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Tumour Budding and Tumour Stroma Ratio are Reliable Predictors for Death and Recurrence in Elderly Stage I Colon Cancer Patients

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ABSTRACT

Aim: Tumour budding (BD) and Tumour stroma ratio (TSR) are considered valuable survival parameters for colon cancer (CC), but it is still unclear whether these parameters predict a poor prognosis. This study aimed to determine the survival effect of TB and TSR in elderly stage I CC patients.

Methods: We evaluated these parameters in eighty-eight stage I CC patients who underwent surgical treatment alone between 1998 and 2015. The relationship between these parameters and age was investigated. Model A was used for methodology.

Results: In elderly patients (≥68), these parameters obtained more successful results for following analysis: relationship between prognostic factors [lymphatic invasion (BD, p = 0.003; TSR, p = 0.003), perineural invasion (BD, p = 0.016; TSR, p = 0.004), tumour deposites (BD, p = 0.005; TSR, p = 0.033), MSI (BD, p = 0.031; TSR, p = 0.012), etc.], correlation of estimates (BD, r = 0.724; TSR, r = 0.703), and reproducibility of study (BD [Kappa (κ)] = 0.53–0.75; TSR (κ) = 0.56–0.71). Also, the cut-off values were useful for BD (area of under ROC (AUC) = 0.816 [0.707-0.925]) and TSR (AUC = 0.810 [0.697-0.924]). In univariate analysis, in elderly patients, these parameters had a poor 5-year survival for RFS (p < 0.001[BD], p = 0.001[TSR]), OS (p < 0.001[BD], p = 0.005[TSR]), and LR (p = 0.008[BD], p = 0.034[TSR]). Multivariate analysis confirmed that these parameters are independent worse predictors for RFS (BD: 1.42[HR], p = 0.002; TSR: 1.50[HR], p = 0.001), OS (BD: 1.38[HR], p = 0.014; TSR: 1.42[HR], p = 0.005), and LR (BD: 1.61[HR], p = 0.034) in elderly patients.

Conclusions: Our results confirm that BD and TSR are reliable indexes for poor survival in elderly stage I CC patients. We recommend using model A for successful results and standardization.

1. What is already known about this subject

Colon cancer (CC) is a leading cause of morbidity and mortality worldwide, approximately one-tenth of the CC patients are diagnosed with an early stage (stage I) of disease and poor clinical progression is seen in 5–10% of stage I patients. CC is a common tumour in elderly patients, especially in patients over 50 years of age. Also, the mortality ratio in elderly patients is higher than in younger patients. In addition, controversy remains as to how ageing affects tumour biology. Because of the increased incidence of elderly patients with CC, understanding the impact of clinical and pathological features on survival in elderly patients has become even more important today. The most promising parameters include tumour budding (BD) and tumour stroma ratio (TSR).

2. What are the new features

1 Firstly, we present two good parameters that have been discussed recently in the literature.
2 Lymph node-negative early-stage patients are common in daily practice and this patient population is highly heterogeneous in survival. Therefore, it would be more useful to examine the early CC subgroups separately.
3 In this study, we evaluated the relationship between BD/TSR and age in CC patients. The benefit of surgery and chemotherapy in elderly patients is limited. Therefore, it is very important to identify high-risk patients in elderly patients. In this study, we found that these two parameters were more significant in elderly patients. Therefore, these two parameters can provide useful information for

Abbreviations: BD, tumour budding; TSR, tumour stroma ratio; CC, colorectal cancer; AJCC, American Joint Cancer Committee; HPF, high power field; K, kappa; ICC, intra-class correlation coefficient; IHC, immunohistochemistry; H&E, hematoxylin and eosin; SD, standard deviation; CI, confidence interval; HR, hazard ratio; MSI, microsatellite instability; MMR, mismatch repair proteins OS overall survival; RFS, relapse-free survival; LR, local recurrence; DR, distant recurrence; Model A, using the 'deeply invasive blocks&hot-spot area&invasive margin'

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predicting survival in elderly patients.

4 The main disadvantage of pathological evaluation is the lack of standardization. Sources of variability include visualization, staining, optimal field, optimal method, etc. Whereas, each of these methods represents a different area of the tumour and may alter the results obtained. This also applies to these parameters, especially TSR. In this study, we used a standard method that we had previously achieved successful results, Zengin (2019) [18]. This method is model A and recommends using deepest invasive block, hot-spot area and invasive margin for evaluation. In this study, we tried to improve standardization in pathological evaluations.

3. How might it impact on clinical practice in the foreseeable future?

Our results confirm the prognostic effect of BD and TSR in elderly stage I CC patients. Based on the present results, these indicators can be a promising risk estimator that can be easily implemented in daily practice. Remarkably, this cell-based parameter has a high predictive power without any additional cost. We also recommend using model A for successful results in future studies.

4. Introduction

Colon cancer (CC) is one of the most common causes of death worldwide, and 5–10% of CC patients have stage I disease [1]. Approximately 90–95% of stage I patients have a good postoperative prognosis and the use of adjuvant chemo-radiotherapy is generally not recommended [2]. However, some patients in this population have a poor prognosis (5–10%), and TNM system does not consider any other evidence that permits risk stratification for these patients [3]. This is a particular clinical challenge and new prognostic markers are needed for better clinical management. On the other hand, life expectancy has increased dramatically worldwide and the number of people over the age of 65 is increasing. In fact, studies show that even 75-year-olds will live for more than 7 years [4]. Also, studies in the literature show that the incidence of CCs increases in the elderly population [4,5]. In addition, CCs in elderly patients is associated with significantly higher mortality rates than in younger patients [5]. Therefore, it has become more important to understand the different clinical and pathological characteristics of CCs in elderly patients today. The most promising factors include Tumor Budding (BD) and Tumor stroma (TSR).

Tumour budding (BD) is defined as the presence of individual/small clusters of tumour cells in the invasive front [6,7]. Initially, it was thought that BD was associated with epithelial-mesenchymal transition and that was the first step in the development of extracellular matrix transition [7]. Subsequently, numerous studies have shown that an increase in the number of tumour buds in CC is associated with lymph node metastasis, distant metastasis and increased risk of recurrence [8,9]. Also, according to the International Tumour Budding Consensus Conference Group, BD should be included in the high-risk indicators of CC [10].

Tumour stroma ratio (TSR) is the qualitative or quantitative ratio of tumour stroma to cancer cells [11]. Recent studies have shown that a high rate of tumour stroma ($\geq$50% stroma) is associated with a better prognosis than tumours with abundant carcinoma tissue ($<$ 50% stroma) [11,12]. Also, studies have shown that the stroma of a tumour supports the epithelial-mesenchymal transition, proliferation of cancer cells, loco-regional metastasis, and resistance to chemotherapy [12]. In addition, several studies have confirmed the prognostic value of TSR for many types of cancer, including cervix [13], oesophagus [14], and breast [15]. Furthermore, many large studies have confirmed the importance of TSR in CCs [16,17].

In this research, we investigated the relationship between BD / TSR and age in patients with stage I CC using a well-established methodology.

5. Materials and methods

Publications on BD and TSR show significant differences in population and methodology [7–17]. In this study, we used model A which is a reliable method for standardization [18]. This method recommends the use of the deepest invasive block, hot spot area, and invasive margin for pathological evaluation. So, this study provides significant improvements in the standardization of pathological evaluation.

This study was prepared under the guidance of REMARK [19] and was summarized in **Supplementary Fig. S1**.

5.1. Ethics statement

This research was approved by the Health Research Ethics Committee of Kırıkkale University. This research was conducted in accordance with the 1964 Helsinki declaration and the ethical standards of the national/institutional research committee. All volunteer patients were informed about the content of the study and informed consent was obtained.

5.2. Study design

This retrospective cohort was performed in a tertiary care university hospital in Kırıkkale, Turkey. Six hundred and eighty-five patients who underwent surgery for stage I-IV CRC between 1998–2015 were included in this study and suitable patients were identified according to the following criteria.

5.3. Patients selection and data sources

Clinical, pathological and survival information was obtained from the database of Kırıkkale University. There were no patients with known distant metastasis. Also, patients with synchronous tumours and other malignancies in the past and death or recurrence within 1 month were excluded from the study. The summary of the exclusion criteria was as follows: patients with other stages of diseases (n = 309), rectum cancers (n = 233), cases with missing tumour blocks (n = 15), cases with inadequate tissue in blocks for examination (n = 12), stage I disease not described in slides (n = 10), patients received adjuvant chemo-/radiotherapy (n = 13), and patients diagnosed with another cancer prior to CC (n = 5). As a result, our study population consisted of 88 patients.

Subsequently, these patients were categorized according to the following criteria: age ($<$ 68 and $\geq$68), size ($<$ 5 and $\geq$5), gender (female/male), lymphatic invasion (yes/no), perineural invasion (yes/no), local inflammatory response (yes/no), margin involvement (yes/no), invasive pattern (yes/no), localization (right/left), tumour deposits (yes/no), microsatellite instability (yes/no), grade (low/moderate grade and high grade) and tumour necrosis (yes/no).

5.4. Tissue collection

Fixed in formalin, embedded in paraffin tumour samples were recognized in the archives of Pathology department. The number of tumour blocks ranged from 4 to 17 per cases. Two tumour blocks were selected from each cases using an x10 objective (4.9 mm^2), one showing the deepest invasive area and one randomly selected. For immunohistochemistry (IHC), tumour blocks that could have both enough tissue and adjacent normal colonic tissue were included in the study. Four sections of 4 microns thick (n = 352) were cut from each block by experienced technicians. Three sections were stained with IHC and the other was stained with hematoxylin and eosin (H&E). MSI status was determined by IHC and was classified into two groups as Mismatch Repair Proteins-deficiency (MMR-D) and MMR- proficiency (MMR-P). The evaluation was performed by three experienced pathologists (M.Z, G.Ö. and S.A.) and the final score was given according to the average of

these observers. The guidelines of the American Joint Committee on Cancer Classification (7th) were used for pathological evaluation [20].

5.5. Evaluation method

Choosing the optimal assessment method in diagnostic tests is one of the most important challenges because it can significantly affect the results. In this study, we used a standard method as model A [18]. As described above, model A means using the deepest invasive block, hotspot area, and invasive margin for pathological evaluation. In addition, optimal cut-off values of BD and TSR were analyzed by ROC curves. The value with the lowest true false-positive ratio and the highest true-positive ratio should be preferred for the best cut-off value. The area under the ROC curve (AUC) generally means a measure of the usefulness of a test, and a small area (AUC → 1) means a less useful test.

5.5.1. Evaluation of BD and TSR

BD and TSR were visually predicted by conventional microscopy (Nikon Eclipse E600, Nikon AG Instruments, Switzerland). These parameters were scored per 5 enhancement per magnification, e.g. 5, 10, 15 for BD and 5%, 10%, 15% for TSR. For TSR, only the percentage of carcinoma were given for clarity, but the complementary value indicates the percentage of stromal tissue, i.e. TSR 60% means a 40% stromal percentage.

Firstly, we examined all sections to see the differences in tumour buds and tumour stroma distribution using an x10 objective. An area within the field of view containing both stromal and tumour tissue for TSR and predominantly tumour buds for BD is selected. Also, tumour cells should be present at all image borders in this selected image area. Then, BD and TSR were noted in 10 high-power fields (HPF) using an x 20 objective according to model A. Finally, all patients were divided into two groups as high-density and low-density according to survival-related cut-off values. Representative examples for BD and TSR are shown in Fig. 1.

For BD, only adenocarcinoma cells with a clearly defined blue hematoxylin-stained nucleus were counted to avoid false staining in the form of brown bud-like cytoplasmic structures. For TSR, mucin-bearing areas, necrosis areas, smooth muscle tissue and major vascular structures were visually removed from the score, whereas lymphocytic infiltration and small vascular tissue were not excluded from the stromal continence. In cases with less than 10 HPFs (n = 3), all available HPFs were counted and the mean value was given according to these areas.

5.6. Relationship between BD/TSR and age

In order to examine the relationship between these parameters and age, the results of the following analyzes were examined separately. Firstly, the relationship between BD/TSR, age and prognostic factors was investigated. Then, the results of correlation, difference, and reproducibility between BD/TSR and age groups were examined, respectively.

5.7. Patients follow-up

Survival and recurrence rates were used for outcome measures. For a better decision on the clinical outcome, the follow-up period was determined as a wide range (seventeen years, range: 10.3 to 205.8 months). Time-to-event endpoints were calculated from the day of primary surgery. All events after sixty months or cases whose last follow-up date was over sixty months were censored at sixty months. Overall survival (OS) was defined as the time from the first surgery day to the day of death for any reason or the last day of contact. Relapse-free survival (RFS) was defined as the time from the first surgery day to the day of death for any reason or to the distant/local-regional relapse day. The clinical, radiological and pathological diagnosis of cancer recurrence was defined as relapse of the disease. This status is called local relapse (LR) if it is limited to the prior treatment area and is named distant relapse (DR) if it is spread to another region, including lung, retroperitoneum and liver.

5.8. Reproducibility of study

For reproducibility of study, agreement of observers and heterogeneity of tumours were considered. Three independent pathologists scored these parameters blindly from clinical and pathological information. Inter- and intra-tumour heterogeneity were investigated by Intra-Class Correlation (ICC) [21]. ICC was accepted as a ratio of the variability in tumour heterogeneity. If the majority of the variation is attributed to inter-tumour variation, such as biological variation, the ICC will be high (ICC → 1). If the majority of the variation is due to intra-tumour variation, such as heterogeneity, the ICC will be low (ICC → 0). Kappa (κ) analysis was used to evaluate the agreement of observers. The κ value is the ratio of variability in inter-observer agreement. Landis et al. classified this ratio as perfect, moderate and substantial for values of 0.81–1, 0.41–0.60 and 0.61–0.80, respectively [22].

5.9. Immunohistochemical study

For immunohistochemical analysis, three sections of 4 microns thick were cut from each block (n = 264) and plated on platinum-coated slides (Dako). To obtained retrieval epitope, deparaffinized tissue sections were incubated in Target Retrieval Solution (Dako) for 20 min in a pressure cooker and sections were cooled for 40 min. To block endogenous peroxidase activity, tissue sections were incubated in 3% H2O2 for 20 min, Avidin Block (Dako) for 15 min and Biotin Block (Dako) for 15 min, respectively. Mouse monoclonal AE1/AE3 (1:250, Dako, clone M3515) was used for primary antibody, and mouse monoclonal PMS2 (1:500, Dako, clone A16-4) and MLH1 (1:100, Dako, clone ES05) antibodies were used for MMR (Since there was no family history of Lynch syndrome in the patient population, MSH2 and MSH6 were not performed). These antibodies were diluted in antibody diluent (Dako) and incubated overnight at 25 °C. To detect bound antibody, secondary anti-mouse antibody (Dako) and Avidin-Biotin conjugate complex (Dako) was applied. Then, sections were visualized with diaminobenzidine for 5 min and were stained with Meyer's hematoxylin for counterstain (Merck, Germany, Darmstadt) and coated with Pertex (Histolab, Sweden, Gothenburg), respectively. For each test run, our tissues had a positive (cancer with overexpression of AE1/AE3) and negative internal control (colonic stromal tissue).

5.10. Statistical evaluation

While descriptive variables were noted, standard deviation (SD), ranges and averages were used for continuous variables, frequency and percentage were used for categorical variables. Chi-Square test was used to evaluate the relationships between these parameters and clinicopathological groups. While continuous variables were analyzed, Wilcoxon Signed Rank test was used for differences and Spearman correlation analysis was used for correlations. As mentioned above, optimal cut-off value was evaluated by ROC analysis, tumour heterogeneity was evaluated by ICC analysis, and inter-observer agreement was evaluated by κ test. Significant differences between univariate survival groups were analyzed by Log-rank test and survival curves were presented by Kaplan-Meier method. To define independent prognostic factors, Cox regression analysis with a 95% confidence interval (CI) and a hazard ratio (HR) of 1.0 as a reference was used. P values less than 0.05 were considered significant. SPSS 21.0 (IBM Institute, North Castle, USA) was used for all analyzes.

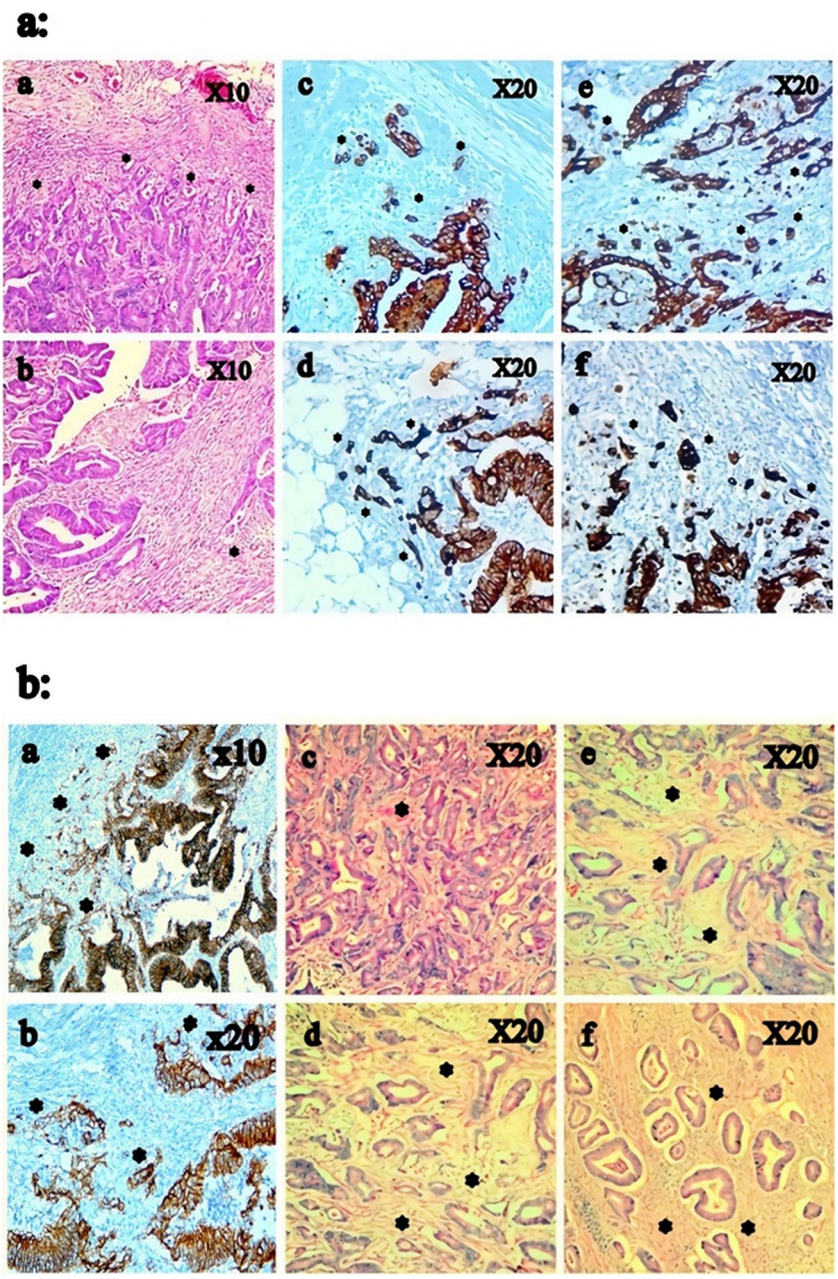

Fig. 1. Representative examples for BD **(1a)** and TSR **(1b).** We initially scanned all the slides to determine areas with the highest and the lowest amount of tumour buds and tumour stroma using an x10 objective. The selected areas in the field of view should include both stromal and tumour tissue for TSR **(asterisks)** and predominantly tumour buds for BD **(asterisks)**. Then, all cases were scored in 10 high-power fields using an ×20 objective for BD (Fig. 1**a)** and TSR (Fig. 1**b)**. In Fig. 1**a**, **a (H&E) - b (H&E) - c (IHC)** shows low BD and **d (IHC) - e (IHC) - f (IHC)** shows high BD. In Figure 1b, **a (IHC) - c (H&E)** - **e (H&E)** shows high TSR and **b (IHC) - d (H&E) – f (H&E)** shows low TSR.
Abbreviations: BD: Tumour budding, TSR: Tumour stroma ratio, H&E: Hematoxylin and eosin, IHC: Immunohistochemistry.

6. Results

6.1. General features

Eighty-eight patients were included in the study. 53 (60.2%) of the cases were male and 35 (39.8%) were female. The mean age and size were 61.48 ± 9.64 (SD) (range: 35–93) and 5.18 ± 1.75 (range: 2–8), respectively. 50 (56.8%) of the cases were in the left colon, 38 (43.2%) were in the right colon; 56 (63.6%) of the patients were low/moderately differentiated and 32 (36.4%) were poorly differentiated. 35 (39.7%) of the patients had high BD, 53 (60.3%) had low BD; 36 (40.9%) of the patients had low TSR and 52 (59.1%) had high TSR. The mean values were 8.27 buds for BD and 39.7% stroma for TSR.

6.2. Estimates of BD and TSR

Firstly, all slides were scanned at low-power magnification (x10 objective), and it was found that tumour buds and tumour stroma were not uniformly distributed in the blocks and that increased at invasive fronts and deeply invasive areas. Two blocks with the best homogeneity were selected from each patient and three gastrointestinal pathologists evaluated these parameters according to model A [18]. Descriptive statistics for BD and TSR are shown in **Supplementary Table S1**. In addition, optimal survival-related cut-off values were useful values and all cases were classified into two groups according to these values as high-density and low-density (BD: ROC = 10.34, AUC = 0.816 [0.707-0.925]; TSR: ROC = 50.43%, AUC = 0.810 [0.697-0.924]) (These values were accepted as 10 buds and 50% stroma to be easy to implement) (Fig. 2).

6.3. Relationship between BD/TSR and age

Since CCs are more common in elderly patients, it is difficult to examine the relationship between CCs and age. Therefore, in this study, we preferred a population of stage I CC patients whose mean age was usually younger. We found interesting relationships between these

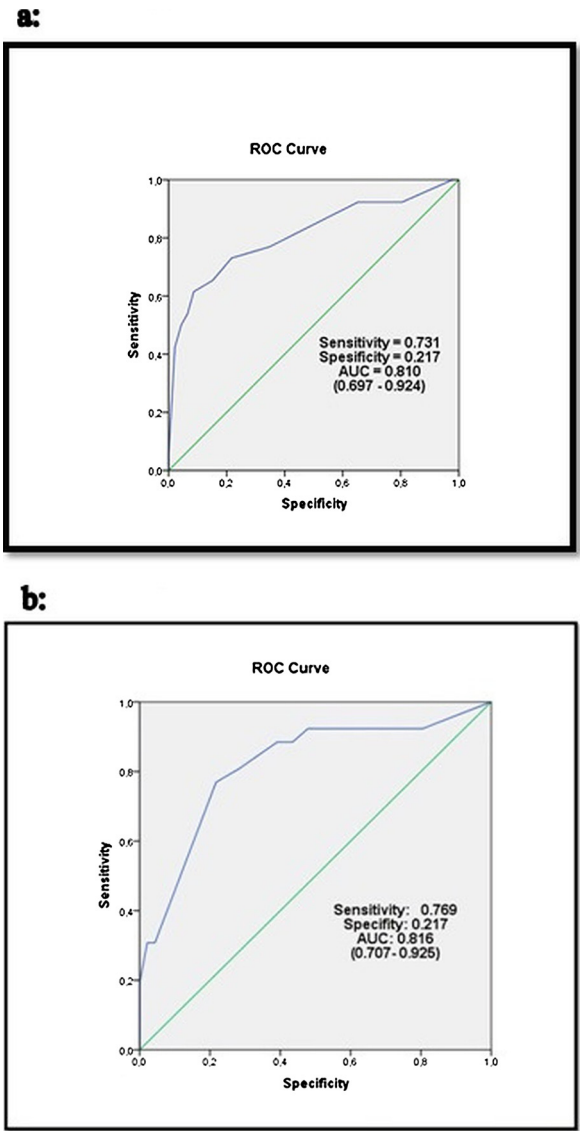

Fig. 2. Optimal cut-off value for BD **(2a)** and TSR **(2b)**.
ROC curves for BD and TSR. AUC analyzed by manual methods. **Abbreviations**: BD: Tumour budding, TSR: Tumour stroma ratio, ROC: Receiver Operating Characteristic, AUC: Areas under the ROC curves.

parameters and elderly patients. At first, the mean age of our population was 61 years, but the survival-related cut-off value was 68 years (ROC = 68.47, AUC = 0.856 [0.744–0.936]). Also, significant relationship with poor prognostic parameters was more advanced in elderly patients (≥68) ([lymphatic invasion (BD, p = 0.003; TSR, p = 0.003), perineural invasion (BD, p = 0.016; TSR, p = 0.004), tumour deposits (BD, p = 0.005; TSR, p = 0.033), MMR-P (BD, p = 0.031; TSR, p = 0.012, etc.)]. Furthermore, an additional analysis was performed with a mean age of 61 years and the significant relationship significantly decreased (Table 1).

When the categorical data were examined, it was seen that most of the high BD/low TSR cases were elderly patients (BD = 74%, TSR = 68%). In fact, the majority of these cases were over 75 years old (BD = 62%, TSR = 56%) (Fig. 3**a**). When the survival analysis was examined, it was seen that most of the high BD/low TSR patients who died within 5 years were elderly patients (BD = 82%, TSR = 73%). In fact, the majority of these cases were over than 75 years old (BD = 73%, TSR = 65%) (Fig. 3**b**). When the continuous data were examined, it was seen that the number of buds and the percentage of stroma increased at older ages (**Supplementary Table S1**). Furthermore, at older ages, correlations between these parameters and age were higher and differences were lower (**Supplementary Table S2**).

6.4. Reproducibility of research

The inter-observer agreement was usually in a clinically useful range (BD, κ = 0.53–0.75; TSR, κ = 0.56–0.71). When the analysis was examined in detail, it was seen that the κ values increased parallel to the age and reached a perfect value at the level of 85–95 years. Similar findings were observed for TSR. That is, since the presence of tumour bud and tumour stroma was more pronounced at older ages, the interobserver agreement was at a higher level as expected (**Supplementary Table S3**).

For tumour heterogeneity, the majority of the variation can be attributed to biological differences between tumours. Because an ICC count of 0.694 (BD) means that 30.6% of the total variance is due to the variation in a single tumour. Similar findings were observed for TSR. Therefore, intra-tumoural variation is considerably lower than inter-tumoural variation. When the details of the analysis were investigated, the magnitude of ICCs at older ages was significantly higher. This means that the number of tumour buds and the ratio of stroma increased at older ages and gave more heterogeneity to the field of view. **(Supplementary Table S3).**

6.5. Follow-up

During follow-up, twenty-three patients died (BD: n = 17 in high BD, n = 6 in low BD; TSR: n = 7 in high TSR, n = 16 in low TSR), and twenty-eight patients had recurrences (BD: n = 21 in high BD, n = 8 in low BD; TSR: n = 9 in high TSR, n = 19 in low TSR). In addition, ten patients had local recurrence (BD: n = 8 in high BD, n = 2 in low BD; TSR: n = 3 in high TSR, n = 7 in low TSR) and seven patients had distant recurrence (BD: n = 5 in high TSR, n = 2 in low TSR; TSR: n = 3 in high TSR, n = 4 in low TSR). For BD, the 5-year RFS and OS ratios were 88% and 90% in high BD cases, versus 75% and 77% in low BD patients, respectively. Also, the 5-year LR and DR rates were 14% and 10% in high BD, versus 4% and 3% in low BD. For TSR, the 5-year RFS and OS rates were 89% and 91% in high TSR patients versus 76% and 78% in low TSR patients, respectively. Also, LR and DR rates were 12% and 10% in low TSR versus 5% and 6% in high TSR (Table 2).

6.6. Survival analyses

In univariate analysis, significant differences between survival groups were observed for RFS (BD, p < 0.001; TSR, p = 0.001), OS (BD, p < 0.001; TSR, p = 0.005) and LR (BD, p = 0.008; TSR, p = 0.034). Also, invasive pattern, MMR-P, and margin involvement were other parameters that were significantly related to an adverse outcome (Table 2, Fig. 3**a**-**b**). Multivariate analysis showed that BD and TSR were independent worse prognostic parameters for RFS (BD: HR = 1.42 [1.13–1.80], p = 0.002; TSR: HR = 1.50 [1.17–1.91], p = 0.001), OS (BD: HR = 1.38 [1.07–1.79], p = 0.014; TSR: HR = 1.42 [1.11–1.82], p = 0.005), and LR (BD: HR = 1.61 [1.03–2.52], p = 0.034). In multivariate analysis, no significant relationship was found between the other parameter and poor survival (Table 3). Also, an additional survival analysis was performed with the age of 75 years in which the tumour buds and tumour stroma were more pronounced and it was found that the significant relationship maintained (Tables 2 and 3).

7. Discussion

In this study, we investigated the prognostic role of BD and TSR in stage I CC patients treated with surgery only. Our findings show that these factors play an important role in the progression of CC, especially

Table 1
Relationship between BD/TSR, age and prognostic factors.

		BD (n = 88) (%)			TSR (n = 88) (%)			BD (n = 88) (%)			TSR (n = 88) (%)		
		< 68 years	≥68 years	P- value	< 68 years	≥68 years	P-value	< 61 years	≥61 years	P-value	< 61 years	≥61 years	P-value
Size				0.132			0.376			0.221			0.276
	< 5 cm	25 (%59)	17 (%41)		28 (%43)	36 (%57)		20 (%47)	22 (%53)		35 (%54)	29 (%46)	
	≥5 cm	20 (%43)	26 (%57)		8 (%33)	16 (%66)		16 (%34)	30 (%66)		10 (%41)	14 (%59)	
Gender				0.293			0.441			0.212			0.400
	Female	27 (%56)	21 (%44)		21 (%44)	26 (%56)		28 (%58)	20 (%42)		26 (%55)	21 (%45)	
	Male	18 (%45)	22 (%55)		15 (%36)	26 (%64)		18 (%45)	22 (%55)		19 (%46)	22 (%54)	
Lymphatic invasion				***0.003****			**0.003***			0.227			0.139
	No	37 (%61)	23 (%39)		28 (%53)	24 (%47)		34 (%56)	26 (%44)		30 (%57)	22 (%43)	
	Yes	8 (%28)	20 (%72)		8 (%22)	28 (%78)		12 (%42)	16 (%58)		15 (%41)	21 (%59)	
Perineural invasion				***0.016****			***0.004****			0.606			0.139
	No	26 (%42)	35 (%58)		27 (%54)	23 (%46)		33 (%54)	28 (%46)		29 (%58)	21 (%42)	
	Yes	19 (%70)	8 (%30)		9 (%23)	29 (%77)		13 (%48)	14 (%52)		16 (%42)	22 (%58)	
LIR				**0.049***			***0.004****			0.458			0.100
	No	24 (%63)	14 (%37)		22 (%57)	16 (%43)		37 (%54)	31 (%46)		38 (%55)	30 (%45)	
	Yes	21 (%53)	29 (%47)		14 (%28)	36 (%72)		9 (%45)	11 (%55)		7 (%35)	13 (%65)	
Invasive Pattern				**0.017***			***0.006****			0.146			0.135
	No	34 (%60)	22 (%40)		29 (%62)	27 (%48)		26 (%46)	30 (%54)		32 (%57)	24 (%43)	
	Yes	11 (%34)	21 (%66)		7 (%21)	25 (%79)		20 (%47)	12 (%53)		13 (%40)	19 (%60)	
Localization				0.139			0.229			0.146			0.296
	Left	29 (%58)	21 (%42)		24 (%46)	28 (%54)		24 (%48)	26 (%52)		29 (%55)	23 (%45)	
	Right	16 (%42)	22 (%58)		12 (%33)	24 (%67)		12 (%31)	26 (%69)		16 (%44)	20 (%54)	
Margin involvement				***0.011****			***0.019****			***0.019****			***0.040****
	No	38 (%59)	26 (%41)		31 (%48)	33 (%52)		31 (%48)	33 (%52)		37 (%57)	27 (%43)	
	Yes	7 (%29)	17 (%71)		5 (%20)	19 (%80)		5 (%20)	19 (%80)		8 (%33)	16 (%67)	
Tumour deposits				***0.005****			***0.033****			0.100			0.202
	No	33 (%63)	19 (%37)		28 (%49)	29 (%51)		25 (%48)	27 (%52)		32 (%56)	25 (%44)	
	Yes	12 (%33)	24 (%67)		8 (%25)	23 (%75)		11 (%30)	25 (%70)		13 (%41)	18 (%59)	
MMR				***0.031****			***0.012****			***0.030****			***0.009****
	MMR-P	39 (%57)	29 (%43)		32 (%48)	34 (%52)		32 (%47)	36 (%53)		39 (%59)	27 (%41)	
	MMR-D	6 (%30)	14 (%70)		4 (%18)	18 (%82)		4 (%20)	16 (%80)		6 (%27)	16 (%73)	
Grade				0.135			0.167			0.389			0.137
	Low grade	32 (%57)	24 (%43)		22 (%47)	24 (%53)		21 (%37)	35 (%63)		27 (%58)	19 (%42)	
	Moderate/ High grade	13 (%40)	19 (%60)		14 (%33)	28 (%67)		15 (%46)	17 (%54)		18 (%42)	24 (%58)	
Tumour necrosis				0.138			0.119			0.437			0.088
	No	31 (%57)	23 (%43)		22 (%49)	23 (%51)		30 (%55)	24 (%45)		27 (%60)	18 (%40)	
	Yes	14 (%41)	20 (%59)		14 (%32)	29 (%68)		16 (%47)	18 (%53)		18 (%41)	25 (%59)	

Two separate analyses were performed for age: 68 years (cut-off value) and 61 years (the mean age). *. P-value is significant at the 0.05 level. Significant results in italics.

Abbreviations: BD: Tumour budding, TSR: Tumour stroma ratio, LIR: Local inflammatory response, MMR-P: Mismatch repair proteins proficiency, MMR-D: Mismatch repair proteins deficiency.

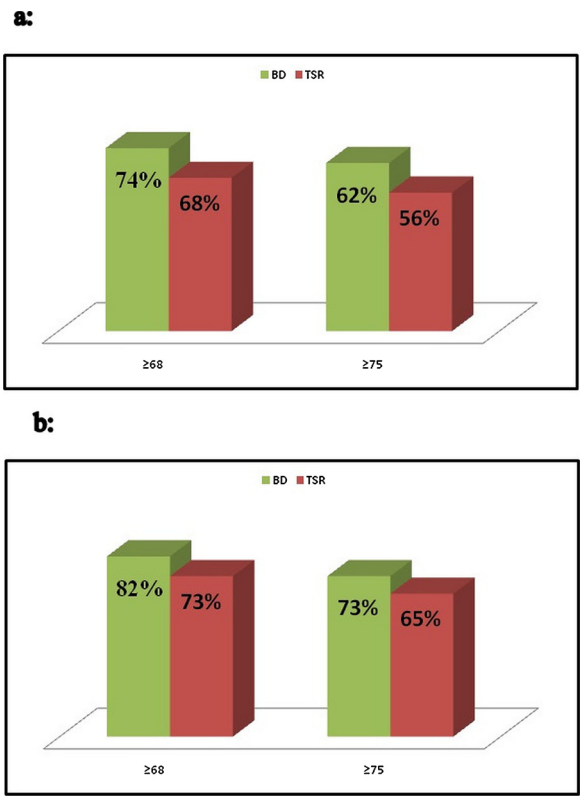

Fig. 3. Association between BD/TSR with elderly patients **(3a)** and survival **(3b).**
Two separate analyses were performed for age: 68 years (cut-off value) and 75 years (BD and TSR were more pronounced). **Abbreviations**: BD: Tumour budding, TSR: Tumour stroma ratio

in elderly patients. Also, we found that model A is a reliable method for standardization.

Although many studies in the literature have shown that BD and TSR in CC are independent prognostic markers [7–12], these studies are highly variable in terms of population, i.e. most studies include different stages of the disease and rectal cancers. For example, Koelzer et al. [8] have recently evaluated BD in a cohort of 150 early-stage colorectal cancer using IHC and 10 HPFs method and they have found that high BD was an independent predictor of worse RFS. However, this study also included rectal tumours and it is not clear in the literature whether the incidence of BD differs between rectal cancers and CCs. Also, Hutchins et al. [23] found that the rate of stroma in early- and late-stage colorectal carcinoma was different and that it was significantly higher in rectum tumours than in CCs. So, in CCs, rectum tumours and early-stages should be examined separately. We investigated a population of patients operated only for stage I CC and we also excluded patients with adjuvant chemotherapy and other known malignancies to prevent potential confusion. Therefore, unlike other studies, our patient population was quite homogeneous.

Many studies in the literature have reported that survival in elderly CC patients is poor than in younger patients and that these poor outcomes may be associated with increased comorbidity rates in elderly patients [24–26]. However, in some studies, similar survival rates were observed in CC patients after curative surgery and no association was reported between age and survival [27]. That is, the relationship between age and survival in CC patients is not clear in the literature. In this study, we selected a population of CC patients with a lower mean age (i.e. stage I CC patients). Thus, the age range of the population increased and the relationship between age, prognosis and tumour morphology was more prominent. We found that the prognosis significantly decreased with increasing age. Also, the number of tumour buds and stroma ratio increased at older ages and these two parameters were more prominent in elderly patients. In addition, we observed that these two parameters were more associated with prognostic factors in elderly patients. Moreover, we performed additional analyzes in young patients and found that these results were significantly reduced. Therefore, our study shows the effect of age on survival, prognostic factors and tumour biology more clearly.

Disadvantages of pathological evaluation are deficiencies in standardization and reproducibility [8–17]. Variability arises from different methods such as staining (H&E, IHC), visualization (x20 objective, x40 objective), scoring method (qualitative, quantitative), and optimal evaluation method (invasive margin, tumour centre). For example, some studies evaluate BD on H&E stained sections and in one HPF [7]. Also, some evaluated TSR using two different methods, 'global' and 'focal' [28]. Whereas, each of the pathological methods described represents a different area of the tumour and may alter the results obtained. In fact, there is now a consensus on BD [10], but current staging systems do not take these parameters into account. There is no consensus about TSR. These show that there are still questions about the prognostic effects of these parameters. Therefore, new studies are needed on these parameters from different aspects. A successful methodology was used in this study [18]. In our opinion, an important reason for the different results in the literature is the lack of standardization. If this difficulty can be overcome, more successful results can be achieved. In this study, we tried to provide this.

Heterogeneity of tumours appears to be a serious problem for CCs in the literature [8,17]. For example, Mesker et al. [17] recommended using the highest PT-stage section of the primary tumour because they documented that the histological sections with the deepest infiltrated areas in the bowel wall had the lowest proportion of adenocarcinoma cell. Also, Kolzer et al. [8] found that heterogeneity in CCs is an important issue. In this study, heterogeneity of the tumour was found to be significantly higher in the elderly. That is, tumour buds and tumour stroma were less at lower ages and, as expected, intra-tumour heterogeneity levels were lower. According to these results, it can be understood that differences between tumour can change precision and accuracy. The heterogeneity of the tumour should be further investigated for future clinical and diagnostic applications in CC.

In the literature, the mean and percentage values of BD and TSR are highly variable and ranges from 7.11 to 8.05 and 19.5% to 45%, respectively [8,29,30]. For example, unlike our 39.7% rate, Koelzer et al. [8] reported a high budding rate of 30%. Also, unlike our finding of 8.24 buds, Horic et al. [29] reported a mean of 7.95 buds, and Koelzer et al. [8] found a mean of 7.11 buds. These differences can be explained by the heterogeneity of tumours, as well as differences in assessment methods. Indeed, we have found inter-tumour heterogeneity, and the use of different tumour sites may partially explain the differences in the results obtained. We have also counted these parameters in 10 HPFs, and this multiple counting method can change the last average number. Also, to avoid false staining, we have counted these parameters only when there is a clearly identifiable blue nucleus, and the results obtained may differ due to this counting rule. As a result, differences arise from the variability of assessment methods, and this problem can be overcome by standardizing the counting methods.

Although the assessment with the IHC stained sections has improved detection rates and inter-observer agreements [31], the current consensus suggests that these parameters should be evaluated using H&E stained sections [32–34]. However, it is not clear whether the values obtained from IHC have the same prognostic effect as H&E. In this study, both H&E and IHC stained sections were used. For BD, we experienced the disadvantage of using H&E by identifying many other bud-like structures, e.g. retraction artefacts around fragmented tumour tissue, fragmentation of tumour tissue induced by abundant inflammatory infiltrates, fragments of tumour glands surrounded by abundant mucin. Also, we experienced the difficulty of using IHC by identifying staining of cells other than adenocarcinoma, e.g. endothelial

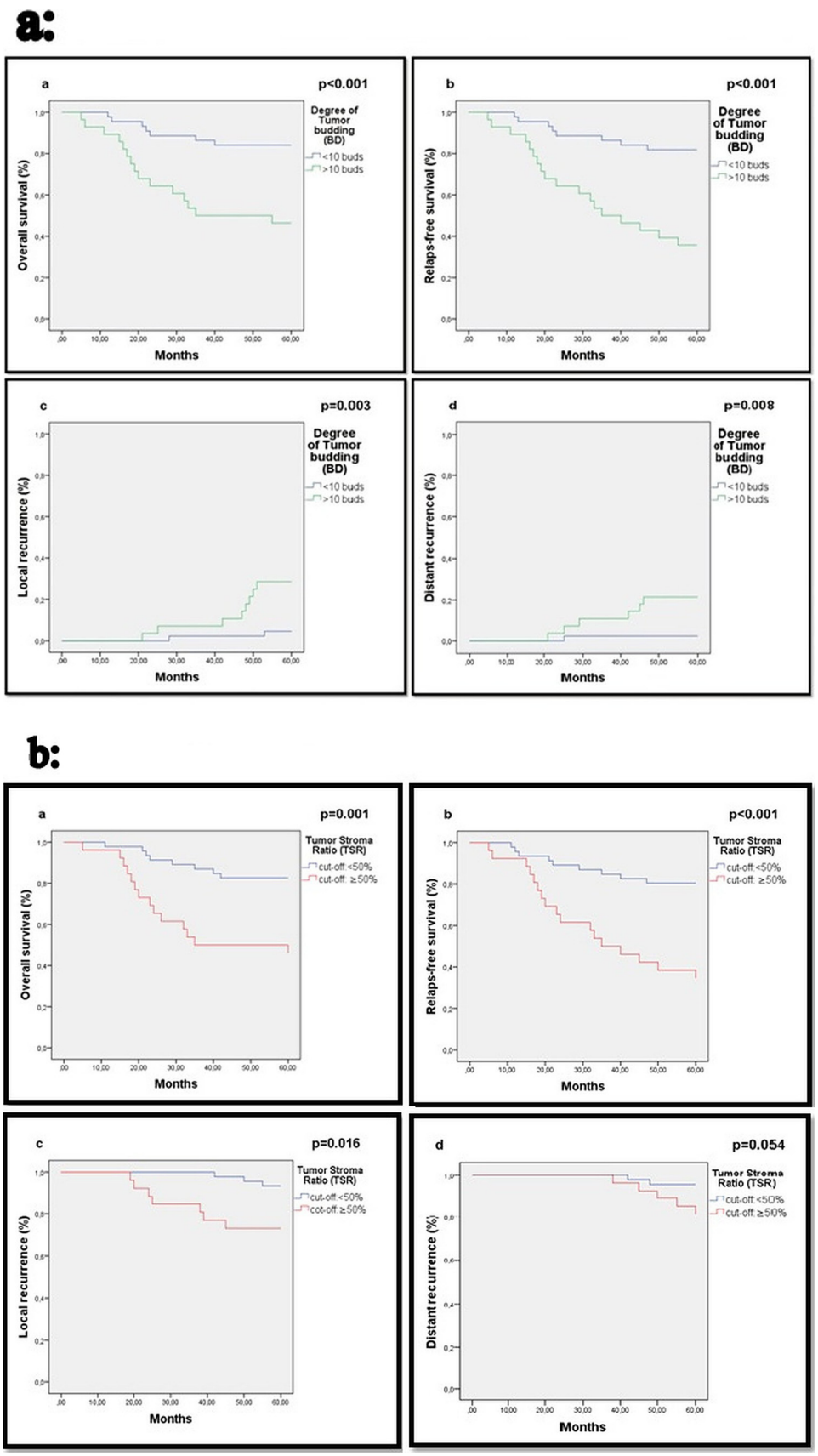

Fig. 4. 5-year survival curves of elderly patients for BD **(4a)** and TSR **(4b)**.
Kaplan-Meier survival curves for BD (Fig. 4**a)** and TSR (Fig. 4**b**). For both two figures, **a** shows overall survival, **b** shows relapse-free survival, **c** shows local recurrence, and **d** shows distant recurrence. P-value is significant at the 0.05 level.
Abbreviations: BD: Tumour budding, TSR: Tumour stroma ratio.

Table 2
Univariate survival analysis of elderly patients for BD and TSR.

		Overall Survival 5-year survival (%)	P-value	Relaps-free Survival 5-year survi val (%)	P-value	Local Recurrence 5-year survival (%)	P-value	Distant Recurrence 5-year survival (%)	P - Value
Size	**< 5 - ≥5**	%85 -%85	0.866	%84-%80	0.445	%3-%9	0.134	%2-%7	0.079
Gender	**Female - Male**	%84 -%86	0.757	%83 -%81	0.564	%7 -%6	0.965	%4 -%6	0.358
Lymphatic invasion	**No-Yes**	%86 -%84	0.757	%85 -%80	0.358	%4 -%9	0.319	%8 -%3	0.095
Perineural invasion	**No-Yes**	%80 -%90	0.082	%90 -%84	0.153	%7 -%6	0.965	%5 -%5	0.998
LIR	**No-Yes**	%83 -%87	0.587	%84 -%84	0.953	%9 -%3	0.134	%9 -%4	0.170
Invasive Pattern	**No-Yes**	%88 -%82	0.480	%88 -%78	***0.042****	%6 -%5	0.899	%4 -%7	0.482
Localization	**Right-Left**	%87 -%83	0.587	%85 -%80	0.358	%3 -%8	0.223	%1 -%6	0.070
Margin involvement	**No-Yes**	% 90-%78	***0.023****	%84 -%79	0.268	%4 -%8	0.653	%4-%6	0.358
Tumour deposites	**No-Yes**	%90 -%80	0.082	%83 -%80	0.186	%8 -%5	0.674	%3 -%7	0.550
MMR	**MMR-D MMR-P**	%87 -%83	0.587	%87 -%77	***0.023****	%6 -%7	0.968	%4 -%6	0.358
Grade	**L grade - M/H grade**	%89 -%83	0.466	%84 -%80	0.245	%3 -%9	0.134	%2-%7	0.079
Tumour necrosis	**No-Yes**	%85 -%85	0.866	%83 -%81	0.564	%6 -%9	0.530	%5-%4	0.823
BD (cut-off 68)	**High-Low**	%77 -%90	< ***0.001****	%75 -%88	< ***0.001****	%14 -%4	***0.003****	%10 -%3	***0.008****
BD (cut-off 75)	**High-Low**	%78 -%89	***0.042****	%77 -%88	***0.023****	%13 -%5	***0.001****	%10 -%4	***0.005****
TSR (cut-off 68)	**High-Low**	%91 -%78	***0.001****	%89 -%76	< ***0.001****	%12 -%5	***0.016****	%10 -%5	0.065
TSR (cut-off 75)	**High-Low**	%89 -%78	***0.041****	%88 -%78	< ***0.042****	%13 -%6	***0.014****	%9 -%4	0.054

Two separate analyses were performed for age: 68 years (cut-off value) and 75 years (BD and TSR were more pronounced). *. P-value is significant at the 0.05 level. Significant results in italics.
Abbreviations: BD: Tumour budding, TSR: Tumour stroma ratio, LIR: Local inflammatory response, MMR-P: Mismatch repair proteins proficiency, MMR-D: Mismatch repair proteins deficiency.

Table 3
Multivariate survival analysis of elderly patients for BD and TSR.

		Overall survival (n = 88) (%)		Relaps-free survival (n = 88) (%)		Local recurrence (n = 88) (%)		Distant recurrence (n = 88) (%)	
		HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95%CI)	P- value	HR (95% CI)	P-value
Invasive pattern	**No**	1	–	1	–	1	–	1	–
	Yes	2.98 (0.37-13.5)	0.299	7.02 (0.47-26.1)	0.568	3.27 (0.02-4.74)	0.357	NC	0.867
Margin involvement	**No**	1	–	1	–	1	–	1	–
	Yes	2.41 (0.68-8.56)	0.172	6.32 (0.54-8.61)	0.470	4.48 (0.28-7.63)	0.639	NC	0.962
MMR	**MMR-D**	1	–	1	–	1	–	1	–
	MMR-P	2.79 (0.46-6.94)	0.397	2.12 (0.78-4.10)	0.349	3.77 (0.34-9.16)	0.492	NC	0.963
BD (cut-off 68)	**Low**	1	–	1	–	1	–	1	–
	High	1.38 (1.07-1.79)	***0.014****	1.42 (1.13-1.80)	***0.002****	1.61 (1.03-2.52)	***0.035****	1.73 (0.98-3.05)	0.058
BD (cut-off 75)	**Low**	1	–	1	–	1	–	1	–
	High	1.48 (1.27-3.11)	***0.016****	1.65 (0.97-5.70)	0.055	2.91 (0.83-4.41)	0.126	2.46 (0.50-4.24)	0.280
TSR (cut-off 68)	**High**	1	–	1	–	1	–	1	–
	Low	1.42 (1.10-1.82)	***0.005****	1.50 (1.11-1.91)	***0.001****	2.53 (0.98-2.43)	0.078	2.61 (0.83-2.53)	0.254
TSR (cut-off 75)	**High**	1	–	1	–	1	–	1	–
	Low	1.53 (1.14-1.89)	***0.006****	1.55 (1.14-1.99)	***0.002****	2.67 (0.90-2.81)	0.081	2.72 (0.78-3.43)	0.318

Two separate analyses were performed for age: 68 years (cut-off value) and 75 years (BD and TSR stroma were more pronounced).*. P-value is significant at the 0.05 level. Significant results in italics.
Abbreviations: BD: Tumour budding, TSR: Tumour stroma ratio, MMR-P: Mismatch repair proteins proficiency, MMR-D: Mismatch repair proteins deficiency, HR: Hazard ratio, CI: Confidence interval, NC: Not calculable.

cells of vascular neoangiogenesis. In addition, the biggest challenge for TSR is to distinguish between stromal tissue and smooth muscle fibres in the H&E stained sections. Morphologically, this two tissue can be distinguished by careful examination of the cell nucleus. The smooth muscle cells have round nuclei (cigar-shaped), while the fibroblasts have more spindle-shaped nuclei. In cases where it is difficult to distinguish, we recommend desmin staining. Further studies are needed to standardize techniques.

This research has many important features. Foremost, we present two reliable parameters that have been recently investigated in many large studies but have low standardization. We conducted this study based on a well-designed cohort in a highly homogeneous population (stage I CC patients without adjuvant therapy). We examined the status of these parameters in elderly patients population, which was the most difficult to treat. We also achieved an improvement in standardization with a successful evaluation method. In addition, we conducted every stage of this study in accordance with REMARK guidelines.

There are some limitations to our study. First of all, there is an internal restriction for all retrospective analysis. Because the tissue examined was previously prepared and examined for diagnostic purposes. Therefore, it was impossible to overcome sampling differences. Although many different areas of primary tumours were evaluated in this study, we were aware that this was only a small part of the whole tumour. Also, since patients are treated according to guidelines before 2015, there may be differences compared to current treatment methods.

7.1. Conclusion

Our results confirm the prognostic effect of BD and TSR in stage I CC patients, especially in the elderly population. Based on the present results, evaluation of these indicators can provide useful survival information to the TNM classification due to its reliability, simplicity and cost-effectiveness. We also recommend using model A for future studies to achieve successful results.

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Declaration of Competing Interest

The author does not report a conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152635.

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