The Journal of Pathology: Clinical Research
J Path: Clin Res July 2017; **3:** 171–178
Published online 12 June 2017 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/cjp2.73

Original Article

Cytokeratin-based assessment of tumour budding in colorectal cancer: analysis in stage II patients and prospective diagnostic experience

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Abstract

Tumour budding in colorectal cancer is an important prognostic factor. A recent consensus conference elaborated recommendations and key issues for future studies, among those the use of pan-cytokeratin stains, especially in stage II patients. We report the first prospective diagnostic experience using pan-cytokeratin for tumour budding assessment. Moreover, we evaluate tumour budding using pan-cytokeratin stains and disease-free survival (DFS) in stage II patients. To this end, tumour budding on pan-cytokeratin-stained sections was evaluated by counting the number of tumour buds in 10 high-power fields (0.238 mm^2), then categorizing counts as low/high-grade at a cut-off of 10 buds, in two cohorts. Cohort 1: prospective setting with 236 unselected primary resected colorectal cancers analysed by 17 pathologists during diagnostic routine. Cohort 2: retrospective cohort of 150 stage II patients with information on DFS. In prospective analysis of cohort 1, tumour budding counts correlated with advanced pT, lymph node metastasis, lymphovascular invasion, perineural invasion (all $p < 0.0001$), and distant metastasis ($p = 0.0128$). In cohort 2, tumour budding was an independent predictor of worse DFS using counts [$p = 0.037$, HR (95% CI): 1.007 (1.0–1.014)] and the low-grade/high-grade scoring approach [$p = 0.02$; HR (95% CI): 3.04 (1.2–7.77), 90.7 versus 73%, respectively]. In conclusion, tumour budding assessed on pan-cytokeratin slides is feasible in a large pathology institute and leads to expected associations with clinicopathological features. Additionally, it is an independent predictor of poor prognosis in stage II patients and should be considered for risk stratification in future clinical studies.

Keywords: tumour budding; colorectal cancer; cytokeratin; prognosis; pathology

Received 14 February 2017; Accepted 6 June 2017

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, royalties or patents received or pending.

Introduction

Tumour budding in colorectal cancers and other gastrointestinal tumours (pancreas, oesophagus) is recognized as an important prognostic factor [1]. In April 2016, a panel of international experts met in Bern, Switzerland to discuss the issue of the diagnostic implementation of tumour budding for colorectal cancer and outlined a set of recommendations based on H&E stains and a three-tiered scoring system [2]. These recommendations were meant as a first step in a dynamic process which over time would yield more evidence regarding some key issues, especially the feasibility of pan-cytokeratin stains in general and clinical relevance specifically in stage II patients.

A Swiss Association of Gastrointestinal Pathology study published in 2016 focused on the inter-observer reproducibility of budding counts on H&E and pan-cytokeratin (AE1/AE3) sections across six different institutes [3]. Several take-home messages were outlined: (1) tumour budding counts were three to six times greater upon pan-cytokeratin staining compared to H&E, (2) the inter-observer reproducibility was markedly improved with pan-cytokeratin staining compared to H&E and (3) a continuous count of the number of tumour buds in 1 or 10 hotspots outperformed a categorical scoring system using a low/high-grade classification. Other authors have also underlined excellent inter- and/or intra-observer agreement with pan-cytokeratin staining in colon, oesophagus, pancreas, and breast cancer as a fast method for tumour budding assessment [4–7]. Based on these studies, the Institute of Pathology, University of Bern has implemented tumour budding counts into all diagnostic reports of primary resected colon cancers from 2013 to 2016 using the 10-hotspot method on pan-cytokeratin.

Only a few studies to date have evaluated the association of tumour budding with clinicopathological data in the highly relevant subgroup of stage II patients using pan-cytokeratin stains [1]. Recent data suggest that stage II colorectal cancers are a particularly heterogeneous group of patients deriving little benefit from post-operative chemotherapy in an unselected setting [8]. Tumour budding has a major impact on the prognostic stratification of patients with this disease and may be an important parameter to identify high-risk patients [9]. Indeed, several studies have shown the impact of tumour budding on overall survival, but few have addressed disease-free survival (DFS) in stage II and only one until now has evaluated DFS and tumour budding with pan-cytokeratin stains.

The aim of this study was to determine the performance of pan-cytokeratin staining for the assessment of tumour budding using a continuous count and a low/high-grade scoring system in two different relevant scenarios: (1) in diagnostic practice, prospectively after more than 2 years of implementation as a routine and (2) in a retrospective stage II collective with the endpoint of DFS.

Methods

Prospective cohort (Institute of Pathology, University of Bern, Switzerland)

From 2013 to 2016, 17 board certified pathologists, including six gastrointestinal experts, evaluated tumour budding counts on pan-cytokeratin (AE1/AE3) stained sections of 236 surgically treated colorectal cancers as part of daily diagnostic routine. Peritumoural budding was defined as single cells or small clusters of less than five cells ahead of the invasive front. An average of six tumour blocks was available per case (median: 5; range 1–14). The block containing the largest number of buds was selected on H&E staining and was immunostained for AE1/AE3 (AE1/AE3; Dako, mouse monoclonal, 1:200, enzyme pre-treatment for 5 min, DAB chromogen) on a Leica Bond III instrument. After scanning the tumour border at low power, 10 high-power fields (40× HPFs, 0.238 mm^2) of densest tumour budding at the invasion front were scored and the total count of tumour buds was recorded [4]. This number along with the following clinicopathological information were extracted from pathology reports: patient gender, age at diagnosis, tumour size and location, histological subtype, pT, pN, pM (UICC seventh edition), lymphatic (L) and venous (V) invasion, perineural invasion (Pn), tumour grade, the number of positive lymph nodes and the percentage of expansive (pushing) tumour border. A pan-cytokeratin stain for tumour budding is shown in Figure 1. This cohort was used to determine whether tumour budding counts on AE1/AE3 in daily diagnostic practice reflects the expected associations of higher counts with more unfavourable prognostic features.

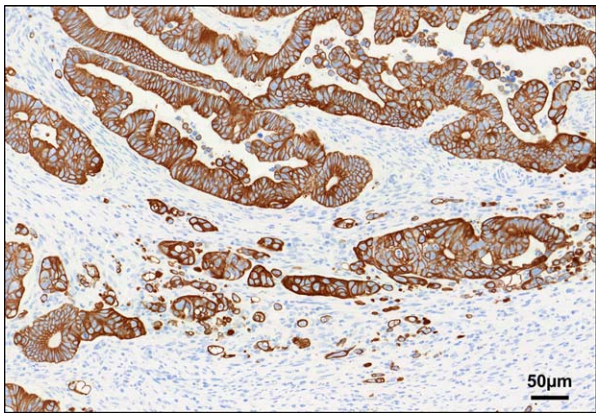


Figure 1. Pan-cytokeratin staining of colorectal cancer, highlighting epithelial cells in brown along with numerous tumour buds at the invasion front.

Retrospective cohort (Mount Sinai Hospital, Toronto, Canada)

Case selection included consecutive cases ($n = 181$) of primary colonic tumours and upper rectal tumours located above the peritoneal reflection in their entirety with sufficient archival material. All patients were treated at the Mount Sinai Hospital between 1992 and 2010. Thirty-one

patients were excluded. Of these, 26 patients were excluded due to death within 6 months of surgery or follow-up of less than 3 years. Further, three pathological stage II patients had clinical evidence of metastatic disease unknown to the pathologist. One patient had a tumour in the pancreas which was thought clinically to represent a second primary rather than a metastasis but this was not proven. One patient had lung metastases within 2 months of diagnosis and was likely to have had stage IV disease from the outset. Final patient number was 150. All slides and gross descriptions were re-reviewed according to the UICC TNM seventh edition by one gastrointestinal pathologist (AG). Clinical information was retrieved from patient records. Mean and median follow up were 63 and 62 months, respectively (min and max: 7–176 months). No patients received neoadjuvant chemotherapy. Patients were followed up in accordance with Cancer Care Ontario recommendations. Specimens were fixed in 10% neutral buffered formalin. Gross assessment and dissection was performed in accordance with standard protocols. An average of six tumour blocks was sampled per case (median: 5; range 1–16). Serial sections were cut at 4-μm from archival tissue blocks and stained for H&E and pan-cytokeratin (AE1/AE3; Dako, mouse monoclonal, 1:200, enzyme pre-treatment 5 min, DAB chromogen; using a Leica Bond Rx instrument). Tumour budding was scored in the same manner as for the prospective cohort, namely in 10 HPFs (40×, 0.238 mm^2) of densest tumour budding at the invasion front. A total count across these 10 fields was then obtained, until 250 buds. Four pathologists or pathologists-in-training scored tumour budding on 59 cases to determine the inter-observer agreement (RK, HD, AG, BM). The aim of this cohort was to determine the effect of tumour budding using the 10 HPF method on pan-cytokeratin on disease-free survival in stage II patients.

Inter-observer variability in block selection

To address inter-observer variability in block selection, a random set of 10 cases from cohort 1 was selected. Two gastrointestinal pathologists (AL and HD) independently reviewed the diagnostic H&E slides and selected the optimal block for budding assessment in a blinded fashion. For discrepant cases, both pathologists were asked to score budding in 10 HPF using a cytokeratin-stained slide to investigate whether the selection of different blocks would strongly impact the budding score.

Statistics

This study was designed in accordance with the REporting recommendations for tumour MARKer prognostic studies (REMARK) [10]. Disease-free survival (DFS) was defined as the time from surgical resection to local or distant recurrence or death, whichever occurred first. Descriptive statistics were performed for all budding counts. Pearson's correlation coefficient was used to determine the strength of the linear relationship between budding counts and continuous normally distributed data (r). The association of tumour budding as a continuous variable with categorical endpoints was analysed with the Wilcoxon Rank Sum Test and with logistic regression. Odds ratios (OR) and 95% confidence intervals (CI) were used to determine effect size with the least aggressive feature used as baseline (OR 1.0). Receiver operating characteristic (ROC) curve analysis and area under the curve (AUC) were used to determine the discriminatory power of tumour budding for the binary endpoint. The intraclass correlation coefficient (ICC) and kappa value (κ) were used to determine the inter-observer agreement in counts and categories, respectively. The Kaplan-Meier method was used to represent survival curves and the log-rank test was used to test significant survival time differences. Multivariate survival time models were analysed using continuous budding counts with the hazard ratio (HR) of 1.0 as baseline and 95% confidence intervals (CI). The Chi-Square or Fisher's Exact tests were used where appropriate. Analyses were performed using SPSS (Version 21) and with SAS (Version 9.4 SAS Institute, Cary, NC). P values <0.05 were considered statistically significant.

Ethics approval

According to the Swiss Law for Research on Humans (Human Research Act, article 2, section 1c) the anonymously collected data of the prospective cohort (Institute of Pathology, University of Bern, Switzerland) does not fall under the scope of the Human Research Act. Research ethics board approval was obtained from Mount Sinai Hospital, Toronto for analysis of the retrospective cohort (Mount Sinai Hospital, Toronto, Canada).

Results

Prospective cohort

Two hundred and thirty-six cases were analysed. Patient characteristics are found in Table 1. The average number of tumour buds was 76.9 across 10 HPFs

Table 1. Associations between tumour budding counts and categories, and clinicopathological features, assessed prospectively during daily diagnostic routine on pan-cytokeratin slides

			Continuous counts			Freq N (%)		
Feature		No.	OR (95% CI)	*P* value	AUC	Low $n = 163$ (69.1)	High $n = 73$ (30.9)	*P* value
Gender	Male	144	1.0	0.3518	0.518	95 (58.3)	49 (67.1)	0.198
	Female	92	0.998 (0.993–1.002)			68 (41.7)	24 (32.9)	
Tumour location	Left + rectum	130	1.0	0.8571	0.502	90 (58.4)	40 (54.8)	0.6039
	Right	97	1.0 (0.996–1.005)			64 (41.6)	33 (45.2)	
Histological subtype	Adenocarcinoma	207	1.0			141 (86.5)	66 (90.4)	0.398
	Mucinous/other	29	0.998 (0.991–1.005)	0.544	0.53	22 (13.5)	7 (9.6)	
pT	pT1-2	59	1.0	<0.0001	0.71	55 (40.7)	4 (11.4)	0.0012
	pT3-4	177	1.019 (1.01–1.028)			108 (59.3)	69 (88.6)	
	pT3	111	1.0	0.0012	0.659	80 (74.1)	31 (44.9)	<0.0001
	pT4	66	1.009 (1.003–1.014)			28 (25.9)	38 (55.1)	
pN	pN0	135	1.0	<0.0001	0.7258	119 (79.3)	37 (50.7)	<0.0001
	pN1-2	87	1.011 (1.006–1.016)			31 (20.7)	36 (49.3)	
V	V0	142	1.0	<0.0001	0.7092	116 (72.1)	26 (36.6)	<0.0001
	V1-2	90	1.013 (1.008–1.018)			45 (28.0)	45 (63.4)	
L	L0	106	1.0	<0.0001	0.6858	88 (54.7)	18 (24.7)	<0.0001
	L1-2	128	1.013 (1.007–1.018)			73 (45.3)	55 (75.3)	
Pn	Pn0	175	1.0	<0.0001	0.742	137 (86.2)	38 (53.5)	<0.0001
	Pn1	55	1.015 (1.009–1.021)			22 (13.8)	33 (46.5)	
R	R0	213	1.0	0.0852	0.595	149 (96.1)	64 (90.1)	0.0727
	R1-2	13	1.007 (0.999–1.016)			6 (3.9)	7 (9.9)	
Grade	G1-2	187	1.0	0.9498	0.514	130 (83.9)	57 (82.6)	0.8143
	G3	37	1.0 (0.994–1.006)			25 (16.1)	12 (17.4)	
pM	pM0	215	1.0	0.0128	0.6872	153 (93.9)	59 (80.8)	0.0022
	pM1	21	1.009 (1.003–1.015)			10 (6.1)	14 (19.2)	
Age		236	−0.057	0.3794	–	113.6*	120.7*	0.4571
Tumour size		222	0.05	0.4275	–	110.3*	112.5*	0.8136
No. pos LN		221	0.31	<0.0001	–	101.1*	132.8*	<0.0001
Expansive TBC		213	−0.44	<0.0001	–	67.6*	125.5*	<0.0001

*Test statistics from rank sum test.
pT, pathological T stage (TNM); pN, pathological lymph node status (TNM); V, venous invasion; L, lymphatic invasion; Pn, perineural invasion; R, resection status; pM, pathological evidence of metastasis; No. pos LN, Number of positive lymph nodes; TBC, tumour border configuration.

(average 7.69 buds/HPF). The distribution of tumour buds found across all patients ranged from 0 to 300.

Continuous counts of tumour buds

Table 1 highlights the OR (95% CI) for tumour budding and clinicopathological features. There was no association between budding counts and age, gender, tumour size, tumour location or histological subtype of the tumour, nor with tumour grade, or resection margin status. A significant and strong association between a higher count of tumour buds was observed with more advanced pT stage (pT1/2 versus pT3/4, $p < 0.0001$; AUC = 0.7555; pT3 versus pT4, $p = 0.0012$), lymph node metastasis ($p < 0.0001$; AUC = 0.7258) and the number of positive lymph nodes ($p < 0.0001$, $r = 0.31$), venous ($p < 0.0001$; AUC = 0.7092) and lymphatic invasion ($p < 0.0001$; AUC = 0.6858), perineural invasion ($p < 0.0001$; AUC = 0.742) and distant metastasis ($p = 0.0128$; AUC = 0.6872; Figure 2). An inverse correlation between the percentage of expansive tumour border (pushing border) and tumour budding counts was observed ($p < 0.0001$; $r = -0.44$).

Cut-off low/high-grade budding

Using a previously published cut-off of 10 buds to assign a low- and high-grade budding score, 163 patients (69.1%) were considered low-grade and 73 (30.9%) were found to have high-grade budding. Associations between the same features were observed (Table 1), namely with pT stage [$p < 0.0001$; OR (95% CI: 8.78 (3.05–25.3)], lymph node metastasis [$p < 0.0001$; OR (95% CI): 3.74 (2.04–6.84)], lymphatic invasion [$p < 0.0001$; OR (95% CI): 3.68 (1.99–6.82)], vascular invasion [$p < 0.0001$; OR (95% CI): 4.46 (2.66–8.07)], perineural invasion ($p < 0.0001$; OR (95% CI): 5.41 (2.82–10.34)], distant metastasis [$p = 0.0022$; OR (95% CI): 3.63 (1.53–8.63)], the number of positive lymph nodes and inversely with the percentage of expansive tumour border (all *P* values <0.005).

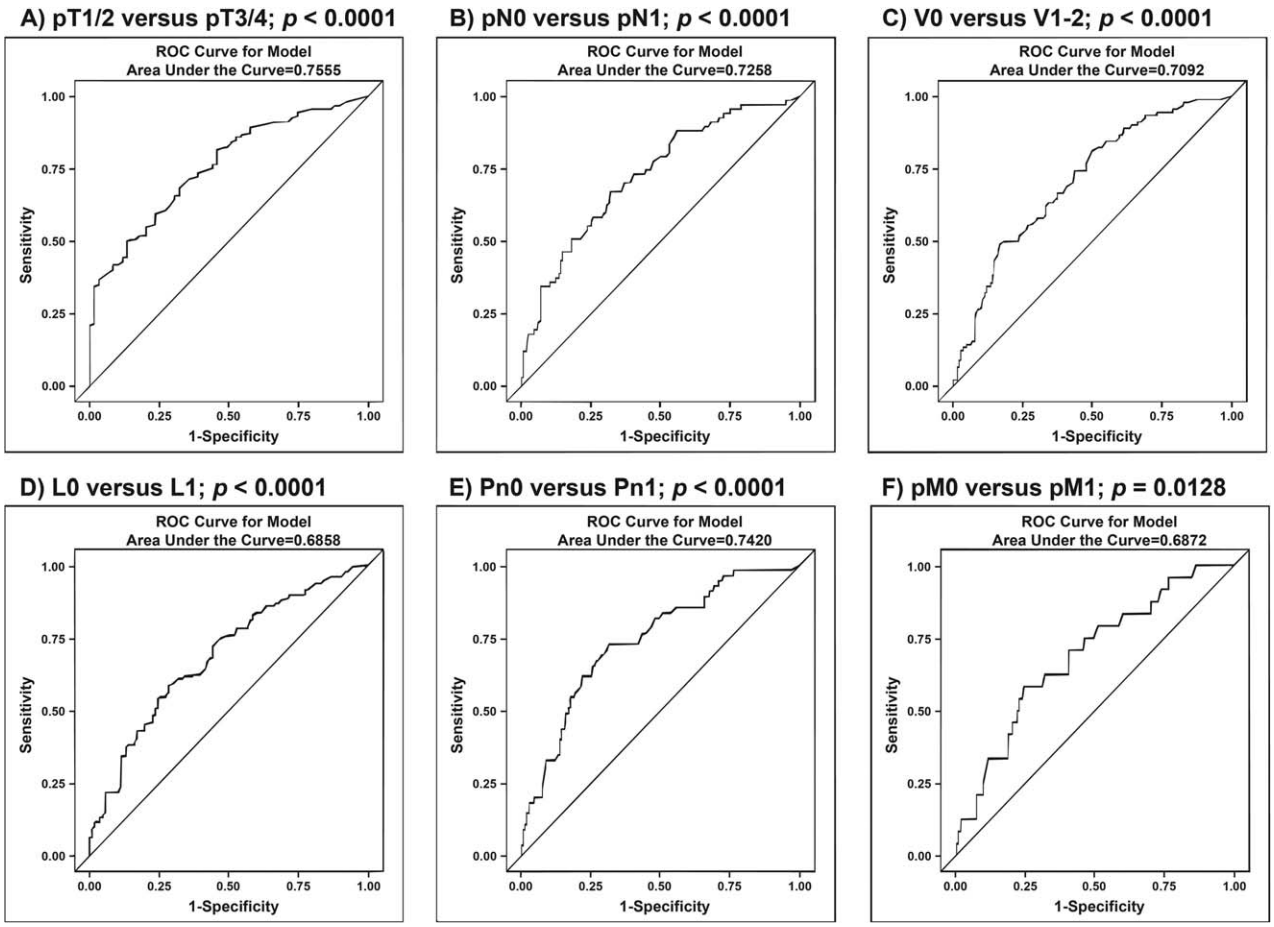


Figure 2. Receiver Operating Characteristic (ROC) curve analysis for tumour budding scores in 10 high power fields using pan-cytokeratin slides. ROC-curves and corresponding area under the curve values for the identification of patients with (A) advanced tumour stage; (B) presence of nodal metastasis; (C) presence of venous invasion; (D) lymphatic invasion; (E) perineural invasion; and (F) distant metastatic disease are shown.

Table 2 compares the performance of both the continuous scores and cut-off scores regarding goodness-of-fit of the logistic regression models used to analyse these results. A lower Akaike Information Criterion (AIC) is interpreted as a better model fit and may translate into more reliable data. In all cases, the AIC values were lower in the analyses using a continuous budding count, implying a more reliable model than using cut-off scores.

Retrospective stage II cohort

The 10 HPF method was applied to determine tumour budding counts on 150 stage II patients. The number of tumour buds across 10 HPFs was 71.1 (average: 7.11 buds per HPF), the median bud count across 10 HPF was 53.5.

Continuous counts of tumour budding

The ICC value used to measure the inter-observer agreement of tumour budding counts for four observers was 0.79. No associations between continuous tumour budding counts or low-/high-grade budding scores were noted with gender, pT3/pT4, tumour location, tumour grade, perforation and the presence of extra-mural venous invasion (EMVI), although numbers for the latter three features are likely underpowered (Table 3).

Table 2. Comparison of goodness-of-fit (continuous and cut-off scores) of models of tumour budding on pan-cytokeratin

	Continuous budding counts	Cut-off at 10 buds
Feature	AIC	AIC
pT	229.309	243.426
pN	245.698	258.038
V	287.039	288.06
L	301.255	307.341
Pn	226.058	229.905
pM	150.423	150.561

AIC, Akaike Information Criterion; pT, pathological tumour stage (TNM); pN, pathological lymph node status (TNM); V, venous invasion; L, lymphatic invasion; Pn, perineural invasion; pM, pathological evidence of metastasis (TNM).

Table 3. Association between tumour budding counts and categories with clinicopathological features in a retrospective stage II cohort assessed on pan-cytokeratin slides

Feature		Freq (%)	Buds Mean	*P* value	Buds Freq (%) LG	Buds Freq (%) HG	*P* value
Gender	Female	62 (41.3)	70.1	0.8772	45 (43.3)	16 (36.4)	0.4353
	Male	88 (58.7)	71.8		59 (56.7)	28 (63.6)	
pT	pT3	127 (85.2)	69.9	0.8076	89 (86.4)	36 (81.8)	0.475
	pT4 (a/b)	22 (14.8)	80.7		14 (13.6)	8 (18.2)	
Location	Right	75 (50.0)	80.4	0.2026	49 (47.1)	24 (54.6)	0.4086
	Left	75 (50.0)	61.8		55 (52.9)	20 (45.5)	
Tumour grade	G1-2	138 (92.0)	68.5	0.1712	98 (94.2)	40 (90.9)	0.4618
	G3	12 (8.0)	100.4		6 (5.8)	4 (9.1)	
Perforation	No	143 95.3)	71.8	0.4435	99 (95.2)	42 (95.5)	1.0
	Yes	7 (4.7)	56.3		5 (4.8)	2 (4.6)	
EMVI	No	121 (86.4)	70.0	0.1467	85 (87.6)	34 (82.9)	0.4638
	Yes	19 (13.6)	86.2		12 (12.4)	7 (17.1)	

LG, low grade; HG, high grade; pT, pathological tumour stage (TNM); G, grade; EMVI, extra-mural venous invasion.

Nonetheless, patients with greater counts of tumour buds had a significantly shorter disease-free survival time in univariate [$p = 0.0382$, HR (95% CI): 1.006 (1.0–1.011)] and multivariable [$p = 0.037$; HR (95% CI): 1.007 (1.0–1.014)] analysis, adjusting for gender, pT, tumour location, and EMVI (Table 4).

Cut-off low/high-grade budding

The kappa values used to measure the inter-observer agreement between observers ranged from 0.61 to 0.83 in pairwise comparisons. Overall, agreement between all four observers was $\kappa = 0.7$ (0.51–0.87). Using a cut-off score of 10 buds across 10 HPFs, we found 104 (69.3%) patients with low-grade and 46 patients (30.7%) with high-grade budding. There were no associations between tumour budding and any of the clinicopathological features evaluated. However, a significantly lower 5-year DFS was found in patients with high-grade budding (73%) compared to low-grade budding (90.7%; $p = 0.0095$; Figure 3). Tumour budding status was again found to be an independent prognostic factor in multivariable analysis [$p = 0.02$; HR (95% CI): 3.04 (1.2–7.77); Table 4] adjusting for potential confounders: gender pT, tumour location, and EMVI.

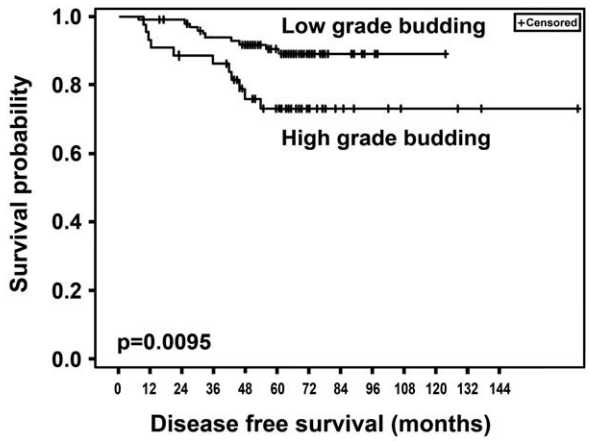
Inter-observer variability in block selection

Two gastrointestinal pathologists (AL and HD) independently reviewed the diagnostic H&E slides and selected the optimal block for budding assessment in a randomly selected set of 10 cases from cohort I. The observers selected the same block for budding assessment in 9 out of 10 cases. One case was discordant. For this case, both pathologists were asked to score budding in 10 HPF using a cytokeratin-stained slide to investigate whether the selection of different blocks would strongly impact the budding score. Adjusting for visual field size, final scores

Table 4. Multivariable analysis of disease-free survival in stage II patients with tumour budding counts and categories

Feature		Counts HR (95% CI)	Counts *P* value		Cutoff HR (95% CI)	Cutoff *P* value
Budding	Baseline	1.0	0.037	LG	1.0	0.02
	Bud count	1.007 (1.0–1.014)		HG	3.04 (1.2–7.77)	
Gender*	Male	1.0	0.178	Male	1.0	0.24
	Female	0.49 (0.17–0.38)		Female	0.54 (0.19–1.52)	
pT*	pT3	1.0	0.465	pT3	1.0	0.366
	pT4	1.55 (0.48–5.0)		pT4	1.7 (0.54–5.42)	
Tumour location*	Right	1.0	0.355	Right	1.0	0.544
	Left	1.37 (0.44–4.03)		Left	1.35 (0.5–3.51)	
EMVI	No	1.0	0.855	V0	1.0	0.864
	Yes	1.13 (0.31–4.06)		V1	1.12 (0.31–4.0)	

*Gender, pT and tumour location were all associated with disease-free survival in univariate analysis.
HR, hazard ratio; LG, low grade; HG, high grade; pT, pathological tumour stage (TNM); V, venous invasion; EMVI, extra-mural venous invasion.



Low grade No. at risk	103	102	100	91	85	66	24	9	3	1	1	0	0
High grade No. at risk	44	40	38	37	27	22	12	7	5	3	3	2	1

Figure 3. Kaplan-Meier curve and log-rank test highlighting survival time differences in patients with low- and high-grade tumour budding on pan-cytokeratin staining in 150 stage II colorectal cancer patients.

correlated well, with an average of 9.8 buds and 11.5 buds per HPF, respectively.

Discussion

The novel findings of this study underscore that the use of pan-cytokeratin (AE1/AE3) immunostaining for the evaluation of tumour budding is feasible in daily diagnostic practice and that DFS in stage II patients can be stratified by tumour budding assessed using this method.

As tumour budding on slides immunostained for pan-cytokeratin evaluated using a 10 HPF method [4] has been used at our institute for more than 2 years, we have for the first time the opportunity to determine the effect of tumour budding in a prospective setting. An association between higher counts of tumour buds, as recorded in diagnostic reports, and more aggressive tumour features was seen, as expected. The use of cytokeratin for the assessment of tumour budding has several advantages over H&E staining. First, immunostaining unmasks three to six times more tumour buds than H&E, better reflecting the biology of the tumour at the invasion front [3]. Fewer buds are being missed in regions of dense peritumoural inflammation and a better discrimination from activated fibroblasts can be made. Pathologists who are less experienced with tumour budding feel more confident with immunostaining and the overall impression given by pathologists is that it is quicker. Reliability is objectively reflected in the greater inter-observer agreement that can be reached with cytokeratin in comparison to H&E [3]. Whether a continuous or categorical scoring system is being applied, tumour budding counts between pathologists are significantly more reproducible with cytokeratin staining. We also provide evidence that selection of blocks for cytokeratin staining is reproducible and consistent between different observers. Taken together, these arguments support the use of pan-cytokeratin for the daily reporting of tumour budding in colorectal cancer.

In a previous study on stage II colorectal cancer, we evaluated the inter-observer reproducibility of tumour budding counts on pan-cytokeratin slides using various scoring systems and the association of tumour budding with overall survival [11]. We showed that continuous budding counts led to a stronger inter-observer agreement. The novel findings of this study demonstrate, using statistical methods, that although the same associations were identified using counts or categories, continuous counts provide better goodness-of-fit to our statistical models. Additionally, observing the ROC curve of tumour budding for all relevant endpoints, no single threshold value should be preferred to split tumour budding counts into low and high groups. The distribution of tumour buds across the range of values also provides no hint for a useful/appropriate threshold value. Several additional arguments supporting a continuous count of tumour buds can be made: the probability of having a clinically relevant outcome (such as lymph node metastasis) will increase as the number of tumour buds increases [9]. Also, if the cut-off 10 buds are used, it seems unreasonable to suggest that tumours with 9 or 11 buds are biologically sufficiently different that they should be placed in different categories. Indeed, from a clinical standpoint, cut-offs are frequently applied to continuous variables in pathology practice despite their limitations (tumour grade, lymphovascular invasion). To benefit from both types of data, both the number of tumour buds and a corresponding category (low-grade, high-grade) could be reported.

Regardless of the scoring method, tumour budding assessed by cytokeratin was a significant and independent factor in DFS analysis in stage II patients. Several other authors, using H&E staining, have highlighted similar results in stage II CRC (reviewed in [9]). Using a modified Ueno method on H&E stain (area 0.95 mm^2; high-grade budding defined as $\geq$10 buds), Betge *et al* found high-grade tumour budding to be associated with significantly worse DFS in a

series of 120 patients (65 versus 91%, high- versus low-grade budding, respectively) [12]. Our study using the 10 HPFs method incorporating densest hotspots has also outlined very similar DFS rates (90.7 and 70.3%) for low-grade and high-grade budding.

Although this appears to be the first study to evaluate pan-cytokeratin staining for the evaluation of tumour budding in a prospective diagnostic setting, these results may be limited by a potential bias, namely that pathologists were not blinded to the TNM stage of the disease or other clinicopathological features at the time of budding counts. Nonetheless, our results demonstrate that even in a setting where tumour budding is reviewed by a multitude of pathologists, it is useful as a prognostic parameter.

To conclude, assessment of tumour budding on pan-cytokeratin slides is feasible in a large pathology institute and leads to expected associations with clinicopathological features. Additionally, it is an independent factor of poor prognosis in stage II patients and should be considered in future studies on tumour budding.

Acknowledgements

We would like to acknowledge Dr. Irene Centeno, Dr. José Galván, and Caroline Hammer from the Translational Research Unit, Institute of Pathology, University of Bern for excellent technical support. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions statement

VHK, NA: contributed to the study concept and design, data acquisition, analysis and interpretation and preparation of the manuscript; HD, BM, DM: contributed to the data acquisition, data interpretation, data quality control and critically reviewed the manuscript; RK, RR, AL: contributed to the study concept and design, data acquisition, data interpretation and preparation of the manuscript; IZ: contributed to the study concept and design, contributed to data acquisition and interpretation, performed statistical analysis and contributed to preparation of the manuscript. All authors critically reviewed and approved the final manuscript.

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