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ORIGINAL ARTICLE

The Proportion of Tumour-Stroma in Metastatic Lymph Nodes is An Accurately Prognostic Indicator of Poor Survival for Advanced-Stage Colon Cancers

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Abstract

The importance of tumour microenvironment in tumour behaviour has now become clearer. This study aimed to determine the prognostic effect of the proportion of tumour-stroma (PTS) in metastatic lymph nodes of advanced-stage colon cancers (CCs). We investigated PTS in positive lymph nodes of stage III-IV CC patients who underwent surgical treatment between 2004 and 2014. We used a standard approach in methodology. PTS was significantly associated with prognostic factors in the metastatic lymph nodes (perineural invasion [$p = 0.031$], lymphatic invasion [$p = 0.032$], invasive margin [$p = 0.043$], advanced pT [$p = 0.020$], and margin involvement [$p = 0.034$]). In addition, the correlations between PTS estimates (R = 0.704 to 0.617, $p < 0.001$), the reproducibility of the research (Kappa = 0.72–0.68) and the usefulness of the cut-off value (ROC: 50.33%; AUC = 0.752 [0.667–0.857]) were successful. In univariate analysis, 5-year survival was poor for RFS ($p < 0.001$), OS ($p = 0.001$) and LR ($p = 0.013$) in high PTS patients. Multivariate analysis confirmed that high PTS was an independent worse parameter for RFS (HR = 1.32, 95% CI: 1.17–2.55, $p = 0.001$) and OS (HR = 1.37, 95% CI: 1.25–1 - 2.56, $p = 0.009$). In this study, we showed that high PTS in metastatic lymph nodes was a successful prognostic marker for advanced-stage CCs. Also, the standard approach we used for the methodology was successful.

Keywords Colon cancers · Proportion of tumour-stroma · Pathology · Prognostic markers · Stage III-IV

Introduction

Colon cancer (CC) is the second most common cause of cancer-related death in both men and women in Europe [1]. Although the outcomes have improved significantly in CCs with improvements in imaging and surgical procedures, relapses still continue in most advanced-stage CC (stage III-IV) [1, 2]. Also, while the traditional pathological staging system remains important for the therapeutic decision in CC, the value of existing pathological parameters alone in showing direct outcome and treatment response is still limited [2, 3]. In addition, although current guidelines recommend adjuvant chemotherapy as a standard treatment for advanced-stage CCs, due to the heterogeneity of CCs, some subpopulations may benefit from targeted therapies [4, 5]. Therefore, new pathological prediction parameters are still needed. One of the most promising pathological parameters recently is the proportion of tumour-stroma (PTS).

Although many biomarkers in the literature have focused on tumour cells, the principle of 'seed-and-soil' has recently been brought up and the role of tumour microenvironment in recurrence and metastasis has been reconsidered [6, 7]. Studies have shown that the normal stromal host tissue surrounding the tumour cells changes complexly during the progression of cancer cells. For example, these stromal cells can play an active role in the growth, progression and invasion of cancer cells by producing various cytokines and growth

Highlights
- *We have presented a parameter that provides reliable findings in many large studies.*
- *We created our population quite homogeneously.*
- *We worked in advanced stage CC, where we encounter most frequently and deaths and relapses are most common.*
- *Unlike other studies, we examined this parameter in lymph nodes.*
- *We tried to provide standardization to pathological evaluation methods.*

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factors. Also, tumours with high intra-tumour stroma can have a more aggressive phenotype. In addition, the stroma of the tumour can affect the response to chemotherapy [7–9]. Therefore, evaluation of the proportion of tumour-stroma (PTS) has been reported as a successful prognostic parameter in many publications and corresponds to the microscopic measurement of the amount of intra-tumour stroma in surgical resection materials. For example, tumours with a high PTS in CC are associated with worse survival than tumours with low PTS [10–13]. Also, this simple, fast and cost-effective pathological technique has been confirmed in many studies in terms of robustness and reliability [10, 11, 13].

As a result, PTS can be used as a reliable marker in CCs to improve risk classification and optimize adjuvant treatment selection. The study aims to investigate the estimated potential of PTS on survival in metastatic lymph nodes of advanced CC patients.

Materials and Methods

One of the most important challenges of the test studies is to decide on the evaluation method. Publications on PTS show significant differences in pathological evaluation [10–13]. In this study, we used a standard approach called model A and method 1 [14, 15]. Model A uses the deepest invasive block, invasive margin, and hot spot area. However, the deepest invasive block is not present in lymph node biopsies. Therefore, this part of the model A is omitted. Method 1 uses ×20 objective, immunohistochemistry and quantitative counting. In other words, this study tried to provide a standard in PTS evaluation.

This study was prepared according to REMARK [16] and summarized in Fig. 1.

Study Design

Ethical approval for this study was given by Kırıkkale University Health Research Ethics Committee (2019.11.11). Also, all procedures were carried out in accordance with the 1964 Helsinki Declaration and the ethical standards of the national / institutional research committee.

This retrospective study was carried out at Kırıkkale University Faculty of Medicine. Four hundred sixty patients who were operated for colorectal cancer between 2004 and 2014 were included in the study. There were no patients in our population who died or relapsed within 1 month, had double tumours and received adjuvant treatment before surgery. Exclusion criteria are summarized as follows: other disease stages ($n = 68$), absence of tumour blocks in archives ($n = 9$), insufficient tumour tissue for examination ($n = 4$), presence of different stages on slides ($n = 12$), rectal tumours ($n = 235$), and three criteria listed in the previous sentence ($n = 20$). Finally, our population consisted of 172 patients. Clinicopathological and survival data were obtained from Kırıkkale University archives. The parameters used in the study were age, size, gender, perineural invasion, lymphatic invasion, margin involvement, invasive margin, local

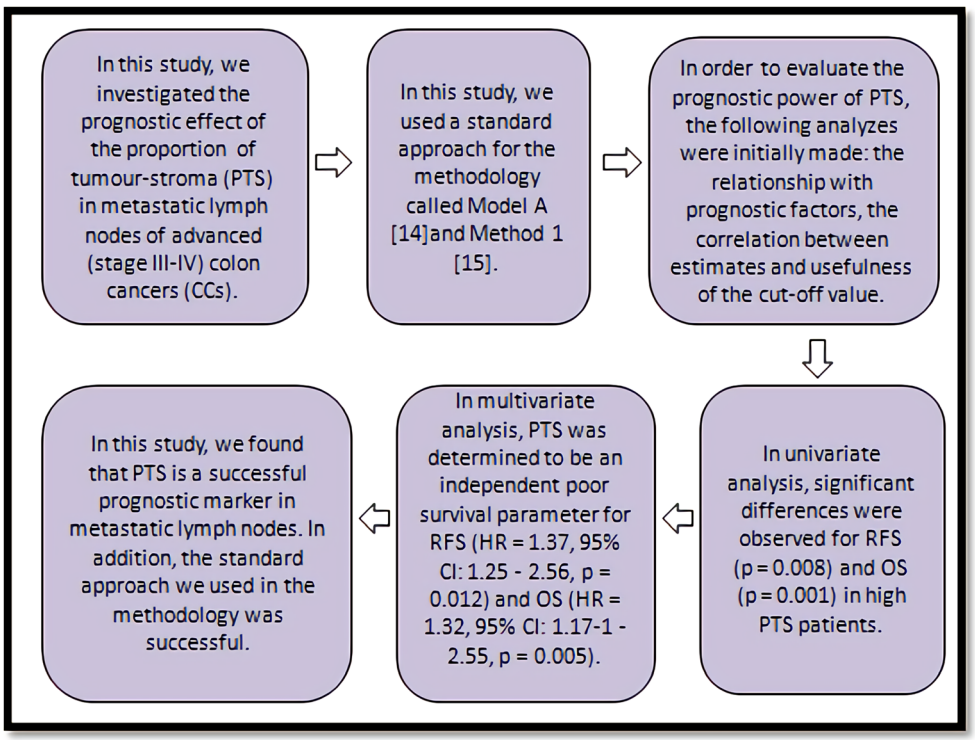

Fig. 1 Summary of the study. Abbreviations: CC: colon cancer, H&E: Hematoxylin and eosin, IHC: Immunohistochemistry, RFS: Relapse-free survival, OS: Overall survival, HR: Hazard ratio, CI: Confidence interval

inflammatory response, localization, tumour necrosis, tumour deposit and grade.

Samples

Tumour samples stored at room temperature embedded in paraffin were taken from the archive of the Pathology Department of Kırıkkale University. Existing tumour blocks ranged from 2 to 22 per patient. The slides of these tumour blocks were scanned and a tumour block representing metastatic lymph node and postoperative biopsy specimens was selected. When selecting tumour blocks, care was taken to have sufficient tumour tissue for future studies. Four 4 μ-thick sections were taken from these blocks ($n = 688$). The two were stained with pan-cytokeratin (AE1/AE3), the rest were stained with hematoxylin and eosin (H&E). The evaluation was done independently by three experienced pathologists, unaware of clinical and pathological information. The average of these observers was taken into account for the final score. The pathological evaluation was based on guidelines from the American Joint Cancer Classification Committee [17].

Histopathological Scoring

PTS was evaluated using a conventional microscope (Nikon AG Instruments, Switzerland, Nikon Eclipse E600). Multiples of 5 were used when PTS was noted, e.g. 5%, 10%, 15%. Only the percentage of stroma was noted and its complementary value showed the percentage of the tumour, i.e. 60% PTS means 40% tumour. Firstly, all sections were scanned to see the distribution of the stroma using an ×10 lens (4.9 mm2). An area containing both stromal and tumour tissue were selected within the field of view. Tumour cells should be at many boundaries of this image area. PTS was noted in 10 high-power fields (HPF) according to the methods mentioned above and averaged. All HPFs were counted for sections with less than 10 HPFs areas ($n = 7$). Finally, all cases were divided into two groups (PTS-low and PTS-high) according to the cut-off value.

In tumours containing necrosis and mucin, these areas were visually excluded. Large vascular structures, smooth muscle tissue, and large nerve structures within the stroma were excluded, but small vascular structures and areas of inflammation were not excluded. Tumour infiltration pattern and local inflammatory response were also evaluated according to model A and method 1. Tumour buds were taken into account while defining the invasive pattern. Tumour budding is defined as the presence of individual and/or small cluster of tumour cells on the invasive front, and areas with tumour buds more than 10 buds are classified as invasive tumour [18]. The local inflammatory response was evaluated according to the recommendations of International Tumor-Infiltrating Lymphocytes Working Group, 2014 [19]. That is, since many non-tumour events can affect the inflammatory response, the mean of the whole area was calculated when evaluating the local inflammatory response. Examples for PTS are available in Fig. 2.

Follow-Up

Survival and recurrence rates were noted for outcomes and primary surgery day was taken into account when calculating endpoints. The follow-up was not interrupted at the end of 60 months and continued for 10 years. However, all events after 60 months were recorded as 60 months. The time between the primary surgery day and the day of death was determined as overall survival (OS). The time between the primary surgery day and local/regional recurrence day or the day of death was defined as recurrence-free survival (RFS). Clinical, radiological or pathological relapse of cancer was defined as recurrence of the disease. This condition is called local recurrence (LR) if it is limited to the treatment area and distant recurrence (DR) if it has spread to a distant area such as liver, lung, retroperitoneum.

Reproducibility

The heterogeneity of tumours and agreement between observer were evaluated in terms of reproducibility. Heterogeneity in tumours was examined by Intra -Class Correlation (ICC) [20]. ICC is a ratio of total variance calculated by differences between tumours. If the difference is due to inter-tumour variabilities, such as biological variation, ICC is expected to be high. If the difference is due to intra-tumour variabilities, such as heterogeneity, ICC is expected to be low. The agreement between the observers was examined by the Kappa test (κ). κ value is a ratio of the total variance calculated by differences between observers and was classified according to Landis in this study [21].

Immunohistochemistry

Immunohistochemical staining was performed on a 4-μm thick histological tissue section of each tumour. Each section had positive control (cancer cells showing AE1/AE3 expression) and negative internal control (normal colon tissue). After deparaffinization and rehydration, antigen recovery was performed by boiling the sections in citrate buffer (pH = 8.0) for 10 min in the microwave, and the sections were cooled to room temperature. To block endogenous peroxidase activity, the sections were incubated for 10 min in 0.3% Hydrogen peroxide-methanol solution at room temperature. Mouse monoclonal AE1/AE3 (Dako, 1:250, clone M3515) was used as the primary antibody. Sections were incubated in the primary antibody overnight at room temperature. The next day,

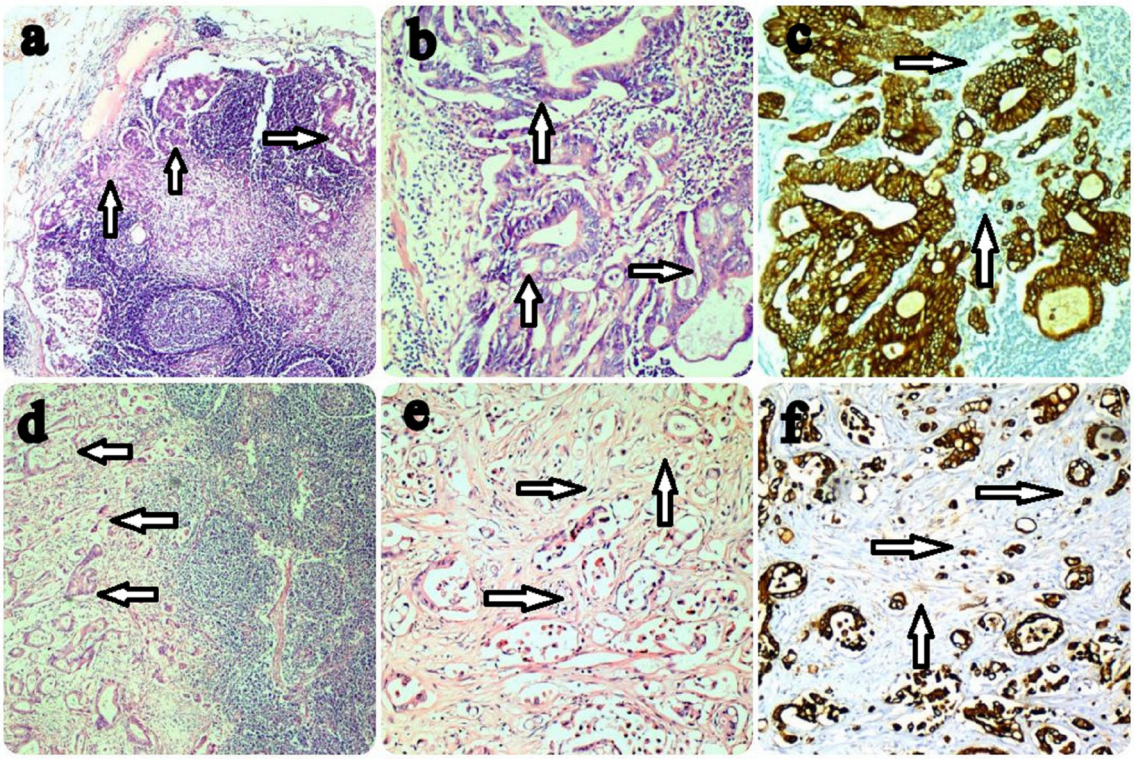

Fig. 2 Examples of microscopic images. Firstly, all slides were scanned using an ×10 objective. An area containing both stromal tissue and tumour tissue (arrows) was selected in the field of view. PTS was scored in 10 high-power fields. Finally, all cases were classified as high PTS (a [×4, H&E], b [×20, H&E], c [×20, IHC]) and low PTS (d [×4, H&E], e [×20, H&E]-f [×20, IHC]). Abbreviations: PTS: Proportion of Tumour Stroma, H&E: Hematoxylin and eosin, IHC: Immunohistochemistry

secondary anti-mouse antibodies (Dako) were applied for 1 h. Then, sections were stained with 3,3′-diaminobenzidine (DAB) for 5 min and counterstained with hematoxylin (Merck, Germany, Darmstadt). Finally, sections were closed with Pertex (Histolab, Gothenburg Sweden).

Statistical Analysis

Frequency, percentage, standard deviation, ranges and averages were used to note statistical variables. Relationships between prognostic parameters were analyzed by Chi-Square analysis. Spearman analysis was used for correlation and Wilcoxon Signed-Rank test was used for differences. Receiver operating characteristic (ROC) curve was used for the optimal cut-off value. The usefulness of the test was evaluated by the area under the ROC curve (AUC). As noted above, the heterogeneity in tumours was assessed by the ICC test and the agreement between the observer was assessed by the κ test. Log-Rank test and Cox regression analysis [95% confidence interval (CI) and 1.0 hazard ratio (HR)] were used for the relationship between univariate and multivariate survival groups. Survival curves were presented by Kaplan-Meier analysis. The significance level for *P* values was accepted as 0.05. SPSS 21.0 (IBM Institute, USA, North Castle) was used for the analysis.

Results

Patient Characteristics

Data of metastatic lymph nodes are given here, data of resection materials are given in the tables. 83 (48.2%) of the patients were female and 89 (51.8%) were male. The mean of size and age were 6.23 ± 1.82 cm (range: 2–11 cm) and 76.27 ± 9.43 years (range: 33–95 years). 84 (48.9%) of the patients were low/moderately differentiated, 88 (51.1%) were poorly differentiated; 85 (49.5%) of the patients were pT1/pT2, 87 (50.5%) were pT3/pT4.

Histopathological Scoring

All slides were scanned at low power magnification to have an idea of the distribution of the tumour stroma. As we expected, the stroma was not homogeneously distributed and was increasing in invasive areas. The stroma was evaluated according to the two standard methods mentioned above. For the biopsy, high PTS was detected in 78 (45.3%) patients and low PTS in 94 (54.7%) patients. The mean percentage of PTS was 50.57 ± 8.39 (range 20–85). Considering the categorical data, there was a significant relationship between high PTS and poor prognostic parameters (perineural invasion [$p = 0.031$], lymphatic invasion [$p = 0.032$], invasive margin [$p =$

0.043], advanced pT [$p = 0.020$], and margin involvement [$p = 0.034$]) (Table 1). Given the continuous data, the cut-off value was useful (ROC: 50.33%; AUC = 0.752 [0.667–0.857]) (This value is considered 50% for practical use) (Fig. 3). Also, analyses of the correlation (R = 0.704 to 0.617, $p < 0.001$) (Table 2) and difference (R = 0.314 to 0.456, $p < 0.001$) was good (Table 2).

Reproducibility

Both continuous and categorical variables were examined separately, but only the best results were mentioned here, as the results were similar. Most of the heterogeneity was due to biological variation between different tumours. For example, the 0.684 ICC value in Table 2 means that the

Table 1 The relationship between PTS and clinicopathological characteristics

		Lymph Node			Resection		
		High PTS	Low PTS	*P*- value	High PTS	Low PTS	*P*- value
Age				0.559			0.382
	<76	40 (51)	44 (46)		37 (52)	47 (46)	
	≥76	38 (49)	50 (54)		33 (48)	55 (54)	
Gender				0.181			0.104
	Female	42 (53)	41 (43)		39 (55)	44 (43)	
	Male	36 (47)	53 (57)		31 (45)	58 (57)	
Size				0.574			0.502
	<6 cm	34 (43)	45 (47)		30 (42)	49 (48)	
	≥6 cm	44 (57)	49 (53)		40 (58)	53 (52)	
Lymphatic invasion				***0.032****			***0.029****
	No	46 (58)	40 (42)		42 (60)	44 (43)	
	Yes	32 (42)	54 (58)		28 (40)	58 (57)	
Perineural invasion				***0.031****			***0.014****
	No	42 (53)	37 (39)		40 (57)	39 (38)	
	Yes	36 (47)	57 (61)		30 (43)	63 (62)	
Tumour necrosis				0.656			0.454
	No	40 (51)	45 (47)		37 (52)	48 (47)	
	Yes	38 (49)	49 (53)		33 (48)	54 (53)	
Invasive margin				***0.043****			**0.011***
	No	41 (52)	35 (37)		39 (55)	37 (36)	
	Yes	37 (48)	59 (63)		31 (45)	65 (64)	
pT-stage				***0.020****			***0.007****
	pT1/pT2	47 (60)	40 (42)		44 (62)	43 (42)	
	pT3/pT4	31 (40)	54 (58)		26 (38)	59 (58)	
Margin involvement				***0.034****			***0.006****
	No	45 (57)	39 (41)		43 (61)	41 (40)	
	Yes	33 (43)	55 (59)		27 (39)	61 (60)	
Grade				0.132			0.236
	Low grade	43 (55)	41 (43)		38 (54)	46 (45)	
	Moderate/High grade	35 (45)	53 (57)		32 (46)	56 (55)	
MSI Status				0.739			0.405
	MMR-P	42 (53)	53 (57)		36 (51)	59 (57)	
	MMR-D	36 (47)	41 (43)		34 (49)	43 (43)	
LIR				0.777			0.252
	Weak	44 (57)	51 (54)		35 (50)	60 (58)	
	Prominent	34 (43)	43 (46)		35 (50)	42 (42)	

*. The statistical significance limit for *P* value was accepted as 0.05 and significant results were written in italics

PTS Proportion of Tumour Stroma, *pT* Pathologic tumour stage, *LIR* Local inflammatory response, *MSI* Microsatellite instability, *MMR-D* Mismatch repair proteins deficiency, *MMR-P* Mismatch repair proteins proficiency

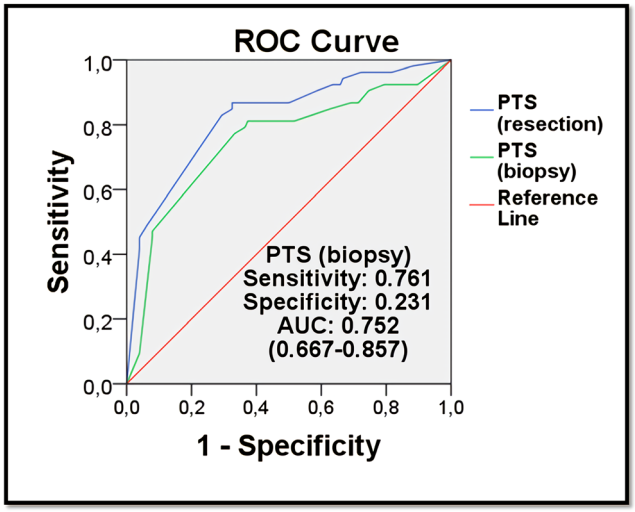

Fig. 3 ROC curves. Areas under the ROC curves (AUC) analyzed by manual methods. Abbreviations: PTS: Proportion of Tumour Stroma, ROC: Receiver Operating Characteristic

variation in a single tumour represents 31.6% of the total heterogeneity. Also, the results of the inter-observer agreement were generally moderate and excellent. On the other hand, the values of the ICC and K were higher in the resection material. Because the area examined in the resection materials was larger and the tumour stroma was more prominent. Therefore, as expected, this event caused the area to appear more heterogeneous and increased the agreement between the observers (Table 2).

Follow-Up

One hundred and five patients died ($n = 63$ in high PTS, $n = 42$ in low PTS) and 132 patients recurred ($n = 70$ in high PTS, $n = 62$ in low PTS). Also, local recurrence was observed in 95 patients ($n = 55$ in high PTS, $n = 40$ in low PTS) and distant recurrence in 75 patients ($n = 40$ in high PTS, $n = 32$ in low PTS). For the biopsy, 5-year RFS and OS rates were 35% and 56% in low PTS cases, 10% and 20% in high PTS cases. In addition, LR and DR ratios were 42% and 34% in low PTS cases, 70% and 51% in high PTS cases (Table 3).

Survival Analyses

In univariate analysis, a significant relationship was observed between high PTS and survival groups of RFS ($p < 0.001$), OS ($p = 0.001$) and LR ($p = 0.013$). In multivariate analysis, high PTS was found to be an independent poor prognosis parameter for RFS (HR = 1.32, 95% CI: 1.17–2.55, $p = 0.001$) and OS (HR = 1.37, 95% CI: 1.25–1 - 2.56, $p = 0.009$). Advanced pT and margin involvement were significantly associated with poor survival in univariate analysis, and margin involvement was another independent poor prognosis parameter in multivariate analysis (Table 4, Fig. 4).

Discussion

In this retrospective study, the role PTS in survival was investigated by examining metastatic lymph nodes in advanced CC cases. We have seen that PTS can be a practical marker in determining patients with poor prognosis in CC. Also, we tried to provide a standard approach to existing pathological assessment methods.

Many current studies report that PTS is an independent poor prognostic marker in colorectal cancer patients [18, 22–25]. However, these studies contain many differences in terms of population. For example, in many studies, colon and rectal cancer patients were handled together [11, 22, 23]. Also, it is not clear whether there is a difference between rectal cancer and CC in terms of PTS in the literature. For example, Hutchins et al. [23] examined PTS in patients with colorectal carcinoma and found that the rate of stroma was significantly higher in patients with rectum cancer than in patients with CC. Also, Park et al. [11] conducted a similar study and determined the same result. That is, PTS should be examined separately in patients with colon and rectal cancer. In this study, we examined PTS in a patient population operated for advanced CC and excluded rectal cancer cases to prevent this confusion. Therefore, our patient population was quite homogeneous.

Differences in assessment methods and low reproducibility rates are the most important challenges to overcome in the

Table 2 The examination of continuous variables and reproducibility

	N	PTS (Correlation)	PTS (Difference)	ICC- Categorical (95% CI)	Kappa values
PTS (A&B) (Resection)	172	0.704, $p < 0.001$	0.314, $p < 0.001$	0.684 (0.442–0.793)	0.72
PTS (A&C) (Lymph Node)	172	0.683, $p < 0.001$	0.332, $p < 0.001$	0.655 (0.408–0.775)	0.69
PTS (B&C) (Resection)	172	0.617, $p < 0.001$	0.456, $p < 0.001$	0.619 (0.398–0.743)	0.68

PTS Proportion of Tumour Stroma, *W* Wilcoxon Signed Rank test, *S* Spearsman correlation analysis, *κ* Kappa values, *ICC* Intra-Class Correlation Coefficient, *CI* Confidence interval, *N* Number, *A* First observer, *B* Second observer, *C* Third observer

Table 3 The relationship between PTS and univariate survival groups

		Overall survival		Relapse-free survival		Local recurrence		Distant recurrence	
		5-year (%)	*P* value	5-year(%)	*P* value	5-year (%)	P- value	5-year (%)	P- Value
Age			0.744		0.637		0.677		0.733
	<76	45		45		49		37	
	≥76	39		39		40		40	
Size			0.428		0.374		0.443		0.526
	<6 cm	51		50		41		37	
	≥6 cm	34		35		56		45	
Gender			0.836		0.788		0.850		0.954
	Female	44		48		45		43	
	Male	41		43		50		42	
Lymphatic İnvasion			0.257		0.196		0.229		0.382
	No	55		47		42		36	
	Yes	33		27		61		47	
Perineural İnvasion			0.219		0.134		0.347		0.449
	No	61		45		40		38	
	Yes	35		25		58		45	
LIR			0.833		0.739		0.856		0.945
	Weak	48		47		46		16	
	Prominent	42		42		51		16	
pT-stage			0.072		***0.033****		0.187		0.277
	pT1/ pT2	63		39		44		9	
	pT3/ pT4	38		15		68		16	
Invasive margin			0.255		0.180		0.452		0.790
	No	60		48		39		14	
	Yes	38		26		54		15	
Margin involvement			***0.042****		***0.014****		0.123		0.165
	No	60		39		45		35	
	Yes	31		11			67	49	
MSI Status			0.867		0.709		0.724		0.931
	MMR-P	45		47		44		42	
	MMR-D	40		43		51		41	
Grade			0.956		0.881		0.849		0.707
	Low grade	46		45		42		35	
	Moderate/	45		46		49		41	
High grade									
Tumour necrosis			0.512		0.442		0.464	0.553	
	No	48		51		40		37	
	Yes	38		38		55		44	
PTS			***0.001****		***<0.001****		***0.013****	0.075	
(Lymph Node)	Low	56		35		42		34	
	High	20		10		70		51	
PTS			***<0.001****		***<0.001****		***0.008****	***0.038****	
(Resection)	Low	58		42		41		38	
	High	18		9		72		65	

*. The statistical significance limit for *P* value was accepted as 0.05 and significant results were written in italics

PTS Proportion of Tumour Stroma, *LIR* Local inflammatory response, *pT* Pathologic tumour stage, *MSI* Microsatellite instability, *MMR-D* Mismatch repair proteins deficiency, *MMR-P* Mismatch repair proteins proficiency

Table 4 The relationship between PTS and multivariate survival groups

		Overall survival		Relaps-free survival		Local recurrence		Distant recurrence	
		(*n* = 172) (%)		(*n* = 172) (%)		(*n* = 172) (%)		(*n* = 172) (%)	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95%CI)	*P* value	HR (95% CI)	*P* value
pT-stage	pT1/ pT2	1	–	1	–	1	–	1	–
	pT3/pT4	2.43 (0.61–9.62)	0.409	2.24 (0.58–9.13)	0.309	3.77 (0.48–8.51)	0.537	NC	0.883
Margin involvement	No	1	–	1	–	1	–	1	–
	Yes	1.65 (0.77–8.43)	0.257	1.44 (1.22–2.71)	***0.045****	5.52 (0.56–10.21)	0.446	7.31 (0.41–15.45)	0.612
PTS (Lymph Node)	Low	1	–	1	–	1	–	1	–
	High	1.32 (1.25–2.56)	***0.009****	1.37 (1.17–2.55)	***0.001****	3.61 (1.32–3.18)	0.061	2.07 (0.89–4.10)	0.128
PTS (Resection)	Low	1	–	1	–	1	–	1	–
	High	1.33 (1.24–3.56)	***0.001****	1.25 (1.22–2.88)	***<0.001****	1.35 (1.17–2.84)	***0.039****	1.72 (0.87–4.05)	0.089

*. The statistical significance limit for P value was accepted as 0.05 and significant results were written in italics

PTS Proportion of Tumour Stroma, *LIR* Local inflammatory response, *pT* Pathologic tumour stage, *MSI* Microsatellite instability, *MMR-D* Mismatch repair proteins deficiency, *MMR-P* Mismatch repair proteins proficiency, *HR* Hazard ratio, *CI* Confidence interval, *NC* Not calculable

evaluation of PTS [10–13, 18, 22–25]. For example, some studies have investigated PTS in two areas, focal and global, and have found better results with the focal method [10, 13]. Whereas, Hynes et al. [24] also used this method, but found better results with the global method [14, 15]. On the other hand, Mesker et al. [10] found that the deepest infiltrated

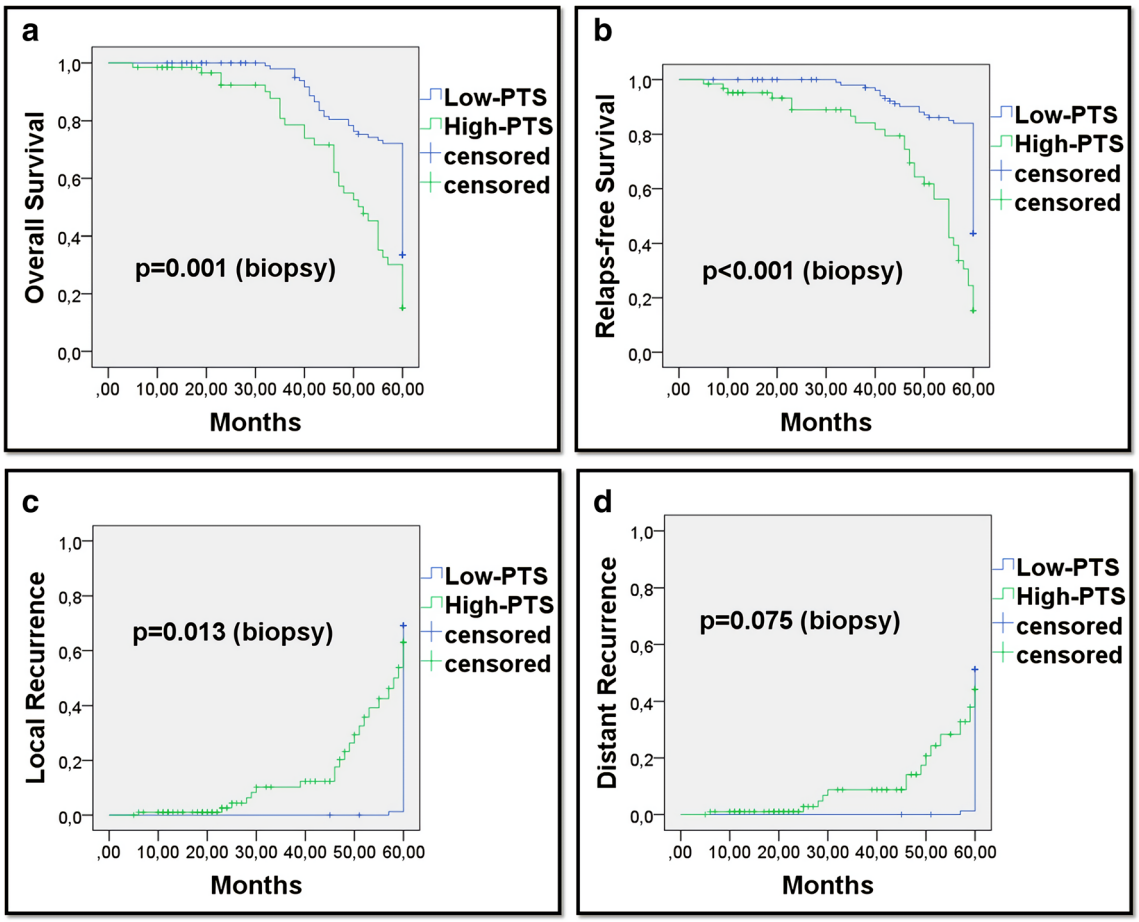

Fig. 4 Curves of survival and recurrence. Kaplan-Meier curves of overall survival (**a**), relapse-free survival (**b**), local recurrence (**c**), and distant recurrence (**d**). The statistical significance limit for *P* value was accepted as 0.05

tumour areas in the bowel wall had the highest stroma rate and therefore recommended the use of the highest pT-stage sections for histological evaluation. In this study, we examined PTS with two standard methods described above. These methods bring a user-friendly, understandable and practical approach to PTS and aim to turn histopathological biomarkers into daily diagnostic practice. Also, the high intra-observer agreement in our study shows that these methods are highly reproducible. In addition, the fact that PTS is significantly associated with survival in metastatic lymph node biopsy specimens, which represent a small area compared to the main tumour, shows the accuracy and sensitivity of these methods. That is, unlike other studies, our study is quite good in terms of standardization in methodology.

Although PTS was significantly associated with most prognostic factors, this relationship with tumour necrosis was not observed. Increased angiogenesis in the region where tumour stroma expands and thus a decrease in tissue hypoxia may be one of the factors explaining this relationship [26]. Also, since necrosis increases systemic inflammation through a series of pro-inflammatory cytokines, such as interleukin-6, this relationship may explain the low local inflammatory response in patients with high PTS [27]. In addition, increased stroma in the tumour may have facilitated the survival of tumour cells by increasing the use of anaerobic metabolism products by tumour cells [28]. That is, more studies are needed on the relationship between PTS and tumour necrosis.

Although the importance of tumour stroma in the progression of cancer cells is known, its relationship with other elements in the tumour microenvironment, such as local inflammatory response and microsatellite status, has not been clearly established. For example, it has been suggested in the literature that tumour stroma can prevent tumour infiltration by immune cells [29, 30]. On the other hand, many studies have reported that MMR-P cases are also associated with a weak local inflammatory response [30]. In our study, weak peritumoral inflammation and MMR-P tendency were observed in patients with high PTS tumours. However, in our study, the effect of TSS on survival remained independent of these two parameters. This indicates that the effect of tumour stroma on these two parameters is not direct and indicates the presence of another mechanism. Consequently, the relationship between tumour stroma and these two parameters should be further characterized.

In this study, a significant relationship was found between high PTS and the presence of infiltrative border. Also, there was a relationship between high PTS and angiolymphatic invasion and perineural invasion. It has been reported in the literature that increased tumour stroma facilitates epithelial-mesenchymal transition (migration of the tumour to normal tissue at the host-tumour interface), gives the tumour an infiltrative appearance and increases aggressive behaviour [31]. Similarly, the increase of immature stroma has been associated with tumour budding, which is seen as an indicator of epithelial-mesenchymal transition [31, 32]. Therefore, our findings support the role of tumour stroma in facilitating tumour progression and in enhancing metastasis ability.

Many studies in the literature suggest that PTS should be evaluated using H&E stained sections [22–25]. However, publications are reporting that the agreement and detection rates between observers are better in studies with IHC stained sections [18]. Also, it is unclear which method is superior in terms of prognostic effects. Although we mainly use the IHC method in this study, we also used the H&E method as an aid. The major disadvantage of H&E stained sections is to distinguish smooth muscle fibres and stromal tissue. To distinguish these two tissues, the cell nucleus must be carefully examined (the smooth muscle cell has a cigar-shaped round nucleus and the fibroblast has a spindle-shaped nucleus). We recommend staining the desmin in case of difficulty. The major disadvantage of IHC stained sections is that some cells other than adenocarcinoma (e.g. endothelial cells of vascular neoangiogenesis) also show reactivity. We think the IHC method is more useful. Larger studies are needed to standardize the counting technique.

The important features of this research are as follows. We have chosen an easy-to-use and reliable parameter that has been studied in many large studies. We conducted our study in a very homogeneous population with a well-designed cohort. We tried to standardize the pathological assessment methods with a fixed approach. And we conducted our research in accordance with REMARK guidelines.

The limitations of our study are as follows. In all retrospective analyzes, there is an internal restriction in the form of sampling bias. Since the area we evaluate was a small part of the tumour, it may not represent the entire tumour. Archive records were used for survival information and individual information was not examined. Since our cases were treated according to guidelines before 2014, there may be differences compared to current treatment methods.

Conclusion

In this study, we showed that PTS is an independent predictor of poor survival in CC. According to our findings, this parameter is a strong biomarker that can be easily used in daily practice to improve the risk stratification of patients. Our study also showed that applying a standard methodology contributes to successful results.

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Compliance with Ethical Standards

Conflict to Interest Statement Authors do not report any conflicts of interest.

Ethical Standards The study was carried out at Kırıkkale University and approved by Kırıkkale University Health Research Ethics Committee (2019.11.11). All procedures performed in our study were consistent with the ethical standard of the national/institutional research committee and the 1964 Helsinki declaration and subsequent adjustment.

Abbreviations *PTS*, Proportion of Tumour Stroma; *CC*, Colon cancer; *H&E*, Hematoxylin and eosin; *IHC*, Immunohistochemistry; *HPF*, High-power field; *SD*, Standard deviation; *HR*, Hazard ratio; *CI*, Confidence interval; *ICC*, Intra-Class Correlation Coefficient; *K*, Kappa; *MSI*, Microsatellite instability; *MMR*, Mismatch repair proteins; *RFS*, Relapse-free survival; *OS*, Overall survival; *LR*, Local recurrence; *DR*, Distant recurrence; *Model A*, Using the 'deepest invasive blocks&hot-spot area & invasive margin'; *Method 1*, Using the '×20 objektive&IHC&quantitative'

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