Tumor Budding, EMT and Cancer Stem Cells in T1-2/N0 Oral Squamous Cell Carcinomas

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Abstract. *Background: Early oral carcinomas have a high recurrence rate despite surgery with clear margins. In an attempt to classify the risk of recurrence of oral squamous cell carcinomas, we explored the significance of tumor budding, epithelial–mesenchymal transition (EMT) and certain cancer stem cell markers (CSC). Materials and Methods: Tumor budding (single cells or clusters of ≤5 cells in the tumor front, divided into high- and low-budding tumors), EMT and CSC markers were studied in 62 immunohistochemically stained slides of T1/2N0M0 oral squamous cell carcinomas. Tissues and records of follow-up were obtained from the Oslo University Hospital, Norway. Tumor budding, EMT and CSC markers were scored and analyzed. Results: The only significant prognostic marker was tumor budding (p=0.043). Expression of the EMT marker E-cadherin was lost from the invasive front and tended to be a prognostic factor (p=0.17), and up-regulation of vimentin in tumor cells in the invasive front was found; this indicates that EMT had occurred. CSC markers were not associated with recurrence rate in the present study. Conclusion: A high budding index was related to poor prognosis in patients with oral cancer. Budding was associated with EMT-like changes. CSC factors were detected but reflected differentiation rather than stemness. Scoring of buds in patients with oral cancer may help discriminate invasive tumors prone to relapse, and thus, provide an indication for adjuvant therapy.*

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The majority of oral cancer cases are squamous cell carcinomas (OSCCs), and are associated with high mortality and morbidity (1). Independent of subsite, surgery and radiotherapy, either alone or in combination, are the main treatment modalities of early oral cancer (2). Currently available therapeutic options have not increased the survival in patients with early OSCC significantly. The simple clinical TNM classification has for years been the universally accepted prognostic marker in predicting the outcome of oral cancer (3). In order to improve therapy, there is a need to explore biological markers which are highly sensitive, specific and easy to apply in daily clinical work.

In spite of the histopathological verdict "complete" resection, some patients, with early-stage oral cancer, go on to have recurrent disease. The reason for this may be due to malignant cells left behind after treatment. These cancer cells might have gained properties which enable them to migrate and metastasize, features that were the objective of this study. Evidently, standard histopathology has limitations for examining surgical margins.

The epithelial to mesenchymal transition (EMT), where carcinoma cells change their properties from epithelial to mesenchymal, is considered a prerequisite for invasion, metastasis and death from disease. Cell–cell adhesions (such as desmosomes, tight junctions and adherence junctions) are lost (4), and a migratory phenotype develops in EMT. Epithelial cells lose expression of proteins such as E-cadherin, keratin and occludin, and features of polarity, and up-regulate expression of vimentin, N-cadherin, α-smooth muscle actin (αSMA) and fibronectin (5, 6). EMT cells can reverse the process and undergo a mesenchymal-to-epithelial transition (MET) in metastases. Thus, epithelial characteristics, after spread of cells to lymph nodes and distant organs, are regained, in order for cells to grow and establish metastases (7). Novel treatments directed at the EMT or MET processes are obvious candidates for improved treatment of cancer (8).

The evidence for the presence of EMT in cancer has accumulated, and most cancer cell biologists accept the EMT hypothesis (9), with only a few researchers arguing against the phenomenon (10). The main reasons for the difficulties in the detection of EMT are plasticity and the transient nature of the EMT process. In addition, it is a challenge to distinguish EMT cells from stromal fibroblasts by light microscopy alone.

Cancer stem cells (CSCs) in solid tumors are believed to represent only a very small fraction of tumor mass and be responsible for tumor progression. This hypothesis has gained considerable support (11) but has also faced opposition (12). Various CSC markers [*e.g.* CD44, CD133, aldehyde dehydrogenase isoform 1 (ALDH1)] have been identified in head and neck squamous cell carcinoma (HNSCC) (13). CD44 has been applied most frequently. However, the use of CD44 as a marker has been questioned due to its abundant expression both in HNSCC and normal head and neck epithelium (13), indicating that CD44 and other stem cell markers have biological functions other than stemness. The detection of stem cells in HNSCC sections has thus been difficult (13, 14). The stem cells are thought to be therapy-resistant, and Brabletz proposed the concept of "migrating cancer stem cells" in the front zone of tumors (15, 16). The invasive front (IF) is considered to be particularly important for tumor progression, as it is the zone of active invasion and important crosstalk between tumor and stroma (17).

Tumor budding is defined as the presence of single carcinoma cells or small clusters of cells (≤5 cells) located at the IF of neoplastic epithelial tumors. Extensive tumor budding is associated with tumor progression and unfavorable prognosis for various types of cancer, as in lung, gastrointestinal, colorectal, anal and esophageal cancer (18-22). Budding was first described in 1954 by Imai (23), but entered mainstream pathology only in the last decade (24). At present, budding is accepted to be an independent prognostic factor in colorectal cancer (20, 25). Budding is associated with loss of cellular adhesion as well as the presence of invasion at the IF, and has been postulated to be closely associated with EMT (24, 26). Budding is related to the prognostic factor, pattern of tumor invasion (27-30). It is also reported to be linked to CSCs, particularly to those migrating at the IF (15, 31).

There are only limited reports on the role of budding in oral cancer (32-34). Our present study confirms the role of tumor budding and provides further characterization of these budding cells by EMT markers, such as loss of E-cadherin expression and up-regulation of vimentin.

Materials and Methods

Patients and tumors. Relevant clinical information, follow-up and cause of death for all patients (with no history of previous head and neck cancer), were recorded prospectively at the Department of Otolaryngology, Oslo University Hospital, Norway, in collaboration with the referral cancer center, Radiumhospitalet, for a period of 15 years (1983-1997). One of the Authors (MEB) has an approved authorization from The Norwegian Data Inspectorate and Ministry of Health to collect and analyze data from patients with head and neck cancer (admitted to the Oslo University Hospital). Classification was initially carried out according to the Union for International Cancer Control, third edition (1982), and later updated to new editions. The database includes 392 patients with T1-2/N0, M0 OSCC (lip and base of tongue tumors were excluded) treated with curative intent. Adequate tumor tissue for further examination was avaliable in only 62 patients, 22 females and 40 males. Fifteen patients had recurrent disease. Tumor sites were: gingiva (N=13), tongue (N=17), floor of mouth (N=25) and other sites (N=7) (buccal mucosa, palate and unspesified mouth region). There were 29 T1 and 33 T2 tumors. Eleven patients were 50 years old or younger, 14 were aged between 51 and 60 years, and 37 were older than 60 years. The study was approved by The Regional Ethical Committee, 2013/1178S-05045.

Treatment. All patients were primarily treated with surgical excision of the tumors. Postoperative radiotherapy was given to 39 patients where suspicion regarding the completeness (defined as at least three high-power fields with no tumor cells) of the excision existed. Two patients were also given preoperative radiation treatment but pre-treatment biopsies were used for the study. External radiotherapy was given using a high-voltage source, and the primary site received a total of 60-70 Gy. Both sides of the neck were irradiated for tongue cancer and cancer affecting the midline. The treatment protocols were the same throughout the whole study period.

Follow-up. The follow-up time (mean=55 months, range=3-151 months) was measured from the time of cancer diagnosis. Complete follow-up information, including disease recurrence and cause of death, were collected from the outpatient and the hospital charts, autopsy reports, family physicians, local hospitals or by interviews with the patients or next of kin. Fifteen patients had recurrent disease during the follow-up period.

Histopathology. Formalin-fixed, paraffin-embedded archival material were processed according to standard procedures for hematoxylin-eosin (HE) staining. As described previously (35), a pathologist (HPD) verified the diagnosis of SCC and evaluated the tumors histopathologically in a blinded fashion, and graded them according to the WHO classification (36), and the IF grading (IFG) system, by taking cellular pleomorphism, keratinization, host response and pattern of invasion into account (Table I) (27, 37).

Immunohistochemistry (IHC). Tumor sections (formalin-fixed, paraffin-embedded, 3 to 5 μm-thick) were pre-treated in PT Link (a procedure combining deparaffinizing and heat-induced epitope retrieval without xylol). The slides were put in a preheated (65˚C) buffer (Flex Target Retrieval Solution, Dako Cytomation Denmark A/S, Glostrup, Denmark), gradually heated to 97˚C, kept for 20 min at 97˚C, cooled to 65˚C (60-70 min), before being washed in Dako Wash Buffer (Dako Cytomation Denmark A/S, Glostrup, Denmark) for 1-5 min. A Dako Autostainer was used, according to the standard staining procedure. The detection system was Dako Envision Flex+/High pH. The primary antibodies used are listed in Table II. Negative controls included replacement of primary antibody with

Table I. *Morphological and immunohistochemical features of the invasive front and their prognostic impact in oral squamous cell carcinomas.*

		N	R	*p*-Value
WHO grading	I	19	7	0.24
	II	33	6	
	III	10	2	
Pattern of invasion	Pushing, well-delineated	13	1	0.13
	Infiltrating solid cords, bands/strands, small groups of cells and single cells	49	14	
IFG	1 (1-7 points)	25	7	0.90
	2 (8-11 points)	29	6	
	3 (12-16 points)	8	2	
αSMA, (N=60)	Negative	15	4	0.85
	Positive	45	11	
N-Cadherin, (N=58)	0-1%	47	12	0.90
	2-10%	1	0	
	11-25%	1	0	
	>25%	9	2	
E-Cadherin, (N=57)	0-10%	45	13	0.17
	>10%	12	1	
Vimentin, (N=59)	0-1%	16	3	0.81
	2-10%	14	3	
	11-25%	4	1	
	>25%	25	7	
Pan-keratin-stained buds* (N=58)	<5 Buds	28	4	0.04
	≥5 Buds	30	10	
Ki67 (% positive cells) (N=59)§	0-66%	29	6	0.53
	≥67%	30	9	
β-Catenin, (N=60)	Positive nuclear staining	52	13	0.92
	No nuclear staining	8	2	
CD24, (N=49)	Negative	43	11	0.80
	Positive	6	2	
CD44, (N=52)	0%	2	0	0.19
	<30%	3	2	
	30%-60%	7	1	
	>60%	40	10	
SOX2 (% positive cells) (N=59)	0-1%	9	2	0.29‡
	2-25%	11	2	
	26-50%	10	5	
	>50%	29	6	
ALDH1, (N=47)	Negative	27	10	0.11
	Positive	20	3	

IFG, Invasive front grading; αSMA: alpha smooth muscle actin; SOX2: SRY (sex determining region Y)-box 2; ALDH1: aldehyde dehydrogenase isoform 1; *bud defined as 1-5 cells; N, number of tumors; R, patients with relapse; §cut-off at median value of 67%; ‡cut-off at 50%.

mouse myeloma immunoglobulin (same subtype and concentration as the monoclonal antibody) and phosphate-buffered saline. Positive controls were tumors known to express the antigens of interest.

Immunoreactivity scoring. The immunostained slides were evaluated using a semiquantitative scale, by three observers (CGA, SK and MB) with a Nikon eclipse 90i microscope and a Nikon DS-Ri1 camera. A consensus score was obtained among the observers in cases of discrepancy. The tumor cells were scored in the IF, defined as approximately two tumor cell layers deepest in the tumor/stroma border, including tumor buds. Areas with the highest antigen expression were selected for scoring, except for E-cadherin staining, where areas with less staining in the front were selected. In addition, for E-cadherin, we also scored the staining in the tumor center.

To evaluate Ki67 expression, we selected the invasive part of the tumor, including buds, with the highest expression, and counted both the positive and the negative cells (objective lens 20×/0.50), and then calculated the percentage of Ki67-positive tumor cells.

Tumor budding was examined in pan-keratin-stained sections and defined as a single cancer cell or a cluster of ≤5 cells in the invasive front. The area with the highest number of buds was selected and counted (objective lens 20×/0.50). The tumors were divided into two groups: high-budding (≥5 buds) and low-budding (<5 buds) tumors.

Statistical analyses. We used IBM SPSS Statistics, version 21. Kaplan–Meier survival analysis was performed, and the differences computed by log-rank test. The endpoints for survival analyses were time to recurrent OSCC, time to last follow-up, or time to death. A

case was censored if death occurred from other diseases and if patients were regarded free of disease at the last consultation or contact. A Cox multivariate survival analysis was performed to examine the prognostic value of different variables simultaneously. Due to a low number of events, it was only possible to include three variables in the Cox regression model. Cross tabulation, including chi-square tests, were performed for testing association between variables. Statistical significance was defined as $p<0.05$.

Results

Tumor budding. The number of high-budding tumors was 30 out of 58. A high number of buds (≥5) was associated with poor prognosis (Figure 1) ($p=0.043$). High-budding cases showed a trend for fewer relapses after postoperative radiation, as only 25% of patients experienced relapse compared to 38% among those who had undergone surgery only.

EMT markers in the IF and their prognostic value. Membraneous E-cadherin was frequently expressed in the tumor center (79% of the tumors had more than 25% E-cadherin-expressing cells), but lost in the tumor front (Figures 2, 3C and 4C). Accumulation of cytoplasmic E-cadherin was frequently observed. Seventeen percent of the tumors had membranous N-cadherin staining in more than 10% of cells in the tumor front, whereas 81% of all the tumors did not express any N-cadherin (Figure 4G). A possible switch from E-cadherin to N-cadherin expression in the IF was observed, as 9 out of 10 tumors with more than 10% N-cadherin-positive cells in the IF expressed little E-cadherin (Table III, Figure 2A). In addition, tumors with more than 10% E-cadherin-positive cells expressed little N-cadherin (except one case) (Table III).

Vimentin was up-regulated in the IF (Figure 3D and 4D), as half of the tumors expressed vimentin in more than 10% of the carcinoma cells. In 27% of the tumors, no vimentin was present in carcinoma cells.

The staining of pan-keratin and keratin 5/6 was rather similar in all cells. A homogenous staining was observed for pan-keratin, whereas staining for keratin 5/6 was positive but occasionally weaker in the IF.

αSMA immunoreactivity was present in tumor cell cytoplasm (Figure 4F) in 75% of the cases. αSMA was expressed in cancer-associated fibroblasts (CAFs) at the IF in 77% of the cases.

β-Catenin was highly expressed (Figure 4E) but with some variation in cytoplasmic immunoreactivity. The central part of the tumor displayed strong staining, closer to the membrane. In 87% of the cases, there were some β-catinin positive nuclei in the IF.

None of these EMT markers had a significant association with prognosis (Table I), but a distinct tendency for more frequent relapse was found for E-cadherin ($p=0.17$) (Figure 2B and 5).

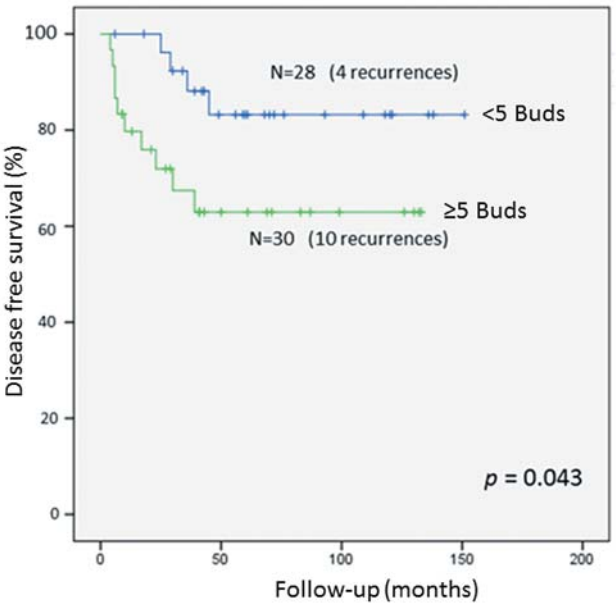

Figure 1. *Kaplan–Meier plot of survival according to tumor budding. A significantly higher number of recurrences (33%) among the high-budding cases was observed compared to recurrences in the low-budding group (14%).*

EMT markers in buds. Vimentin was up-regulated in tumor buds, and observed in 26 out of the 30 high-budding tumors. Vimentin was also expressed in stromal cells, making observations challenging (Figures 3D and 4D). Membranous E-cadherin was generally lost in tumor buds (Figures 2 and 4C; Table IV). Only two out of 30 high-budding tumors showed membranous E-cadherin immunoreactivity. Twenty out of 24 tumors with fewer than 10% E-cadherin-positive tumor cells in the IF revealed presence of vimentin immunostaining in buds. Eight (40%) recurrences were observed among these 20 cases compared to 24% relapses for the whole cohort (N=62). N-Cadherin was expressed in 17% of the tumors, mostly in the front. Six out of 10 of these expressed N-cadherin also in the tumor buds (Figure 2A). Staining for pan-keratin (Figure 2B and 4B) and keratin 5/6 was found in the buds. Nuclear β-catenin expression in buds was observed in 77% of the high-budding tumors. In high-budding cases, 40% of the tumors displayed αSMA expression, and 31% of these cases relapsed.

Proliferative index in buds. Ki-67 was expressed in all tumors (Figure 4H), particularly in the IF (Table I), and in a fraction of buds in 10 cases.

Stem cell markers. We scored the expression of CD44, SOX2, OCT3/4, ALDH1 and CD24, (Figure 4). CD44 was expressed in most front cells and buds (mostly at cell

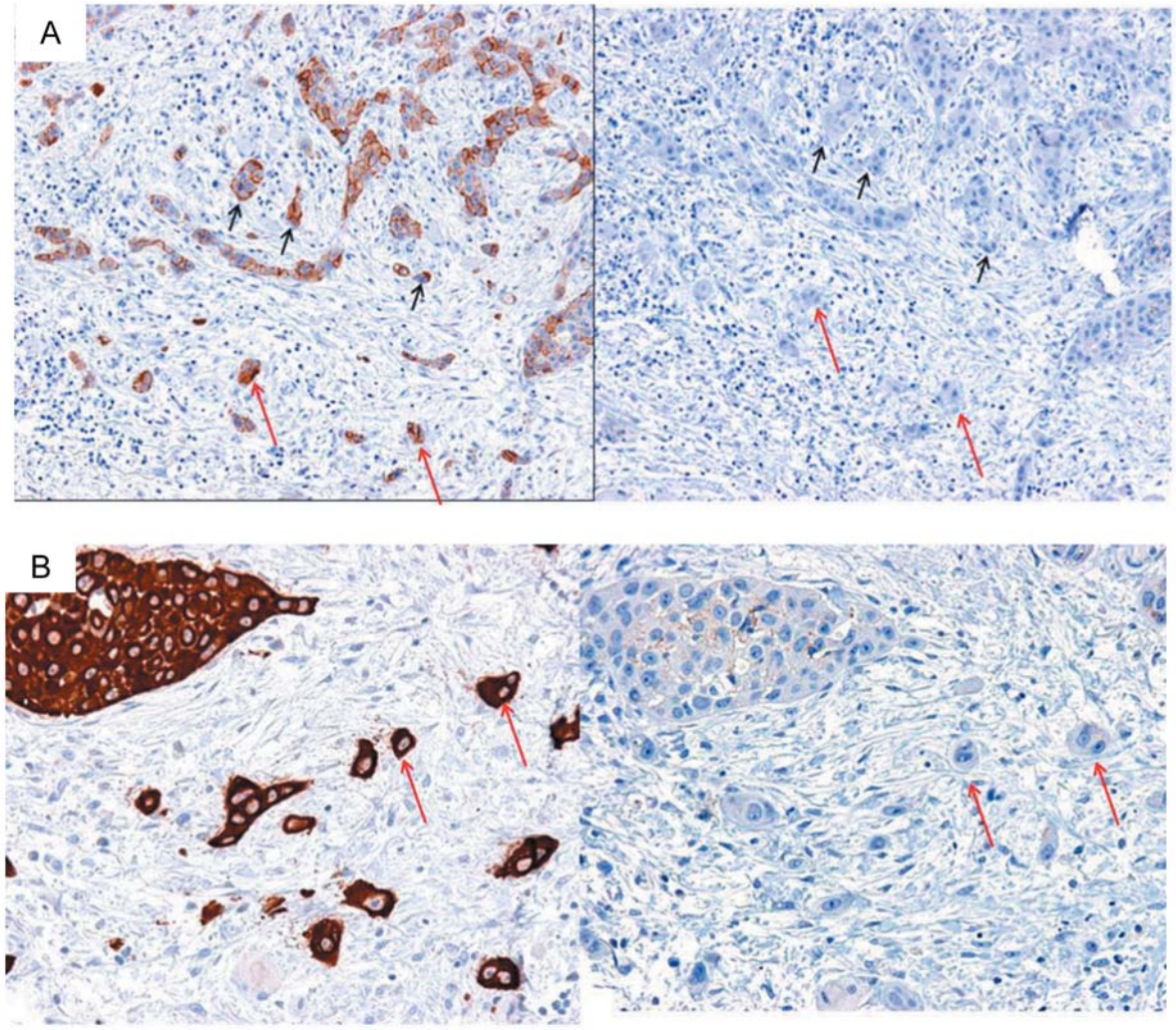

Figure 2. *A: Cadherin switch. Both images are from the same tumor area. Left: Buds positive for N-cadherin; right: Buds negative for E-cadherin. Red arrows indicate buds, black arrows indicate the same area. Objective lens 10×/0.50. B: Tumor budding. Left: Pan-keratin-stained tumor with positive buds; right: E-cadherin staining of same tumor area as in the left image. Negative buds and cells of the front. Red arrows indicate buds. Objective lens 20×/0.50.*

Table II. *The antibodies used for immunohistochemistry in the study.*

Antibody (mouse monoclonal)	Isotype	Dilution	Producer	City, Country
Pan-keratin	IgG1	1:100	Thermo Scientific	Fremont, CA USA
Keratin 5/6	IgG1	1:300	Dako	Glostrup, Denmark
Vimentin	IgG, kappa	1:500		
Alpha smooth muscle actin, (α-SMA)	IgG2a, kappa	1:500		
Ki67	IgG	1:200		
E-Cadherin	IgG1	1:3000	Zymed Laboratories Inc.	South San Franscisco, CA, USA
N-Cadherin	IgG1-K	1:300	Invitrogen	Camarillo, CA, USA
β-Catenin	IgG1	1:6000	BD Transduction Laboratories	Lexington, Kentucky, USA
Aldehyde dehydrogenase isoform 1, (ALDH1)	IgG1	1:3000		
SRY (sex determining region Y)-box 2, (SOX2)	IgG2A	1:500	R&D Systems	Minneapolis, USA
CD44	IgG1	1:200	Santa Cruz Biotechnology, Inc	Dallas, TX, USA
OCT3/4	IgG2b	1:400		

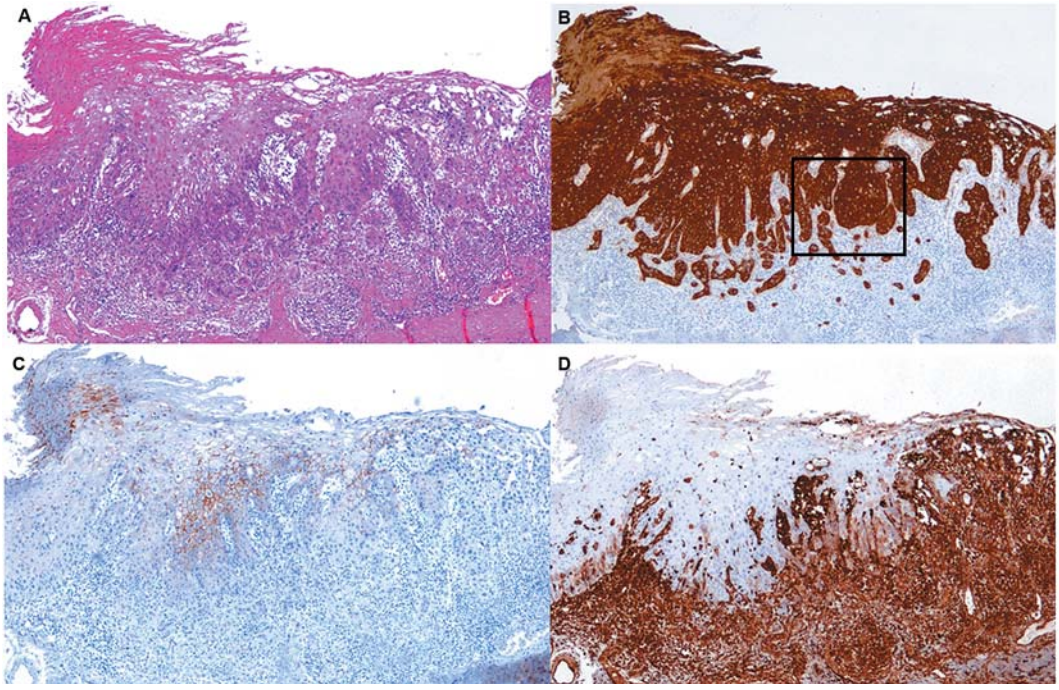

Figure 3. *Different stainings of the same field. A: Hematoxylin-eosin, B: pan-keratin, C: E-cadherin, D: vimentin. Objective lens 4×/0.50 for all stainings. The square outlined in B is shown in Figure 4.*

Table III. *Correlation of N-cadherin and E-cadherin expression, highlighting the cadherin switch. The table indicates the phenomenon of cadherin switch in the invasive front, as cases with high N-cadherin expression had low-E-cadherin expression. However, most cases had little expression of both N- and E-cadherin.*

	E-Cadherin (recurrence), n		Total, n
	<10%	>10%	
N-Cadherin			
<10%	35 (11)	10 (1)	45
>10%	9 (2)	1 (0)	10
Total, n	44	11	55

membranes) in nearly all carcinomas and SOX2 was expressed in 88% of the tumors at the IF. OCT3/4 was detected in a few nuclei in the IF in 39% of the cases but had no prognostic value in the current study. Fourty percent of the tumors had positive ALDH1 staining in cells in the IF. Some highly differentiated cells were CD24-positive, and those cells were found in the IF in 12% of the cases.

Morphology. The classification according to IFG and WHO was not of prognostic significance (Table I). However, regarding the pattern of invasion, tumors with 'pushing/collective' invasion tended to be associated with a better prognosis than did the remaining tumors (p=0.15) (Table I). The pattern of invasion correlated significantly with tumor budding (Pearson chi-square, p=0.002) (Table III).

Multivariate analysis showed that budding was the only significant prognostic factor in comparison with E-cadherin and tumor size, showing 3.5 times higher risk of recurrence associated with high-budding tumors. The pattern of invasion was not included in the multivariate analyses due to high correlation with budding.

Discussion

The recurrence rate of early oral cancer is high despite surgery with free resection margins. This may be due to cancer cells being left behind in the surrounding tissue. These cells might be CSCs or cells having undergone EMT. Frozen sections are often used to determine the completeness of a resection but may not be able to detect these cells in the specimens.

High tumor budding is associated with a poor prognosis, particularly in colorectal cancer, and has been suggested to be included in therapy planning (20). Our tumor budding results are in accordance with other studies, which have reported budding to be a prognostic factor in oral cancer (32, 33, 38-40).

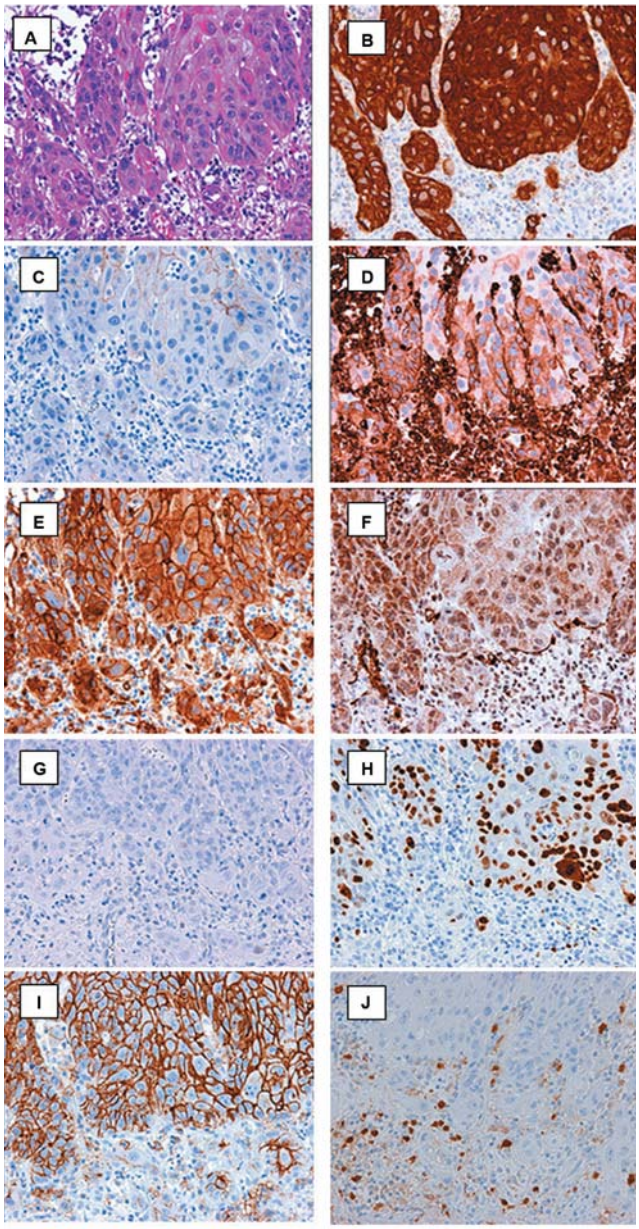

Figure 4. *High-power view of the area outlined in Figure 3B. Objective lens 20×/0.50 for all stainings, A-J. A: Hematoxylin-eosin, B: pan-keratin, C: E-cadherin, D:vimentin, E: β-catenin, F: alpha smooth muscle actin (α-SMA), G: N-cadherin, H: Ki-67, I: CD44, J: Aldehyde dehydrogenase isoform 1 (ALDH1). (Staining for CD24 and OCT3/4 was negative in this area, not shown).*

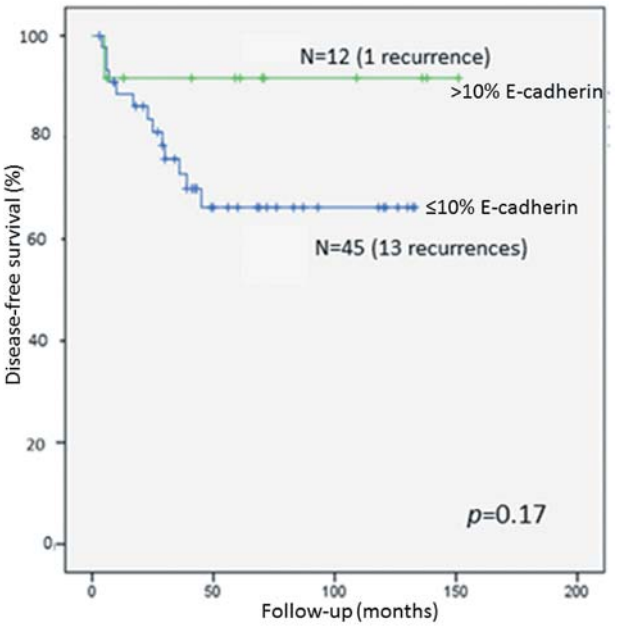

Figure 5. *Kaplan–Meier plot of survival according to membranous E-cadherin expression in the invasive front, using a cutoff of 10% positivity. In low-E-cadherin-expressing tumors, there were 13 relapses among 45 patients (29%). In cases with more than 10% E-cadherin-stained cells, there was only one relapse among 12 cases (8%) (p=0.17). This shows a clear trend for more relapses in the low-E-cadherin group.*

Table IV. *Characteristics of high- and low-budding tumors. Low-budding tumors with 'pushing' pattern of invasion were associated few relapses. The correlation between pattern of invasion and the number of buds was significant (p=0.002). When comparing the amount of E-cadherin with number of buds, tumors with a high number of buds and little E-cadherin expression were associated with more relapses. In low-budding cases with high E-cadherin expression, there were no relapses.*

	Number of buds (recurrence), n	
	<5	≥5
Pattern of invasion		
Pushing	10 (1)	1 (0)
All other patterns	18 (3)	29 (10)
Total	28 (4)	30 (10)
E-Cadherin*		
0-10%	20 (4)	24 (9)
>10%	8 (0)	4 (1)
Total	28 (4)	28 (10)

*Membranous, invasive front.

Scoring of buds is reproducible and relatively easy to perform, and may therefore be of clinical value for oral cancer. Both HE and pan-keratin immunostainings have been used in scoring (41). We found pan-keratin to be more sensitive and easy to score than HE, as also reported by Zlobec and Lugli (41), and that pan-keratin staining reveals more buds than routinely stained HE sections. However, van Wyk and co-workers reported that pan-keratin neither is more reproducible, nor gives a higher prognostic value than HE scoring in colorectal cancer (42).

In our material, high-budding cases that received postoperative radiotherapy had fewer relapses than those who underwent surgery only. This indicates that pan-keratin-positive tumor buds are radiosensitive. Some tumors with few buds recurred despite postoperative radiation. This might be due to migrating pan-keratin-negative cells with EMT or CSC properties, prone to being radioresistant. This and other studies suggest that patients with tumors with a high-budding-score should receive adjuvant therapy (20, 31, 38, 41).

Tumor budding is associated with the pattern of invasion, which has proven to be the best morphological prognostic indicator in oral cancer (28). In our present study, tumors with pushing/collective invasion were associated with a favorable prognosis. The pattern of invasion correlated significantly with tumor budding but budding proved to be a stronger prognosticator. Tumor buds have been associated with little proliferation, and little apoptosis (20, 43). However, in the present study, we observed several Ki67-positive buds, indicating that proliferation occurs in a heterogeneous manner in buds.

Typical EMT characeristics are loss of E-cadherin, gain of N-cadherin and up-regulation of vimentin. The expression of EMT markers in oral cancer has been investigated in some studies (6, 44-47). One report found a down-regulation of E-cadherin and matrix metallopeptidase 9 (MMP9), the epithelial phenotype, and up-regulation of vimentin and MMP2, the mesenchymal phenotype, in OSCC cell lines, in addition to vimentin immunolocalized in the cytoplasm of OSCC cells in the IF (45). Pectasides *et al*. studied HNSCCs, although only a few oral carcinomas, and found a longer 5-year progression-free survival in high-E-cadherin expressors compared to patients with low-E-cadherin expression (46). Chaw *et al*. reported a trend for decreased E-cadherin and increased vimentin expression, and that epithelial β-catenin localization shifted from being membranous to cytoplasmic or nuclear with increasing histopathological grade. They stated that a change in β-catenin localization and subsequent activation of vimentin provides one possible rationale on how an epithelial cell can gain a mesenchymal phenotype, and supports the role of β-catenin and vimentin in EMT and malignant transformation (47). The nuclear expression of β-catenin, linked to the Wnt signaling pathways and DNA transcription, is an established prognostic factor (48).

We observed loss of membranous E-cadherin and up-regulation of vimentin in most cases, particularly in the IF, suggesting that this tumor area is important for tumor invasion, as reported elsewhere (17, 37). Crosstalk between tumor cells and stroma in the IF is also of importance for tumor invasion (49). However, we did not find loss of keratin expression and general up-regulation of N-cadherin, indicating that only partial EMT was present in these early carcinomas. The EMT marker αSMA is believed to be expressed in both cells having undergone EMT and in CAFs in the stroma, and we found this marker in tumor cells in the IF in 75% of the tumors, also suggesting that at least partial EMT occurs. Since it is difficult to distinguish cells fully having undergone EMT from CAFs in the IF, we may have overlooked keratin-negative cancer cells. The presence of keratin-negative cells having undergone EMT, particularly in the IF, may be important in tumor progression. N-Cadherin-positive cells have been shown to be more migratory and less adherent (50). The cadherin switch, from E-cadherin to N-cadherin (51), was only observed in a fraction of the tumors in our series, also suggesting that only partial EMT takes place. Neither EMT characteristics, nor clinical and histological features were statistically significantly associated with recurrent disease, most likely due to the limited number of patients.

In our material, budding was associated with EMT markers, especially down-regulation of E-cadherin and up-regulation of vimentin. Similar observations have been found in esophageal (52) and in tongue (38) SCC. In the present series, 77% of the buds were nuclear β-catenin-positive, but with no prognostic significance, which is in contrast to findings in colorectal cancer (48). More than half of the N-cadherin-expressing tumors were high-budding cases, indicating a role in the invasive process.

CSCs have been associated with EMT (6) and tumor buds (31), and are expected to be present in the IF (15). The CSC markers analyzed varied between different tumor types (53). For HNSCC, various CSC markers are reviewed in studies of Major *et al*. (13) and Patel *et al*. (54) without finding any reliable stem cell marker or panel of markers. Biddle and associates reported two distinct phenotypes, a proliferative one that retained epithelial characteristics (non-EMT CSCs), and a migratory phenotype with mesenchymal traits (55). They showed that non-EMT and EMT CSCs could switch their epithelial or mesenchymal traits to reconstitute the cellular heterogeneity which was characteristic of CSCs. However, only ALDH1-positive cells having undergone EMT had the ability to seed a new epithelial tumor (55). Brabletz *et al*. suggested that so-called migrating CSCs are frequent in buds in the IF (15). In our current study, high expression of the stem cell markers CD44, ALDH1 and SOX2 in normal epithelial cells, as well as in tumor cells, may indicate that these markers also have a role in differentiation (35).

To conclude, we report a high tumor-budding index to be related to poor prognosis in patients with oral cancer. Tumor budding was associated with EMT-like changes, and at least partial EMT was found in our series. CSC factors were detected but may reflect differentiation rather than stemness. Scoring of buds in patients with oral cancer can help discriminate invasive tumors that are prone to relapse.

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Conflicts of Interest

None.

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