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ORIGINAL ARTICLE

Tumor budding in colorectal cancer revisited: results of a multicenter interobserver study

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Abstract Tumor budding in colorectal cancer (CRC) is recognized as a valuable prognostic factor but its translation into daily histopathology practice has been delayed by lack of agreement on the optimal method of assessment. Within the context of the Swiss Association of Gastrointestinal Pathology (SAGIP), we performed a multicenter interobserver study on tumor budding, comparing hematoxylin and eosin (H&E) with pan-cytokeratin staining using a 10 high power field (10HPF) and hotspot (1HPF) method. Two serial sections of 50 TNM stage II-IV surgically treated CRC were stained for H&E and pan-cytokeratin. Tumor buds were scored by independent observers at six participating centers in Switzerland and Austria using the 10HPF and 1HPF method on a digital pathology platform. Pearson correlation (r) and intra-class correlation coefficients (ICC) comparing scores between centers were calculated. Three to four times more tumor buds were detected in pan-cytokeratin compared to H&E slides. Correlation coefficients for tumor budding counts between centers ranged from r=0.46 to r=0.91 for H&E and from r=0.73 to r=0.95 for pan-cytokeratin slides. Interobserver agreement across all centers was excellent for pan-cytokeratin [10HPF: ICC=0.83 and 1HPF: ICC=0.8]. In contrast, assessment of tumor budding on H&E slides reached only moderate agreement [10HPF: ICC=0.58 and 1HPF:

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ICC=0.49]. Based on previous literature and our findings, we recommend (1) pan-cytokeratin staining whenever possible, (2) 10HPF method for resection specimens, and (3) 1HPF method for limited material (preoperative biopsy or pT1). Since tumor budding counts can be used to determine probabilities of relevant outcomes and as such more optimally complement clinical decision making, we advocate the avoidance of cutoff scores.

Keywords Tumor budding · Colorectal cancer · Interobserver · TNM stage · Prognostic factor · Lymph node metastasis

Abbreviations

CRC	Colorectal cancer
H&E	Hematoxylin and eosin
HPF	High power fields
ICC	Intra-class correlation coefficient
ITB	Intra-tumoral budding
r	Pearson correlation coefficient
SAGIP	Swiss Association of Gastrointestinal Pathology
UICC	Union for International Cancer Control
TNM stage	Tumor Node Metastasis stage

Background

Tumor budding in colorectal cancer (CRC) is recognized by the UICC as an important additional prognostic factor [2, 5]. In recent years, the number of studies investigating the clinical outcome of patients with various degrees of tumor budding has rapidly expanded. Consistent associations of tumor budding with poor overall survival and increased probability of lymph node and/or distant metastasis across these studies have been reported [6, 10, 11, 16, 19, 22]. The challenge now lies in the translation of this knowledge into daily diagnostic application.

There are at least three different relevant clinical scenarios in which tumor budding may refine management of CRC patients. First, evidence suggests that tumor budding may help identify patients with submucosally invasive (pT1) tumors or malignant polyps treated by endoscopic resection who may be at "high-risk" for lymph node metastasis [1, 15, 24, 26, 28]. Although lymph node involvement in this setting is low (10–15 %), histomorphological predictors of metastasis are highly sought after to identify patients requiring surgical resection. Second, stage II patients are heterogeneous and comprise subgroups group with dissimilar outcome. Tumor budding has been suggested as an additional indicator for the possible selection of patients for adjuvant therapies [9, 16, 27]. Finally, tumor budding has been detected in preoperative biopsies of patients with rectal cancer and linked to lymphatic vessel invasion and lymph node metastasis, supporting the possible inclusion of tumor budding as a preoperative feature for identifying patients for neoadjuvant therapy [14, 21].

Despite the abundance of evidence highlighting the relevance of tumor budding for CRC patients, the implementation of this feature into histopathology reports has been slow. The reasons for this are first and foremost the lack of studies providing evidence for its interobserver agreement and secondly the absence of collaborative efforts for method standardization. It has been postulated that the use of cytokeratin immunohistochemistry facilitates the visualization of tumor buds and therefore should lead to an improvement of agreement over standard hematoxylin and eosin (H&E) stains [17]. The most comprehensive study to our knowledge investigating these issues was recently published by Puppa and colleagues [20]. Using a digital pathology approach, CRCs from all stages of disease were evaluated using five different scoring methods using conventional H&E and pan-cytokeratin immunostained slides. The methods selected were all based on previously published works and included subjective impression with predominant budding patterns, semiquantitative assessment of budding (low/moderate/high), or using cutoff scores of 10 buds or even three buds depending on the field of vision [6, 16, 25, 27]. Findings from this study showed only a fair degree of agreement with cytokeratin staining facilitating the detection of buds but not leading to any improvement in interobserver agreement.

The aim of this study was to compare the usefulness and interobserver agreement of pan-cytokeratin staining versus conventional H&E staining for the visualization and interpretation of tumor budding in CRC. To this end, we launched a Swiss Association for Gastrointestinal Pathology (SAGIP) study including six independent centers from Switzerland (Bern, Basel, Liestal, Geneva, and Lausanne) and Austria (Feldkirch). We systematically evaluated tumor budding counts and agreement after scoring 10 high power fields (HPF) or a single densest hotspot of tumor buds.

Methods

Patients and selection of tissue blocks

A retrospective cohort of 198 CRC patients treated between 2006 and 2011 at the Insel Hospital, Bern Switzerland provided the case series upon which the study was based. All cases had been grossed and processed according to the national quality guidelines of the Swiss Society of Pathology (SGPath) [3]: A minimum of one tumor block per 1 cm of tumor diameter was embedded for each case including a

minimum of one section each of the transition zone between invasive cancer and normal mucosa, tumor center, invasive front, and relation to the serosa. The histopathological slides were reviewed by an experienced gastrointestinal pathologist (AL) and two residents (VHK and HD). Surgical resection specimens were classified according to the TNM classification of malignant tumors, seventh edition [23]. In order to obtain an even distribution of cases with a wide variation in the number of tumor buds, cases with TNM stage II-IV disease were identified (n=142); 50 of which were randomly selected for further study. Patient characteristics are summarized in Table 1. One representative block from the tumor center of each case was selected for pan-cytokeratin staining based on standardized selection criteria: (a) Full tissue section containing the invasive front with (b) highest degree of budding as estimated during diagnostic review of H&E stained slides. The study design is outlined in Supplemental Fig. 1.

Assay methods

Tissue blocks from all cases were retrieved from the archives of the Institute of Pathology, University of Bern. These surgical resection specimens had been processed according to the guidelines of the Swiss Association of Pathology, fixed in 10 % buffered formalin and paraffin-embedded at the Institute of Pathology, University of Bern, Switzerland. Two 4-μm-thick sections serial sections were cut; one was stained with H&E while the other underwent immunohistochemistry for pan-cytokeratin marker AE1/AE3 (Dako, mouse monoclonal, 1:200, enzyme pretreatment 5 min; DAB chromogen) using a Leica Bond III instrument. Double staining with CD8+ on a hematoxylin background was performed but was not evaluated for this study.

Table 1 Clinicopathological features of the 50 colorectal cancers

Feature		Frequency N (%)
Gender	Male	31 (62.0)
	Female	19 (38.0)
Patient age	Mean, median	68.6, 70.4
Tumor location	Left	20 (40.0)
	Rectum	4 (8.0)
	Right	26 (52.0)
Histological subtype	Adenocarcinoma	40 (80.0)
	Mucinous	6 (12.0)
	Other	4 (8.0)
pT	pT2	2 (4.0)
	pT3	38 (76.0)
	pT4	10 (20.0)
pN	pN0	25 (50.0)
	pN1-2	25 (50.0)
pM	pM0	43 (86.0)
	pM1	7 (14.0)
Lymphatic vessel invasion	L0	10 (20.0)
	L1-2	40 (80.0)
Venous vessel invasion	V0	17 (34.0)
	V1-2	33 (66.0)
Tumor grade	G1-2	33 (66.0)
	G3	16 (32.0)
Perineural invasion	Pn0	44 (88.0)
	Pn1	6 (12.0)

Evaluation of tumor budding

An introductory meeting for all participating centers in Switzerland and Austria (Institutes of Pathology in Liestal, Bern, Geneva, Lausanne, Basel, and Feldkirch) was held, and criteria for scoring were discussed. Tumor budding was defined as single cells or clusters of up to five cells present at the invasive front of CRC. For pan-cytokeratin stained slides, tumor buds needed to show clear cytoplasmic cytokeratin reactivity and a nucleus. Cytoplasmic pseudofragments or areas of necrosis were excluded.

Digital pathology approach and data analysis

All slides (n=100) were scanned using an Aperio Scan Scope platform to a series of mobile hard drives then distributed to each center. Observers were blinded to clinicopathological and patient data and scored H&E and pan-cytokeratin-stained slides separately. Slides were viewed using Aperio Image scope software (Fig. 1a). Tumor budding at the invasive front was first identified using low power magnification. Standardized HPF (HPF field diameter 600 μm, area 1.13 mm^2) were simulated using a fixed size annotation and placed by each observer individually in areas of highest budding density. Tumor buds were then counted in a total of 10HPF, and counts for each HPF were recorded in an Excel file. The average number of tumor buds counted with H&E and pan-cytokeratin-stained sections was calculated and compared for agreement across centers. To obtain a hotspot count for each case, the densest HPF across the 10 fields was utilized.

Statistics

Descriptive statistics were performed for each center and scoring method. Pearson correlation coefficients (r) were used to determine the strength of the linear relationships between two centers and plotted as a correlation matrix. Interobserver agreement was assessed using the intra-class correlation coefficients (ICC), with values approaching 1.0 indicative of more favorable agreement. Analyses were performed using SPSS (Version 21) for descriptive statistics and correlations and with

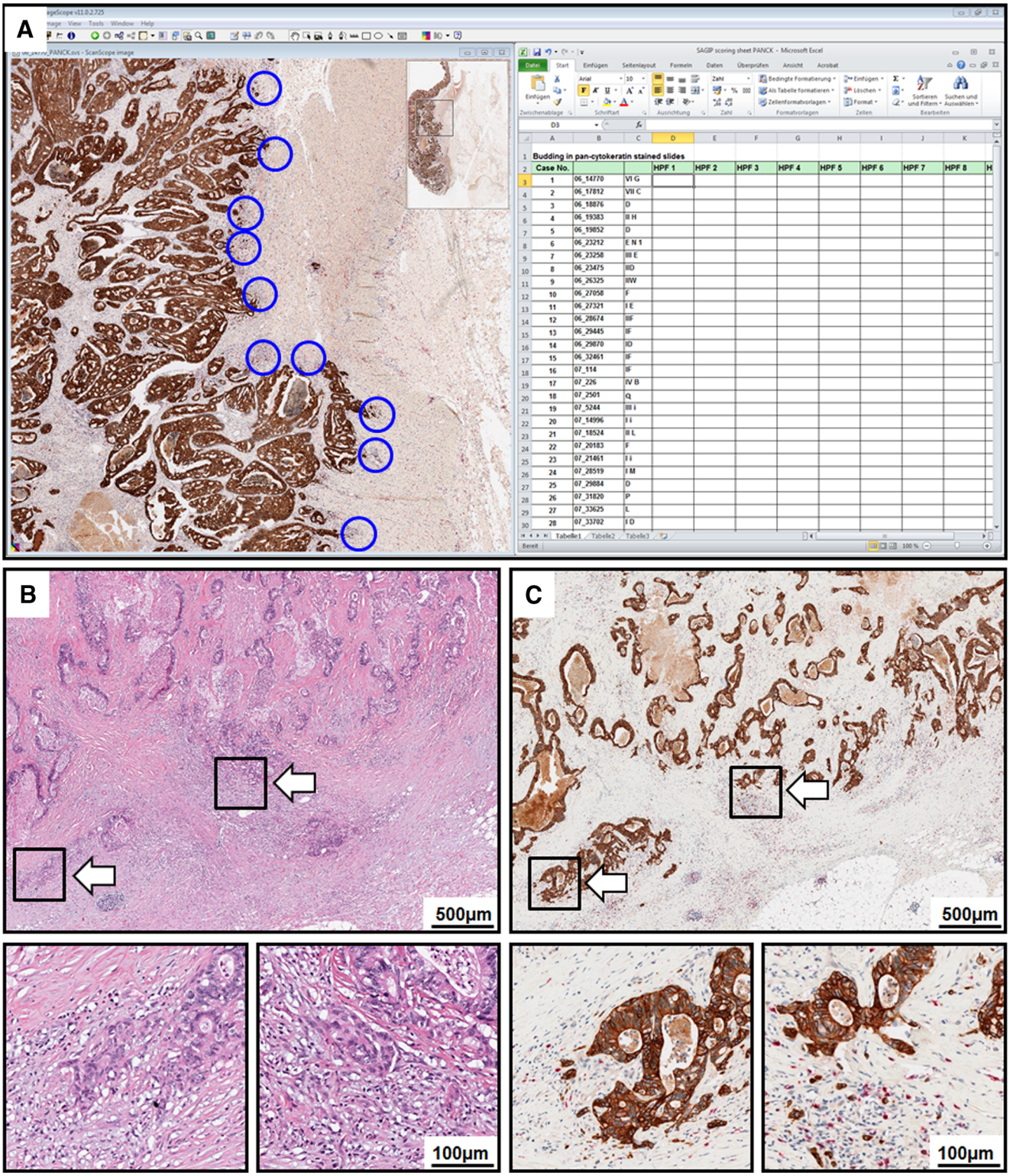


Fig. 1 **a** Digital simulation of tumor budding assessment on a pan-cytokeratin stained slide. Slides were first scanned at low magnification to identify tumor budding hotspots. HPF of standardized size (HPF field diameter 600 µm, area 1.13 mm^2) were simulated using a round fixed size annotation and placed by each observer individually in areas of highest budding density. Tumor buds were then counted in a total of 10HPF. Raw data for each HPF for both HE and pan-cytokeratin were recorded in an Excel file. The average number of tumor buds counted with HE and pan-cytokeratin stained sections was calculated and compared for agreement across centers. **b**, **c** Visualization of tumor budding in colorectal cancer by H&E and pan-cytokeratin stain. Pan-cytokeratin immunohistochemistry facilitates the identification of tumor buds in the peritumoral stroma. Even at high magnification, tumor buds can be hard to differentiate from reactive stromal fibroblasts in a background of peritumoral inflammation and desmoplasia in the standard H&E stain (*insets*)

SAS (Version 9.4 SAS Institute, Cary, NC) for interobserver agreement across multiple centers.

Results

Comparison of pan-cytokeratin and H&E tumor budding counts across six centers

We performed a multi-institutional interobserver study of tumor budding. To this end, each center evaluated 10HPFs of densest budding on 50 H&E slides and 50 matched pan-cytokeratin slides. Raw data for each case per center (6,000 data points total) are provided in Supplemental Table 1. Representative photos of tumor budding are presented in Fig. 1b, c.

Average tumor budding counts across centers ranged from 6.08 to 12.61 using the 10HPF method on pan-cytokeratin stained slides. These values were on average 3.7 times higher than with the 10HPF method on H&E slides (Table 2).

Similar results were observed using a hotspot method. The single densest 1HPF containing tumor buds on a pan-cytokeratin stain ranged from 13.1 to 21.3 in comparison to 2.4 and 10.98 on H&E. Again on the pan-cytokeratin stain 3.3 times more tumor buds were identified in comparison to H&E.

Correlation of tumor budding counts between centers

In order to visualize the relationship between tumor budding scores across all centers, a correlation matrix was performed (Fig. 2). Each center was compared to the five others.

For H&E stained slides assessed using the 10HPF method, correlation coefficients ranged variably from $0.46 \leq r \leq 0.91$, with an overall average of $r=0.64$. The 1HPF method for H&E slides performed markedly worse: with the exception of one comparison (centers 5 and 2) showing $r=0.93$, the remaining correlations had values of 0.37 to 0.69, and overall r of 0.55 (Fig. 2a).

Table 2 Comparison of average 10HPFs and 1 Hotspot counts using pan-cytokeratin (pan-CK) and H&E stains and the fold-change between these 2 methods

	10HPF		Fold-change	1 Hotspot		Fold-change
Centers	pan-CK	H&E		pan-CK	H&E	
1	10.12	3.01	3.36	18.18	6.34	2.87
2	9.12	3.83	2.38	18.18	9.04	2.01
3	9.9	2.03	4.87	18.16	4.38	4.15
4	6.08	0.93	6.57	13.14	2.44	5.39
5	8.65	5.66	1.53	18.66	10.98	1.7
6	12.61	3.84	3.28	21.3	6.12	3.48

For pan-cytokeratin assessment of tumor budding using the 10HPF approach, strong correlations were found throughout with values of $0.73 \leq r \leq 0.95$ and an average overall $r=0.85$. 1HPF performed similarly well with a range of values from 0.74 to 0.93 and an overall correlation coefficient of 0.83 (Fig. 2b).

Interobserver agreement of tumor budding counts

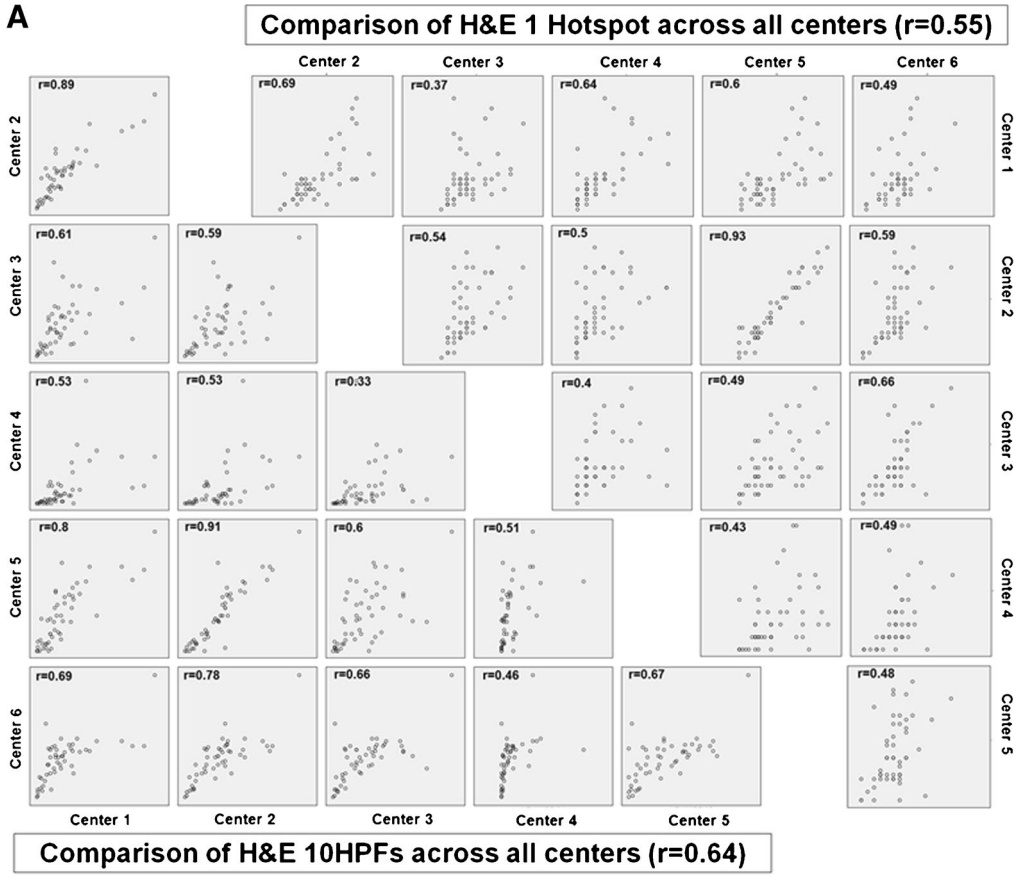
Correlation coefficients as a measure of linear relationship do not represent interobserver agreement. Therefore, to assess the agreement across all centers, the ICC values were calculated. In descending order, ICC values were 0.83, 0.8, 0.58, and 0.49 for pan-cytokeratin in 10HPFs, pan-cytokeratin in one hotspot, H&E in 10HPFs, and H&E in one hotspot.

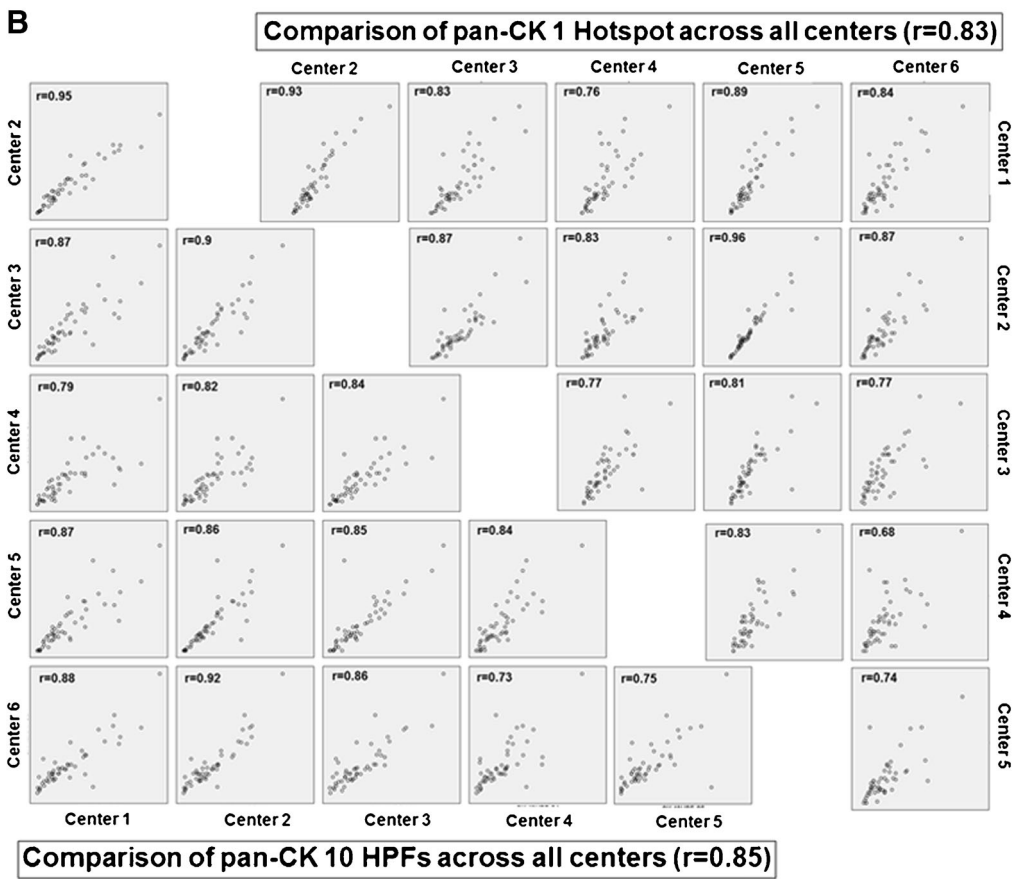
Discussion

We present the first multi-institutional study to investigate interobserver agreement of tumor budding counts using two approaches (10HPF and 1HPF methods) on both conventional H&E and pan-cytokeratin stained slides. Our results clearly demonstrate that the use of pan-cytokeratin in comparison to H&E stained sections (1) facilitates the visualization of tumor buds in CRCs and (2) leads to a marked improvement in interobserver agreement.

In a first step, we compared the average number of tumor buds evaluated by each center using pan-cytokeratin and H&E stains. The blocks for the evaluation of tumor budding were selected based on standardized criteria in a diagnostic scenario: assessment of routine CRC cases grossed according to national quality guidelines. One block containing the (a) invasive front with (b) the highest degree of budding as suspected during standard diagnostic slide review was chosen for the quantification of tumor buds in H&E and immunohistochemistry. Regardless of the approach, either 10HPFs or 1HPF, counts on pan-cytokeratin stains resulted in more than three times the number of tumor buds in comparison to H&E stains. This result underlines the difficulty in assessing budding using conventional slides, particularly under the following scenarios: dense peritumoral inflammation which may obscure the differentiation of cancer cells from other cell types like histiocytes and activated fibroblasts or fragmentation of neoplastic glandular structures mimicking the presence of tumor budding [13]. Nonetheless, caution should still be used for interpretation. The assessment of immunohistochemistry can be complicated by the presence of cytokeratin-positive microvesicles and membrane fragments, which may lead to overcounting. It is therefore important to verify the presence of a nucleus for the identification of a tumor bud.

In a second step, we found strong linear correlations between each center using pan-cytokeratin stains. Moreover, the


A
Comparison of H&E 1 Hotspot across all centers (r=0.55)
Center 2
Center 3
Center 4
Center 5
Center 6
r=0.89
r=0.69
r=0.37
r=0.64
r=0.6
r=0.49
r=0.61
r=0.59
r=0.54
r=0.5
r=0.93
r=0.59
r=0.53
r=0.53
r=0.33
r=0.4
r=0.49
r=0.66
r=0.8
r=0.91
r=0.6
r=0.51
r=0.43
r=0.49
r=0.69
r=0.78
r=0.66
r=0.46
r=0.67
r=0.48
Center 1
Center 2
Center 3
Center 4
Center 5
Comparison of H&E 10HPFs across all centers (r=0.64)


B
Comparison of pan-CK 1 Hotspot across all centers (r=0.83)
Center 2
Center 3
Center 4
Center 5
Center 6
r=0.95
r=0.93
r=0.83
r=0.76
r=0.89
r=0.84
r=0.87
r=0.9
r=0.87
r=0.83
r=0.96
r=0.87
r=0.79
r=0.82
r=0.84
r=0.77
r=0.81
r=0.77
r=0.87
r=0.86
r=0.85
r=0.84
r=0.83
r=0.68
r=0.88
r=0.92
r=0.86
r=0.73
r=0.75
r=0.74
Center 1
Center 2
Center 3
Center 4
Center 5
Comparison of pan-CK 10 HPFs across all centers (r=0.85)

◀ **Fig. 2** Comparison of tumor budding scores across all 6 centers using H&E (**a**) and pan-cytokeratin stains (**b**) in a correlation matrix. Correlation coefficients (*r*) for each comparison are *inset*. On the *left*, the linear relationship using 10HPFs between centers is depicted, while on the *right* the relationship using 1HPF

overall correlation coefficients and intra-observer agreements were similar for the 10HPF method (r=0.85; ICC=0.83) and 1HPF method (r=0.83; ICC=0.8). In a previous investigation by our group, we compared seven different scoring methods for tumor budding counts after pan-cytokeratin staining including methods proposed by Hase, Nakamura, Wang, Ueno, and a 10HPF as well as 1HPF approach [7]. In this study, findings from three independent observers identified the 10HPF (ICC=0.91) and 1HPF (ICC=0.83) methods as most reproducible in addition to having similar values. Agreement was markedly less favorable for assessment of tumor budding counts after H&E staining. The distribution of scores between centers was considerably more dispersed and led to ICC values of only 0.58 for 10HPF and 0.49 for the single hotspot. These results clearly underline the benefit of pan-cytokeratin stains for the assessment of tumor budding in CRCs.

Our study offers novel insight into the assessment of tumor budding. The interobserver agreement using a 10HPF and 1HPF method by pan-cytokeratin was similar. This result underlines the possible application of tumor budding to the preoperative biopsy specimen, which usually provides only a limited amount of tumor material. Intra-tumoral budding (ITB), i.e., budding within the main tumor body, is highly correlated with budding counts found at the invasion front [12] and therefore unsurprisingly ITB has been associated with lymphatic vessel invasion, lymph node metastasis, unfavorable overall survival, and low rates of tumor regression after neoadjuvant therapy [4, 14, 21]. Recently, we showed that the number of intra-tumoral buds directly correlates with the probabilities for regional and distant metastases [29]. This alternative method for describing the impact of tumor budding on patient outcome was preferred over scores assigned by a cutoff (e.g. low/high). Firstly, assigning probabilities to raw counts of tumor budding may add more value to the clinical discussion on patient management, giving more flexibility to treating physicians. Secondly, using cutoff scores to determine high- or low-grade budding counts may yield more harm than good: a single bud on either side of the cutoff could lead to dramatically different decision. Additionally, the use of cutoffs has a significant detrimental effect on interobserver agreement as was seen when using an average of 10 buds across 10HPFs [8]. This may also be the explanation for the differences seen in this study as compared to the one conducted by Puppa and colleagues who, using pan-cytokeratin stains, included scoring systems based solely on categorical grading schemes [20]. Thirdly, it is clear that the more buds are detected at the invasion front, the worse is the patient outcome and the greater the likelihood of metastasis regardless of the scoring system [6, 15, 16, 18, 19, 27].

This multi-institutional study on the assessment of tumor budding in CRC demonstrates a significant advantage for pan-cytokeratin staining and demonstrates that high rates of interobserver agreement can be achieved independent of whether a 10HPF or hotspot method is used for assessment. Strong interobserver reproducibility is an essential prerequisite for implementation of any histomorphological prognostic factor in diagnostic practice. Therefore, we suggest the following: (1) pan-cytokeratin staining for the assessment of tumor budding wherever possible, (2) a 10HPF method for assessment of budding in resection specimens, (3) a 1HPF method for

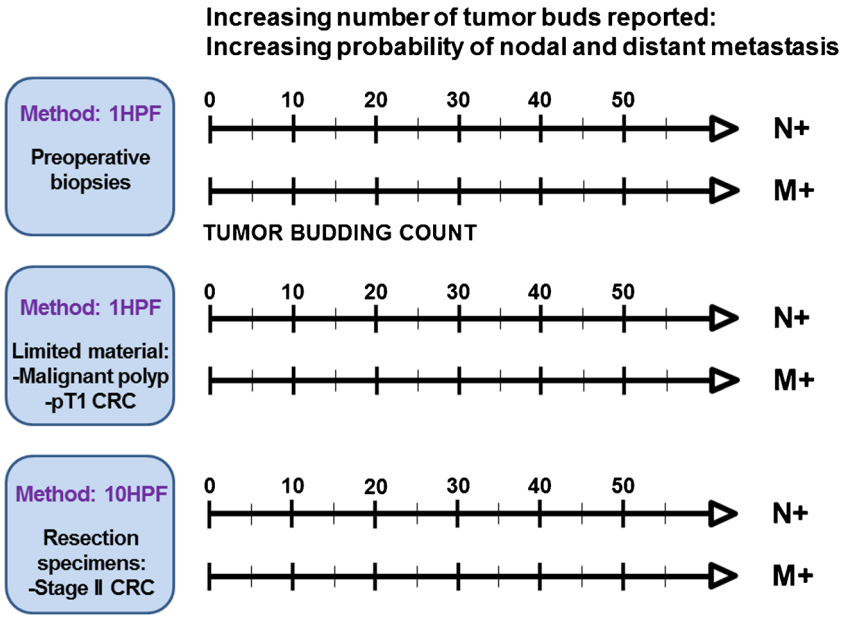


Fig. 3 Theoretical probability scales based on, for example, logistic regression or Cox regression models to determine the likelihood of certain outcomes using the actual number of tumor buds in preoperative biopsies, early invasive CRC, and resection specimens

assessment of budding in the preoperative biopsy, and (4) the avoidance of cutoff scores. This methodological approach will help to provide reliable data for risk assessment of CRC patients in the pathology report and may impact on clinical management in defined scenarios.

Conclusions

The prognostic relevance of tumor budding in CRC patients has consistently been demonstrated. We foresee that the actual tumor budding counts, which can be used to determine the probabilities of relevant outcomes such as lymph node metastasis, distant metastasis, or survival endpoints, will contribute significantly to clinical decision making. Figure 3 outlines a theoretical unifying approach for future implementation of tumor budding as a continuous feature by the histopathologist: As shown in previous studies, small preoperative biopsy fragments presence of tumor budding may be best assessed by the 1HPF method. Based on tumor budding counts, a defined risk estimate for nodal and distant metastasis can be reported to the clinician to support personalized patient management in the preoperative setting (e.g. rectal cancer). In pT1 CRC and malignant polyps which provide a limited amount of material, tumor budding counts as assessed by a 1HPF hotspot method may allow precise risk estimation of nodal and distant metastatic relapse. Together with well-based risk indicators such as lymphovascular invasion, invasion depth, and margin status, this information on tumor budding may support the clinician when considering segmental resection. Last, tumor budding as assessed by the 10HPF method in a resection specimen is a proven prognostic indicator for stage II CRC patients. In this scenario, tumor budding counts may help to guide clinical follow up and support personalized decisions for adjuvant therapy on an interdisciplinary basis.

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Authors contributions VHK obtained, reviewed, and categorized histopathological data, scored tumor budding, performed data interpretation, and together with IZ drafted the manuscript; IZ obtained and categorized clinicopathological data, performed data interpretation and statistical analysis. AL reviewed cases and together with VHK conceived the study and study design and performed manuscript editing; MDB, GC, KD, FO, GP, WS, and LT scored the cases, and reviewed and approved the final manuscript. HD obtained, reviewed, and categorized histopathological data, reviewed and approved the final manuscript. MDB, DI, MH, and BS obtained, reviewed, and categorized clinical data, and reviewed and approved the final manuscript. All authors have read and given approval of the final manuscript.

Compliance with ethical standards The use of patient material has been approved by the local ethics committee of the Insel University Hospital (16-03-12).

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