Assessment of Tumour Budding in Colorectal Adenocarcinomas

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ABSTRACT

Objective: To calculate tumour budding *p*T2 colorectal carcinomas and study its association with other prognostic indicators.
Study Design: Cross-sectional study.
Place and Duration of Study: Shaukat Khanum Memorial Cancer Hospital and Research centre, from 2018-2019.
Methodology: Hematoxylin and eosin-stained slides of 50 Patients using 4-5 microns' thick sections were prepared using Leica Peloris for processing, Leica ST 5020 for staining and Leica CV 5030 for cover slipping.
An Olympus cx31 microscope was used to assess tumour budding. The "Hotspot method" (as proposed by ITBCC) was used.
Results: 26 (52%) slides showed low tumour budding (BD1), 8 (16%) showed intermediate tumour budding (BD2), and 16 (32%) showed high tumour budding (BD3). one patient out of 26 had positive nodal status in the low tumour budding category (3.8%). However, at the initial diagnosis, this number was significantly higher in the intermediate (50%) and high tumour budding (37.5%) categories. The mean survival in patients with low tumour budding was 22.615 months, which was significantly higher than 12.250 months and 13.188 months for intermediate and high tumour budding, respectively, with overall mean survival of 17.94 (±5) months. The overall survival rate in our study was 92.30% (24/26 patients), 25% (2/8 patients) and 12.5% (2/16 patients) for BD1, BD2 and BD3 patients, respectively (p=0.001).
Conclusion: Our study supports the inclusion of tumour budding in colorectal tumour checklists because of its association with survival and lymph node metastases.

Keywords: Colon, Colorectal carcinomas, Prognostic indicators.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy worldwide.[1,2] According to Pakistan Annual Cancer Registry Report, it is the second most common cancer in males and the sixth most common cancer amongst females.[3]

Multiple histopathological factors impact prognosis and survival in CRC. The Union for International Cancer Control (UICC) and the American Joint Committee of Cancer (AJCC) have provided us with a TNM classification, the most important prognostic predictor in these cases.[1,2] Although this TNM classification remains the gold standard for stratifying patients into subgroups based on prognosis, extreme survival and patient behaviour vary within the same stage, indicating a need for additional predictive and prognostic biomarkers. The additional features of prognostic significance include perineural invasion, lymphovascular invasion, infiltrating tumour borders, poorly defined clusters, Extramural venous invasion (EMVI) and tumour budding.[1,4]

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Tumour budding (TB) is a histomorphological phenomenon. It reflects the tumour cells detached from the primary tumour and Epithelial-Mesenchymal transition(EMT).[4,5] It is defined as a single neoplastic cell or a group of up to 4 neoplastic cells present at the invasive front of the primary tumour. Tumour budding is further subdivided into two categories: Peritumoral budding(PTB), defined as tumour bud-ding at the invasive front, and Intratumoral budding (ITB), defined as tumour budding in the centre of the tumour.

According to multiple studies, tumour budding has been regarded as an independent adverse prognostic marker associated with lymph node metastasis in pT2 colonic adenocarcinomas.[4,5,6] Other studies have also suggested that tumour budding is directly associated with higher tumour grade, infiltrating tumour borders, and lymphatic and perineural invasion. Therefore, tumour budding may be an early warning for subsequent adverse tumour behaviour. Although tumour budding has been regarded as an important prognostic marker, it was not routinely reported due to a standardized scoring system/criteria unavailability. An International Tumor Budding Consensus Conference (ITBCC) was held in 2016, in which a strong

consensus was reached on a single method for assessment of tumour budding and its reporting.[1-3,5,6]

The primary purpose of our study was to calculate tumour budding according to the method proposed by ITBCC in *p*T2 colorectal carcinomas and study its association with other prognostic indicators.

METHODOLOGY

Study approval was obtained from our Internal Review Board (IRB Number EX-02-08-19-08). We retrospectively reviewed 50 cases from 2018-to 2019 of pT2 colorectal carcinomas, regardless of lymph node status and presence of metastasis at the time of diagnosis, identified via a search of the database in our hospital (Shaukat Khanum Memorial Cancer Hospital and Research Centre, SKMCH & RC). The patients were included in our study sample through purposive non-probability sampling.

Inclusion Criteria: Patients with primary colorectal carcinomas, pT2 tumours, surgically resected specimens, were included in the study.

Exclusion Criteria: Patients with background inflammatory bowel disease or cases with prior neo-adjuvant chemotherapy, radiotherapy or combined chemoradiotherapy or specimens with poor fixation or processing artefacts or cases with pure signet ring cell morphology were excluded from the study.

Hematoxylin and eosin-stained slides of 4-5 microns thick sections were prepared using Leica Peloris for processing, Thermo Histostar for Embedding, Leica RM 2245 for microtomy, Leica ST 5020 for staining and Leica CV 5030 for coverslipping.

An Olympus cx31 microscope was used to assess tumour budding, and each case was evaluated by at least two histopathologists of our institute. The specimen area on the microscope was normalized to 0.785 mm2. The "Hotspot method" (as proposed by ITBCC) was used; that is, ten different fields were scanned at 20x objective along the invasive front, the field with the most extensive tumour budding (hotspot) was selected and tumour buds were counted.

A three-tiered system as proposed by ITBCC to facilitate prognostic stratification was used: 0-4 Tumor buds-Low budding (Bd 1), 5-9 Tumor-buds Intermediate budding (Bd 2),10 or more than 10 Tumor Buds-high budding (Bd 3).

Cytokeratin immunhistochemistry was performed in all the cases for budding categorization (Figure).

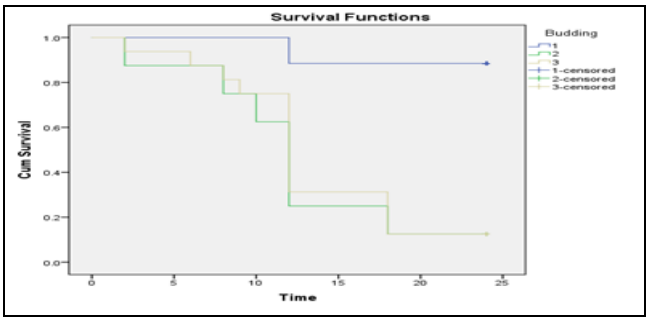

Figure: Graphical illustration of survival with respect to tumor budding.

A two year follow up was obtained from all patients, and a correlation between disease-free survival and lymph node metastasis was done. Statistical Package for Social Sciences (SPSS) version 20.0 was used for the data analysis. In addition, survival was assessed using the Kaplan Meier method, and lymph node status was assessed using two by two table.

RESULTS

Out of 50 patients, 20 were female (40%), and 30 were male (60%), ranging in age from 25 years to 91 years, with a mean age of 50 years. All kinds of resection specimens were included in the study. 25 (50%) were right hemicolectomy specimens, with 20 (40%) having cecal and ascending colon tumours and 5 (10%) with tumours involving hepatic flexure. 2 were transverse colectomies with mid transverse colon tumours, and 23 were abdominoperineal resections and lowered anterior resections with tumours present in the rectosigmoid junction or rectum.

On hematoxylin and eosin (H&E) stained slides, 37 cases showed well to moderately differentiated adenocarcinomas, and 13 showed poorly differentiated adenocarcinoma. All 13 poorly differentiated adenocarcinomas had intermediate to high tumour budding. Signet ring cells and mucinous adenocarcinoma were excluded from the study.

Margin status was evaluated, and only two specimens had positive distal resection margins. Both

Table-I: Lymph node status relative to tumour budding and high tumour budding.

Budding Grade	Total Number of Cases	Cases with Positive Lymph Nodes (Percentage)	Cases with Negative Lymph Nodes (Percentage)
Low tumor budding (BD1)	26	1 (3.8%)	25 (96.2%)
Intermediate tumor budding (BD2)	8	4 (50%)	4 (50%)
High tumor budding (BD3)	16	10 (62.5%)	6 (37.5%)

were abdominoperineal resections with high tumour budding.

Of all the evaluated patients, 26 (52%) showed low tumour budding (BD1), 8 (16%) showed intermediate tumour budding (BD2), and 16 (32%) showed high tumour budding (BD3).

The lymph node status regarding budding was shown in the Table-I. Only one patient out of 26 had positive nodal status in the low tumour budding category (3.8%). However, this number is significantly higher in the intermediate (50%) and high tumour budding (37.5%) categories at the initial diagnosis.

The frequency of cases with lymph node involvement was higher in BD 2 and BD 3. Also, the pN stage was relatively higher in the BD 3 category. Details of pN staging with respect to tumour budding were given in the Table-II.

The recurrence rate was 25 % in BD 2 patients and 31.25% in BD 3 patients. Compilation of data according to the overall stage into two groups also supports the earlier results. The first group comprises stage 1 and 2 tumours, and the second group comprises stage 3 and above. Thirty-five cases fall into group 1 with overall survival of 92% and 10% in low tumour budding and high tumour budding cases. In group 2, with 15 cases, all cases belonged to the high tumour budding category with only an overall survival of 13.3% (p=0.001).

These results pointed toward an adverse outcome in terms of survival in patients with high and intermediate tumour budding.

Figure showed the relationship of cumulative survival in all three categories of tumour budding. The cumulative survival with respect to tumour budding decreases significantly in BD 2 and BD 3 patients. Both show overlapping curves.

Table-II: pN staging (AJCC 8) with respect to tumor budding grade.

Budding Grade	Total number of cases	Cases with *p*N0	Cases with *p*N1a	Cases with *p*N1b	Cases with *p*N2a	Cases with *p*N2b
BD1	26	25 (96.2%)		1 (3.8%)		
BD2	8	4 (50%)	2 (25%)	2 (25%)		
BD3	16	6 (37.5%)	3 (18.75%)	3 (18.75%)	3(18.75%)	1(6.25%)

A 2 year follow up of the patients was done, which revealed that patients with low tumour budding had better outcomes in terms of overall disease-free survival and life span than intermediate and high tumour budding. The mean survival in patients with low tumour budding was 22.615 months, significantly higher than 12.250 months and 13.188 months for intermediate.

The overall survival rate in our study was 92.30% (24/26 patients) , 25% (2/8 patients) and 12.5% (2/16 patients) for BD1 , BD2 and BD3 patients , respectively (p=0.001).

None of the BD 1 category patients had developed recurrence or metastasis. One patient with BD1 status died due to unknown causes after one year of treatment, and another patient early in the course of treatment. Two patients from the BD 2 category presented with disease recurrence and subsequent deaths, and 4 died early in the post-operative period. Five patients from BD 3 category presented with recurrence or metastatic disease after treatment. Four patients passed away, and one went through pulmonary metastasectomy and survived.[10] BD3 patients died in the post-operative period.

The Table-III showed the mean survival times with respect to budding. The Table-IV represented the correlation of overall stage with tumour budding and survival.

Table-III: shows mean of survival times with respect to budding.

Budding grade	Mean Survival ± SD (Months)	95% Confidence Interval	
		Lower Bound	Upper Bound
BD 1	22.6 (± 0.7)	21.14	24.08
BD 2	12.2 (± 2.16)	8.01	16.49
BD 3	17.9 (± 1.44)	10.3	19.86

Table-IV: Association of overall stage with survival.

Group 1 (Stage 1 & 2)		Group 2 (Stage 3 & Above)	
Survival in low tumor budding category	Survival in high tumor budding category	Survival in low tumor budding category	Survival in high tumor budding category
23/25 (92 %)	1/10 (10%)	No cases	2/15 (13.3%)

DISCUSSION

Tumour budding is a microscopic finding that refers to the process of dissociation of tumour cells at the invasive front of carcinomas. It was shown to be of prognostic significance in previous literature with

respect to lymph node involvement recurrence and survival.[1,2,7,8]

Previous methods were employed to assess tumour budding because no definite method, tumour budding cut-off, or microscopic field area for its assessment was defined. Due to its prognostic significance, a standardized method was required, leading to the 2016 consensus meeting.[1,2] The meeting aimed to standardize the method of assessment of tumour budding. Tumour budding was defined as a single cell or a cluster of up to 4 cells at the invasive front on H&E sections (A group of more than four cells was termed a poorly differentiated cluster). H & E sections were preferred due to cost-effectiveness compared to the use of cytokeratin staining. The counting is to be done in one hotspot, decided after scanning at least ten different fields, in a microscopic field size of 0.785mm^2. The results are then compiled, and tumour budding is graded into High (>10 buds), Intermediate (5-9 buds) and Low (0-4 buds).[1,2,3]

The role of Cytokeratin stain is still controversial. Whereas some Authors argue that assessment of tumour budding should be done on H&E slides only due to the cost and impracticality of performing the immunohistochemical stain, other authors believe that Cytokeratin immunostain should be routinely used to improve accuracy and decrease interobserver variability.[9,10] More qualified pathologists may not require this as an aid. However, we performed cyto-keratin stain on all cases. According to our observa-tions, it helps better assess tumour budding, speci-fically in cases with obscuring factors, such as inflammation.

Tumour budding and poorly differentiated cluster both probably fall into the sequential spectrum of the same process. This thought is based on the findings that both entities show diminished/absent membranous E-cadherin and EpCAM expression, increased nuclear beta-catenin staining and reversed pattern of MUC 1 expression. However, no definite/ universal assessment method of poorly differentiated clusters has yet been devised.[11,12,13]

Many studies have been done to assess the relation of tumour budding with nodal status, vascular invasion, histological grade and survival. However, up till now, more work has been done on pT3 tumours.

In 2019, Demir *et al,* compared survival between low-intermediate and high tumour budding groups with a median disease-free survival of 43 months and 28 months out of 60 months followed up of 75 patients operated for rectal adenocarcinoma.[5] They did not find any relation with lymph node involvement. The main difference that came up in our study is the poorer survival in the intermediate category and the association of both intermediate and high category with lymph node involvement. The difference may be since the patients selected for their study were pretreated with neoadjuvant chemotherapy, and our patients were not. In addition, our follow up period was 24 months compared to theirs, which was 60 months. Demir *et al,* used a tumour regression grading system. However, the pathological staging was not taken into account.

In a study of 138 patients, Tanaka *et al*. demonstrated 98% and 74% survival in BD1 and BD2 tumours, respectively, in stage 2, T3 colorectal carcinomas using a two-tiered instead of a three-tiered approach.[14] It is noteworthy that we used a three-tiered system and analyzed pT2 patients regardless of stage. Since stage 2 tumours do not have lymph node metastasis, and a large percentage of our cases had positive lymph node status, the exact comparison could not be made. However, as mentioned in the results, the overall survival stage 2 tumours are 88% and 10% in low and high tumour budding categories, respectively.

Wang *et al,* 2009 analyzed 128 patients in the T3N0M0 stage and demonstrated a survival rate of 63% and 91% in patients with high and low tumour budding, respectively.[15] Our study matches with respect to decreased survival in high tumour budding cases and better survival in low tumour budding cases, i-e, 12.50% and 92.30%, respectively. However, the patients evaluated in their study had higher T stage, and a follow up of 5 years was done. Also, they used a two-tiered approach without an intermediate category. In our study, we used a three-tiered approach, evaluated pT2 tumours and a follow up of 2 years was obtained.

In 2008, Ohtsuki et al. analyzed 149 patients with tumours having wall penetration, i-e, T2, T3 and T4. He demonstrated that high tumour budding was directly associated with disease recurrence and lymph node metastasis.[13] Out of 24 patients with tumour budding, 15 patients had positive lymph nodes. In our study, 15 patients had positive lymph nodes out of 50 cases with tumour budding. However, as opposed to their study, only *p*T2 tumours were analyzed in our study and our categorization was based on all three grades of tumour budding. Furthermore, they categorized and studied the association with lymph node

metastasis based on the presence or absence of tumour budding, regardless of its grade. Ohtsuki *et al,* also suggested that the utility of cytokeratin stain renders better results in the assessment of tumour budding and the analysis of other features like micro lymphatic invasion.[13] This is in definite concordance with our observation that cytokeratin stain aids in better evaluation of tumour budding.

Lymph node metastasis is one of the most important prognostic indicators in colorectal carcinomas.[16,17] It has been reported that tumour budding helps predict lymph node and hematogenous metastasis. It seems to be the initial histological event in invasion and metastasis. Bektas *et al,* evaluated 73 patients regardless of pathological stage and reported that the frequency of lymph node metastasis was 30.3% in low tumour budding cases and 77.5% in high tumour budding cases.[18] In our study of 50 patients with *p* T2 tumours, this frequency was 3.8% and 37.5%, respectively. Although the overall percentage of lymph node metastasis is somewhat less in our study, the difference between both grades of tumour budding is still significant in both studies.

Cappellesso *et al,* in 2017, analyzed pT1 colorectal tumours and found that nodal metastasis was found in 28.5% of patients with tumour budding. The percentage of patients with positive lymph nodes in our study is 38.76%.[19] Nevertheless, it is worth mentioning here that our study included only patients with pT2 tumours, regarding which not many studies have been done until now. In 2002, Okuyama *et al.* studied 101 patients and assessed the relation of tumour budding with lymphovascular invasion and risk for lymph node metastasis 20. Out of 101 patients, tumour budding was present in 42 patients, and lymphovascular invasion was present in 39 cases. Their study, however, mainly dealt with lymphovascular invasion as a risk factor for nodal metastasis and its correlation with the presence or absence of tumour budding. We, in contrast, evaluated the number of positive lymph nodes in all three categories of tumour budding. In 2002, Park *et al,* detected isolated tumour cells by using pan-cytokeratin stain in 335 lymph nodes from 71 patients in node-negative colorectal carcinomas with tumour budding regardless of pT stage compared to which our study dealt with pT2 tumours specifically and relationship of tumour budding category with lymph node status. They also employed cytokeratin stain on the primary tumour slide and lymph nodes to detect isolated tumour cells.[21] Cytokeratin proves to be a helpful marker in this regard.[22,23] However, we used cytokeratin to evaluate the tumour budding category better.

Tumour budding has also been evaluated and regarded as an independent prognostic indicator in other gastrointestinal (oral, pancreatic and oesophagal carcinomas etc.) and non-gastrointestinal tumours (lung, larynx, skin and breast cancers etc.) but it is yet to be a formal part of staging checklists.[24,25]

CONCLUSION

Our study supports the inclusion of tumour budding in colorectal tumour checklists because of its association with survival and lymph node metastases. We also think that cytokeratin will facilitate counting, at least for the pathologists with no experience in reporting tumour budding.

Conflict of Interest: None.

Authors' Contribution

HM: Writing, Statistics, SM: Idea, Proof reading, UH:, MH: Proof reading, NA: Idea, IK: Revision analysis.

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