMed: 3766864]
7. Ho CM, Lam KH, Wei WI, Lau SK, Lam LK. Occult lymph node metastasis in small oral tongue cancers. *Head Neck.* 1992; 14:359–363. [PubMed: 1399568]
8. Bånkfalvi A, Piffkő J. Prognostic and predictive factors in oral cancer: the role of the invasive tumour front. *J Oral Pathol Med.* 2000; 29:291–298. [PubMed: 10947243]
9. Bryne M, Koppang HS, Lilleng R, Kjaerheim A. Malignancy grading of the deep invasive margins of oral squamous cell carcinomas has high prognostic value. *J Pathol.* 1992; 166:375–381. [PubMed: 1517891]
10. Bryne M. Is the invasive front of an oral carcinoma the most important area for prognostication? *Oral Dis.* 1998; 4:70–77. [PubMed: 9680893]
11. Kawashiri S, Tanaka A, Noguchi N, et al. Significance of stromal desmoplasia and myofibroblast appearance at the invasive front in squamous cell carcinoma of the oral cavity. *Head Neck.* 2009; 31:1346–1353. [PubMed: 19373786]
12. Kaneoya A, Hasegawa S, Tanaka Y, Omura K. Quantitative analysis of invasive front in tongue cancer using ultrasonography. *J Oral Maxillofac Surg.* 2009; 67:40–46. [PubMed: 19070746]
13. Kurokawa H, Zhang M, Matsumoto S, et al. The high prognostic value of the histologic grade at the deep invasive front of tongue squamous cell carcinoma. *J Oral Pathol Med.* 2005; 34:329–333. [PubMed: 15946179]
14. Po Wing Yuen A, Lam KY, Lam LK, et al. Prognostic factors of clinically stage I and II oral tongue carcinoma-A comparative study of stage, thickness, shape, growth pattern, invasive front malignancy grading, Martinez-Gimeno score, and pathologic features. *Head Neck.* 2002; 24:513–520. [PubMed: 12112547]
15. Goto M, Tsukamoto T, Inada K, et al. Loss of p21WAF1/CIP1 expression in invasive fronts of oral tongue squamous cell carcinomas is correlated with tumor progression and poor prognosis. *Oncol Rep.* 2005; 14:837–846. [PubMed: 16142340]
16. Brandwein-Gensler M, Teixeira MS, Lewis CM, et al. Oral squamous cell carcinoma: histologic risk assessment, but not margin status, is strongly predictive of local disease-free and overall survival. *Am J Surg Pathol.* 2005; 29:167–178. [PubMed: 15644773]
17. Brandwein-Gensler M, Smith RV, Wang B, et al. Validation of the histologic risk model in a new cohort of patients with head and neck squamous cell carcinoma. *Am J Surg Pathol.* 2010; 34:676–688. [PubMed: 20414102]
18. Wang X, Zhang J, Fan M, et al. The expression of E-cadherin at the invasive tumor front of oral squamous cell carcinoma: immunohistochemical and RT-PCR analysis with clinicopathological correlation. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2009; 107:547–554. [PubMed: 19327640]
19. Liu LK, Jiang XY, Zhou XX, Wang DM, Song XL, Jiang HB. Upregulation of vimentin and aberrant expression of E-cadherin/beta-catenin complex in oral squamous cell carcinomas: correlation with the clinicopathological features and patient outcome. *Mod Pathol.* 2010; 23:213–224. [PubMed: 19915524]
20. Bello IO, Vilen ST, Niinimaa A, Kantola S, Soini Y, Salo T. Expression of claudins 1, 4, 5, and 7 and occludin, and relationship with prognosis in squamous cell carcinoma of the tongue. *Hum Pathol.* 2008; 39:1212–1220. [PubMed: 18547615]
21. Akimoto S, Nakanishi Y, Sakamoto M, Kanai Y, Hirohashi S. Laminin 5 beta3 and gamma2 chains are frequently coexpressed in cancer cells. *Pathol Int.* 2004; 54:688–692. [PubMed: 15363037]
22. Franz M, Richter P, Geyer C, et al. Mesenchymal cells contribute to the synthesis and deposition of the laminin-5 gamma2 chain in the invasive front of oral squamous cell carcinoma. *J Mol Histol.* 2007; 38:183–190. [PubMed: 17390227]
23. Ueno H, Murphy J, Jass JR, Mochizuki H, Talbot IC. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology.* 2002; 40:127–132. [PubMed: 11952856]

24. Hayes BD, Maguire A, Conlon N, Gibbons D, Wang LM, Sheahan K. Reproducibility of the rapid bud count method for assessment of tumor budding in stage II colorectal cancer. *Am J Surg Pathol*. 2010; 34:746–748. [PubMed: 20414097]
25. Prall F, Ostwald C, Linnebacher M. Tubular invasion and the morphogenesis of tumor budding in colorectal carcinoma. *Hum Pathol*. 2009; 40:1510–1512. [PubMed: 19751876]
26. Wang LM, Kevans D, Mulcahy H, et al. Tumor budding is a strong and reproducible prognostic marker in T3N0 colorectal cancer. *Am J Surg Pathol*. 2009; 33:134–141. [PubMed: 18971777]
27. Kanazawa H, Mitomi H, Nishiyama Y, et al. Tumour budding at invasive margins and outcome in colorectal cancer. *Colorectal Dis*. 2008; 10:41–47. [PubMed: 18078460]
28. Prall F. Tumour budding in colorectal carcinoma. *Histopathology*. 2007; 50:151–162. [PubMed: 17204028]
29. Koike M, Kodera Y, Itoh Y, et al. Multivariate analysis of the pathologic features of esophageal squamous cell cancer: tumor budding is a significant independent prognostic factor. *Ann Surg Oncol*. 2008; 15:1977–1982. [PubMed: 18408975]
30. Miyata H, Yoshioka A, Yamasaki M, et al. Tumor budding in tumor invasive front predicts prognosis and survival of patients with esophageal squamous cell carcinomas receiving neoadjuvant chemotherapy. *Cancer*. 2009; 115:3324–3334. [PubMed: 19452547]
31. Brown M, Sillah K, Griffiths EA, et al. Tumour budding and a low host inflammatory response are associated with a poor prognosis in oesophageal and gastro-oesophageal junction cancers. *Histopathology*. 2010; 56:893–899. [PubMed: 20636792]
32. Yamaguchi Y, Ishii G, Kojima M, et al. Histopathologic Features of the Tumor Budding in Adenocarcinoma of the Lung: Tumor Budding As an Index to Predict the Potential Aggressiveness. *J Thorac Oncol*. 2010; 5:1361–1368. [PubMed: 20631633]
33. Ohike N, Coban I, Kim GE, et al. Tumor budding as a strong prognostic indicator in invasive ampullary adenocarcinomas. *Am J Surg Pathol*. 2010; 34:1417–1424. [PubMed: 20871215]
34. Sarioglu S, Acara C, Akman FC, et al. Tumor budding as a prognostic marker in laryngeal carcinoma. *Pathol Res Pract*. 2010; 206:88–92. [PubMed: 19959297]
35. Landis JR, Koch GG. An application of hierarchical kappa-type statistics in the assessment of majority agreement among multiple observers. *Biometrics*. 1977; 33:363–374. [PubMed: 884196]
36. Masugi Y, Yamazaki K, Hibi T, Aiura K, Kitagawa Y, Sakamoto M. Solitary cell infiltration is a novel indicator of poor prognosis and epithelial-mesenchymal transition in pancreatic cancer. *Hum Pathol*. 2010; 41:1061–1068. [PubMed: 20413143]
37. Uchikado Y, Natsugoe S, Okumura H, et al. Slug Expression in the E-cadherin preserved tumors is related to prognosis in patients with esophageal squamous cell carcinoma. *Clin Cancer Res*. 2005; 11:1174–1180. [PubMed: 15709186]
38. Bryne M, Jenssen N, Boysen M. Histological grading in the deep invasive front of T1 and T2 glottic squamous cell carcinomas has high prognostic value. *Virchows Arch*. 1995; 427:277–281. [PubMed: 7496597]
39. Tanaka M, Hashiguchi Y, Ueno H, Hase K, Mochizuki H. Tumor budding at the invasive margin can predict patients at high risk of recurrence after curative surgery for stage II, T3 colon cancer. *Dis Colon Rectum*. 2003; 46:1054–1059. [PubMed: 12907899]
40. Bryne M, Nielsen K, Koppang HS, Dabelsteen E. Reproducibility of two malignancy grading systems with reportedly prognostic value for oral cancer patients. *J Oral Pathol Med*. 1991; 20:369–372. [PubMed: 1719194]
41. Sawair FA, Irwin CR, Gordon DJ, Leonard AG, Stephenson M, Napier SS. Invasive front grading: reliability and usefulness in the management of oral squamous cell carcinoma. *J Oral Pathol Med*. 2003; 32:1–9. [PubMed: 12558952]
42. Piffkò J, Bãnkfalvi A, Ofner D, Rasch D, Joos U, Schmid KW. Standardized demonstration of silver-stained nucleolar organizer regions-associated proteins in archival oral squamous cell carcinomas and adjacent non-neoplastic mucosa. *Mod Pathol*. 1997; 10:98–104. [PubMed: 9127314]

Figure 1. Histopathological analyses of tumor budding in tongue squamous cell carcinoma
Histopathological analyses were performed as described in Material and Methods to identify tumor buds in the invasion front of the TSCC. **(A)** Tumor budding at the invasive front in tongue squamous cell carcinoma (H&E, 20×). **(B)** Tumor budding with hyperchromatic nuclei in an H&E section (40×). Tumor buds were identified with arrowheads.

Figure 2. The effects of tumor budding on prognosis

Kaplan-Meier plots of overall survival in patient groups defined by intensity of tumor budding (High-intensity tumor budding: ≥ 5 tumor buds, Low-intensity or No tumor budding: < 5 tumor buds or no tumor bud detectable). The difference in survival rates is statistically significant ($p < 0.001$).

Figure 3. Immunohistochemistry analyses of E-cadherin and Vimentin expression in the tumor budding cells

Immunohistopathological analyses were performed as described in Material and Methods to evaluate the expression of E-cadherin and Vimentin expression in tumor budding cells. Strong staining of E-cadherin protein was detected at the cytoplasmic membrane and the intercellular borders in the adjacent non-cancerous epithelium and in the center/superficial tumor parts (A and B, 40 $\times$). In contrast, cancer cells at the tumor budding site showed negative or weak (dot-like) membranous immunostaining for E-cadherin (C, 40 $\times$, arrow). As shown in D and E, no Vimentin staining was observed in adjacent non-cancerous epithelium or in the center/superficial tumor parts (20 $\times$). In contrast, strong Vimentin

staining was detected in the ITF and tumor budding cells (**F**). A subset of cancer cells at budding site also exhibits a spindle-like morphology (40×, arrow).

Table 1

Correlations among clinical and histopathological features of primary TSCC #

	Age	Sex	Tumor size	Grade	pT stage	pN stage	Clinical stage	Tumor budding
Age	0.08456		-0.038	0.06086	-0.0728	-0.0016	-0.0294	-0.0019
Gender			0.15257*	0.11917	0.14313*	0.02571	0.1179	-0.0539
Tumor size				0.10703	0.89649**	0.41054**	0.80261**	0.22149*
Grade					0.03091	0.07592	0.07389	0.1543*
pT stage						0.36165**	0.83668**	0.11509
pN stage							0.74313**	0.3178**
Clinical stage								0.22814*
Tumor budding								

Spearman correlation coefficients were presented.

pT: pathological T-stage; pN: pathological N-stage

* $P < 0.05$;** $P < 0.01$

Table 2

The effects of clinical and pathohistological parameters on prognosis*

	Univariate analysis			Multivariate analysis		
	HR	95%CI	P value	HR	95%CI	P value
Gender	Female	1				
	Male	1.470	0.740 – 2.921	0.2711		
Age	≤ 55	1				
	> 55	1.386	0.751 – 2.555	0.2963		
Grade	Well	1				
	Mediate	1.935	0.959 – 3.904	0.0652		
	Poor	2.359	0.854 – 6.516	0.0979		
Tumor Size	≤ 4 cm	1				
	> 4cm	2.681	1.488 – 4.830	0.0010	0.806	0.251 – 2.588
pT	pT1–2	1				
	pT3–4	3.395	1.872 – 6.158	<.0001	3.990	1.283 – 12.404
pN	Negative	1				
	Positive	2.546	1.410 – 4.597	0.0019	1.209	0.457 – 3.200
Clinical stage	I–II	1				
	III–IV	3.427	1.766, 6.651	0.0003	1.510	0.401 – 5.683
Tumor budding	Low	1				
	high	3.350	1.774, 6.323	0.0002	3.029	1.535 – 5.977

* Analysis was done with Cox proportional hazard regression. HR: hazard ratio; 95% CI: 95% confidence interval.

Table 3

Association of the tumor budding intensity and the expression of E-cadherin and Vimentin

Genes	Number	Intensity of tumor budding		P value
		Low	High	
E-cadherin	Preserved	60	48	12
	Reduced	73	26	47
Vimentin	Negative	70	59	11
	Positive	63	15	48