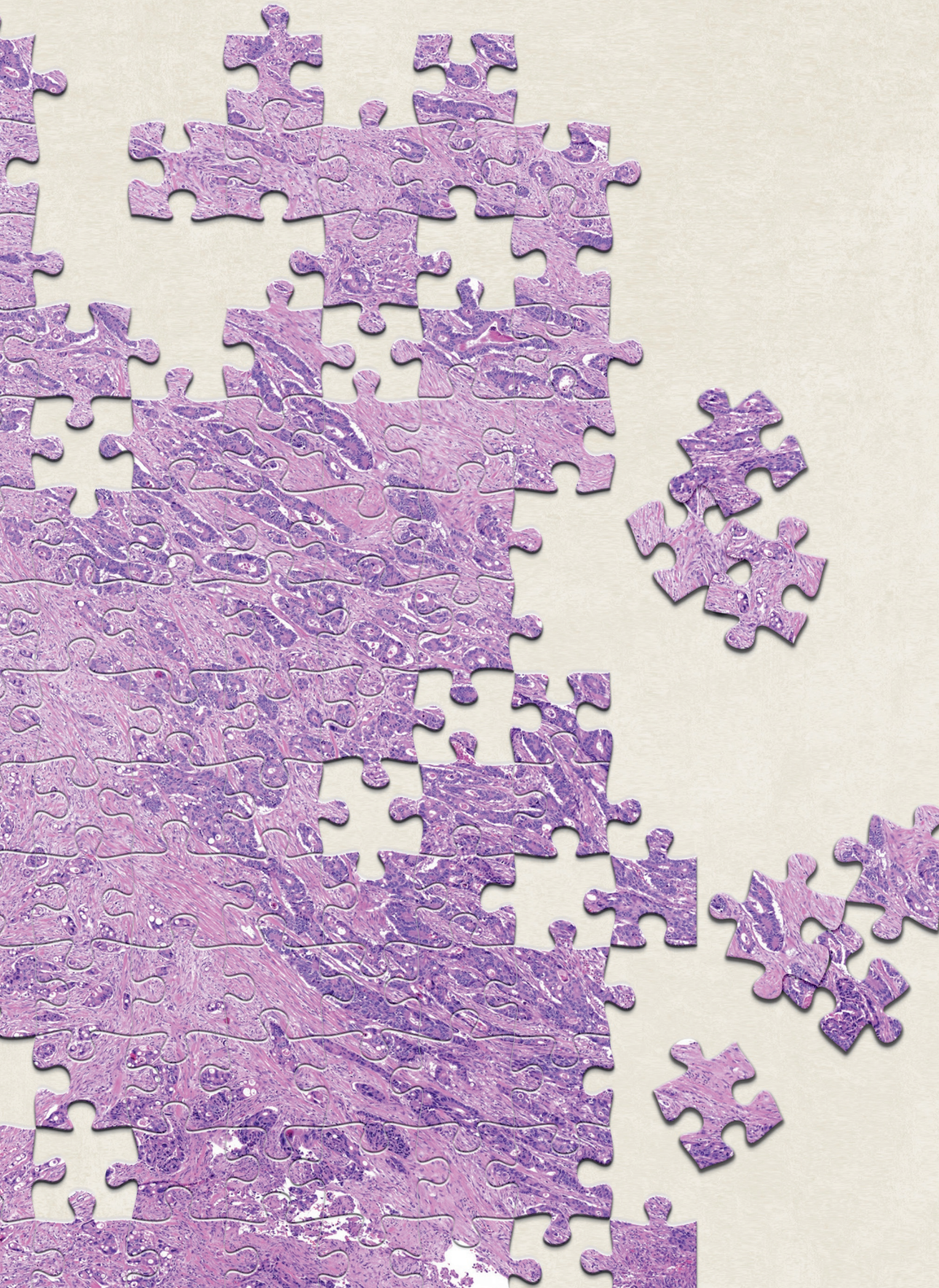
The tumor-stroma ratio in epithelial cancer types: towards implementation in diagnostic pathology

Smit, M.A.



CHAPTER 7

Tumor-stroma ratio outperforms tumor budding as biomarker in colon cancer; a cohort study

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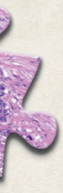
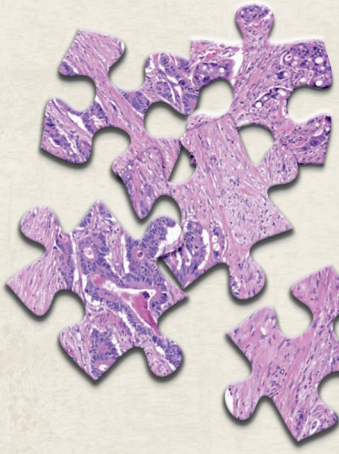
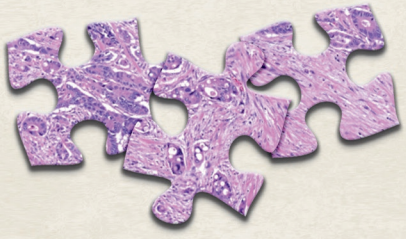




Marloes A Smit | Gabi W van Pelt | Valeska Terpstra
Hein Putter | Rob AEM Tollenaar | Wilma E Mesker
J Han JM van Krieken



Abstract

The tumor-stroma ratio (TSR) and tumor budding (TB) are two high-risk factors with potential to be implemented in the next TNM classification. The aim of the current study was to evaluate the practical application of the two biomarkers based on reproducibility, independency and prognostic value.

Patients diagnosed with stage II or III colon cancer who underwent surgery between 2005 and 2016 were included. Both TSR and TB were scored on hematoxylin and eosin stained tissue sections. The TSR, based on the relative amount of stroma, was scored in increments of 10%. TB was scored following the consensus guidelines; a bud was defined as ≤4 tumor cells. For analysis three categories were used. Cohen's kappa was used for reproducibility. The prognostic value was determined with survival analysis.

In total 246 patients were included. The TSR distribution was: N=137 (56%) stroma-low and N=109 (44%) stroma-high. The TB distribution was; TB-low N=194 (79%), TB-intermediate N=35 (14%) and TB-high N=17 (7%). The reproducibility of the TSR was good (interobserver agreement Kappa = 0.83 and intra-observer agreement Kappa = 0.82), whereas the inter- and intra-observer agreement for scoring TB was moderate (Kappa 0.47 and 0.45, respectively). The survival analysis showed an independent prognostic value for disease free survival for TSR (HR 1.57; 95% CI 1.01-2.44; p=0.048) and for TB-high (HR 2.01; 95% CI 1.02-3.96; p=0.043).

Based on current results, we suggest the TSR is a more reliable parameter in daily practice due to better reproducibility and independent prognostic value for disease free survival.

Introduction

The prognosis and selection for adjuvant treatment of colon cancer patients is largely based on the Tumor Node Metastasis (TNM) classification (1). Patients diagnosed with stage III or stage II with one or more high-risk (ASCO) criteria will usually be selected for adjuvant chemotherapy (1). However, among patients staged II without any high-risk factors, approximately 30% will suffer from recurrent disease within three to five years after surgery (2). To better predict which patients will develop recurrence, additional high risk factors next to the ASCO criteria have been described (1). These "new" high-risk factors should improve the selection of patients who will likely benefit from adjuvant therapy. Thus, high-risk criteria should not only select stage II patients at high risk for recurrence, but also select patients at stage III who are likely to be overtreated with adjuvant therapy.

New prognostic parameters have been identified on the basis of molecular pathology (for example CMS analysis) (3), lymph node assessment (for example one-step nucleic acid amplification assay (OSNA)) (4, 5), but also on simple morphologic parameters. Morphologic parameters are tissue based and can be evaluated during routine pathology practice.

The tumor-stroma ratio (TSR) is a biomarker based on the microenvironment of the tumor and has proven to be a strong prognostic parameter (6, 7). The TSR is based on the relative amount of stroma in the primary tumor. Patients with a tumor containing >50% stroma (stroma-high) have a worse prognosis, compared to patients with a tumor of ≤50% stroma (stroma-low). The TSR is validated by many international study groups and is prognostic in multiple epithelial cancer types (6, 7). The TSR is scored on hematoxylin and eosin (H&E) stained sections used in routine diagnostics, the scoring method is easy to learn, well reproducible and takes about 1-2 minutes (8).

Tumor budding (TB), the propensity of the primary tumor to bud off single cells and cell clusters (≤4 cells) at the invasive front, correlates with prognosis and is also frequently evaluated as a new biomarker in colon cancer. According to the guidelines, TB scoring should be performed at the invasive front of a tumor on an H&E stained section (9). The reproducibility of TB on H&E sections shows highly variable results (10-13). Therefore, some studies use cytokeratin stained sections to identify the tumor buds for better interobserver agreement (14, 15). Various studies showed TB to be an independent prognostic biomarker for overall survival (OS) and disease free survival (DFS) in stage I and stage II colon cancer patients (14, 16, 17). Patients with tumors with high budding have a worse prognosis compared to patients with low budding. Recently it was recommended to report TB in T1 tumors for decision-making about additional resection after biopsy or removal of a polyp (18).

Both TSR and TB have shown to be prognostic biomarkers in several series of colon cancer patients and both seem potentially suitable to use in routine pathology diagnostics. In order to implement TSR and/or TB as prognostic factors in daily clinical practice, their robustness and reproducibility should be thoroughly assessed (19). TB has recently been added to the guidelines for locally advanced colon cancer (18). The prospective validation of the TSR as a biomarker is currently under investigation in the UNITED study (20, 21).

Here we analyze the value of TSR and TB by comparing their reproducibility, independency from one another and the prognostic value in stage II and stage III colon cancer patient samples.

Materials & methods

Patient selection

Patients who underwent curative surgery for colon cancer, between January 2005 up to and including December 2016 at the LUMC were retrospectively included in this cohort study. Patients were included when they met the following inclusion criteria: pathological stage II or stage III colon cancer and age ≥18 years. The following exclusion criteria were met: rectal cancer, neo-adjuvant treatment, a medical history of cancer 10 years prior to colon cancer (except for basal cell skin cancer or cervical carcinoma in situ) or any colon cancer in history, double tumors, and/or deceased within 3 months after surgery (***Supplementary Table 1***). The H&E stained slides used for routine diagnostics were collected from the Department of Pathology and the slides were anonymized and scanned with the Panoramic 250 scanner (3DHistech, Hungary) (tissue level pixel size ~0.33 µm/pixel) for digital analysis. The observers were blinded for clinical and pathological data and for each other's results during biomarker scoring.

Tumor-stroma ratio

The TSR was scored on H&E stained sections from the primary tumor by two observers (MS and GvP, Leiden University Medical Center, Leiden). The TSR was scored at a 100x magnification. The stroma percentage was scored in increments of 10, in a field with as much as possible tumor-stroma and with tumor cells on four opposite sides of the vision field (8, 22, 23). If no agreement was reached, a third observer was consulted (HvK, Radboud University Medical Center, Nijmegen). One of the observers (MS) scored the TSR also digitally, using a circular annotation of 3.4mm2 to mimic the field of view of a 100x magnification. For analysis the TSR was dichotomized. A tumor with an amount of stroma of ≤50% was classified as stroma-low, a percentage >50% was classified as stroma-high, in line with previous studies (8, 22-24). In ***Figure 1*** an example of a stroma-low (A) and a stroma-high tumor (B).

Tumor budding

TB was scored, on exactly the same slides as the TSR, by two observers (VT (Haaglanden Medical Center, the Hague) and HvK) as recommended by the consensus (9). HvK scored TB both microscopically and digitally, VT scored TB only digitally. A tumor bud was determined as a single cell or a small cluster of cells to a maximum of four cells. TB was scored at the invasive front, at a single vision field by a magnification of 200x. The number of buds was normalized as described in the conversion table in the consensus. When TB was scored digitally, an annotation with an area of 0.785mm2 was used. For survival analysis the microscopic numbers were used, and the continuous numbers were categorized for statistical analysis. The three categories were: TB-low (0-4 buds), TB-intermediate (5-9 buds) and TB-high (≥10 buds) (9). In ***Figure 1*** an example of a TB-low (C) and a TB-high tumor (D).

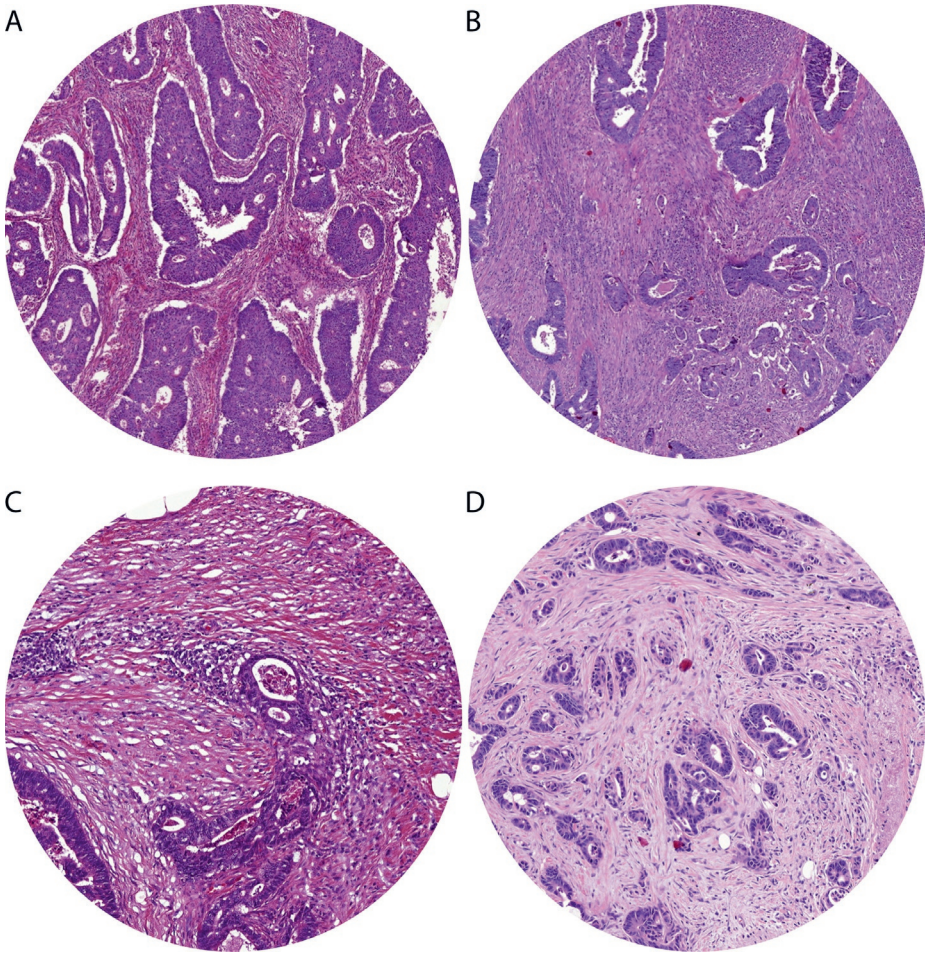


Figure 1. Examples of the 4 µm hematoxylin & eosin stained slides of colon carcinomas. In A) a stroma-low tumor, in B) a stroma-high tumor. Both viewed at a 100x magnification with an area of 3.4mm2. In C) a tumor-budding low tumor, in D) a tumor-budding high tumor. Scored in an area of 0.785mm2.

Statistics

Descriptive variables are presented with mean and standard deviation (SD) for normally distributed continuous variables. Non-normally distributed continuous variables are presented by median and range. The Chi-square test is used for measuring associations between categorical variables. Cohen's kappa is used to determine the interobserver agreement of scoring TSR and TB (digitally), and to determine the intra observer agreement for scoring TSR and TB (microscopic vs digital).

The prognostic value of the two individual parameters was explored. DFS was defined as the time from surgery to recurrence or death, depending on what occurred first. OS was defined as the time from surgery to death of any cause.

Univariate survival analysis was performed using a Kaplan Meijer curve and a Log rank test. Cox regression analysis was performed for univariate and multivariate analysis for Hazard Ratio's (HR) and the 95% confidence interval (95% CI).

All tests were 2-sided and a p-value of < 0.05 was considered to be significant. Statistical analyses were performed using SPSS version 25 (SPSS Inc., Chicago, IL, USA).

Results

Patient cohort

In total 381 colon cancer stage II or stage III patients underwent surgery in the time period 2005-2016. Of these, 135 patients were excluded because one of the exclusion criteria was met, most often (N=70) due to a medical history of cancer, and 246 patients were included in the cohort (***Figure 2***). The tumors of these patients were scored for both TSR and TB.

The patient population in the cohort had a mean age of 68 years (SD 12 years) and 54% males (N=134). 53% (N=131) of the patients had pathological stage (p-Stage) II and 92 patients (37%) received adjuvant therapy. The TSR distribution was: N=137 (56%) stroma-low and N=109 (44%) stroma-high. TB was divided in 3 categories; TB-low (N=194 (79%)), TB-intermediate (N=35 (14%)) and TB-high (N=17 (7%)). All baseline characteristics are displayed in ***Table 1***.

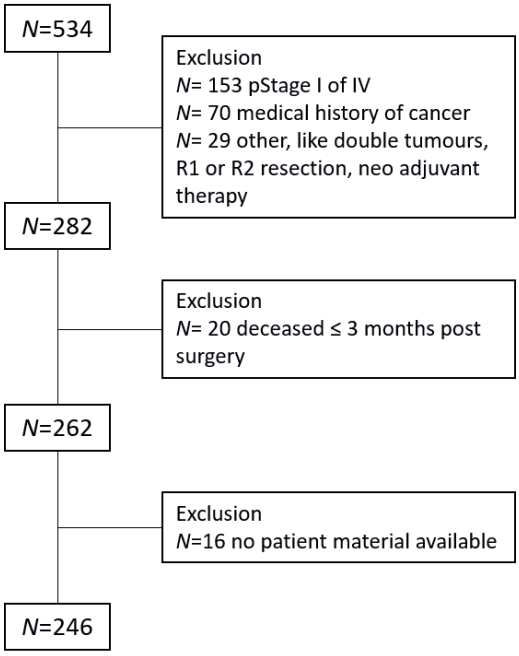


Figure 2. Flowchart of the patient selection.

Interobserver variability

The interobserver agreement for scoring TSR between the two observers was good to almost perfect (Kappa = 0.83). The TSR was also scored digitally by one observer (MS), a good to almost perfect intra observer agreement was reached (Kappa = 0.82).

The interobserver agreement for scoring TB was moderate with a Kappa of 0.47. One of the observers (HvK) scored the sections microscopically and digitally for TB, with a moderate intra observer agreement of Kappa 0.45. A wide variety of scoring was observed when reviewing the discrepancies, even within one case, and no trends or obvious reason for discrepancy could be detected that could explain the inter- or intra-observer variation.

Association

Of the 246 patients, 120 (49%) were categorized as stroma-low and TB-low (low-risk patients), and 10 (4%) patients were classified as stroma-high and TB-high (high-risk patients). The distribution of TSR and TB was shown in ***Supplementary Table 2***. An association between TSR and TB was found (Chi-square p=0.001).

Table 1. **Patient and tumor characteristics of 246 patients with colon cancer.**

Characteristics	**All**	**Tumor-stroma ratio**			**Tumor Budding**			
		Stroma-low (≤50%)	Stroma-high (>50%)		Low (0-4 buds)	Intermediate (5-9 buds)	High (≥10 buds)	
	N=246 (%)	137 (56)	109 (44)	*p-value*	194 (79)	35 (14)	17 (7)	*p-value*
Age (years), mean (SD)	68 (12)	68 (12)	67 (12)	0.349	68 (12)	65 (11)	69 (13)	0.282
Gender				0.166				0.456
Male	134 (54)	80 (58)	54 (50)		109 (56)	18 (51)	7 (41)	
Female	112 (46)	57 (42)	55 (51)		85 (44)	17 (49)	10 (59)	
pTNM stage				0.298				0.987
II	131 (53)	77 (56)	54 (50)		104 (54)	18 (51)	9 (53)	
III	115 (47)	60 (44)	55 (51)		90 (46)	17 (49)	8 (47)	
pT status				0.566				0.506
T1 + T2	18 (7)	17 (12)	54 (50)		17 (9)	1 (3)	0 (0)	
T3	196 (80)	106 (78)	36 (33)		152 (78)	30 (86)	14 (82)	
T4	32 (13)	14 (10)	19 (17)		25 (13)	4 (11)	3 (18)	
pN status				0.566				0.986
N0	131 (53)	77 (56)	54 (50)		104 (54)	18 (51)	9 (53)	
N1	74 (30)	38 (28)	36 (33)		59 (30)	10 (29)	5 (29)	
N2	41 (17)	22 (16)	19 (17)		31 (16)	7 (20)	3 (18)	
Localization				0.002*				0.859
Right	117 (48)	77 (56)	40 (37)		93 (48)	17 (49)	7 (41)	
Left	129 (52)	60 (44)	69 (63)		101 (52)	18 (51)	10 (59)	
Adjuvant therapy				0.553				0.106
No	154 (63)	88 (64)	66 (61)		124 (64)	17 (49)	13 (77)	
Yes	92 (37)	49 (36)	43 (39)		70 (36)	18 (51)	4 (24)	

* Significant result.

N Lymph nodes, p Pathological, SD Standard deviation, T tumor

Survival analysis

The median follow-up time was 47 months (range 4-158). During follow-up 48 (20%) patients had recurrence of disease, and 68 (28%) patients died. In total 83 (34%) DFS events occurred, due to the fact that some patients deceased with recurrence.

There was no significant difference in OS for TSR (HR 1.36; 95% CI 0.84-2.19; p=0.206). However, the TSR was prognostic for DFS (HR 1.59; 95% CI 1.03-2.45; p=0.036). Univariate analysis showed that TB was prognostic for OS (TB-High HR 2.36; 95% CI 1.16-4.81; p=0.018) and for DFS (TB-High HR 2.40; 95% CI 1.23-4.70; p=0.011). Kaplan-Meijer survival curves for TSR and TB are shown in ***Figure 3*****.**

Based on the results from the univariate Cox regression analysis (***Table 2***), in the multivariate Cox regression model the results were corrected for age and pT-status. TSR remained a significant prognostic parameter for DFS (HR 1.57; 95% CI 1.01-2.44; p=0.048), but this prognostic value was not found for OS. For TB the prognostic value did not retain significance in multivariate analysis for OS, but for DFS TB-high remained prognostic (HR 2.01; 95% CI 1.02-3.96; p=0.043) (***Table 3***).

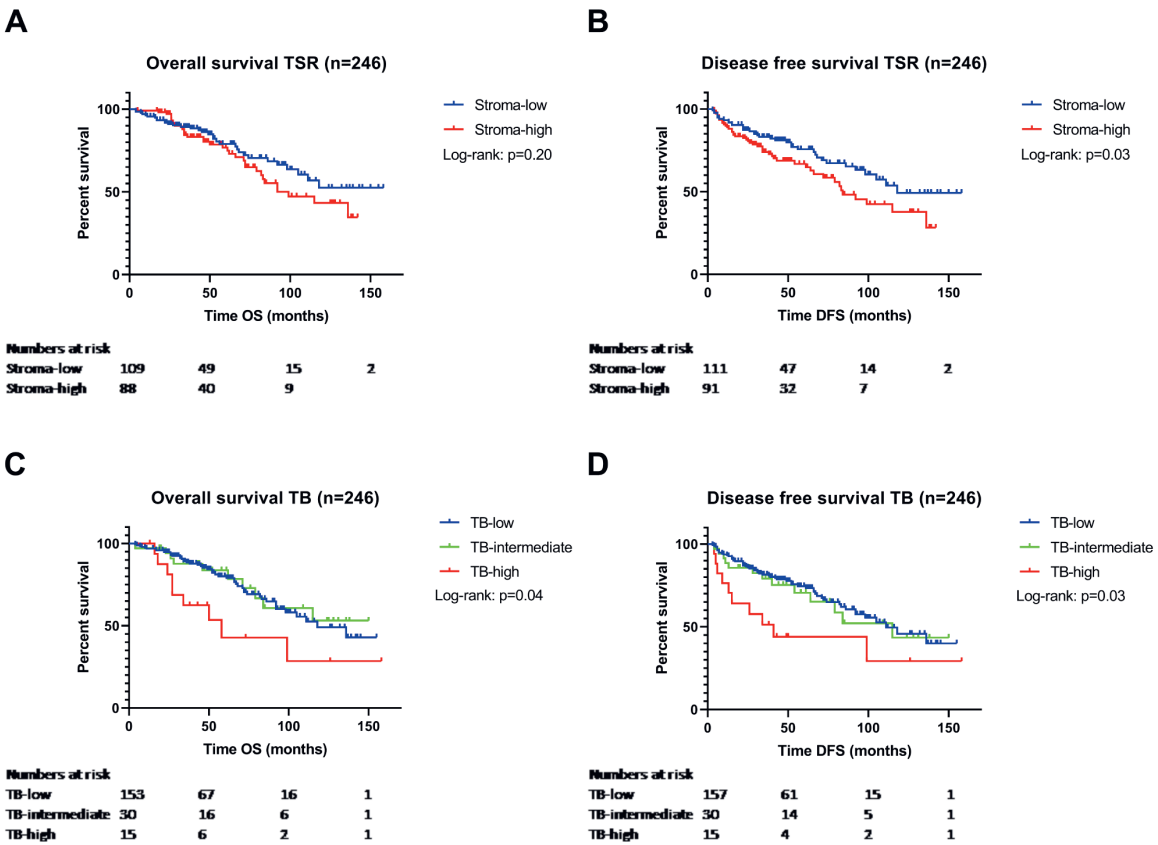


Figure 3. The Kaplan-Meijer survival curves of the 246 patients with colon cancer. Survival curves for TSR in A) for overall survival (p=0.20), and B) for disease free survival (log rank p=0.03). Survival curves for TB in C) for overall survival (p= 0.04), and in D) for disease free survival (p=0.03).

Table 2. Cox univariate analysis for overall and disease free survival.

		Overall survival			Disease free survival		
	N (%)	HR	95% CI	p-value	HR	95% CI	p-value
Age	246 (100)	1.069	1.041-1.097	<0.001	1.052	1.029 - 1.076	< 0.001
Gender							
Male	134 (54)	REF		0.888	REF		0.418
Female	112 (46)	0.966	0.597-1.563		1.196	0.776-1.842	
pTNM stage							
II	131 (53)	REF		0.621	REF		0.369
III	115 (47)	1.128	0.700-1.817		1.219	0.792-1.875	
pT status							
T1 + T2	18 (7)	REF		0.191	REF		0.051
T3	196 (80)	1.271	0.396-4.841	0.686	1.813	0.569-5.777	0.315
T4	32 (13)	2.138	0.613-7.460	0.233	3.276	0.954-11.256	0.059
pN status							
N0	131 (53)	REF		0.687	REF		0.604
N1	74 (30)	1.034	0.598-1.786	0.906	1.161	0.711-1.894	0.551
N2	41 (17)	1.330	0.691-2.560	0.393	1.334	0.739-2.408	0.339
Localization							
Right	117 (48)	REF		0.955	REF		0.127
Left	129 (52)	0.986	0.612-1.590		0.946	0.434-1.10	
Adjuvant therapy							
No	154 (63)	REF		0.005*	REF		0.127
Yes	92 (37)	0.441	0.249-0.783		0.694	0.434-1.110	
TSR							
Stroma-low	137 (56)	REF		0.206	REF		0.036*
Stroma-high	109 (44)	1.359	0.844-2.188		1.589	1.032-2.447	
TB							
Low	194 (79)	REF		0.051	REF		0.039
Intermediate	35 (14)	0.924	0.467-1.828	0.820	1.116	0.612-2.035	0.721
High	17 (7)	2.358	1.157-4.805	0.018*	2.397	1.225-4.693	0.011*

* Significant result.

95% CI 95% confidence interval, N Lymph nodes, p Pathological, SD Standard deviation, T tumor, TB tumor budding, TSR tumor-stroma ratio.

Table 3. Cox multivariate analysis for overall and disease free survival, corrected for age and pT-status.

		Overall survival			Disease free survival		
	N (%)	HR	95% CI	p-value	HR	95% CI	p-value
TSR							
Stroma-low	137 (56)	REF		0.151	REF		0.048*
Stroma-high	109 (44)	1.432	0.877-2.338		1.565	1.005-2.437	
TB							
Low	194 (79)	REF		0.144	REF		0.103
Intermediate	35 (14)	1.21	0.601-2.442	0.592	1.358	0.736-2.505	0.328
High	17 (7)	2.069	1.000-4.283	0.050	2.013	1.022-3.964	0.043*

* Significant result.

Discussion

In the current study two morphology based histological parameters were evaluated and correlated with the prognosis of stage II and stage III colon cancer patients. Both parameters are easy to assess in daily routine pathology, as they are scored on H&E stained sections. This study showed that TSR was an independent prognostic parameter for DFS, but not for OS. TB was a prognostic parameter for OS as well as for DFS in the univariate analysis, but did not remain significant as an independent prognostic parameter after multivariate analysis. No clear explanation could be found why the OS for TSR was not significantly different between the stroma-low and stroma-high group. When observing the survival curves, in the first year after surgery more people died in the stroma-low group. At baseline, the stroma-low group was slightly older and more often at stage III, however these groups were not significantly different. Elderly patients are generally at higher risk for developing late surgery-related complications and may die due to these complications (25, 26). TB was probably not prognostic due to the fact that the group TB-high was small (N=17 (7%)). However, TB is recommended by the ESMO guidelines for localized colon cancer to score in daily diagnostics (18). The prognostic significance of TB was evaluated by Landau et al. in a cohort of stage III colon cancer patients, showing TB to be an independent prognostic parameter for recurrence free survival (27). In contrast, analyzing the prognostic effect of TB in all stages of colon cancer TB failed to be significant as an independent prognostic factor, except when stage II patients were analyzed separately (28). In the current study we did not analyze stage II and stage III separately, due to the low number of patients with TB-high score (stage II 10 patients, stage III 7 patients).

The TSR and TB were both scored by two observers, as is preferred in research setting. The interobserver agreement of scoring TSR was good to almost perfect (Kappa = 0.83) and this result is comparable with current literature (8). The interobserver agreement for scoring TB has shown to be moderate (Kappa = 0.47), as was the intra observer agreement (Kappa =

0.45). The interobserver variability for TB is diverse (10-13), and our results are consistent with previous research (29, 30).

In daily pathology practice there is currently a shift towards digital microscopy. Therefore we compared the microscopical and digital scoring for both TSR and TB. The TSR was well reproducible (intra observer agreement of Kappa = 0.82). TB however showed only a moderate agreement between the microscopical assessment and the digitalized image assessment (intra observer agreement of Kappa = 0.45).

It is remarkable that TB-low and TB-intermediate show similar overall survival curves. Only TB-high showed a significant worse prognosis compared to the other two groups. In our study the TB-high group is small with 7% (N = 17) of the cases in this group, which is comparable with the findings of Eriksen et al. (15). In their study TB was scored on cytokeratin stained sections and the score was divided into two groups with the cut-off point at 10 buds (≥10 buds = budding-high). Here TB was not significant for survival. We may conclude that TB-high is a prognostic factor, but only for a small subgroup of the patient population. Eriksen et al. also investigated the prognostic value of the TSR, and showed that the TSR was independently prognostic for survival (DFS and OS).

An association between TSR and TB was found. The hypothesis is that patients who are stroma-high and TB-high have a significant worse survival compared to stroma-low and TB-low patients. It would be interesting to investigate this combined parameter for impact on survival, but the patient groups in our study were too small to draw reliable conclusions.

As all retrospective cohort studies this design is a limitation of current study. As a benefit of the retrospective design long-time follow-up data was available for all patients in the cohort. The number of patients in the cohort should preferentially be larger and needs validation in an independent validation cohort. The UNITED study, a multicenter prospective study, could serve as a good potential (25).

Both TB and TSR are scored on H&E stained sections and can thus be scored during routine diagnostics. Comparing both methods, TSR is a fast and easy parameter to score and is highly reproducible compared to TB. Some pathologists prefer to score TB after the slide is stained for cytokeratin for better visualization of the tumor buds. This certainly helps to increase the reproducibility, but also makes the scoring more costly and time consuming.

Regarding the simplicity and consistency of assessing TSR and its independent prognostic value for disease free survival of stage II and III colon cancer patients, we suggest that adding TSR as a biomarker in the pathology report could be of value in clinical decision policy.

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Supplementary material

Supplementary Table 1. In- and exclusion criteria.

Inclusion criteria	Exclusion criteria
pStage II and III	Age <18
H&E slides available	Rectal cancer
	Neoadjuvant treatment
	Medical history of cancer 10 years prior to colon cancer or any colon cancer in history
	Double tumors
	Deceased within 30 days after surgery

Supplementary Table 2. The association between tumor-stroma ratio and tumor budding. (Chi-square p=0.001).

		Tumor-stroma ratio		
		Stroma-low	Stroma-high	Total
Tumor budding	Low	120 (88%)	74 (68%)	194
	Intermediate	10 (7%)	25 (23%)	35
	High	7 (5%)	10 (9%)	17
Total		137	109	246