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Original article

Evaluation of Immunohistochemistry and H&E Staining for Detection of Tumor Budding in Colorectal Cancer

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Abstract

Tumor budding (TB) is an established prognostic marker in colorectal cancer (CRC) and is defined as single tumor cells or clusters of up to four cells at the invasive front. While the International Tumor Budding Consensus Conference (ITBCC) recommends hematoxylin and eosin (H&E) as the standard for TB evaluation, immunohistochemistry (IHC) with pan-cytokeratin may improve detection in histologically challenging cases. The current study aims to compare TB assessment using H&E and pan-cytokeratin IHC and evaluate its association with clinicopathological parameters in CRC.A retrospective analysis was conducted on 98 CRC cases resected between 2017 and 2022. TB was graded according to ITBCC 2016 criteria on H&E and cytokeratin-stained sections (clone AE1/AE3). Associations with clinicopathological features were analyzed using Chi-square or Fisher's exact tests, with $p \leq 0.05$ considered significant.On H&E, TB was positive in 61 cases (62.2%): Bd1 (29.6%), Bd2 (15.3%), Bd3 (17.3%). On IHC, TB was positive in 67 cases (68.4%): Bd1 (32.7%), Bd2 (19.4%), Bd3 (16.3%) ($p < 0.001$ for increased detection). High TB (Bd2–Bd3) correlated significantly with advanced pathological stage ($p < 0.001$), higher T stage ($p < 0.001$), lymph node metastasis ($p < 0.001$), vascular invasion ($p = 0.002$), perineural invasion ($p = 0.003$), and desmoplasia ($p = 0.017$). Pan-cytokeratin IHC improves TB detection compared with H&E, especially in morphologically complex areas, and high TB is strongly associated with aggressive tumor features. Selective IHC use may enhance diagnostic accuracy and prognostic assessment in CRC.

Keywords: tumor budding, colorectal cancer, immunohistochemistry, pan-cytokeratin, H&E, prognosis

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Introduction

Colorectal cancer (CRC) ranks as the third most prevalent cancer and the fourth leading cause of cancer-related mortality (Marcellinaro et al., 2023). It frequently arises from benign neoplastic lesions, including adenomatous and serrated polyps, due to the gradual accumulation of genetic and epigenetic alterations, some of which are implicated in the activation of oncogene signaling pathways (Mármol et al., 2017). Despite advancements in diagnostic and therapeutic strategies, the ability of CRC cells to invade surrounding tissues and metastasize remains a major clinical challenge (Bian et al., 2020). The invasion-metastasis cascade is a complex process involving multiple biological mechanisms, including tumor budding (TB), which are increasingly recognized as critical factors in CRC progression (Peng et al., 2023).

TB is a well-known histopathological hallmark in CRC and has been identified as an independent unfavorable prognostic marker (Graham et al., 2015). It is defined as the presence of single tumor cells or small clusters of up to four cells at the invasive front of carcinomas, representing a morphological manifestation of epithelial–mesenchymal transition (EMT) (Lugli et al., 2017) and early metastatic potential (Zlobec et al., 2021). Numerous studies have indicated that TB is associated with lymph node metastasis (Dawson et al., 2019), local recurrence, distant metastases, and decreased overall survival, making it an essential factor in patient risk assessment (Rogers et al., 2016).

Despite its prognostic significance, the evaluation of tumor budding in routine diagnostic practice has encountered persistent challenges due to the absence of standardized definitions and grading criteria (Mitrovic et al., 2012). This issue was discussed by the International Tumor Budding Consensus Conference (ITBCC) in 2016, which suggested a three-tier scoring system based on hematoxylin and eosin (H&E) staining: Bd1 (0–4 buds), Bd2 (5–9 buds), and Bd3 (≥10 buds) per "hotspot" field (Neppl et al., 2020). These recommendations enhanced reproducibility and simplified the incorporation of TB into CRC reporting guidelines (Lugli et al., 2017).

Nevertheless, H&E evaluation of TB remains challenging in specific histological situations, including desmoplastic stroma, inflammatory cell infiltration, necrosis, and mucin pools, where tumor buds may be hidden (Pihlmann Kristensen et al., 2024). Immunohistochemical (IHC) staining using pan-cytokeratin has been recommended to improve detection accuracy by focusing epithelial tumor cells, thereby differentiating them from stromal or inflammatory components (Fisher et al., 2022). This method could improve sensitivity and interobserver consistency, especially in conditions of borderline or ambiguous budding on H&E. However, an overdependence on IHC raises the risk of inflating budding grades, which may affect treatment decisions (Takamatsu et al., 2019).

Taking these factors into consideration, a direct comparison of TB detection employing H&E versus pan-cytokeratin IHC in CRC can yield significant insights for

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the improvement of diagnostic techniques. This study aims to evaluate the agreement between these two methodologies, examine their correlation with clinicopathological parameters, and determine whether IHC provides significant diagnostic superiority over traditional H&E in TB assessment.

Methods

Ethical Approval

This study was approved by the Ethics Committee of the University of Duhok and the Duhok Directorate General of Health (Approval Number: 31072024-6-44 in July 31, 2024). As this was a retrospective study using archival material, informed consent was waived.

Study Design and Cases

This retrospective study involved 98 patients who received surgical resection for histologically confirmed colorectal adenocarcinoma from January 2017 to December 2022. Formalin-fixed paraffin-embedded (FFPE) tissue specimens were obtained from the archives of the Department of Pathology, Duhok Central laboratory, Duhok, Iraq. Data regarding clinical and pathological aspects, such as patient demographics, tumor location, histological type, grade, and stage, has been collected from pathology reports and medical records.

Exclusion Criteria

Patients who had previously received systemic chemotherapy or radiotherapy for their colorectal cancer were excluded from the study. This restriction was applied to eliminate potential confounding effects of prior treatments, as both chemotherapy and radiotherapy can alter tumor morphology, induce tissue necrosis or fibrosis, and modify molecular signaling pathways

Histopathological Evaluation of Tumor Budding

Slides stained with H&E for each case were independently examined by two experienced pathologists who were unaware of the clinical outcomes. TB was evaluated following the 2016 guidelines of ITBCC, as follow:

- The “hotspot” area at the invasive front was identified under low magnification.
- Tumor buds, defined as single tumor cells or clusters of up to four tumor cells, were counted in one hotspot field measuring 0.785 mm² at ×20 objective magnification.
- Bud counts were categorized into three grades: Bd1 (low budding, 0–4 buds), Bd2 (intermediate budding, 5–9 buds), and Bd3 (high budding, ≥10 buds).

Cases where inflammation, necrosis, mucin pools, or desmoplastic stroma made it difficult to identify tumor buds were recorded separately for additional IHC analysis.

Immunohistochemistry for Pan-Cytokeratin

Sections measuring 4 μM from representative tumor blocks underwent IHC staining for pan-cytokeratin (clone AE1/AE3, Dako, Denmark) using the EnVision detection system. Antigen retrieval was conducted with citrate buffer (pH 6.0) in a pressure cooker for a duration of 20 minutes. The chromogen employed was diaminobenzidine (DAB), with hematoxylin used as the counterstain. Cytokeratin IHC was applied to distinguish between epithelial tumor cells at the invasive front, facilitating improved visualization of tumor buds in regions where H&E assessment was challenging. Budding was assessed according to the ITBCC criteria applied to cytokeratin-stained sections.

Statistical Analysis

Data analysis was conducted using SPSS software version 24.0 (IBM Corp., Armonk, NY, USA). The relationship between tumor budding grade (assessed via H&E and IHC) and clinicopathological variables was analyzed using the Chi-square test or Fisher’s exact test, as applicable. A p-value of 0.05 or less was considered statistically significant.

Results

Clinicopathological Characteristics of the Patients

The clinicopathological features of the 98 colorectal cancer cases are summarized in Table 1. The median age fell within the older adult category, with approximately two thirds of patients aged 50 years or older. There was nearly an equal number of males and females. Just over fifty percent of the tumors were situated in the left colon. The predominant type was conventional adenocarcinomas, with a minor percentage exhibiting mucinous or signet-ring cell histology. The majority of tumors exhibited moderate differentiation. Stage II and stage III diseases were the most prevalent, with over two-thirds of tumors classified as T3 or T4. More than 40% of patients presented with nodal metastases, while a minority exhibited distant metastases (M1). Perineural and vascular invasion were observed in approximately fifty percent and nearly seventy-five percent of cases, respectively. Lymphocytic infiltration was noted in more than two-thirds of tumors, and desmoplasia was also prevalent.

Table 1. Demographic and clinicopathological characteristics of the study cohort

Variable	Category	n	%
Age	< 50 years	30	30.6
	≥ 50 years	68	69.4
Sex	Male	52	53.1
	Female	46	46.9
Tumor location	Right colon	42	42.9
	Left colon	56	57.1
Histology	Adenocarcinoma NOS	85	86.7
	Mucinous adenocarcinoma	12	12.2
	Signet-ring cell carcinoma	1	1.0
Grade	Well differentiated	8	8.2
	Moderately differentiated	74	75.5
	Poorly differentiated	16	16.3
Stage	I	23	23.5
	II	32	32.7
	III	36	36.7
	IV	7	7.1
T stage	T1	5	5.1

Variable	Category	n	%
	T2	26	26.5
	T3	53	54.1
	T4	14	14.3
N stage	N0	56	57.1
	N1	26	26.5
	N2	16	16.3
M stage	M0	91	92.9
	M1	7	7.1
Perineural invasion	Positive	49	50.0
	Negative	49	50.0
Vascular invasion	Positive	71	72.4
	Negative	27	27.6
Lymphocytic infiltration	Positive	67	68.4
	Negative	31	31.6
Desmoplasia	Positive	63	64.3
	Negative	35	35.7

Tumor Budding on H&E Staining

According to ITBCC 2016 criteria, tumor budding was identified in nearly two-thirds of cases on H&E-stained sections. Low-grade budding (Bd1) was the most common pattern among positive cases, followed by intermediate (Bd2) and high-grade (Bd3) budding. Over one-third of tumors showed no detectable budding on H&E. Representative photomicrographs of the three budding grades are shown in Figure 1, and the detailed distribution is summarized in Table 2.

Table 2. Tumor budding grades on H&E-stained sections

TB Grade (H&E)	n	(%)
Bd1 (0–4 buds)	29	(29.6)
Bd2 (5–9 buds)	15	(15.3)
Bd3 (≥10 buds)	17	(17.3)
Negative	37	(37.8)
Total	98	(100)

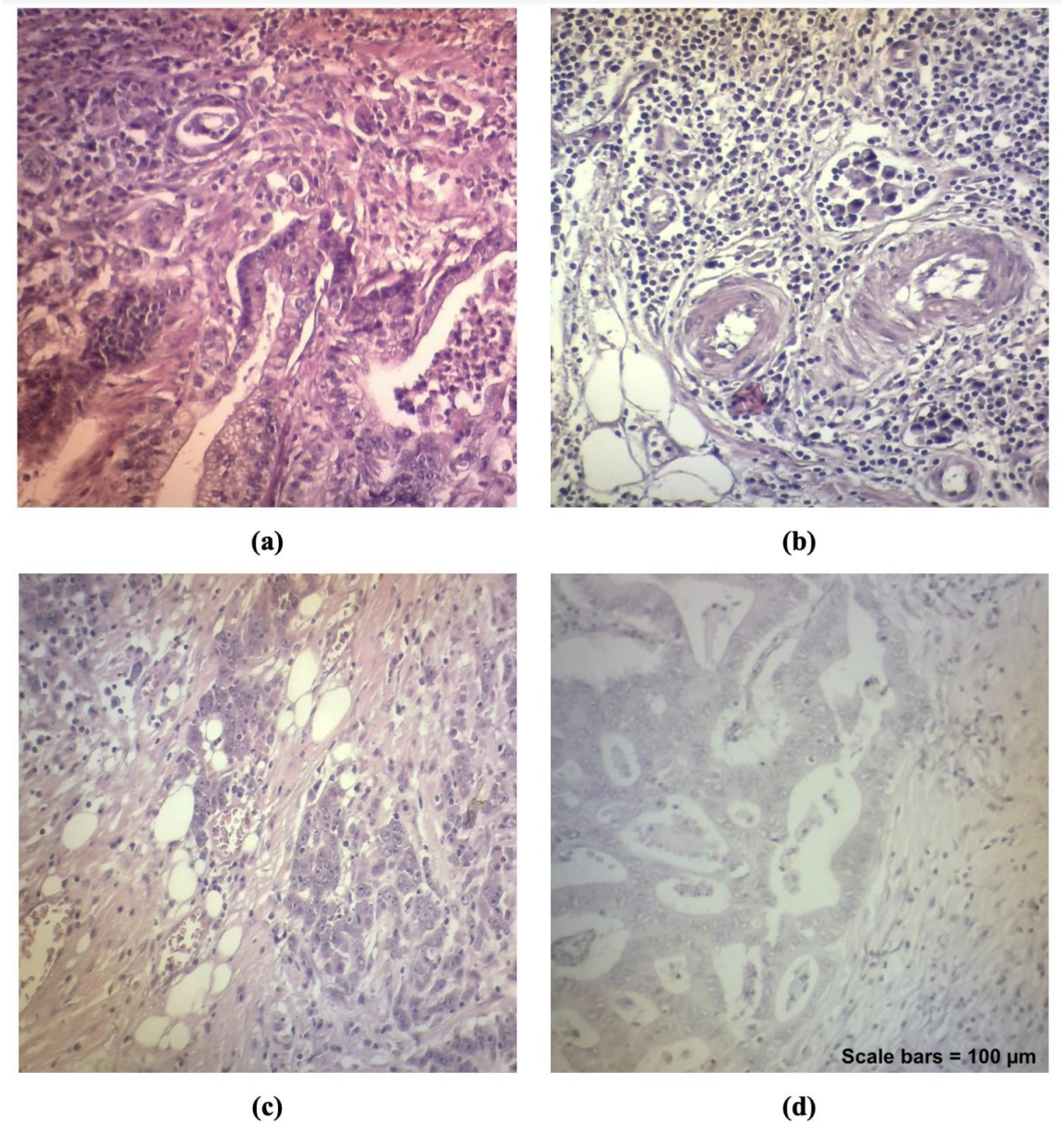

Fig. 1. Representative photomicrographs of tumor budding grades on H&E staining according to ITBCC 2016 criteria. (a) Bd1: low-grade budding (0–4 buds); (b) Bd2: intermediate-grade budding (5–9 buds); (c) Bd3: high-grade budding (≥10 buds); (d): Negative. Tumor buds are identified as single cells or small clusters of up to four cells at the invasive front. Scale bars = 100 µm.

Tumor Budding on Pan-Cytokeratin–Stained Sections

Pan-cytokeratin (AE1/AE3) immunostaining was positive in over two-thirds of cases. In positive cases, staining markedly improved the visibility of tumor buds, particularly in areas with dense stromal reaction or prominent inflammatory infiltrates. Among positive cases, low-grade budding (Bd1) was most frequent, followed by intermediate (Bd2) and high-grade (Bd3) budding. The detailed distribution is shown in Table 3, with representative microscopic images in Figure 2.

Table 3. Tumor budding grades on pan-cytokeratin–stained sections

TB Grade (IHC)	n	(%)
Bd1 (0–4 buds)	32	(32.7)
Bd2 (5–9 buds)	19	(19.4)
Bd3 (≥10 buds)	16	(16.3)
Negative	31	(31.6)
Total	98	(100)

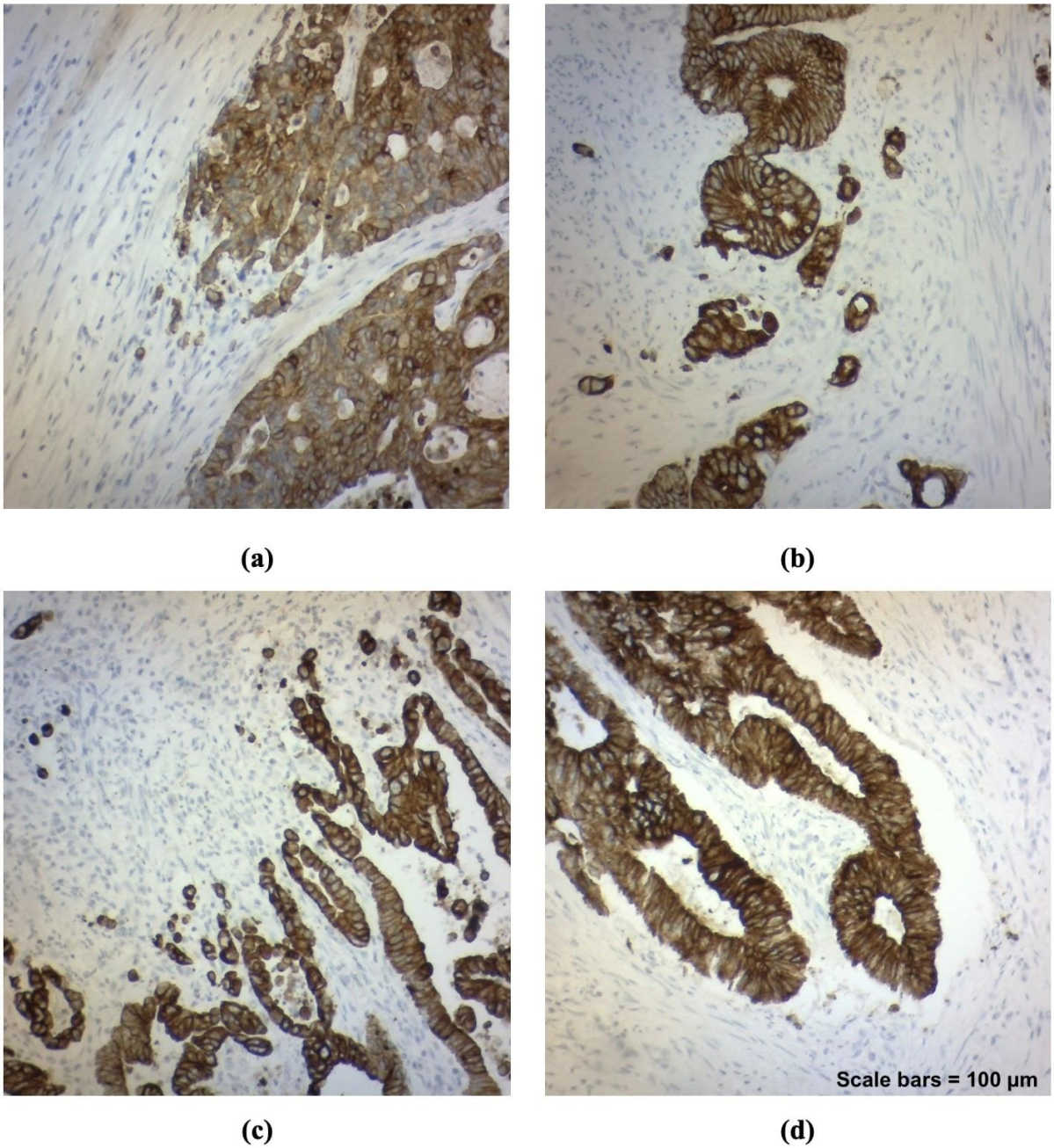

Fig. 2. Representative photomicrographs of tumor budding grades on IHC staining according to ITBCC 2016 criteria. (a) Bd1: low-grade budding (0–4 buds); (b) Bd2: intermediate-grade budding (5–9 buds); (c) Bd3: high-grade budding (≥10 buds); (d): Negative. Tumor buds are identified as single cells or small clusters of up to four cells at the invasive front. Scale bars = 100 µm.

Comparison between H&E and pan-cytokeratin detection

Pan-cytokeratin immunostaining significantly increased the detection of tumor budding compared with H&E, reducing the proportion of negative cases from 37.8% to 31.6% (p = 0.04) as shown in Figure 3. While the relative proportions of Bd1–Bd3 grades remained similar between the two staining methods, pan-cytokeratin facilitated the identification of buds in cases with dense stromal reaction or inflammatory background.

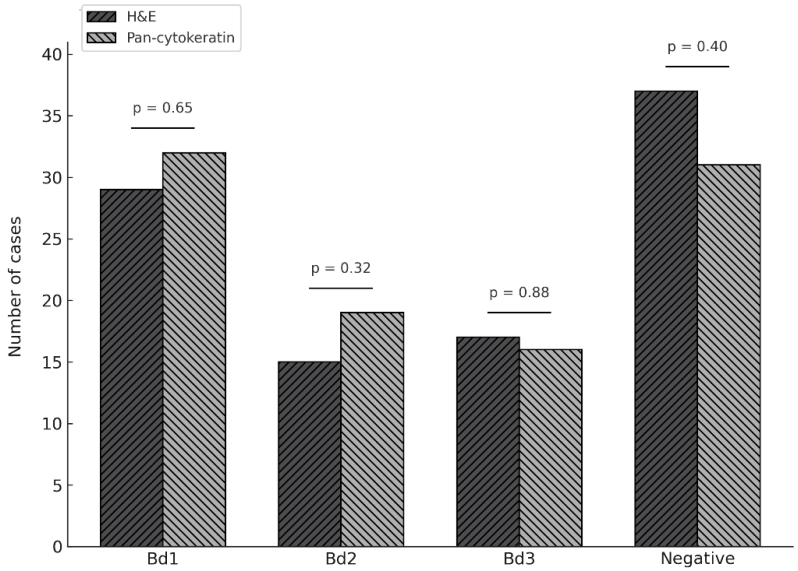

Fig. 3. Comparison of tumor budding grades assessed on H&E-stained sections and pan-cytokeratin (AE1/AE3)–stained sections according to ITBCC 2016 criteria. The number of cases in each budding category (Bd1, Bd2, Bd3, and negative) is shown for both staining methods. Pan-cytokeratin staining significantly increased tumor budding detection compared with H&E (p = 0.04).

Table 4. Association between tumor budding grade (H&E) and clinicopathological variables.

Variants		Tumor Budding		
		Positive (%)	Negative (%)	p- value
Age	**<50**	23 (76.7%)	7 (23.3%)	0.70
	≥50	38 (55.9%)	30 (44.1%)	
Sex	**Male**	35 (67.3%)	17 (32.7%)	0.302
	Female	26 (56.5%)	20 (43.5%)	
Location of Tumor	**Right**	26 (61.9%)	16 (38.1%)	1.000
	Left	35 (62.5%)	21 (37.5%	
Histological type	**Ad**	52 (61.2%)	33 (38.8%)	1.000 [a]
	Mucinous	8 (66.7%)	4 (33.3%	
	Signet ring cell Ad	1 (100.0%)	0 (0.0%)	
Grade	**Well**	3 (37.5%)	5 (62.5%)	0.108 [a]
	Moderate	45 (60.8%)	29 (39.2%)	
	Poor	13 (81.3%)	3 (18.8%)	
Stage	**I**	4 (17.4%)	19 (82.6%)	**0.000** [a]
	II	18 (56.3%)	14 (43.8%)	
	III	33 (91.7%)	3(8.3%)	
	IV	6 (85.7%)	1 (14.3%)	
Tumor invasion	**T1**	1 (20.0%)	4 (80.0%)	**0.001** [a]
	T2	11 (42.3%)	15 (57.7%)	
	T3	36 (67.9%)	17 (32.1%)	
	T4	13 (92.9%)	1 (7.1%)	
Lymph node involvement	**N0**	22 (39.3%)	34 (60.7%)	**0.000**
	N1	23 (88.5%)	3 (11.5%)	
	N2	16 (100.0%)	0 (0.0%)	
Metastasis	**M0**	55 (60.4%)	36 (39.6%	0.249 [a]
	M1	6 (85.7%)	1 (14.3%)	
Vascular invasion	**Positive**	51 (71.8%)	20 (28.2%	**0.002**
	Negative	10 (37.0%)	17 (63.0%)	
Perineural invasion	**Positive**	38 (77.6%)	11 (22.4%)	**0.003**
	Negative	23 (46.9%)	26 (53.1%)	
Lymphocytic infiltration	**Positive**	41 (61.2%)	26 (38.8%)	0.825
	Negative	20 (64.5%)	11 (35.5%)	
Desmoplasia	**Positive**	45 (71.4%)	18 (28.6%)	**0.017**
	Negative	16 (45.7%)	19 (54.3%)	
Total		61	37	

Correlation between Tumor Budding and Clinicopathological Parameters

On H&E-stained sections, high TB (Bd2–Bd3) showed significant associations with advanced pathological stage ($p < 0.001$), higher T stage ($p < 0.001$), lymph node metastasis ($p < 0.001$), vascular invasion ($p = 0.002$), perineural invasion ($p = 0.003$), and desmoplasia ($p = 0.017$). No significant correlations were found with age, sex, tumor location, histological subtype, or lymphocytic infiltration (Table 4).

On pan-cytokeratin–stained sections, high TB was also significantly associated with advanced pathological stage ($p = 0.001$), lymph node metastasis ($p = 0.001$), vascular invasion ($p = 0.003$), perineural invasion ($p = 0.029$), and desmoplasia ($p = 0.012$). A significant correlation was also observed with histological grade ($p = 0.046$), while no association was detected with age, sex, tumor location, or lymphocytic infiltration (Table 5).

Overall, both staining methods demonstrated strong correlations between high TB and aggressive tumor features, with pan-cytokeratin slightly enhancing the detection of these associations.

Table 5. Association between tumor budding grade (IHC) and clinicopathological variables.

Variants		Pan-Cytokeratin expression		
		Positive (%)	Negative (%)	p- value
Age	<50	25 (83.3%))	5 (16.7%)	0.058
	≥50	42 (61.8%)	26 (38.2%)	
Sex	Male	38 (73.1%)	14 (26.9%)	0.384
	Female	29 (63.0%)	17 (37.0%)	
Location of Tumor	Right	30 (71.4%)	12 (28.6%)	0.663
	Left	37 (66.1%)	19 (33.9%)	
Histological type	Ad	60 (70.6%)	25 (29.4%)	0.290 [a]
	Mucinous	6 (50.0%)	6 (50.0%)	
	Signet ring cell Ad	1 (100.0%)	0 (0.0%)	
Grade	Well	3 (37.5%)	5 (62.5%)	0.046 [a]
	Moderate	5067.6%)	24 (32.4%)	
	Poor	1487.5%)	2 (12.5%)	
Stage	I	9 (39.1%)	14 (60.9%)	**0.001** [a]
	II	21(65.6%)	11 (34.4%)	
	III	32 (88.9%)	4 (11.1%)	
	IV	5 (71.4%)	2 (28.6%)	
Tumor invasion	T1	2 (40.0%)	3 (60.0%)	**0.206** [a]
	T2	15(57.7%)	11 (42.3%)	
	T3	39 (73.6%)	14 (26.4%)	
	T4	11 (78.6%)	3 (21.4%)	
Lymph node involvement	N0	30 (53.6%)	26 (46.4%)	**0.001**
	N1	22 (84.6%)	4 (15.4%)	
	N2	15 (93.8%)	1 (6.3%)	
Metastasis	M0	62 (68.1%)	29 (31.9%)	0.1000 [a]
	M1	5 (71.4%)	2 (28.6%)	
Vascular invasion	Positive	55 (77.5%)	16 (22.5%)	**0.003**
	Negative	12 (44.4%)	15 (55.6%)	
Perineural invasion	Positive	39 (79.6%)	10 (20.4%)	**0.029**
	Negative	28 (57.1%)	21 (42.9%)	
Lymphocytic infiltration	Positive	45 (67.2%)	22 (32.8%)	0.817
	Negative	22 (71.0%)	9 (29.0%)	
Desmoplasia	Positive	49 (77.8%)	14 (22.2%)	**0.012**
	Negative	18 (51.4%)	17 (48.6%)	
Total		67 (68.4%)	31 (31.6%)	

Discussion

In this study, TB in CRC was evaluated using both H&E staining and pan-cytokeratin IHC, comparing detection rates and assessing correlations with clinicopathological parameters. The data showed that cytokeratin IHC significantly increased the detection of TB compared with H&E alone, and high-grade TB correlated with multiple adverse clinicopathological features, including advanced stage, higher T and N classification, vascular and perineural invasion, and desmoplasia.

The results are consistent with multiple recent investigations that have demonstrated the prognostic importance of TB in CRC. High TB has been linked to poor overall survival (Petrelli et al., 2015) and disease-free survival (Zhang et al., 2025) across all disease stages in previous studies. However, because survival analysis was not available in our cohort, our findings should be interpreted as pathological associations rather than direct prognostic validation. In particular, studies have shown that high TB correlates with lymph node metastasis, even in early-stage CRC (Satoh et al., 2014), highlighting its role in guiding surgical and adjuvant therapy decisions (Van Wyk et al., 2019).

A major strength of the present study was the direct comparison between H&E and pan-cytokeratin IHC for TB assessment. While ITBCC 2016 recommends H&E as the primary method, several authors have demonstrated that IHC can improve bud detection in morphologically challenging cases. For example, EL-Gendi (2011) (El-

Gendi and Al-Gendi, 2011) and Caie et al. (2014) (Caie et al., 2014) reported that cytokeratin staining increased TB counts, particularly in cases with dense inflammatory infiltrates or desmoplastic stroma, and broken glands, similar to the findings of the current study. Importantly, our findings suggest that the added value of IHC may be greatest in specific clinical scenarios. In early-stage disease (particularly pT1 and stage II CRC), where TB assessment may influence surgical strategy or adjuvant therapy decisions, IHC can provide greater diagnostic certainty. Likewise, in borderline cases near the Bd1/Bd2 threshold, IHC may clarify grading and reduce ambiguity. In post-neoadjuvant settings, where fibrosis, necrosis, or mucin pools can obscure buds, cytokeratin IHC offers an advantage in visualizing residual tumor cells (Ichimasa et al., 2021).

However, increased sensitivity of immunohistochemistry (IHC) in detecting cytokeratins can indeed lead to potential drawbacks, particularly in the context of overestimating tumor grade (Barak et al., 2004). While cytokeratins are valuable biomarkers for diagnosing various cancers, their expression can also highlight non-budding epithelial elements, complicating the interpretation of results (Cserni et al., 2006). Therefore, we recommend selective use of IHC only when H&E assessment is equivocal, to maximize accuracy without compromising specificity.

The findings of this study also support the growing body of evidence suggesting that TB should be routinely incorporated into pathology reports as part of CRC risk assessment (Berg and Schaeffer, 2018), particularly in early-stage disease where therapeutic decisions are nuanced (Cho and Kakar, 2018; Graham et al., 2015). However, definitive prognostic conclusions in our cohort cannot be made without survival data, and future multicenter prospective studies are warranted.

Limitation

This study has several limitations. It was retrospective and single-center in design, which may restrict generalizability. Survival outcomes were not available, limiting our ability to directly validate the prognostic value of TB in this cohort. Reliance on IHC carries the potential risk of overestimating bud counts, as cytokeratin can highlight non-budding epithelial fragments. Finally, interobserver reproducibility was not formally assessed, which could influence consistency in TB reporting.

Conclusion

Pan-cytokeratin IHC enhances the detection of tumor budding compared with H&E staining, particularly in histologically complex areas. High-grade TB was strongly associated with multiple adverse pathological features in our cohort. While our findings align with prior literature supporting the prognostic relevance of TB, survival analysis was not available, and conclusions regarding patient outcomes should therefore be drawn cautiously. Selective use of cytokeratin IHC may nonetheless improve diagnostic accuracy and facilitate more reliable assessment in clinically relevant scenarios such as early-stage CRC, borderline budding cases, and post-neoadjuvant settings.

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