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Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I–II colon cancer patients *,**

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Abstract. *Background*: For stage I–II colon cancer a significant number (5–25%) of patients has recurrent disease within 5 years. There is need to identify these high-risk patients as they might benefit from additional treatment.

Stroma-tissue surrounding the cancer cells plays an important role in the tumor behavior. The proportion of intra-tumor stroma was evaluated for the identification of high-risk patients. In addition, protein expression of markers involved in pathways related to stroma production and epithelial-to-mesenchymal transition (EMT) was analyzed: β-catenin, TGF-β-R2 and SMAD4.

Methods: In a retrospective study of 135 patients with stage I–II colon cancer, the amount of stroma was estimated on routine haematoxylin–eosin stained histological sections. Sections were also immunohistochemically stained for β-catenin, TGF-β-R2 and SMAD4.

Results: Of 135 analyzed patients 34 (25.2%) showed a high proportion of stroma (stroma-high) and 101 (74.8%) a low proportion (stroma-low). Significant differences in overall-survival and disease-free-survival were observed between the two groups, with stroma-high patients showing poor survival (OS $p < 0.001$, HZ 2.73, CI 1.73–4.30; DFS $p < 0.001$, HZ 2.43, CI 1.55–3.82). A high-risk group was identified with stroma-high and SMAD4 loss (OS $p = 0.008$, HZ 7.98, CI 4.12–15.44, DFS $p = 0.005$, HZ 6.57, CI 3.43–12.56); 12 of 14 (85.7%) patients died within 3 years. In a logistic-regression analysis a high proportion of stroma and SMAD4 loss were strongly related (HZ 5.42, CI 2.13–13.82, $p < 0.001$).

Conclusions: Conventional haematoxylin–eosin stained tumor slides contain more prognostic information than previously fathomed. This can be unleashed by assessing the tumor–stroma ratio. The combination of analyzing the tumor–stroma ratio and staining for SMAD4 results in an independent parameter for confident prediction of clinical outcome.

Keywords: Colon cancer, primary tumor, high-risk patients, stroma, prognosis

1. Introduction

The five year survival rate for colon cancer stage I–II patients (AJCC staging) is 93% for stage I, 85% for stage IIa and 72% for stage IIb [2]. The high surgical cure rate for patients with "low-risk" stage II and the outcome of clinical trials and meta-analysis give debatable recommendations for or against adjuvant chemotherapy [3–7]. For Northern European countries the current advice by the ESMO (European Society for

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Medical Oncology) is "no adjuvant treatment". Nevertheless 5–25% of stage I–II patients will have recurrence of disease within 5 years [2]. Therefore there is a strong need for additional parameters to select patients for additional therapy.

Pathological characterization as recommended by the ASCO (American Society of Clinical Oncology) serves as an indication for chemotherapy for "high-risk" stage II patients, identified on the basis of clinical features as T4, obstruction or perforation and low number of removed lymph nodes ($n < 12$) [8].

Recent models on metastatic pathways which focus on invasion include the "tumor–host" interface and in particular focus on the role of the stroma tissue. The proportion and the composition of tumor stroma differ between tumors, and are distinct from normal tissue stroma [9].

A number of key parameters involved in intra-tumor stroma production that may support our finding can be found in the transforming growth factor-β (TGF-β) and Wnt-signaling pathway.

For the Wnt-signaling pathway, the main oncoprotein in colorectal cancer is the Wnt pathway effector β-catenin. Accumulation in the nucleus of β-catenin is indicative of activation of the *wnt*-signaling pathway through mutation of the APC-gene, which occurs at early step of colorectal carcinogenesis [10,11]. β-catenin is involved in two fundamental processes; EMT (epithelial-mesenchymal-transition) and stem cell formation. Loss of membraneous E-cadherin in adherens junctions results in translocation of β-catenin from adherens junctions to the nucleus which in turn triggers the loss of E-cadherin and subsequently the EMT. β-catenin nuclear staining was found upregulated in the invading area of colorectal cancer and seems to correlate with metastasis and poor survival [12].

For the TGF-β pathway, growth factors produced by tumor cells, cause the tumor surrounding stroma to become "reactive" upon which tumor cell proliferation, migration and angiogenesis is promoted. A molecular mimicry in tumor resembles stroma injury, which occurs in wound healing. Amongst others the TGF-β signaling is a key regulator of this process [13]. Fibroblasts – the main cell type in stroma – may differentiate into so-called cancer-associated fibroblasts (CAFs) during the progression to invasive carcinoma [14,15]. EMT is engaged by several cytokines associated with proteolytic digestion of the basal membrane (by metalloproteinases) upon which the epithelium resides. The role of the TGF-β signaling pathway relates to both the primary tumor and the stroma. In addition, its role is dual: in early stages it blocks tumor growth, whereas in progressed stages it stimulates invasion and metastasis [16]. TGF-β exerts its function by binding to specific transmembrane receptors, for which receptor II is found mutated in colorectal cancer [17]. Furthermore Bhowmick et al. found that the loss of TGF-β responsiveness in fibroblasts resulted in intraepithelial neoplasia and an increased abundance of stroma cells [1].

Smad proteins are key signal-transducers of the TGF-β pathway and are essential for the growth suppression function of TGF-β [18]. Smad proteins are regulators of transcription and act as tumor suppressor molecules whose inactivation by mutation or silencing is associated with pancreatic and colon cancer. For colon cancer, SMAD4 coded at 18q21.1, plays a key role; 18q deletion is observed in 30% of invasive colorectal carcinoma and has been described as an independent prognostic parameter [19–21].

In a former study we have investigated the proportion of intra-tumor stroma, on haematoxylin–eosin (H&E) stained histological sections, as prognostic parameter for colon carcinoma. In a set of 122 patients (stage I–III) a significant difference in survival time was observed between patients with a high amount of intra-tumor stroma (stroma-high) and patients with less stroma (stroma-low). Stroma-high patients showed a significantly worse survival [22].

The current study is based on stage I–II patients only, to identify a subgroup of patients with bad prognosis who might benefit from adjuvant therapy. We have analyzed 135 stage I–II colon cancer patients with at least 11 years of follow-up for the proportion of tumor related stroma and the protein expression of markers involved in pathways related to stroma production and EMT; TGF-β-R2, SMAD4 and β-catenin. It was found that in particular SMAD4 allows for further prognostic stratification of stage I–II colon cancer patients.

2. Methods

2.1. Patient recruitment

We included 139 colon cancer patients with stage I–II tumors (clinically staged according to the classification of the AJCC) [23], who underwent curative surgery at the Leiden University Medical Center between 1980 and 2001. Fifty-eight patients were part of two consecutive series formerly published for H&E

analysis only [22]. For this study, additionally 77 patients were obtained from a case-control series.

Case-control series: Cases ($n = 27$) considered with regional or distant recurrent disease between three months and five years after the date of diagnosis of primary colon carcinoma. Regional metastases were considered intra-abdominal or intrapelvic metastases in lymph nodes or in connective tissue. Fifty controls were selected with no locoregional or distant disease within five years after diagnosis of primary colon cancer. For each case two controls were matched for TNM stage, date of incidence and date of birth.

None of the patients had pre- or postoperative chemo- or radiation therapy. Patients with synchronous second tumors, other malignancies in the past and death or recurrence (distant or loco-regional) within 1 month, were excluded.

All samples were handled in a coded fashion, according to National Ethical Guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

2.2. Histopathological protocol

Pathological examination entailed routine microscopic analysis of 5 μm H&E stained sections of the primary tumor. The amount of intra-tumor stroma, visually scored by three investigators (V. Smit, K.V. Leeuwen, W. Mesker), was estimated on the most invasive part of the tumor. For a detailed protocol see Mesker et al. [22].

Four of the 139 selected patients were rejected on the basis of poor quality of the histological material, leaving H&E sections from 135 patients for analysis.

For the identification of microsatellite instability-high (MSI-H) patients, 5 μm slides were immunohistochemically stained for MLH1 and PMS2 markers [24].

2.3. Immunohistochemical staining for TGF-β-R2, SMAD4 and β-catenin

For 17 patients the paraffin embedded tissue blocks, necessary for staining with the monoclonal antibodies, were not available in the pathology archives, leaving blocks from 118 patients for analysis.

Four-micron-thick sections from paraffin embedded tissue were positioned onto silane-treated Starfrost slides (Klinipath, Duiven, Netherlands) and left to dry overnight. Antigen retrieval was performed at low pH 6.0 citrate buffer (0.01 M) for TGF-β-R2 and β-catenin and at high pH 9.0 Tris 0.01 M/EDTA buffer (0.001M) for SMAD4 for 10 min. Subsequently, slides were incubated at room temperature for 15 min (TGF-β-R2) or overnight (SMAD4, β-catenin) using antibodies to TGF-β-R2 (rabbit polyclonal antibody, ab28383, prediluted; Abcam, Cambridge, United Kingdom), SMAD4 (mouse monoclonal antibody, sc-7966, dilution 1 : 400 in 5% non-fat milk in PBS; Santa Cruz Biotechnology, Santa Cruz, CA) or β-catenin (mouse monoclonal antibody, clone 14, dilution 1 : 1600 in 1% PBS/BSA; BD Biosciences Transduction Laboratories, Lexington). After the primary antibody step, slides were incubated for 30 min with EnVision-horseradish peroxidase anti-mouse or anti-rabbit (DakoCytomation, Heverlee, Belgium) followed by incubation with diaminobenzidine (liquid DAB + Substrate Chromogen System, K3468, DakoCytomation, Heverlee, Belgium) for 5 min. Control specimens were processed without primary antibodies. Internal positive control for SMAD4 consisted of normal epithelium and stroma for TGF-β-R2. For the β-catenin we have used a set of known positive colorectal tumors with nuclear expression as positive control.

The intensity and pattern of the immunohistochemical staining was visually evaluated. In case of TGF-β-R2 membranous staining, four categories were applied; from negative (0) to positive (3). For nuclear SMAD4 staining we used three categories (0 = negative, 1 = positive and 2 = mixed (neg/pos)) and for β-catenin four categories from membranous (0) to all nuclear expression (3).

3. Statistics

Statistical analysis was performed using SPSS software version 14.0. Overall-Survival (OS) was defined as the time period between the date of primary surgery and the date of death from any cause or the date of last follow-up. Metastases-Free-Survival (MFS) was defined as the time period between the date of primary surgery and the date of first loco-regional or distant metastases or the date of last follow-up. Disease-Free-Survival (DFS) was defined, according to proposed guidelines, as the time from the date of primary surgery until the date of death or to the date of first loco-regional or distant recurrence or the date of a second primary tumor [25].

Stroma-high was defined as: $<$50% tumor cells including the values 10, 20, 30 and 40% tumor and stroma-low: $\geqslant$50% tumor cells including the values 50, 60, 70, 80 and 90%. Analysis of the survival curves

was performed using Kaplan–Meier Survival Analysis and differences in survival distributions were tested using Log Rank Statistics. The Cox proportional hazards model was used to determine the Hazard Ratio (HZ) of explanatory variables on OS and DFS. The logistic regression analysis was used to determine the interaction between the variables intra-tumor stroma and SMAD4.

Of the various staining patterns the following categories were statistically evaluated: TGF-β-R2 0 versus 1, 2, 3; SMAD4 0 versus 1, 2; β-catenin 0, 1 versus 2, 3.

4. Results

4.1. Patient demographics

The study consisted of 74 men (54.8%) and 61 women (45.2%), with a mean age of 68.2 years (SD 11.5; range 30.1–85.0 years). From 135 primary tumors 63 (46.7%) were located left sided and 72 (53.3%) right sided.

Left-sided tumors were defined as: flexura lienalis ($n = 3$), colon descendens ($n = 5$), colon sigmoideum ($n = 43$) and rectosigmoideum ($n = 12$), and right-sided as: coecum ($n = 34$), colon ascendens ($n = 15$), flexura hepatica ($n = 10$) and colon transversum ($n = 13$) (Table 1).

Table 1
Patient characteristics

	Total N (%)	Stroma-high N (%)	Stroma-low N (%)
Gender			
Male	74 (54.4)	20 (57.1)	54 (53.5)
Female	61 (45.2)	14 (41.2)	47 (46.5)
Mean age (yrs)	68.2	68.5	68.0
Location tumor			
Left	63 (46.7)	22 (61.1)	43 (42.6)
Right	72 (53.3)	14 (38.9)	58 (57.4)
T status			
T1	4 (3.0)	1 (3.0)	3 (3.0)
T2	84 (62.2)	23 (67.6)	61 (60.4)
T3	41 (30.4)	10 (29.4)	31 (30.7)
T4	6 (4.4)	0	6 (5.9)
Stage			
I	24 (17.8)	2 (5.9)	22 (21.8)
IIA	105 (77.8)	32 (94.1)	73 (72.3)
IIB	6 (4.4)	0	6 (5.9)
Grading (differentiation)			
Well	23 (17.0)	6 (17.6)	17 (16.8)
Moderate	77 (57.0)	22 (64.7)	55 (54.5)
Poor	31 (23.1)	6 (17.7)	25 (24.8)
Unknown	4 (2.9)	0 (0)	4 (3.9)
MSI			
MSS	108 (80.0)	32 (94.1)	76 (75.2)
MSI-H left sided	2 (1.5)	0	2 (2.0)
MSI-H right sided	23 (17.0)	2 (5.9)	21 (20.8)
Unknown	2 (1.5)	0	2 (2.0)

4.2. Histopathology

Routine H&E stained sections from the most invasive part of the tumor were microscopically analyzed for the presence of stroma involvement using 5× and 10× microscope objectives. The variation in scoring for the individual pathologists for stroma-high versus stroma-low was 6.9% (range 4.4–8.8%) with low inter-observer variation between the three pathologists (Kappa range 0.596–0.702, $p < 0.0001$).

Immunohistochemical staining of the antibodies we did not result in any background staining. Also the negative controls were clean. Internal positive controls for SMAD4 consisted of normal epithelium and stroma for TGF-β-R2. These were all found positive. For the β-catenin we have used a set of known positive colorectal tumors with nuclear expression as positive control. The normal tissue as negative control was found negative.

The immunohistochemical stained slides were analyzed blindly and independent. There was a low variation in the scoring of the slides between two observers (5.1%).

For the analysis of the results we have combined different categories mentioned as most predictive in the cited literature and reported in our study as most informative.

For examples of the scoring and immunological staining see Suppl. Fig. 3: http://www.qub.ac.uk/isco/JCO.

4.3. Correlation with prognosis

Of 135 analyzed patients 34 (25.2%) were scored stroma-high and 101 (74.8%) stroma-low. Significant differences were found for overall (OS), disease free (DFS) and metastasis free (MFS) survival between stroma-high and stroma-low patients (OS $p < 0.001$; HZ 2.73, DFS $p = 0.001$; HZ 2.43, MFS $p < 0.001$) (Table 2, Suppl. Table 1: http://www.qub.ac.uk/isco/

Table 2
p Values (univariate) for stroma-high versus stroma-low patients and TNM parameters defined per site

	Total $n = 135$	Left $n = 63$	Right $n = 72$
Univariate			
Stroma			
OS	<0.001	0.001	0.001
DFS	<0.002	0.002	0.007
MFS	<0.001		
HZ			
OS	2.73	2.85	2.99
DFS	2.43	2.63	2.50
95% Conf. int.			
OS	1.73–4.30	1.52–5.33	1.49–6.00
DFS	1.55–3.82	1.42–4.90	1.26–4.97
T-status*			
OS	0.772	0.006	0.396
DFS	0.632	<0.001	0.550
Stage**			
OS	0.752	0.685	0.387
DFS	0.895	0.693	0.502

*T2 versus T3: $p = 0.47$; **Stage I versus IIa + IIb: $p = 0.84$, Stage I versus IIa: $p = 0.79$; For the T-status and Stage hazard ratio's contain value 1.0 and are therefore not relevant.

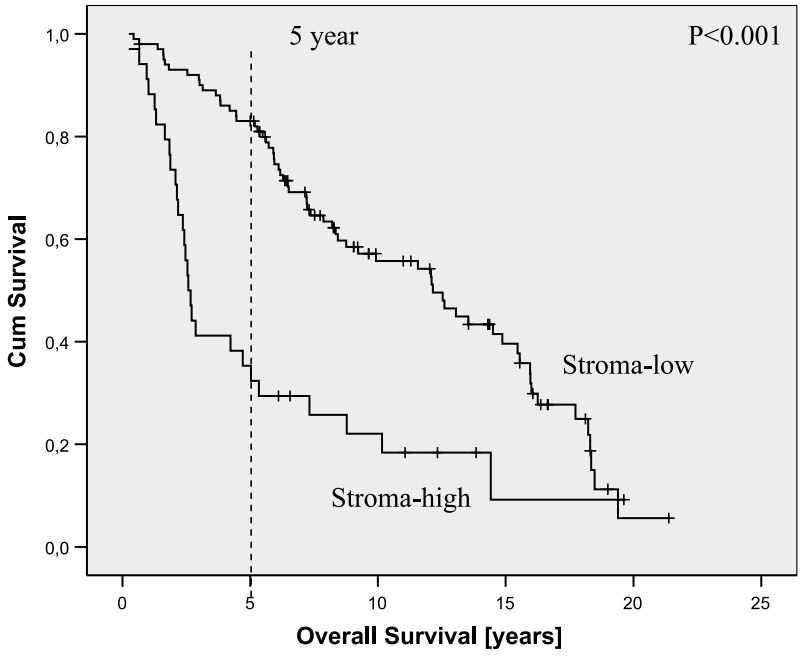
JCO, and Fig. 1). Please note that the Kaplan–Meier curves display a long follow-up period of 25 years.

For stage IIa, 32 (30.5%) out of 105 patients were scored stroma-high. Of these twenty-one (65.6%) patients died within 5 years and 11 (34.4%) were still alive after 5 years (OS $p < 0.001$; HZ 2.7 (range 1.64–4.45), DFS $p = 0.001$; HZ 2.30 (range 1.41–3.74)). Twenty of 21 patients died due to their disease, 15 developed metastases to the liver, 4 to the peritoneum and one to the lung. Remarkably, none of the 21 "high-risk" patients (defined as stroma-high, death $\leqslant$5 years) fulfilled the ASCO "high-risk" criteria for T4, obstruction or perforation.

Six patients with stage IIb were included in this series. All patients were scored as stroma-low. The mean survival for these patients was OS: 6.39 years, DFS 6.08 years.

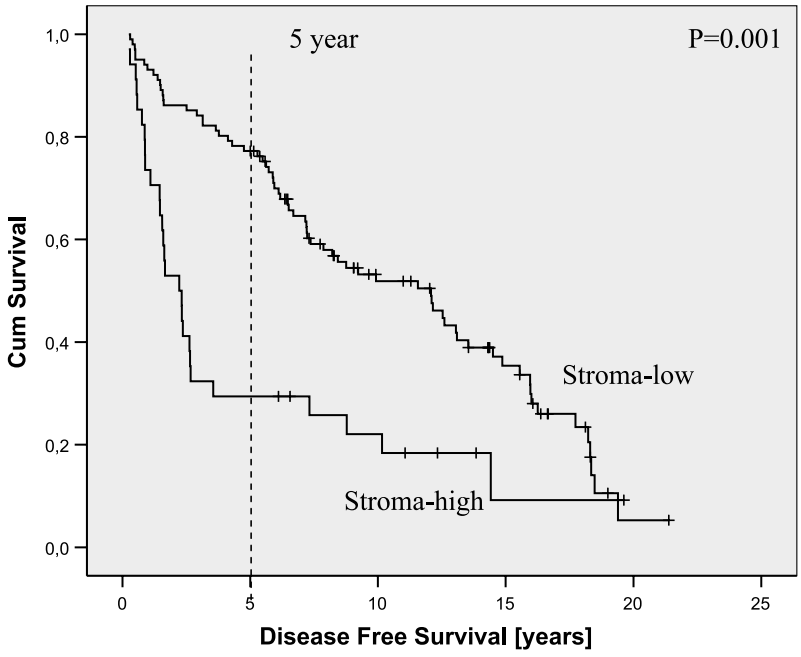
For stage I, 8.3% ($n = 2$) of the patients were scored as stroma-high. The survival for these two patients was respectively: OS/DFS both 2.67 years, 2.08/0.89 years. The mean overall-survival time for the stroma-low group was 10.8 years.

Within all stages no correlation was observed between the proportion of stroma and the tumor differentiation grade (ASCO recommendations).

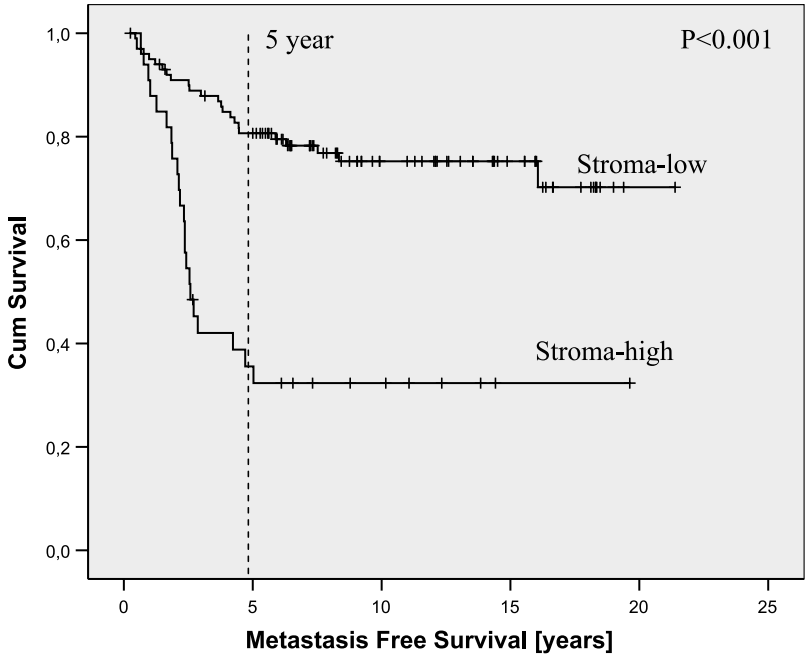


(a)

Fig. 1. Kaplan–Meier survival curves for stroma-high and stroma-low patients: (a) OS, (b) DFS, (c) MFS. Notably, the mean age of the analyzed patient group was 68.2 years with a mean follow-up time of 10.9 years. As some patients have a follow-up period of 20 years the full survival time was displayed.



(b)



(c)

Fig. 1. (Continued).

4.4. Topography

We investigated the topography separately, known that this parameter effects prognosis. From a total of 135 patients 46.7% ($n = 63$) had a tumor located left sided in the colon and 53.3% ($n = 72$) right sided.

Twenty (31.7%) of the left sided tumors were stroma-high and 43 (68.3%) stroma-low. Survival anal-

ysis showed significant differences between both groups (OS $p < 0.001$; HZ 2.85, DFS $p = 0.002$; HZ 2.63) (Table 2 and Suppl. Table 1: http://www.qub.ac.uk/isco/JCO).

Fourteen (19.4%) of the patients with a right-sided tumor were stroma-high and 58 (80.6%) stroma-low. Significant differences between both groups were observed (OS $p = 0.001$; HZ 2.99, DFS $p = 0.007$; HZ 2.50).

Although the number of patients with stroma-high differed per location (left or right), the prognosis for stroma-high patients was similar: HZ 2.85 versus 2.99 and HZ 2.63 versus 2.50.

Twenty-five patients were MSI-H of which 23 (92%) were located right sided and 2 (8%) left sided. Five-year survival for the total MSI-H (microsatellite instability-high) group was 90% compared to the microsatellite stable (MSS) group with 70% (OS; $p = 0.887$, DFS: 0.895). The stroma percentage evaluated for the MSS group only resulted in comparable results as for the MSS including the MSI-H group (OS, DFS: $p < 0.001$). Two patients with MSI-H had a high stroma percentage. One of these two patients showed abrogation for SMAD4.

4.5. Immunostaining for TGF-β receptor 2

Staining for TGF-β-R2 resulted in a positive membranous staining of the tumor cells. When no membranous staining was observed it was concluded that TGF-β-R2 was abrogated. From 117 patients stained for TGF-β-R2, 104 (88.9%) showed positive membranous expression and 13 (11.1%) were negative. No significant difference in survival time was observed between both groups (OS $p = 0.079$, DFS $p = 0.106$).

Between the stroma-high and stroma-low group no significant difference in survival times were observed for patients with and without abrogation of TGF-β-R2 (Table 3).

4.6. Immunostaining for β-catenin

Staining for β-catenin resulted in membranous staining, nuclear staining, or showing both; these patients were counted as nuclear staining. The number of patients with nuclear staining was 59. There was no significant difference in survival time for patients with and without nuclear expression (OS $p = 0.227$, DFS $p = 0.116$). From 117 patients stained for β-catenin 29 were stroma-high of which 18 (62.1%) had expression of the protein in the nucleus and 11 (36.7%) showed expression in the cytoplasmic membrane. No significant correlation was observed between the stroma-high and stroma-low group and either nuclear or membraneous β-catenin expression (Table 3).

4.7. Immunostaining for SMAD4

In case of active TGF-β signaling, SMAD4 positive nuclear staining is expected. Nuclear and cytoplasm negative staining indicates abrogation of the SMAD4 gene expression leading to changes in the TGF-β pathway. From 118 patients stained for SMAD4, positive nuclear staining was seen in 90 cases, 17 were negative and 11 patients showed both positive and negative areas (haplo-insufficiency) within the tumor; these latter patients were counted as negative [26]. The total number of patients with negative staining for SMAD4 was 28 (23.5%). There was a significant difference in survival time between the SMAD4 positive and the SMAD4 negative patients (OS $p = 0.006$, DFS $p = 0.022$).

The proportion of SMAD4 positive and SMAD4 negative patients within the stroma-high group was about equal but a distinct difference in survival time between both groups was observed, with stroma-high/SMAD4-negative patients showing a worse prognosis (OS $p = 0.008$, DFS $p = 0.005$). Twelve of the 14 (85.7%) stroma-high/SMAD4-negative patients

Table 3
Characteristics of immunostaining for TGF-β-R2, SMAD4, β-catenin in relation to the amount of intra-tumor stroma of the primary tumor

	TGF-β-R2* ($n = 117$)		SMAD4* ($n = 118$)		β-catenin* ($n = 117$)	
	Negative	Positive	Negative	Positive	Nuclear	Membrane
Stroma-high	2	26	14	14	18	11
	(1.7%)	(22.2%)	(11.9%)	(11.9%)	(15.4%)	(9.4%)
Stroma-low	11	78	14	76	41	47
	(9.4%)	(66.7%)	(11.9%)	(64.4%)	(35.0%)	(40.2%)
Chi-square	$p = 0.444$		$p < 0.001$		$p = 0.148$	

*Percentage is based on the total number for markers analyzed patients.

Table 4
Results of SMAD4 staining relative to the amount of stroma in the primary tumor

	Stroma-high*	Stroma-low**
SMAD4-negative		
Percentage of patients	11.9%	11.9%
Percentage at 5-year	**7.1%**	85.7%
SMAD4-positive		
Percentage of patients	11.9%	64.4%
Percentage at 5-year	57.1%	80.3%

Notes: Percentage is based on the total number of patients that were analyzed for markers ($n = 118$).
*Significance for Smad staining within the stroma-high group: OS $p = 0.008$, HZ 7.98, CI 4.12–15.44; DFS $p = 0.005$, HZ 6.57, CI 3.43–12.56.
**Significance for Smad staining within the stroma-low group: OS $p = 0.937$, HZ 1.56, CI 0.76–3.21; DFS $p = 0.685$, HZ 1.37, CI 0.67–2.80.
The series that was analyzed consists partly of a consecutive and partly of a case-control set. Calculated hazard ratio's (HZ) are valid and meaningful but the 5-year survival time can not be used to generalize. In our data set 30% of the patients had a recurrence within 5 years; the actual rate for stage I–II patients is 25%.

died within 3 years. For the stroma-low group this difference was not significant (OS $p = 0.937$, DFS $p = 0.685$). Percentages of 5 year follow up were 7.1% for stroma-high/SMAD4-negative patients and 80.3% for stroma-high/SMAD4-positive patients (Tables 3 and 4).

Combined use of H&E staining and SMAD4 immunohistochemistry as prognostic marker (stroma-high/stroma-low with positive or negative staining of SMAD4) showed significantly different Kaplan–Meier curves (OS $p < 0.001$, DFS $p < 0.001$) (Fig. 2). A group of "high-risk" patients with low survival time showing a high amount of intra-tumor stroma and negative SMAD4 staining could be distinguished with additional independent prognostic value.

In a univariate Cox-regression analysis, the amount of stroma appeared to be an independent factor for survival ($p < 0.001$, HZ 2.73).

In a logistic regression analysis the interaction between the variables high intra-tumor stroma and loss of SMAD4 were found to be strongly related (HZ 5.42, CI 2.13–13.82, $p < 0.001$) indicating that SMAD4 staining can be a specific marker to select "high-risk "patients.

No significant relationship between the amount of stroma and β-catenin staining or the amount of stroma and TGF-β-R2 was found.

5. Conclusions

In a former study we investigated the tumor–stroma ratio as prognostic parameter for stage I–III colon cancer patients. Significant differences in survival time were found for patients showing different amounts of intra-tumor stroma within the primary tumor [22]. Patients with a high percentage of stroma were found to have a worse prognosis.

In the current study we focus on stage I–II patients aiming at the identification of a subgroup who might benefit from additional therapy.

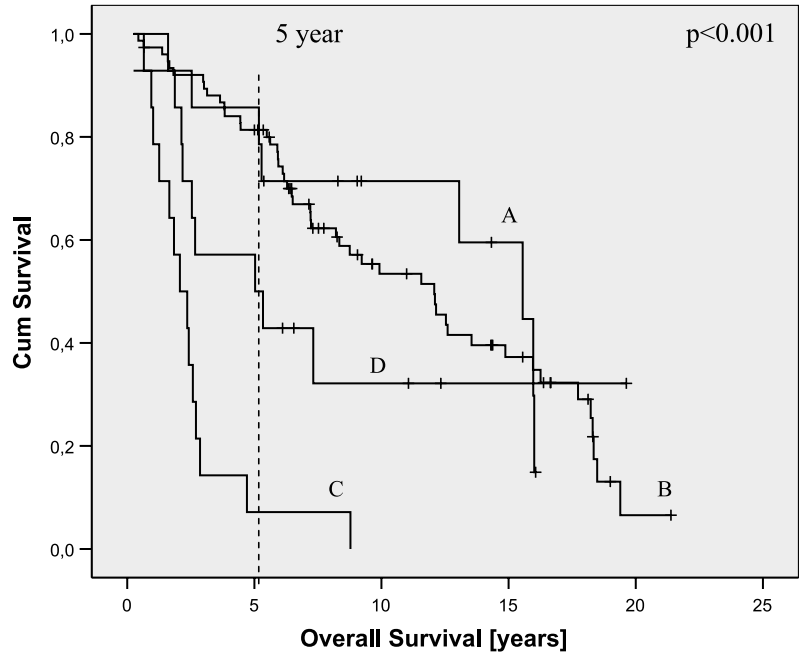
Additionally, we investigated three elements involved in the signaling pathways related to tumor–stroma interactions: TGF-β-R2, SMAD4 and β-catenin. The already strong prognostic information provided by the tumor–stroma ratio was further refined by adding information regarding the SMAD4 status, which loss selects for a specific group of patients with more aggressive tumors. This specific group of patients with stroma-high/loss of SMAD4 showed a low 5-year survival of 7.1% compared to 80.3% for patients with stroma-low/SMAD4 positive staining.

Several studies report a higher frequency of SMAD4 inactivation in patients presenting unfavorable survival, which is in agreement with our observations [20, 27,28]. Although other groups give evidence that increased nuclear β-catenin expression is independently associated with higher N stage and worse survival [29, 30], we did not find β-catenin to correlate with either overall survival or associated with stroma involvement.

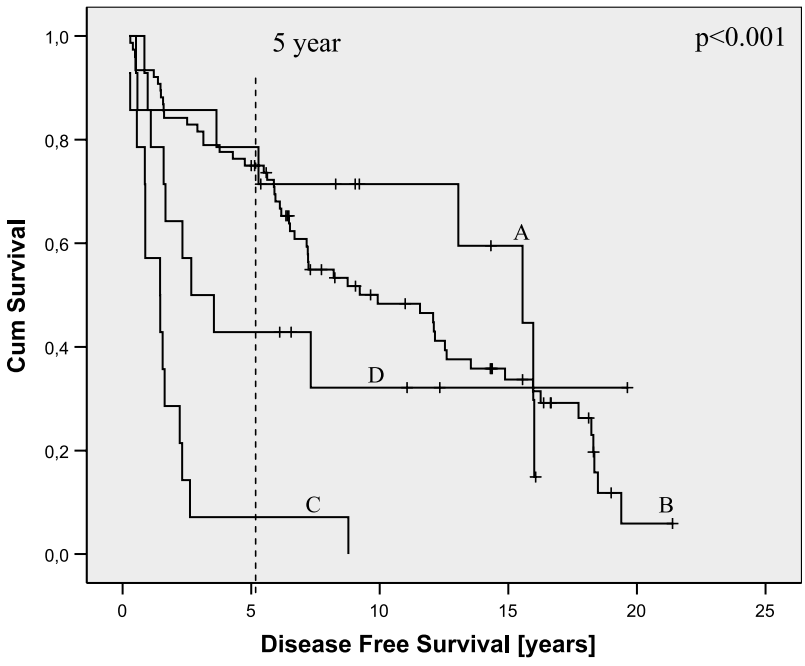
Currently there is no univocal policy for standard treatment of stage II patients. Treatment of the complete group is not meaningful, although for high-risk patients, the ASCO recommends adjuvant treatment [31]. A recently published paper by the QUASAR Collaborative Group reports that treatment of this group would result in an absolute benefit from an 18% reduction in mortality of 5.4% for high-risk patients compared to 3.6% in low-risk patients [32].

According to literature 25% of colon cancer stage II patients have recurrence within 5 years [2]. Within our analyzed group this percentage was 30%. Of the patients with a high amount of stroma, 62% had recurrence of disease, whereas for patients with stroma-high in combination with SMAD4 abrogation this was 86% within 5 years.

These results show that tumor–stroma ratio as single parameter or in combination with SMAD4 immunohistochemistry can further select for a patient population



(a)



(b)

Fig. 2. Kaplan–Meier survival curves for stroma-high patients and stroma-low patients with positive and negative SMAD4 staining: (a) OS, (b) DFS. Notably, the mean age of the analyzed patient group was 68.2 years with a mean follow-up time of 10.9 years. As some patients have a follow-up period of 20 years the full survival time was displayed. A. Stroma-low/SMAD4-negative; B. Stroma-low/SMAD4-positive; C. Stroma-high/SMAD4-negative; D. Stroma-high/SMAD4-positive.

with specific bad prognosis. When confirmed in series from other institutions our approach might contribute to a better selection of high-risk stage I and II patients that might benefit from adjuvant treatment. Con-

sequently, prospective studies to select patients for a randomized clinical study in which adjuvant therapy is selectively applied in stage I and II colorectal cancer should follow.

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