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The prognostic value of tumor budding in a thoroughly characterized stage II colon cancer population in the context of a national screening program

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Abstract

Tumor budding as a prognostic marker in colorectal cancer has not previously been investigated in a cohort of screened stage II colon cancer patients. We assess the prognostic significance of tumor budding in a thoroughly characterized stage II colon cancer population comprising surgically resected patients in the Region of Southern Denmark from 2014-2016. Tumors were re-staged according to the 8th edition of UICC TNM Classification, undergoing detailed histopathological evaluation and tumor budding assessment following guidelines from the International Tumor Budding Consensus Conference. Prognostic evaluation utilized Kaplan-Meier curves, log-rank tests, and Cox proportional hazard models for time to recurrence (TTR), recurrence-free survival (RFS), and overall survival (OS).

Out of 497 patients, 20% were diagnosed through the national colorectal cancer screening program. High-grade tumor budding (Bd3) was found in 19%, and tumor budding was associated with glandular subtype, perineural invasion, mismatch repair proficient tumors, and tumor recurrence ($p<0.001$, $p<0.001$, $p=0.045$ and $p=0.007$ respectively). In multivariable Cox regression, high-grade tumor budding (Bd3) was a significant prognostic factor for TTR compared to low-grade (Bd3 HR 2.617; $p=0.007$). An association between tumor budding groups and RFS was observed, and the difference was significant in univariable analysis for high-grade compared to low-grade tumor budding (Bd3 HR 1.461; $p=0.041$). No significant differences were observed between tumor budding groups and OS.

High-grade tumor budding is a predictor of recurrence in a screened population of patients with stage II colon cancer and should be considered a high-risk factor in a shared decision-making process when stratifying patients to adjuvant chemotherapy.

1. Introduction

Colorectal cancer (CRC) is the third most common type of cancer and the second most deadly cancer worldwide with more than 1.8 million new cases each year and more than half a million estimated cancer-related deaths[1]. Approximately one-third of individuals diagnosed with colon cancer fall into the Union for International Cancer Control (UICC) stage II [2] and are offered curative surgery. Despite radical resection, some of these patients experience recurrence.

UICC stage II colon cancer represents a heterogeneous group of tumors with varying prognosis suggesting a beneficial effect of adjuvant chemotherapy to only a subgroup of patients. Identifying this group of patients with increased risk of recurrence is pivotal in a modern oncologic setting treating each patient based on a personalized oncologic approach. Despite this, adjuvant chemotherapy to patients with UICC stage II colon cancer remains controversial[3]. Several randomized trials have tested the survival benefit of adjuvant chemotherapy in stage II colon cancer, but the gain in recurrence-free survival (RFS) as well as overall survival (OS) has been either absent, small, or questionable[4–6]. While these trials are based on cohorts in the late 90s and early 2000s, it is essential to note that great improvements have been made in pre-operative staging, surgery, and pathological examination in more recent decades. Thus, there is a need for more updated patient cohorts, especially as high-income countries recommend CRC screening[7].

National and international guidelines for the adjuvant treatment of stage II colon cancer recommend considering adjuvant chemotherapy in the presence of high-risk factors[8–10]. Recent meta-analyses have demonstrated a significantly improved OS and disease-free survival (DFS) in patients with high-risk stage II colon cancer receiving adjuvant chemotherapy[11,12]. Investigating the prognostic value of individual high-risk factors remains a matter of debate with conflicting results[9,13]. However, the survival advantages of adjuvant chemotherapy on RFS and OS in patients with a T4 tumor have been demonstrated consistently[14,15], T4 thus being the only high-risk factor decisively prompting adjuvant chemotherapy in the updated ASCO guideline[9]. Other risk factors ought to be considered

in a shared decision-making process, discussing the potential benefits and risks of adjuvant chemotherapy.

Tumor budding represents a promising biomarker and is included as a histological prognostic factor in the 8th edition of UICC TNM Classification[16]. High grade tumor budding is a poor prognostic marker in stage II colon cancer[17–21] and has not been investigated in a screened stage II colon cancer cohort. This retrospective cohort study was initiated to evaluate the prognostic value of tumor budding in an up-to-date, screened population of patients with UICC stage II colon cancer. The Danish CRC screening program started in March 2014, inviting all citizens between 50 and 74 years of age to participate in screening every second year, allowing us to collect a cohort of screened patients with a long clinical follow-up period.

The objectives were:

i) to determine the distributions of stage II colon cancer in the three tumor budding groups Bd1, Bd2, and Bd3, defined at the International Tumor Budding Consensus Conference (ITBCC), in a screened population of stage II colon cancer patients

ii) to describe clinicopathological characteristics in the three tumor budding groups

iii) to test the differences in time to recurrence or death in the three tumor budding groups with and without controlling for prespecified prognostic clinicopathological factors (T category, mismatch repair (MMR) status, and histological type).

2. Materials and Methods

2.1. Patient selection

A retrospectively collected, multicenter cohort consisting of patients with resected colon cancers from 2014-2016 in the Region of Southern Denmark (constituting a fifth of the Danish population) was identified using the Danish Colorectal Cancer Group database[22] and the Danish Pathology System (n=739). Patients included met the following criteria: Histologically verified colonic adenocarcinoma UICC stage II, complete resection (R_0 resection), age≥18 years, and a Danish personal identification

number. Exclusion criterias: neo-adjuvant chemotherapy, a history of malignant disease up to 10 years before colon cancer (except non-melanoma skin cancer) or any colon cancer in history, synchronous tumors (up to 4 months post colon cancer diagnosis), death within 3 months post-surgery, or hereditary cancer (familial adenomatous polyposis or Lynch syndrome).

In total, 242 patients were excluded and 17 patients fulfilled more than one exclusion criteria. Finally, the study population comprised 497 patients (Fig. 1).

2.2. Histopathological and clinical characterization

Archived hematoxylin and eosin stained tissue slides used for routine diagnostic purposes were retrieved from the four pathology departments in the Region of Southern Denmark. All tumor slides (3-49 slides per patient, mean 9) were reviewed and re-staged according to the UICC TNM Classification (8^{th} edition). A comprehensive histopathological characterization was performed, and the following pathological features were assessed: T category, histological subtype, differentiation level, lymphatic, venous, and perineural invasion. A resident and a senior pathologist performed the evaluation and re-staging, consulting a second senior pathologist in cases of doubt.

From the Danish Pathology System, we retrieved information on MMR status (evaluated by immunohistochemistry), number of removed lymph nodes, status of histologically verified recurrence, and any metachronous cancer in the follow-up time.

We conducted a retrospective review of electronic patient records, and extracted clinical data including age, sex, postoperative chemotherapy, radiology confirmed recurrence, metachronous cancer, and survival status. Information on the surgery-related variables, anastomotic leakage, acute surgical approach (obstructing or perforating tumors), and tumor localization were obtained from the DCCG registry based on the surgeon´s report. Right-sided tumors included tumors from cecum, the ascending colon, and transverse colon whereas left-sided tumors were in the descending colon or sigmoid colon. Status of screening was extracted from the DCCG registry and confirmed by a review of the electronic patient record.

In the re-classification process, 32 patients did not fulfill the criteria for UICC stage II and were excluded (Fig. 1). Twenty-five of these patients were originally classified as stage II but re-staged as stage III or IV due to confirmation of nodal or distant metastases.

2.3. Evaluation of tumor budding

A tumor bud is defined as a single cell or cell cluster of up to four tumor cells budding off the primary tumor and was evaluated by the hotspot method as per recommendations by ITBCC[21]. In short, the complete invasive front was scanned and tumor budding was counted in the selected hotspot normalized to the field size of 0.785mm^2 and graded into Bd1 (low) 0-4 buds, Bd2 (intermediate) 5-9 buds, and Bd3 (high) ≥10 buds (Fig. 2). The scoring was done by one observer, MPK, blinded to all clinicopathological variables.

2.4. Intra- and interobserver reliability

A total of 50 randomly selected patients representing both T3 and T4 tumors were selected to estimate the intra- and interobserver variation in the tumor budding scoring. Two observers, SKF and MPK, re-evaluated the score of tumor budding independently blinded to the clinical and histopathological information including the original tumor bud count. Intra- and interobserver variability was calculated based on weighted kappa statistics and was 0.70 and 0.52, respectively.

2.5. Survival endpoints

Survival endpoints were adopted from the consensus agreement[23]. Time to recurrence (TTR) was defined as the time from date of primary surgery to date of local or distant recurrence of colon cancer or to date of death from colon cancer. RFS was defined as the time from surgery to date of local or distant recurrence of colon cancer or death of any cause, whichever occurred first. OS was defined as the time from primary surgery to death of any cause or last follow-up. All records were censored either at the point of loss to follow-up (n=2) or upon the end of the study period (May 15, 2023).

2.6. Statistical analysis

Continuous variables were presented with means and standard deviation for normal-distributed variables and median and interquartile range for non-normally distributed variables. Categorical variables were presented as numbers and percentages. The distribution of the numerical variables was visualized by histograms and compared graphically by a quantile-quantile plot.

The association between tumor budding and clinicopathological characteristics was analyzed using the chi-squared test or Fisher´s exact test (variables with < 5 observations) as to the categorial variables, and kruskal-Wallis test was used when variables were continious.

The survival rates, TTR, RFS, and OS, were visualized by Kaplan-Meier curves. The log-rank test compared the differences in survival functions between the tumor budding groups.

Univariable and multivariable Cox regression models were performed with Bd1 as the reference group and reported as hazard ratios (HR) with a 95% CI.

A causal directed acyclic graph (DAG) was drawn using dagitty.net to define the minimal adjustment set to include in the multivariable analysis (Suppl. Fig. 1)[24].

The multivariable analysis was adjusted for the potential confounders identified by the DAG: T category, MMR status, and histologic type. According to the DAG, no adjustments are made for the mediators (venous, lymphatic, and perineural invasion) as well as the colliders (adjuvant chemotherapy and tumor localization). Potential confounding from sex was blocked when adjusting for MMR status, and potential non-collapsibility bias from age, number of lymph nodes, anastomotic leakage, and surgical approach was blocked by the collider adjuvant chemotherapy.

A sensitivity analysis tested the robustness of the association between the tumor budding groups and TTR using the Fine-Gray competing risk method, with death treated as competing event.

Schoenfeld residuals checked the proportional hazard assumption for each regression analysis and did not violate it. However, patients were stratified according to T category status in the model for RFS to fit the assumption.

Due to the low amount of missing data (MMR status not assessed in one tumor), all multivariable analyses were performed on complete cases (n=496).

Values of $p<0.05$ were considered statistically significant, and all analyses were performed using Stata software (version 18.0 BE). All data were recorded in a Research Electronic Data Capture database with an automatically generated entry check via the Open Patient Data Explorative Network organization.

2.7. Ethical statement

This study was conducted under the Declaration of Helsinki and approved by The Regional Committees on Health Research Ethics for Southern Denmark (S-20190164) with dispensation from obtaining informed consent from the study patients. No patients were excluded due to sign up in the Danish Registry of Tissue Utilization. The manuscript is in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in medical Journals. The study was reported in accordance with the Reporting recommendations for Tumor MARKer prognostic studies[25].

3. Results

3.1. Patient characteristics

The study population comprised 497 patients (Fig. 1) with a mean age of 73 years (SD 10 years), Males constituted 47% of the population. Fourteen percent of the patients received adjuvant chemotherapy and 9% experienced recurrence of the colon cancer within the follow-up period. In 98% of the tumors, at least 12 lymph nodes were collected and the median lymph node yield was 26. High-grade tumor budding (Bd3) was found in 19% of patients, intermediate-grade budding (Bd2) in 28% of patients, and low-grade budding (Bd1) in 53% of patients. Table 1 illustrates the baseline characteristics.

3.2. Association between tumor budding and clinicopathological characteristics

Tumor budding was associated with glandular subtype, perineural invasion, mismatch repair proficient tumors, and tumor recurrence ($p<0.001$, $p<0.001$, $p=0.045$, and $p=0.007$ respectively).

Postoperative recurrence was more frequent in patients with Bd3 than in the other tumor budding groups. A weak association between tumor budding groups and sex (p=0.054), and between tumor budding groups and lymphatic invasion (p=0.074) was found. Table 2 presents the relationships between tumor budding and all the investigated clinicopathological variables.

3.3. Survival analysis

The median follow-up time was 7.2 years (range 0.4 – 9.3 years). Within the follow-up period, 44 patients experienced recurrence and 28 patients died of colon cancer. In total, 149 patients died of other causes. During the follow-up period, 81 patients was diagnosed with a metachronous cancer (11 CRC, 14 lung, 14 mammary gland, 9 prostate, and 34 other).

The Kaplan-Meier survival curve for TTR showed significant differences between the tumor budding groups ($p_{\text{log-rank}}$ =0.004) (Fig. 3). No significant difference between the tumor budding groups and the endpoint RFS or OS was found ($p_{\text{log-rank}}$=0.093 and $p_{\text{log-rank}}$=0.185, respectively).

Results from the univariable Cox regression (Table 3) showed that high-grade tumor budding was prognostic for TTR (Bd3 HR 2.566; p=0.005) and RFS (Bd3 HR 1.461; p=0.041) compared to low-grade budding. We noted a correlation between tumor budding groups and overall survival (OS). However, the observed difference did not reach statistical significance (Bd3 HR 1.401; p=0.080).

In the multivariable Cox regression analysis (Table 4), high-grade tumor budding (Bd3) remained a significant prognostic factor for TTR (Bd3 HR 2.617; p=0.007). For RFS, the prognostic value did not remain significant (Bd3 HR 1.416; p=0.068).

A competing risk analysis (death as competing event) reported an almost identical HR for TTR as compared to the univariable and multivariable Cox regression, and the conclusions based on estimates and p-values did not differ (data not shown).

Subgroup analysis of patients not receiving adjuvant chemotherapy post-surgery showed similar results for all survival endpoints, however with lower statistical power (data not shown).

The follow-up time in this study is long compared to other studies[20,21] and most colon cancer recurrences occur within the first 5 years after surgery[26]. A post hoc analysis reduced the follow-

up time to 5 year and resulted in increased HR for TTR, RFS, and OS. For RFS the differences among the tumor budding groups became significant in multivariable analysis (Bd3 HR 1.725; p=0.021).

4. Discussion

In this retrospective cohort study, we determined the distribution of tumor budding in a well-characterized stage II colon cancer cohort from a screened population. With the clinicopathological characteristics, most variables were similar among the tumor budding groups. However, a positive correlation between the tumor budding category and venous, lymphatic, and perineural invasion was significant for the latter. Tumors with a low degree of budding were more often mismatch repair deficient (dMMR) and had a mucinous phenotype than tumors with intermediate- or high-grade budding. The recurrence rate was higher, and the time to recurrence was significantly shorter for tumors with a high degree of budding compared to those with a lower degree.

Several previous studies have reported a prognostic value of tumor budding in UICC stage II colon cancer[17–19,27,28]. To our knowledge, no previous research has assessed the prognostic value of tumor budding in a UICC stage II colon cancer within the framework of a national screening program. We were able to show a substantial difference in TTR and RFS among the tumor budding groups.

We find that Bd1 and Bd2 show similar survival curves for all three endpoints. Only Bd3 showed a worse prognosis compared to the two other groups and Bd3 seems to be remarkably different from the other two groups (Fig. 3). Tumor budding, as an intensity-dependent biomarker, is supported by the ITBCC reporting Bd3 to be associated with increased risk of recurrence and mortality in stage II colon cancer[21].

Survival endpoints are often poorly defined in prognostic studies of stage II colon cancer. This leads to a lack of comparability among studies. Metachronous cancers are infrequently addressed, likely due to the absence of available data. Previous research by Birgisson et al. found that including secondary primary cancers (excluding second CRC) as events in disease-free survival (DFS) led to reduced survival (62% vs. 58% after 5 years for stage I-III CRC), particularly in stage II CRC (68% vs. 60%)[29]. Since secondary primary non-CRC is more common in CRC patients than the general

population[30] and occurs more frequently in stage II patients[29], it's crucial to consider these events in survival analyses. A slightly higher percentage of metachronous cancers were present in our cohort compared to Birgisson et al.'s findings (16% vs. 12%). The events of metachronous cancer were ignored from the TTR and RFS analyses to align with survival endpoint definitions by Punt et al[23] and to maintain a real-life cohort representative of the actual stage II population.

A secondary analysis, censoring patients with an event of metachronous cancer in the follow-up period, resulted in a significant difference between the tumor budding groups and the endpoint RFS ($p_{\text{log-rank}}$=0.011). On multivariable analysis, the estimated HR for RFS became significant for Bd3 (Bd3 HR 1.561; p= 0.031), clearly demonstrating how the estimated HR changes depending on whether we ignore or censor these events, and underscores the importance of having precise definitions for endpoints in survival analysis.

The ITBCC recommends evaluation of tumor budding by the hotspot method[21]. To identify the slide with the highest degree of budding, one must screen the complete invasive front. In 37 % of the tumors in our evaluation, we identified hotspots in slides outside the most invasive part of tumor with a higher tumor budding category than the most invasive tumor slide. This emphasizes the importance of a thorough review of all the tumor slides, not solely the most invasive slide, as indicated by ITBCC[21]. This is of relevance in a clinical setting where more slides from the invasive front are available, and highlights the importance of clearly specifying which tissue sections have been examined in tumor budding studies in order to compare results.

Populations-based screening programs lead to colon cancer patients with more favorable histopathological features[31] thus changing the patient population. Screening behavior and different sex compositions should be considered in this modern cohort. Twenty percent of the patients were diagnosed during screening before they experienced symptoms. Screening is expected to alter the T category distribution with a shift towards a lower T category[32]. The overall proportion of tumors in the T4 category in our population was 12 %, but it was only 6% in the screened subgroup emphasizing the effect of screening. More women than men were diagnosed in screening (57%) and women were overrepresented in the lower budding group. Women represent 71% of the dMMR

tumors, and as these tumors have a lower budding tendency, this contributes to the association between sex and tumor budding.

Evaluation of tumor budding should be made with caution in mucinous adenocarcinomas. Mucinous adenocarcinomas were included in this study in order to demonstrate the prognostic value of tumor budding in a heterogeneous setting. A post hoc subgroup analysis done exclusively on the glandular adenocarcinomas omitting the mucinous and low-differentiated adenocarcinomas increased the estimated HR for TTR and showed similar results for RFS and OS, however with lower statistical power (data not shown).

4.1. Methodological consideration/Strengths and weaknesses

This study did not have any of the weaknesses typically seen in retrospective data collection. No subjective data were used and the researchers were blinded to the outcome status of study subjects. Selection of patients was not exclusively based on databases but included the regional pathology system making the cohort representative of the entire stage II colon cancer population. Nationwide recommendations exist regarding radiology, surgery, pathologic examination, and oncologic treatment, so the regional setup in this study was not seen as a limitation. Instead, the close collaboration between the departments in the Region of Southern Denmark made the exchange of tissue flexible and complete.

This post-screened cohort has undergone a comprehensive characterization, and we suggest that it is better characterized than the majority of stage II populations used for research, as it represents validated stage II patients only. The lymph node sampling (with 98% having >12 lymph nodes collected as recommended[9]) is superior to other cohorts[18,28]. Further, the current approach, which includes comprehensive characterization and a higher sampling yield, appears to have contributed to a smaller recurrence rate compared to similar cohorts[17,18] but equal to a recent study by Xiong et al[33].

The thorough, microscopic characterization and data extraction directly from electronic medical records are unique. This has completed the tumor and patient classification and brought it up to date.

As a result, we have a well-characterized, post-screened UICC stage II colon cancer cohort representative of a modern patient population. Along with the completeness of data, we propose our results to have broad applicability in the assessment of tumor budding in patients with stage II colon cancer.

The interobserver agreement in assessing tumor budding was moderate (kappa = 0.70), and align with similar studies [18,19]. It is worth noting that despite the modest agreement in tumor budding evaluation, we have a substantial signal in our results underlining the robustness and eligibility of tumor budding as a prognostic marker. It is relevant to re-consider tumor budding as a prognostic biomarker in the emerging field of molecular and circulating tumor biomarkers[34]. However, we still believe that this histological marker is eligible and relevant for prognostic purposes. First and foremost, tumor budding is a histological marker evaluated immediately after surgery as an integral part of the routine pathological evaluation without being major cost- or time-consuming. Second, tumor budding is an immediate prognostic marker in contrast to, for example, circulating tumor DNA which necessitates evaluation over time at more time points. Evaluated together the markers could potentially improve our selection of high-risk patients in relation not only to adjuvant chemotherapy but also in relation to follow-up regime. In addition to quantitative biomarkers, tumor budding is a morphological characteristic of the tumor microenvironment and is associated with epithelial mesenchymal transition[35]. The morphology and microenvironment surrounding the budding cells bring us additional information on the tumor-stroma interplay and the biology in the cancer setting.

In conclusion, we demonstrated the prognostic value of tumor budding in this thoroughly characterized stage II colon cancer population. We evaluated the clinicopathological characteristics and survival rates in the context of a national screening program. The recurrence rate in a modern setting is less than 10% and consequently, the survival analysis must rely on more cancer-specific endpoints. This brings perspectives with other biomarkers, and it would be relevant to evaluate other prognostic biomarkers in a modern stage II cohort as well as assess tumor budding by immunohistochemistry to diminish interobserver variability.

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Conflict of interests

The authors declare no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval

This study was approved by The Regional Committees on Health Research Ethics for Southern Denmark (S-20190164) with dispensation from obtaining informed consent from the study patients. The study was performed in accordance with the Declaration of Helsinki.

Author contribution

Maria P. Kristensen: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing-original draft, writing – review and editing, visualization, funding acquisition, project administration. **Ulrik Korsgaard**: investigation, review and editing. **Signe Timm**: methodology, formal analysis, review and editing. **Torben F. Hansen**: conceptualization, methodology, review and editing. **Inti Zlobec:** conceptualization, review and editing. **Henrik Hager**: conceptualization, methodology, review and editing, supervision. **Sanne Kjær-Frifeldt**: conceptualization, methodology, validation, investigation, review and editing, supervision.

The statistical consultation was provided by **Signe Timm**.

All Authors have read and approved the final manuscript.

Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Figure legends

Figure 1. Flow chart. The selection of patients resected for UICC stage II colon cancer from 2014-2016 in the Region of Southern Denmark.

Figure 2. Tumor budding. Hematoxylin and eosin stained high-power images (x40) of tumor budding at the invasive front in stage II colon cancer. Tumor budding is defined as single tumor cells or clusters of up to four cells budding of the primary tumor. Black arrows indicate tumor buds. (A) Low-grade budding (Bd1 0-4 buds). (B) Intermediate budding (Bd2 5-9 buds). (C) High-grade budding (Bd3 ≥10 buds).

Figure 3. Kaplan Meyer curves illustrating the association between tumor budding groups and survival endpoints A) time to recurrence (TTR), B) recurrence-free survival (RFS), and C) overall survival (OS).

Supplementary information is available at Human Pathology's website.

Table 1 Baseline clinicopathological characteristics in a cohort of 497 patients with UICC stage II colon cancer

Characteristics			Characteristics	Number	(%)
Age at surgery			**Histological type**		
Mean (SD)	73	(10)	Glandular	388	78%
Examined lymph nodes			Mucinous	70	14%
Median (IQR)	26	(18)	low differentiated	39	8%
			Tumor differentiation		
	Number	(%)	Well, moderate	458	92%
Sex			Poor	39	8%
Male	233	47%	**Venous invasion**		
Female	264	53%	Yes	116	23%
Screening			No	381	77%
Yes	100	20%	**Lymphatic invasion**		
No	397	80%	Yes	25	5%
Surgical approach			No	472	95%
acute (obstruction/perforation)	48	10%	**Perineural invasion**		
elective	449	90%	Yes	59	12%
Anastomotic leakage			No	438	88%
yes	16	3%	**MMR**		
no	481	97%	pMMR	374	75%
Postoperative adjuvant chemotherapy			dMMR	122	24%
Yes	69	14%	missing	1	<1%
No	428	86%	**Tumor budding**		
Localization			Low	262	53%
Right	251	51%	Intermediate	142	28%
Left	246	49%	High	93	19%
T category			**Postoperative recurrence**		
pT3	437	88%	Yes	44	9%
pT4	60	12%	No	453	91%

Abbreviations: MMR, mismatch repair; pMMR, mismatch repair proficient; dMMR, mismatch repair deficient

Table 2 The relationship between tumor budding and clinicopathological characteristics in a cohort of 497 patients with UICC stage II colon cancer

	Tumor budding								
	Low		Intermediate		High		Total		p-value
Age at surgery									
mean (SD)	73	(9)	72	(11)	73	(9)	73	(10)	0.929
Examined lymph nodes									
Median (IQR)	28	(18)	25	(22)	25	(11)	26	(18)	0.108
	n (%)		n (%)		n (%)		n (%)		
Sex									0.054
Male	110	(42)	77	(54)	46	(49)	233	(47)	
Female	152	(58)	65	(46)	47	(51)	264	(53)	
Screening									0.934
Yes	52	(20)	30	(21)	18	(19)	100	(20)	
No	210	(80)	112	(79)	75	(81)	397	(80)	
Surgical approach									0.187
acute (obstruction/perforation)	20	(8)	15	(11)	13	(14)	48	(10)	
elective	242	(92)	127	(89)	80	(86)	449	(90)	
Anastomotic leakage									0.582
yes	9	(3)	3	(2)	4	(4)	16	(3)	
no	253	(97)	139	(98)	89	(96)	481	(497)	
Tumor localization									0.797
Right	136	(52)	69	(49)	46	(49)	251	(51)	
Left	126	(48)	73	(51)	47	(51)	246	(49)	
Histological type									<0.001*
Glandular	185	(71)	121	(85)	82	(88)	388	(78)	
Mucinous	56	(21)	12	(9)	2	(2)	70	(14)	
low differentiated	21	(8)	9	(6)	9	(10)	39	(8)	
Tumor differentiation									0.641
Well, moderate	241	(92)	133	(94)	84	(90)	458	(92)	
Poor	21	(8)	9	(6)	9	(10)	39	(8)	
T category									0.436
pT3	228	(87)	129	(91)	80	(86)	437	(88)	
pT4	34	(13)	13	(9)	13	(14)	60	(12)	
Venous invasion									0.359
Yes	55	(21)	35	(25)	26	(28)	116	(23)	
No	207	(79)	107	(75)	67	(72)	381	(77)	
Lymphatic invasion									0.074
Yes	10	(4)	6	(4)	9	(10)	25	(5)	
No	252	(96)	136	(96)	84	(90)	472	(95)	
Perineural invasion									<0.001*
Yes	17	(6)	22	(15)	20	(22)	59	(12)	
No	245	(94)	120	(85)	73	(78)	438	(88)	
MMR[a]									0.045*
pMMR	185	(71)	113	(80)	76	(82)	374	(75)	
dMMR	76	(29)	29	(20)	17	(18)	122	(25)	
Postoperative adjuvant chemotherapy									0.721
Yes	39	(15)	17	(12)	13	(14)	69	(14)	
No	223	(85)	125	(88)	80	(86)	428	(86)	
Postoperative recurrence									0.007*
Yes	19	(7)	9	(6)	16	(17)	44	(9)	
No	243	(93)	133	(94)	77	(83)	453	(91)	

[a] numbers may vary due to missing data for 1 patient

* statistical significance

Table 3 Cox univariable analysis for time to recurrence, recurrence-free survival, and overall survival (n=497)

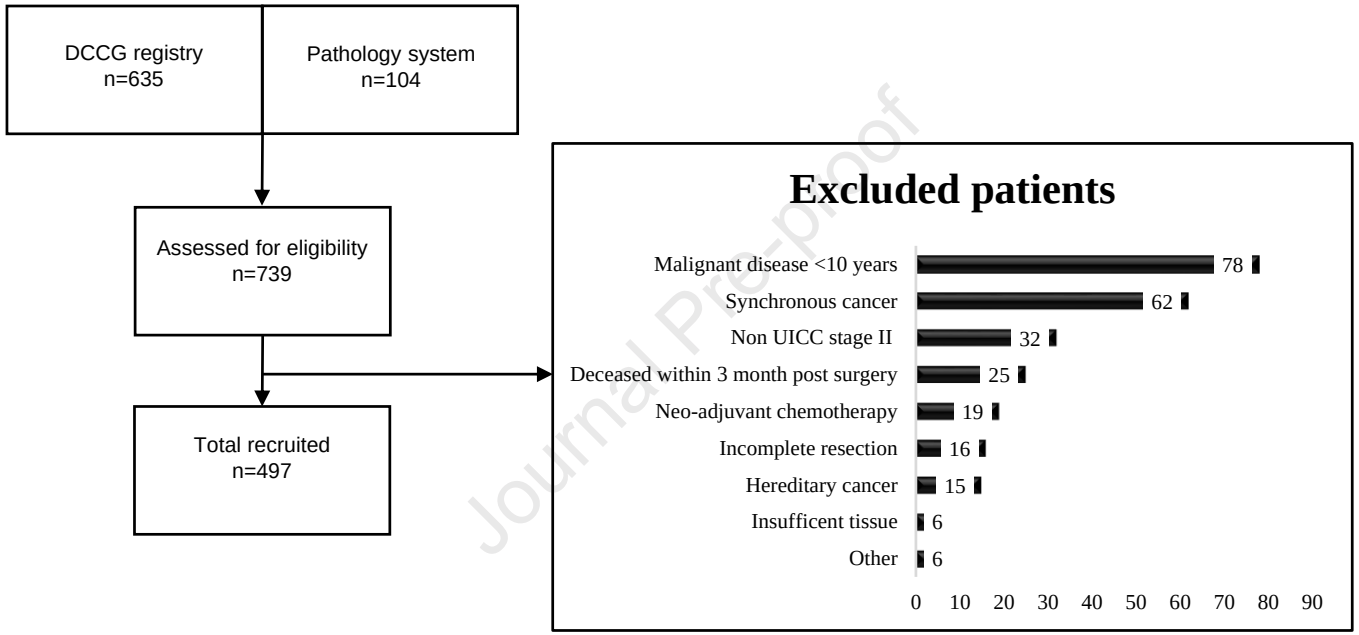
Characteristics		Time to recurrence			Recurrence-free survival			Overall survival		
	n (%)	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Tumor budding										
Low	262 (53%)	ref			ref			ref		
Intermediate	142 (29%)	0.867	0.392-1.917	0.725	0.998	0.710-1.404	0.992	1,015	0.716-1.438	0.934
High	93 (18%)	2,566	1.319-4.991	0.005*	1,461	1.015-2.103	0.041*	1,401	0.961-2.041	0.080

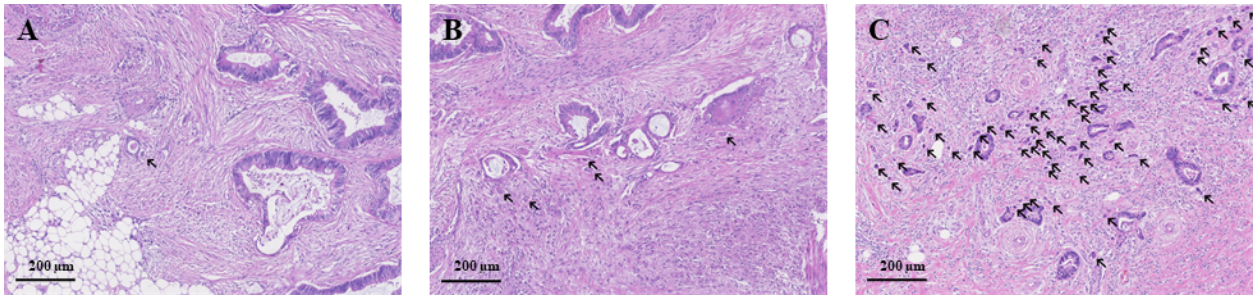
* Statistical significance

Table 4 Cox multivariable analysis for time to recurrence, recurrence-free survival, and overall survival, corrected for MMR status, T category, and histologic type (n=496)

Characteristics		Time to recurrence			Recurrence-free survival			Overall survival		
	n (%)	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Tumor budding										
Low	261 (53%)	ref			ref					
Intermediate	142 (29%)	0.934	0.416-2.097	0.869	1,000	0.707-1.414	1,000	1,029	0.721-1.468	0.875
High	93 (18%)	2,617	1.295-5.288	0.007*	1,416	0.974-2.059	0.068	1,361	0.924-2.004	0.118

* Statistical significance





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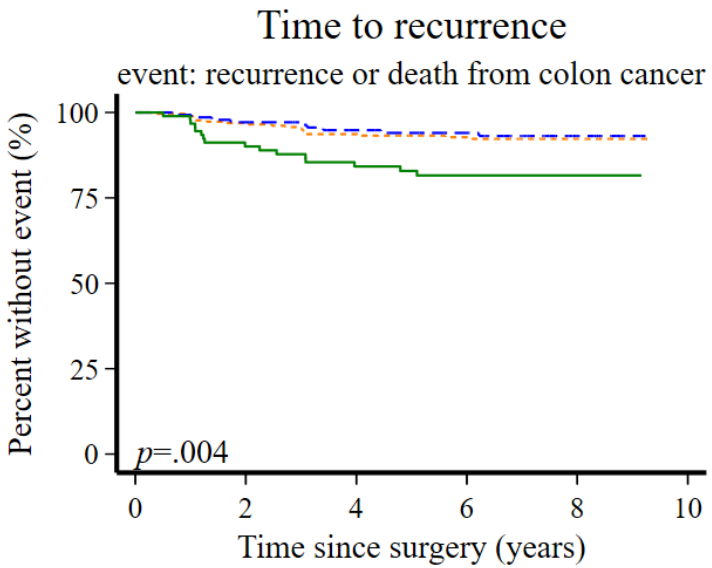
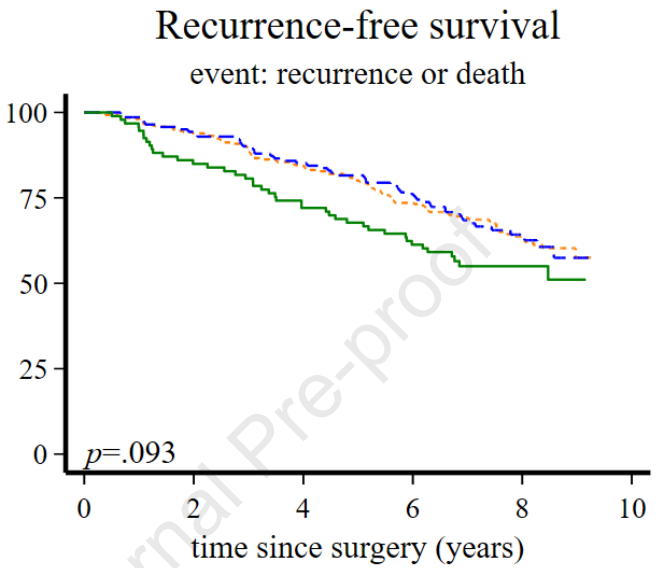


Number at risk

Bd1	262	246	219	191	84
Bd2	142	133	120	107	41
Bd3	93	79	67	57	20

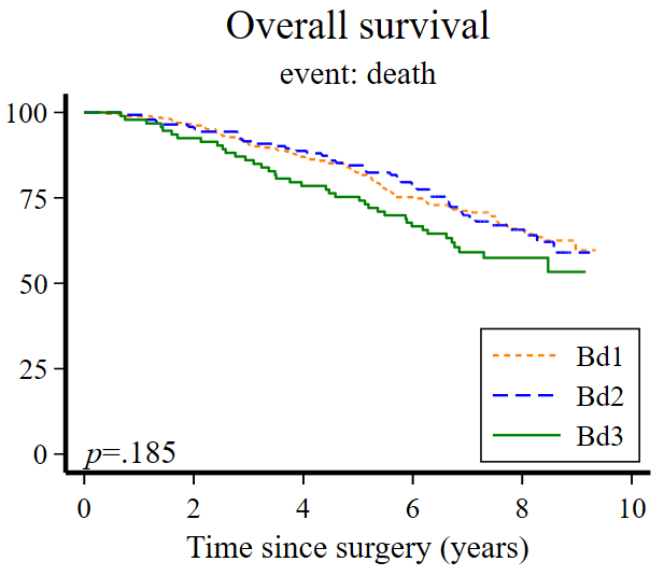
B



Number at risk

Bd1	262	246	219	191	84
Bd2	142	133	120	107	41
Bd3	93	79	67	57	20

C



Number at risk

Bd1	262	252	228	197	88
Bd2	142	136	126	112	42
Bd3	93	86	73	62	21

Highlights

- Tumor budding evaluation in a screened stage II colon cancer cohort
- Minimal adjustment set identified from causal directed acyclic graph
- Tumor budding a predictor of recurrence in patients with stage II colon cancer