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Tumor budding and prognosis in gastric adenocarcinoma

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Abstract

Tumor budding has been associated with poor prognosis in several cancer types, but its significance in gastric cancer is unknown. The aim of this study was to assess the prognostic significance of tumor budding in gastric adenocarcinoma, and its main histological types. Some 583 gastric adenocarcinoma patients who underwent surgery in Oulu University Hospital during the years 1983-2016 were included in this retrospective cohort study. Tumor budding was counted per 0,785mm^2 fields from the slides originally used for diagnostic purposes. Patients were divided into low- (<10 buds) and high budding (≥10 buds) groups. Tumor budding was analyzed in relation to 5-year survival and overall survival. Cox regression was used to calculate hazard ratios (HR) with 95% confidence intervals (CI), adjusted for confounders. Determining tumor budding was difficult in diffuse type cancer due to the uncohesive growth pattern of these tumors. Patients with high tumor budding had worse 5-year survival compared to patients with low tumor budding (adjusted HR 1.55, 95% CI 1.20 – 2.01). In intestinal type adenocarcinomas, the high budding group had significantly poorer 5-year survival compared to the low budding group (adjusted HR 1.57, 95% CI 1.14-2.15). There were no differences in 5-year survival between the budding groups in the diffuse type adenocarcinoma. In conclusion, high tumor budding is an independent prognostic factor in gastric adenocarcinoma, but its value is limited to intestinal type of gastric adenocarcinoma. In diffuse type gastric adenocarcinoma, the assessment of tumor budding is hardly feasible and it does not have prognostic relevance.

Introduction

Gastric cancer is the third deadliest cancer in the world,[1] with high rates of recurrence even after potentially curative surgery.[2] Tumor-node-metastasis (TNM) -classification based on infiltration depth of tumor, number of lymph node metastases and presence or absence of distant metastases is the most commonly used way to estimate the prognosis of gastric cancer patients. However, many early stage gastric cancer patients still die due to cancer.[3] Assessment of histological patterns might be useful to increase the prognostic accuracy of TNM-classification and to help identify high-risk patients that benefit from intensive therapy.

Tumor budding is a novel prognostic factor that has been under interest especially in colorectal cancer.[4] It is defined as presence of single cells or clusters of two to four cells, called tumor buds, in the invasive front of tumor.[4] High tumor budding is a well-established marker of poor prognosis in colorectal cancer,[4] but it has been linked with poor prognosis with various other cancer types, like breast cancer,[5] head and neck squamous cell carcinoma[6] and pancreatic adenocarcinoma.[7]

The prognostic value of tumor budding in gastric cancer is poorly known. Two of the four earlier small studies have suggested that budding may associate to poor prognosis in gastric cancer,[8-11] but none of the four studies counted buds from a single field of vision on hematoxylin-eosin stained slides, which is now recommended in colorectal cancer.[4]

The aim of this study was to evaluate the reproducibility of the assessment of tumor budding with the contemporary methods and to clarify the prognostic value of tumor budding in gastric adenocarcinoma and its histological subsets in a large cohort.

Materials and methods

Study design

This study was a retrospective cohort study in a single institution in a tertiary care hospital in Northern Finland. There were 601 patients that underwent gastrectomy for gastric cancer in Oulu University Hospital between years 1983 and 2016. Of these, 583 gastric adenocarcinoma patients had diagnostic glass slides available for analysis and were included in the study. The study was approved by the Oulu University Hospital Ethics Committee. The need to obtain a written or oral consent from the patients was waived by the Finnish National Authority for Medicolegal Affairs (VALVIRA).

Data collection

The patients were identified from the archives of the Department of Pathology at the Oulu University Hospital, Finland. Clinical data for each patient was obtained from patient records, including operation charts and pathology reports. The immutable national personal numbers assigned to each resident in the country were used to combine data from the patient records and the 100% complete follow-up data from the Causes of Death Registry at the Statistics Finland, available until the end of 2016.

The original, prospectively collected hematoxylin-eosin diagnostic glass slides used for clinical decision-making were retrieved from the pathology archive and reviewed. Multiple HE-stained sections from each patient were viewed with light microscope, and a representative section with deepest invasion was used for further analysis.

Exposure (Tumor budding)

Sections were scanned and digitized using Aperio AT2 (Leica Biosystems, Wetzlar, Germany), and tumor budding was analyzed from scanned slides using Aperio ImageScope by two independent researchers (N.K. and M.E.) blinded to the clinical and outcome data.

Tumor budding was analyzed from invasive parts of tumor using the hotspot method that is considered to be the most useful method for assessing tumor budding in colorectal cancer.[4] A bud was defined as single tumor cell or a cluster of two to four tumor cells at invasive edge of tumor that appeared to be detached from the main tumor. First, the invasive front of tumor was screened with low magnification to find the area with most tumor budding. If it was unclear clear which area had most tumor budding and if the first area that was screened had under 15 buds, tumor budding was assessed from several areas and the area with most budding was used for analysis. The number of buds was counted from a single field of view of 0.398mm^2 using 200x total magnification, and the number of buds was multiplied by 1.97 to achieve the number of buds per area of 0.785mm^2, as recommended for colorectal cancer.[4] The resulting number of buds per 0.785mm^2 high power field (HPF) is used for all analyses.

The median number of tumor buds per standardized HPF was 14, and therefore it was decided to divide budding into low (<10 buds per standardized HPF) and high (≥10 buds per standardized HPF) budding groups, combining the two lowest categories of tumor budding in colorectal cancer (0-4 buds and 5-9 buds per HPF) into the low budding group in the present study. Examples of low and high tumor budding are shown in Figure 1. Patients that would have been placed into different subgroups by the two investigators had their slides reanalyzed in consultation with an expert gastrointestinal pathologist (T.J.K.), and consensus was reached.

Outcomes

Primary outcome of the study was 5-year survival, defined as death for any cause during the time between date of surgery and death of patient during 5 years or the end of 5-year follow up.

Secondary outcome of the study was overall survival, defined as death for any cause during the time between date of surgery and death of patient or the end of follow up.

Statistical analysis

All statistical analyses were decided *a priori*. Cohen's kappa was calculated to analyze interobserver agreement. χ^2-test was used to obtain p-values when comparing categorical variables. Continuous variables were compared, and p-values were obtained by T-test. Kaplan-Meier method was used to compare survival between groups, and log rank test was used to determine statistical significance of differences between groups. Cox regression model was used to perform multivariable analysis, providing hazard ratios (HR) with 95% confidence intervals (CI). Cox regression was adjusted for potential confounding variables: 1) year of surgery (<2000 or ≥2000), 2) age at diagnosis (continuous variable), 3) sex (male or female), 4) administration of perioperative chemotherapy (yes or no), 5) tumor stage (stage 0-II or stage III-IV), 6) Lauren classification (intestinal, diffuse or mixed) and 7) radical resection (R_0 or $R_{1/2}$). Subgroup analyses were performed in Laurén intestinal, and diffuse type gastric adenocarcinomas separately, adjusted for other confounders listed above, and additionally for histological grade (I-II, or III) in the intestinal type subgroup. IBM SPSS Statistics 24.0 (IBM corp., Armonk, NY) was used for all statistical analyses.

Results

Patients

After the retrieval of diagnostic glass slides, a total of 583 surgically treated patients diagnosed with gastric carcinoma were included in the study. Median age of the patients was 69 years (range 27 to 90 years, interquartile range 15.4), with 352 (60.4%) of patients being men and 231 (39.6%) women. Only 22 (3.8%) of patients underwent perioperative chemo(radio)therapy. Of these 583 patients, 437 (75.0%) underwent microscopically confirmed R_0 resection, and 146 (25.0%) had $R_{1/2}$ resection. The patients with $R_{1/2}$ resection included patients with non-curative intent, as well as 34 (5.8%) patients that had distant metastases at the time of surgery. Median follow-up time, including the patients that died during follow-up, was 26 months (range 0-396 months).

Assessment of tumor budding

Tumor budding was analyzed successfully for each patient. Defining tumor budding for the diffuse and mixed subtype tumors turned out to be difficult, as according to most recent definition by WHO diffuse subtype cancers include areas with isolated or small groups of tumor cells.[12] Some tumors of diffuse or mixed carcinoma had fewer cell clusters fulfilling definition of budding outside the main tumor area than others. They included for example intramucosal signet ring cell carcinomas and tumors consisting of cell mass with overall uncohesive growth but remaining of some cell-cell contacts, and low number or absence of detached cells in the invasive front (Figure 1C).

The patients were divided into low budding (<10 buds) and high budding (≥10 buds). 86 slides (14.8% of cases) needed re-assessment to reach consensus because they had been placed in different groups by different researchers. Main reasons for reassessments were difficulties in recognizing the

area with most buds and distinguishing tumor buds from stroma or immune cells in certain cases. Cohen's kappa vas calculated to analyze interobserver agreement after dividing patients to low, moderate and high budding groups. Kappa value calculated before the re-assessment was 0.706.

After the assessment, 262 (44.9%) of patients had low tumor budding and 321 (55.1%) had high tumor budding. The patients in high budding group were more often operated before year 2000, younger and female, and had more often advanced TNM-stage, diffuse-type histology, high histological grade and unradical resection (Table 1).

Primary outcome: 5-year survival

The primary outcome occurred in 387 (66.4%) of the 583 patients. The 5-year survival was significantly higher in low budding group (41.0%) compared to high budding group (23.0%, log rank test $p<0.001$, Figure 2). In the univariable analysis, high budding group had significantly worse survival compared to low budding group (HR 1.67, 95% CI 1.36-2.05, Table 2). In multivariable analysis, high budding group had significantly worse survival compared to low budding group (adjusted HR 1.55, 95% CI 1.20 – 2.01, Table 2).

In the subgroup analysis of the patients with intestinal histological type the 5-year survival was 37.4% in the low budding group and 13.2% in the high budding group (Figure 2). The difference between groups was statistically significant (log rank test $p<0.001$). In the univariable analysis, high budding group had significantly worse survival compared to low budding group (HR 1.92, 95% CI 1.43 – 2.57 Table 2). In the multivariable analysis, the high budding group had significantly poorer 5-year survival compared to the low budding group (adjusted HR 1.57, 95% CI 1.14-2.15, Table 2)

In the diffuse type histology subgroup 5-year survival was 53.1% in low budding group and 26.4% in high budding group. In the univariable analysis, the difference between the low and high budding groups was statistically significant (HR 2.00, 95% CI 1.27 – 3.16). However, adjustment for confounders mitigated this difference in 5-year survival for diffuse type adenocarcinoma (Table 2).

Secondary outcome: overall survival

In univariable analysis, patients with high tumor budding had significantly worse survival (HR 1.46, 95% CI 1.22 – 1.75, Table 2) compared to those with low tumor budding. In multivariable analysis, patients with high tumor budding had significantly worse survival (adjusted HR 1.40, 95% CI 1.12 – 1.76, Table 2) compared to those with low budding.

In the subgroup analysis of intestinal histological type, patients in high tumor budding group had significantly worse survival (adjusted HR 1.39, 95% CI 1.03 – 1.87, Table 2) compared to low tumor budding group.

In the diffuse type histology subgroup, the patients with high tumor budding had borderline worse survival compared to those with low budding in the multivariable analysis (adjusted HR 1.54, 95% CI 1.01 – 2.34, Table 2).

Discussion

The results of the present study suggest that tumor budding is a reproducible and independent prognostic factor in intestinal type gastric cancer. In diffuse type gastric cancer, the assessment of tumor budding might not be feasible and does not seem to have prognostic relevance.

Strengths and limitations of present study should be considered before interpreting results. This study was a retrospective single-institution study, which can limit its applicability for larger populations. Nevertheless, this study is much larger than any of the previous studies on the topic.[8-11] The long study period between years 1983-2016 of our study, during which the treatment of gastric cancer has evolved, as well as other confounding, might also be considered a limitation. However, these limitations were counteracted by adjusting multivariate analysis for year of surgery and all other confounders. Patients with unradical resections were also included to minimize selection bias and maximize the power of this study, and multivariate analyses were adjusted for radicality of resection instead. Another strength of the present study is its contemporary hotspot method of assessing tumor budding. The four earlier studies on tumor budding in gastric cancer used the method of screening several fields of vision and counting the average amount of buds per field of vision.[8-11] However, this average budding method is no longer recommended in colorectal cancer, and hotspot method, in which a single standardized 0,785mm^2 field of vision with highest amount of tumor budding is assessed.[4] Multiplying the number of buds counted from smaller field of vision to estimate buds per standardized HPF might reduce the accuracy of the assessment to some extent, compared to actually counting tumor buds on this area, but the difference is likely small. Furthermore, using the established 0,785mm^2 area of assessment is justifiable based on the evidence in colorectal cancer where this has been suggested as more replicable and comparable.[4]

Four previous small studies on tumor budding and prognosis in gastric cancer have been conducted. A Turkish study including 126 patients with T1 gastric cancer reported that tumor budding positive patients had more lymph node metastases compared to lymph node negative group (OR 8.87, 95% CI 2.79-22.16).[11] A Danish study (n=52) including only patients with intestinal histology reported a HR of 1.60 for recurrence in high budding group (defined as average number of buds in 10 fields being ≥1) compared to low budding (<1 bud in 10 fields on average) group in multivariate analysis, but the result was not statistically significant (p=0.08).[10] A Japanese study (n=153) using cytokeratin stained slides for analysis suggested that high budding (> 10 buds in a single field hotspot with 40x objective) is associated with poor prognosis (HR of 1.61, 95% CI 1.12–2.41 in univariate analysis) but tumor budding was not an independent prognostic factor based on multivariate analysis in their study.[8] A Chinese study (n=296) also suggested that high tumor budding, defined as ≥5 buds in 10 high power fields on average, is associated with poor survival (HR 1.57, 95% CI 1.04-2.35 in multivariate analysis).[9] In the present study, the HR for 5-year mortality in high budding group was 1.55 (95% CI 1.20-2.01) in high budding group compared to low budding group in multivariate analysis, which is similar to the two previous studies comparing survival between low and high budding groups.[8, 9] Taken together, the studies suggest that tumor budding is an independent prognostic factor in gastric cancer.

Tumor budding in different histological types of gastric cancer has not previously been analyzed in depth in previous studies.[8-11] In two studies, the diffuse type cancers were excluded because of the difficulties in defining tumor budding in those cases.[8, 10] The Chinese study of 296 patients included all histological subtypes, but did not provide subgroup analysis.[9] The Turkish study focusing on only early gastric cancer also included both histological types but did not provide subgroup analysis.[11]In the present study, high tumor budding was a strong 5-year- and overall prognostic factor in intestinal type subgroup, while its value was limited in the diffuse type subgroup and not

significant for 5-year survival after adjustment. Because low budding was observed often in intramucosal diffuse tumors, other factors than tumor budding, such as tumor stage and unradical surgery, are more likely to explain association between high budding and poor prognosis in diffuse type cancers. As there are also clear problems with defining tumor budding in the diffuse histological type, tumor budding should probably be used as a prognostic factor only in the intestinal type gastric cancer.

Tumor buds have been shown to arise from parts of tumor expressing markers that are associated with epithelial-to-mesenchymal transition (EMT).[13] EMT has been shown to increase invasive and metastatic capabilities of cancer cells,[14] which might explain why patients with high tumor budding have worse outcomes than those who with low tumor budding. Several potential treatments that target EMT in different cancer types are being developed,[15] and could benefit gastric cancer patients with high tumor budding.

There are some clinical and research implications for the present study. Tumor budding can be routinely analyzed with HE-stained slides, making it easy and cost-efficient to adopt for clinical use to estimate prognosis and need for adjuvant therapies in those with intestinal type gastric cancer. However, large retrospective and/or prospective studies are needed firstly to establish and validate an optimal cutoff for tumor budding in intestinal type gastric cancer and to assess the value of tumor budding in clinical decision-making. Further studies or clinical use of tumor budding in diffuse type gastric cancer are not recommended.

In conclusion, high tumor budding is an independent prognostic factor in gastric adenocarcinoma, more specifically in intestinal type gastric adenocarcinoma. Assessment of tumor budding in diffuse type gastric adenocarcinoma is not recommended.

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References

1. Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Allen C, et al. Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015: A Systematic Analysis for the Global Burden of Disease Study. *JAMA Oncol* 2017;**3**:524-48.

2. Yoo CH, Noh SH, Shin DW, et al. Recurrence following curative resection for gastric carcinoma. *Br J Surg* 2000;**87**:236-42.

3. Ikoma N, Blum M, Chiang YJ, et al. Survival rates in T1 and T2 gastric cancer: A Western report. *J Surg Oncol* 2016;**114**:602-6.

4. 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;.

5. Liang F, Cao W, Wang Y, et al. The prognostic value of tumor budding in invasive breast cancer. *Pathol Res Pract* 2013;**209**:269-75.

6. Almangush A, Salo T, Hagstrom J, et al. Tumour budding in head and neck squamous cell carcinoma - a systematic review. *Histopathology* 2014;**65**:587-94.

7. O'Connor K, Li-Chang HH, Kalloger SE, et al. Tumor budding is an independent adverse prognostic factor in pancreatic ductal adenocarcinoma. *Am J Surg Pathol* 2015;**39**:472-8.

8. Tanaka K, Shimura T, Kitajima T, et al. Tropomyosin-related receptor kinase B at the invasive front and tumour cell dedifferentiation in gastric cancer. *Br J Cancer* 2014;**110**:2923-34.

9. Che K, Zhao Y, Qu X, et al. Prognostic significance of tumor budding and single cell invasion in gastric adenocarcinoma. *Onco Targets Ther* 2017;**10**:1039-47.

10. Olsen S, Jin L, Fields RC, et al. Tumor budding in intestinal-type gastric adenocarcinoma is associated with nodal metastasis and recurrence. *Hum Pathol* 2017;**68**:26-33.

11. Gulluoglu M, Yegen G, Ozluk Y, et al. Tumor Budding Is Independently Predictive for Lymph Node Involvement in Early Gastric Cancer. *Int J Surg Pathol* 2015;**23**:349-58.

12. Lauwers GY, Carneiro F, Graham DY et al. Gastric Carcinoma. In: Bosman FT, Carneiro F, Hruban RH, et al. *WHO classification of tumours of the digestive system, 4th ed*. Lyon: IARC Press; 2010:48–58.

13. Gurzu S, Silveanu C, Fetyko A, et al. Systematic review of the old and new concepts in the epithelial-mesenchymal transition of colorectal cancer. *World J Gastroenterol* 2016;**22**:6764-75.

14. Suarez-Carmona M, Lesage J, Cataldo D et al. EMT and inflammation: inseparable actors of cancer progression. *Mol Oncol* 2017;**11**:805-23.

15. Davis FM, Stewart TA, Thompson EW, et al. Targeting EMT in cancer: opportunities for pharmacological intervention. *Trends Pharmacol Sci* 2014;**35**:479-88.

Tables

Table 1. Associations between tumor budding and clinicopathological variables in 583 surgically resected patients with gastric adenocarcinoma.

	Low budding (n=262)	High budding (n=321)	P-value
Year of surgery			**<0.001**
≥2000	92 (35.1%)	161 (50.2%)	
<2000	170 (64.9%)	160 (49.8%)	
Mean age at diagnosis	70,2	64.1	**<0.001**
Sex			**0.022**
Man	172 (65.6%)	180 (56.1%)	
Woman	90 (34.4%)	141 (43.9%)	
Perioperative chemotherapy			0.16
Yes	7 (2.7%)	15 (4.7%)	
No	255 (97.3%)	306 (95.3%)	
Tumor stage			**<0.001**
1 or 2	207 (79.0%)	152 (47.4%)	
3 or 4	55 (21.0%)	169 (52.6%)	
Lauren class			**<0.001**
Intestinal	209 (79.8%)	84 (26.2%)	
Diffuse	45 (17.2%)	225 (70.1%)	
Mixed	8 (3.1%)	12 (3.7%)	
Histological grade in intestinal type			**0.004**
I or II	143 (68.4%)	40 (47.6%)	
III	66 (31.6%)	44 (52.4%)	
Radicality of resection			**<0.001**
R0	230 (87.8%)	207 (64.5%)	
R1 or R2	32 (12.2%)	114 (35.5%)	

Table 2. Univariable and multivariable analysis of tumor budding and prognosis in 583 patients with gastric adenocarcinoma.

	Number of patients	Low budding HR (95% CI)	High budding HR (95% CI)
5-year survival			
All patients (Crude)	583	1.00 (Reference)	1.67 (1.36-2.05)
All patients (Adjusted)[a]	583	1.00 (Reference)	1.55 (1.20-2.01)
Subgroup analysis			
Intestinal type (Crude)	293	1.00 (Reference)	1.92 (1.43-2.57)
Intestinal type (Adjusted)[b]	293	1.00 (Reference)	1.57 (1.14-2.15**)**
Diffuse type (Crude)	270	1.00 (Reference)	2.00 (1.27-3.16)
Diffuse type (Adjusted)[c]	270	1.00 (Reference)	1.39 (0.85-2.27)
Overall survival			
All patients (Crude)	583	1.00 (Reference)	1.46 (1.22-1.75)
All patients (Adjusted)[a]	583	1.00 (Reference)	1.40 (1.12-1.76)
Subgroup analysis			
Intestinal type (Crude)	293	1.00 (Reference)	1.58 (1.20-2.08)
Intestinal type (Adjusted)[b]	293	1.00 (Reference)	1.39 (1.03-1.87)
Diffuse type (Crude)	270	1.00 (Reference)	2.10 (1.41-3.13)
Diffuse type (Adjusted)[c]	270	1.00 (Reference)	1.54 (1.01-2.34)

a Adjusted for year of diagnosis, age, sex, tumor stage, Lauren classification, perioperative chemotherapy and radical resection

b Adjusted for year of diagnosis, age, sex, tumor stage, tumor grade, perioperative chemotherapy and radical resection

c Adjusted for year of diagnosis, age, sex, tumor stage, perioperative chemotherapy and radical resection

Figure legends

Figure 1. The photomicrographs show examples of low tumor budding in intestinal type cancer (A), high tumor budding in intestinal type cancer (B), low tumor budding in intramucosal diffuse type cancer (C) and high tumor budding in diffuse type cancer (D), with 200x total magnification. Arrows indicate examples of individual tumor buds.

Figure 2. The Kaplan-Meier figures presenting 5-year survival stratified by tumor budding in gastric adenocarcinoma (A), 5-year survival stratified by tumor budding in the intestinal type gastric adenocarcinoma (B), and 5-year survival stratified by tumor budding in the diffuse type gastric adenocarcinoma (C).

Figures