RESEARCH ARTICLE

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Histopathological Significance and Prognostic Impact of Tumor Budding in Colorectal Cancer

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Abstract

Background: Colorectal cancer (CRC) is a heterogeneous disease with a complex etiology. New prognostic factors need to be investigated. Our present focus is on histopathological significance and prognostic impact of tumor budding in CRC. **Material and Methods:** A total of 60 treatment-naive consecutive patients undergoing surgical resection of CRCs during the period of January 2011 to December 2013 were included in the study. Details of each related to their demographic and tumor profile were recorded. Hematoxylin and Eosin (H and E) and pan-cytokeratin details of each "case" immunohistochemically stained sections were examined for tumor budding assessment along with clinical features. **Results:** The most frequent site of involvement was the rectosigmoid and sigmoid colon (31.6%). The majority of the cases were moderately differentiated (75%), showed tumor invasion into the pericolic/subserosal fat (66.6%) and stage III (38.3%). Nodal involvement was present in 47%. Correlations between tumor budding and nodal involvement (p-value 0.039) and AJCC stage (p-value 0.021) were found to be statistically significant. **Conclusion:** Tumor budding is a promising and powerful predictor of lymph nodal metastasis and a higher stage of tumor and can be used as a marker for high-risk CRC. Routine H and E staining aided by cytokeratin immunostaining allows reproducible grading of tumor budding in CRC cases.

Keywords: Tumor budding- colorectal cancer- prognostic marker

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Introduction

Colorectal cancer (CRC) is the third most common form of cancer and the leading cause of cancer-related deaths in the Western world, causing 655,000 deaths worldwide per year (Jemal et al., 2006). In India, the average annual incidence rate in men and women is 7.7 and 5.1 per million population respectively with the occurrence of 36917 male and 27415 female cases (Ferlay et al., 2013). The mortality rate in men and women is 7.8 and 6.4 per 100,000 cases respectively (Ferlay et al., 2013). The majority of patients undergo surgical resection as a primary modality of treatment. The essential prognostic factors according to the "International Union against Cancer" are TNM staging, lymphatic and venous invasion, whereas tumor grade, perineural invasion, tumor budding and tumor border configuration are proposed as additional prognostic factors (Lugli et al., 2012).

"Tumor budding", defined by the presence of tiny cords of neoplastic epithelium (five or less tumor cells) that extend from the neoplastic glands into the adjacent stroma at the invasive front, is a strong, reproducible and independent prognostic marker of outcome and represents a distinct component of tumor invasion reflects the biological aggressiveness of the tumor (Prall, 2007). In CRC, around 20-40% cases demonstrate this feature which is strongly correlated to local and distant metastases and hence poor prognosis (De Smedt et al., 2016). It can also be used a criterion to identify patients with early-stage tumor requiring mucosal resection after endoscopic resection and thus has a bearing upon the management options (Ueno et al., 2004; Tytherleigh et al., 2008). Moreover, stage II patients with tumor budding experience significantly worse outcomes, prompting some authors to suggest that adjuvant chemotherapy should be considered in these patients (Hase et al., 1993; Okuyama et al., 2003; Nakamura et al., 2008). The detection of budding in malignant polyps appears as a risk factor for lymph node metastasis and hence, surgery is required in such patients. This also indicates the radical resection need for neoadjuvant chemotherapy in patients at high-risk, if the budding is detected in the biopsy material (Koelzer et al., 2016). The present study was conducted to assess the histopathological significance and prognostic impact of tumor budding in CRC.

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Materials and Methods

A total of 60 consecutive patients undergoing surgical resection for CRC during the period of January 2011 to December 2013 were included in the study. None of these patients had received any pre-operative chemotherapy, radiotherapy or combined chemo-radiation. The histopathological grade was evaluated using WHO grading system by two pathologists independently (Hamilton et al., 2010). Only histopathologically confirmed cases were included in the study. The clinical staging as per American Joint Committee on Cancer 7th edition (AJCC) was obtained from electronic medical records (Edge et al., 2010). Demographic details were obtained from medical records. The study was approved by the ethical committee of the Institute and was carried out in accordance with the principles of the Helsinki Declaration.

H and E stained slides were prepared using 4 micron thick sections stained on an autostainer (Medite Slide Stainer TST 44C, Germany). All the tumor sections were examined and studied thoroughly, twice. The tumor was graded according to the WHO grading criteria (Hamilton et al., 2010). Pan-cytokeratin (using clone AE1/AE3, by Dako) immunohistochemistry was performed, stained on automated immunostainer Ventana Benchmark XT (Roche/Ventana, Tucson, AZ, USA), applied to chosen sections to clearly delineate budding foci. Tumor budding was graded using Ueno method (Figure 1). Both the average bud count in 10 consecutive fields and the highest bud count in one field (hotspot) were assessed under 200x magnifications (field area=0.950 mm^2) using Nikon Eclipse Ci. The field area was reduced to 0.785 mm^2 and budding was graded as low, moderate and marked with respect to the bud count, <10, 10-19 and >20 respectively (Ueno et al., 2004).

Statistical Analysis

All statistical analyses were performed using SPSS software (Version 22, SPSS Inc, Chicago, IL, USA). The reported hospital-based annual prevalence of CRC at Rajiv Gandhi Cancer Institute and Research Centre, Delhi was found to be 4% and the optimum sample size was calculated considering the same prevalence. The descriptive statistics were presented in frequencies for categorical variables. Fischer's Exact Test or Chi-Square Tests were used according to the nature of data. Survival analysis was performed using the Kaplan-Meier method (Kaplan and Meier, 1958). Log-Rank test was used to compare the difference in survival among the groups. A two-sided p-value <0.05 was considered as significant.

Results

Of the 60 cases of CRC included in the study, the age distribution of cases was from 21 to 80 years with a peak incidence in the age range of 51-60 years (41.6%). The patient and tumor details are shown in Table 1. A total of 71.7% of the cases were male patients. The most frequent site of involvement was rectosigmoid and sigmoid colon (31.6%) followed by caecum (23.3%). The right side of colon was involved in 40% cases, left side of the colon was involved in 38.3% cases and the transverse colon, splenic flexure and hepatic flexure were involved in the remaining 21.7 % cases. Moderately differentiated tumor grade was observed in 75% patients whereas around 67% patients showed tumor invasion into the pericolic/ subserosal fat. Stage III tumor was reported in 38% patients. Nodal involvement was present in 47% cases (Table 1). The median number of lymph nodes removed was 21 (range 11-59). The mean number of metastatic lymph nodes was 5.9 (range 1-19) in the group of patients showing lymph node involvement. Metastasis, recurrence and death were reported in 21.7%, 3.3% and 6.7% patients, respectively.

Table 1. Patient and Tumor Details

Characteristic	N
Sex	
Male	43
Female	17
Tumor site	
Right colon	24
Transverse colon	13
Left colon	23
Tumor size (cm)	
<5	13
5-10	41
>10	6
Nodal involvement	
Present	28
Absent	32
Histologic type	
Well differentiated	1
Moderately differentiated	45
Poorly differentiated	12
Undifferentiated	2
Extent of invasion	
Muscularis propria	5
Subserosal and pericolic fat	40
Serosa	15
AJCC Stage	
1	4
2	21
3	23
4	12
Metastasis	
Present	13
Absent	47
Local recurrence	
Present	2
Absent	58
Vital status	
Dead	4
Alive	56

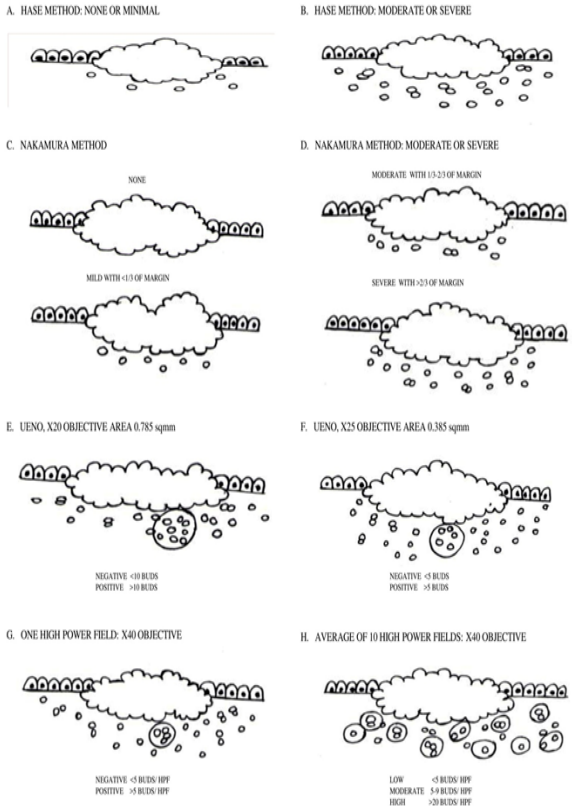

Figure 1. These Free Hand Illustrations Describe the Various Methods of Enumerating. "Tumor Budding". The present study utilized the Ueno method as shown from E-H.

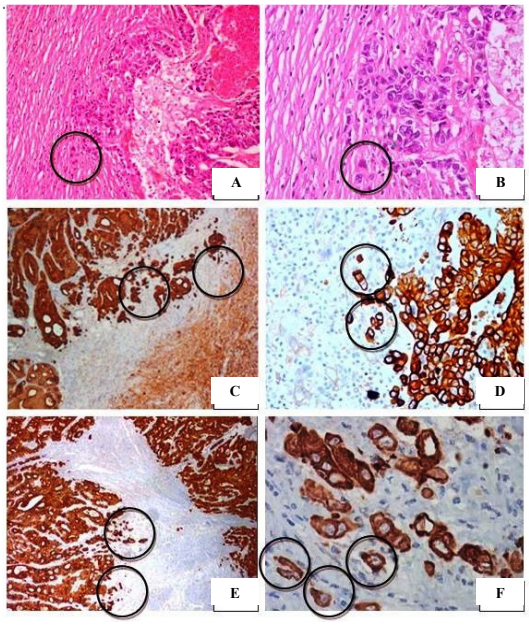

Figure 2. This Composite Image Demonstrates Tumor Budding on "H and E" Staining and Immunohistochemistry with Pan-cytokeratin. A) Tumor budding seen on 100x in an H and E stained section from a case of colorectal carcinoma (highlighted by black circle). B) Same area seen under 400x in H and E stained section. C) and D) are 100x and 200x view of high grade tumor budding as seen after immunohistochemistry staining with pan-cytokeratin. E) and F) are images of pan-cytokeratin stained intermediate grade budding with epithelial-mesenchymal transformation (as seen in image f within red circle) under 100x and 400x respectively.

Metastasis was observed in 5 cases in liver and 3 cases in lung followed by 4 cases involving pelvic and abdominal peritoneum and 1 case with soft tissue involvement of presacral fossa.

Figure 2 demonstrates tumor budding on H and E staining and immunohistochemistry with pan-cytokeratin. In terms of assessment of tumor budding, when the average count in 10 consecutive fields was taken into consideration, (as shown in Table 2) low-grade tumor budding (<10/200X) was seen in 43 (71.6%) cases and high-grade tumor budding (≥10/200X) was seen in 17 (28.4%) cases. The cases showing high-grade tumor budding were further graded into moderate (10-19/200X) (21.7%) and marked (≥20/200X) (6.7%). However, when the highest bud count in one 200X field in the entire section was assessed (Table 2), low-grade budding was seen in 13 cases (21.7%) and high-grade budding in 47 (78.3%) cases. Out of the cases with high bud count, moderate budding was seen in 26 (43.33%) cases and marked budding in 21 (35%) cases.

Correlation of various characteristics with highest tumor budding is given in Table 3. Considering the average and highest bud count, no statistically significant correlation was observed between tumor budding and site of involvement by tumor (p-values 0.936 and 0.052, respectively), histological grade of tumor (p-values 0.808 and 0.645, respectively), extent of invasion (p-values 0.330 and 0.643, respectively), distant metastasis (p-values 0.332 and 0.667, respectively) and local recurrence (p-values 0.407 and 0.323, respectively). Association between tumor budding and nodal involvement (p-value 0.039 for highest bud count

Table 2. Tumor Budding Based on Average and Highest Count

Budding Intensity (Average Count)		No. of cases (%)
Low grade budding		43 (71.6%)
High grade budding	Moderate budding	13 (21.7%)
	Marked budding	4 (6.7%)
Budding Intensity (Highest Count)		
Low grade budding		13 (21.7%)
High grade budding	Moderate budding	26 (43.3%)
	Marked budding	21 (35%)

Table 3. Correlation of Various Characteristics with Tumor Budding

Characteristic	Tumor budding (average count) (N)		p-value	Tumor budding (highest count) (N)		p-value
	Low	Moderate-Marked		Low	Moderate-Marked	
Tumor Site						
Right colon	16	8		1	23	
Transverse colon	10	3	0.936	4	9	0.052
Left colon	17	6		8	15	
Tumor Size						
<5	5	0		1	4	
5-10	28	12	0.319	10	30	0.235
> 10	10	5		2	13	
Nodal involvement (Present/Absent)						
Present	15	13	0.008**	3	25	0.039**
Absent	28	4		10	22	
Histologic Type						
Adenocarcinoma	34	16		8	42	
Mucinous adenocarcinoma	5	1	0.510	3	3	0.128
Signet ring cell carcinoma	2	0		1	1	
Undifferentiated carcinoma	2	0		1	1	
Histologic grade						
WD*	1	0		0	1	
MD*	30	15	0.808	8	37	0.645
PD*	10	2		4	8	
U*	2	0		1	1	
Extent of invasion						
Muscularis propria	5	0		1	4	
Subserosal and pericolic fat	28	12	0.330	10	30	0.643
Serosa	10	5		2	13	
AJCC stage						
1	4	0		0	4	
2	17	4	0.358	9	12	0.021**
3	14	9		2	21	
4	8	4		2	10	
Metastasis						
Present	9	4	0.332	2	11	0.667
Absent	34	13		11	36	
Local Recurrence						
Present	2	0	0.407	1	1	0.323
Absent	41	17		12	46	
Status						
Dead	3		0.878	1	3	0.867
Alive	40			12	44	
Overall survival						
90% (59 months)			0.984	90% (59 months)	87% (144 months)	0.904
Recurrence free survival						
93% (59 months)			0.390	91% (59 months)	96% (144 months)	0.391

*WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; U, undifferentiated; **p-values <0.05 were considered as significant.

and p-value 0.008 for average bud count), and AJCC stage (p-value 0.021 for highest bud count) were found to be statistically significant. Further, survival analysis was also done. The overall survival (OS) was 87% at 59 months and four deaths were reported whereas the recurrence free survival (RFS) was 94% at 59 months and two cases of

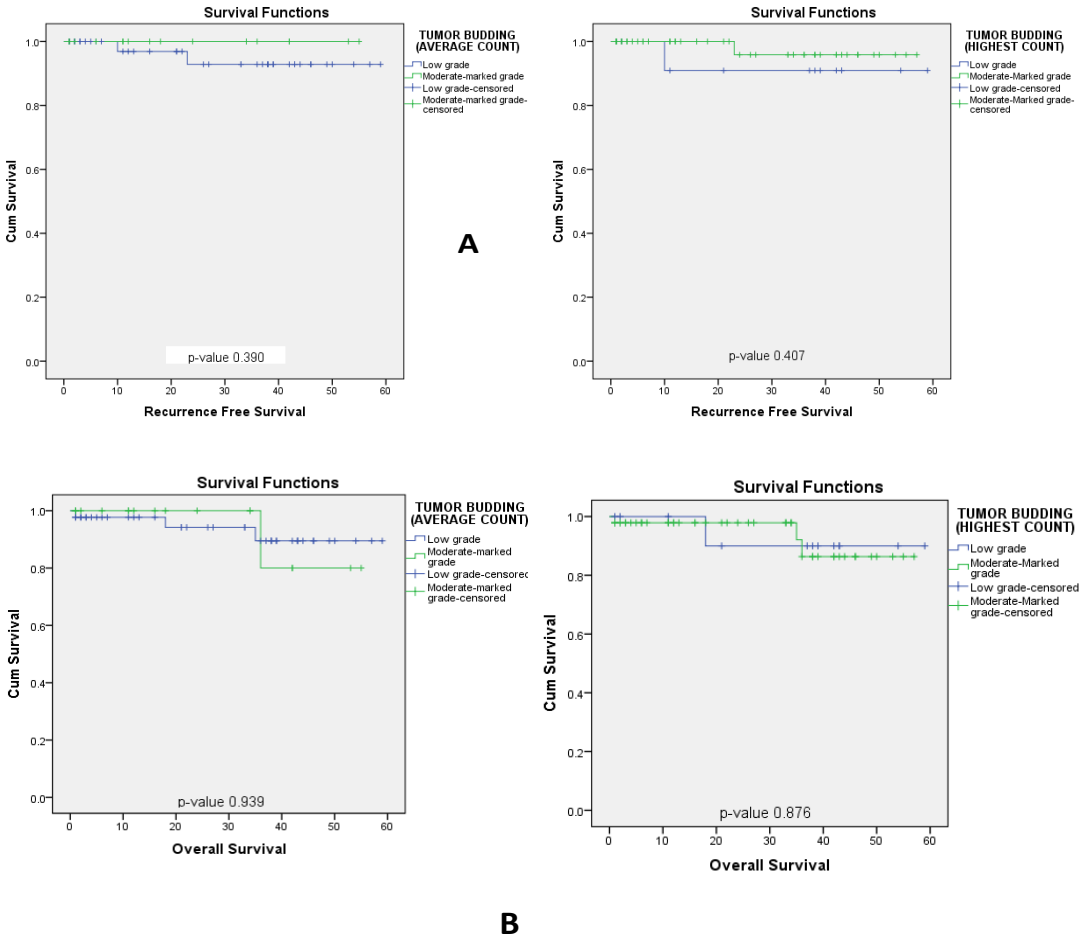

Figure 3. Kaplan-Meier Curves for (A) Recurrence Free Survival and (B) Overall Survival

recurrence had been reported. The correlations between tumor budding and survival (OS and RFS) was observed to be statistically not significant (Figure 3).

Discussion

Tumor budding has shown promise as an emerging prognostic marker in addition to conventional TNM stage, lymphovascular embolization, indeterminate margin and microsatellite instability.

Tumor buds are identifiable on H and E staining but their appreciation can be enhanced by cytokeratin immunohistochemistry. While the experienced pathologist can identify tumor budding reliably and reproducibly, a pan-cytokeratin staining is a useful aid and eases the experience of identifying and counting tumor budding. Kai et al., (2016) have proposed that cytokeratin immunohistochemistry may improve the interobserver variability in the evaluation of tumor budding especially when the assessment is performed by a non-gastrointestinal pathologist. This is more evident, especially in T1 CRC patients. The results of the study showed a tendency of assigning higher budding rates by experienced pathologists (Kai et al., 2016). Based on our experience, cytokeratin staining definitely improves the assessment of tumor budding and is recommended as an ancillary technique.

An important question to be answered concerns how many tumor buds are significant and how they shall be enumerated. To be relevant in clinical practice for prognostication and therapeutic decision making, it is necessary that a reproducible method with a definite cut point be used. The densest area (hot spot) for budding combined with Ueno method is considered most appropriate because it is objective, has the well-standardized field size of 0.785 mm^2 and provides numerical cutpoints to delineate negative, mild, moderate, and high budding (Ueno et al., 2004).

In our study, when the highest bud count (hotspot) in the entire tumor section was counted, 47 (78.3%) cases exhibited high-grade budding. This is in concordance with a study by El-Gendi and Al-Gendi (2011), who also used the hotspot method of bud count and recorded high-grade budding in 77.3% of tumors examined. Conversely, when the average bud count per 200X field was taken into consideration, of 60 cases, only 17 (28.4%) cases showed high-grade budding (>10 buds), which is concordant with a study by Morodomi et al., (1989) using average count option. They identified high grade budding in 27.5% tumors. However, the findings of the study by Tanaka et al., (2003) was not concordant with our results, as the method of grading in BD-1 and BD-2 was distinct from ours and based on subjective assessment. Moreover, the use of cytokeratin staining helped us define the budding more effectively. This complete reversal of tumor budding intensity on average and highest count can be attributed to more aggressive clones at the hotspot which

are undergoing epithelial-mesenchymal transition and separating out into individual cells more readily.

Tumor budding was correlated with different clinical and histological parameters like tumor site, tumor size, histologic type, histologic grade, the extent of invasion, nodal involvement, AJCC stage, distant metastasis and local recurrence. No correlation could be established between histological grade and budding intensity in our study and similar results have been reported (Ohtsuki et al., 2008; Wang et al., 2009; El-Gendi and Al-Gendi, 2011). However, Sevda et al., (2012) found a correlation between higher histologic grade and higher budding intensity, which is contrary to our findings. Can this be ascribed to the single cell morphology in poorly differentiated tumors which may all be falsely counted as tumor buds? Also, Zhang et al., (2016) have proposed the correlation of tumor budding with high recurrence rate, lymph node metastasis, chemoresistance and poor prognosis of CRC.

When the T stage of the tumors was correlated with tumor budding, it was found that for bud count <10, 76.9% tumors were stage T3 tumors whereas, for bud count ≥10, 63.8% tumors were of stage T3. It is, however stated that majority of patients in our cohort were T3 tumors. Similarly, in the studies by El-Gendi and Al-Gendi (2011) and Sevda et al., (2012) in low grade as well as high-grade budding cases, the majority of the tumors were of T3 and there was no definite correlation with the intensity of budding and T stage of the tumor.

Association between tumor budding and nodal involvement (p-value 0.039 for highest bud count and p-value 0.008 for average bud count) has been found to be statistically significant in this study. This finding is similar to many other studies which have demonstrated a statistically significant correlation between high grade tumor budding density and lymph node metastasis (Morodomi et al., 1989; Tanaka et al., 2003; Ueno et al., 2004; Guzinska-Ustymowicz, 2005; Kanazawa et al., 2008; Wang et al., 2009).

When tumor budding was correlated with distant metastasis and local recurrence, no statistically significant correlation was found. In other studies, a statistically significant correlation between local recurrence as well as distant metastasis with a higher grade of tumor budding has been observed (Tanaka et al., 2003; Guzinska-Ustymowicz, 2005; Ohtsuki et al., 2008). The reason for this contradictory result is hard to explain but may be related to the unique biology of Indian CRC or different stage distribution of study cohort.

Rogers et al., (2016) performed a systematic review and meta-analysis of the impact of tumor budding in CRC and suggested it as strongly predictive of recurrence and cancer-related death at 5 years. In terms of survival, tumor budding was observed to be associated with worse survival in stage II CRC, and more particularly in pathological T3N0M0 patients and especially for considering the option of administering adjuvant chemotherapy in high-risk node negative CRC patients (Petrelli et al., 2015). In our study, OS was better in patients with low-grade of tumor budding whereas RFS was better in patients with moderately to marked tumor budding. Petrelli et al., (2015) showed that high-grade budding was observed in patients with poor OS at 5 years. They also observed that presence of tumor budding was linked to an increased risk of death. Therefore, budding may be a negative prognostic factor and responsible for adverse outcomes in CRC patients.

In conclusion, overall, tumor budding, a reflection of epithelial-mesenchymal transition, is an effort of the tumor cells to separate out from main tumor mass and create metastasis. It is seen to be a promising and powerful predictor of nodal metastasis and a higher stage of the tumor. Long term follow-up may show tumor budding as a marker for increased aggressiveness of CRC and may help in identifying candidates for adjuvant therapy in stage II disease and for prognostication. Routine H and E staining aided by cytokeratin immunostain can aid in the grading of tumor budding, a practice that should be employed regularly in histopathological reporting of CRC. Our experience substantiates tumor budding as a significant predictor of lymph nodal metastasis.

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