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The carcinoma–stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage

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Abstract. *Background:* Tumor staging insufficiently discriminates between colon cancer patients with poor and better prognosis. We have evaluated, for the primary tumor, if the carcinoma-percentage (CP), as a derivative from the carcinoma-stromal ratio, can be applied as a candidate marker to identify patients for adjuvant therapy. *Methods:* In a retrospective study of 63 patients with colon cancer (stage I–III, 1990–2001) the carcinoma-percentage of the primary tumor was estimated on routine H&E stained histological sections. Additionally these findings were validated in a second independent study of 59 patients (stage I–III, 1980–1992). (None of the patients had received preoperative chemo- or radiation therapy nor adjuvant chemotherapy.) *Results:* Of 122 analyzed patients 33 (27.0%) had a low CP and 89 (73.0%) a high CP. The analysis of mean survival revealed: overall-survival (OS) 2.13 years, disease-free- survival (DFS) 1.51 years for CP-low and OS 7.36 years, DFS 6.89 years for CP-high. Five-year survival rates for CP-low versus CP-high were respectively for OS: 15.2% and 73.0% and for DFS: 12.1% and 67.4%. High levels of significance were found (OS $p < 0.0001$, DFS $p < 0.0001$) with hazard ratio's of 3.73 and 4.18. In a multivariate Cox regression analysis, CP remained an independent variable when adjusted for either stage or for tumor status and lymph-node status (OS $p < 0.001$, OS $p < 0.001$). *Conclusions:* The carcinoma-percentage in primary colon cancer is a factor to discriminate between patients with a poor and a better outcome of disease. This parameter is already available upon routine histological investigation and can, in addition to the TNM classification, be a candidate marker to further stratify into more individual risk groups.

Keywords: Colon cancer, TNM classification, primary tumor, stroma, prognosis

1. Introduction

Colorectal cancer (CRC) is the fourth most common form of cancer occurring worldwide, with an estimated 1.02 million new cases diagnosed each year. It affects men and women almost equally. Large differences exist in survival, associated with disease stage. It is estimated that 529,000 deaths from colorectal cancer occur worldwide annually, causing colorectal cancer to be the second most common cause of death from cancer in men in the European Union and the United States [1].

The current method for staging of colorectal cancer is according to the TNM classification. TNM is the most widely used system for classifying the anatomic extent of cancer spread and important for decision making in therapy [2]. Information on nodal involvement is an important part of CRC staging since metastasis to regional lymph nodes (LNs) is one of the most important factors relating to the prognosis of colorectal carcinomas. Patients with metastatic LNs have a shorter survival and require adjuvant systemic chemotherapy. Despite this, nodal involvement alone is not considered sensitive enough to discriminate be-

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tween patients with poor and better prognosis, because up to 20–40% of patients with invasive tumors, but without demonstrated nodal involvement, die of their cancer [3].

The five year survival for colon cancer stage II patients (AJCC staging) is 85% for stage IIA and 72% for stage IIB [4]. There is controversy in the necessity of adjuvant treatment as is shown in several studies [5–9]. During the ASCO Annual Meeting (June 2–6, 2006, Atlanta, GA) recommendations for treatment of stage II disease were proposed. Experts in GI cancer reported the results of a meta-analysis on 7 randomized trials (3,732 patients) and concluded that there is no rational to routinely apply adjuvant therapy, with the exception of high risk cases based on clinical features (T4, obstruction or perforation), nodal sampling (number of LNs resected) and prognostic factors. For some prognostic factors data exist supporting the role to select patients at risk: loss of chromosome 18q, DCC (deleted in colorectal cancer-gene) expression, DNA mismatch repair status (MMR), microsatellite instability (MSI), p53 and k-ras mutations, high thymidylate synthese (TS) expression, and circulating tumor cells in bone marrow and blood.

Currently extensive research is performed to distinguish patients with low/high risk profiles on basis of molecular techniques. Methods aiming at genomic or expression analysis using array technology or proteomics predominantly focus on the analysis of the primary tumor [10,11]. So far, genomic and expression profiling has not led to a clear set of prognostic factors that can be used for individual patient management.

Recent models on metastatic invasion focus on the tumor-"host" interface, in particular the role of the stromal tissue. The biological meaning of the stromal compartments are thought to be part of the process of wound healing in cancer, but there is also strong emphasis that CAFs (cancer-associated fibroblasts) are important promotors for tumor growth and progression [12,13].

Assuming these models are correct we anticipate that changes in the proportion of stromal compartment in the primary tumor probably reflect progression. We therefore have determined the carcinoma percentage (CP), as a derivative from the carcinoma-stromal proportion, and tested this parameter for survival. Surprisingly in a set of patients with a good and bad survival a clear difference in CP for both groups was observed. This finding stimulated us to extend our patient group for further analysis with respect to CP.

In a study of 63 colon patients (stage I–III, 1990–2001, neither pre-operative chemo- or radiation therapy nor adjuvant chemotherapy) with a mean follow up of 9.03 (SD 3.1) years we have estimated the CP on, for diagnostics used, H&E stained sections of the primary tumors and investigated its relation to overall (OS) and disease free (DFS) survival. The results of this study were then validated in a second independent study of 59 colon patients (stage I, III. 1980–1992, neither pre-operative chemo- or radiation therapy nor adjuvant chemotherapy) with a mean follow up of 16.1 (SD 4.3) years. Since for both studies OS and DFS did not differ (OS $p = 0.96$, DFS $p = 0.53$) they were also analyzed as one series.

2. Material and methods

2.1. Patient recruitment

We selected 63 unspecified colon cancer patients with stage I–III tumors (clinically staged according to the tumor-node-metastasis classification of the AJCC [2]), who underwent curative surgery at the Leiden University Medical Center between 1990 and 2001.

For the validation study an additional 59 patients with colon cancer stage I–III were selected who also underwent curative surgery at the Leiden University Medical Center between 1980 and 1992.

None of the patients had received preoperative chemo- or radiation therapy nor adjuvant chemotherapy. Unlike the situation in the US where patients are being treated with adjuvant therapy more common, our patients were not adjuvantly treated. There were no patients included in this study with known distant metastases at surgery. Further, patients with double tumors, other malignancies in the past and death or recurrence (distant or loco-regional) within 1 month, were excluded. HNPCC patients were also 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). For detailed patient characteristics see Table 1.

2.2. Histopathological protocol

Pathological examination entailed routine microscopic analysis of 5 μm H&E stained sections from the most invasive part of the primary tumor. The carcinoma percentage was visually estimated by two persons (HM, WM) on the whole tumor area, on basis

Table 1
Patient characteristics

Characteristics	Original series		Validation series	
	CP-low	CP-high	CP-low	CP-high
Gender	N (%)	N (%)	N (%)	N (%)
Male	9 (50.0)	25 (55.6)	10 (66.7)	28 (62.2)
Female	9 (50.0)	20 (44.4)	5 (33.3)	16 (36.4)
Mean age (yrs)*,**	69.6 (sd 15.3)	67.6 (sd 12.3)	65.3 (sd 12.6)	66.8 (sd 12.5)
Location tumor				
Left	6 (33.3)	14 (31.1)	10 (62.5)	18 (41.9)
Right	12 (66.7)	31 (68.9)	6 (37.5)	25 (58.1)
T status				
T1	0 (0)	4 (8.9)	0 (0)	0 (0)
T2	2 (11.1)	6 (13.3)	0 (0)	24 (54.5)
T3	12 (66.7)	27 (60.0)	10 (66.7)	20 (45.5)
T4	4 (22.2)	8 (17.8)	5 (33.3)	0 (0)
N status				
N0	4 (22.2)	38 (84.4)	2 (13.3)	22 (50.0)
N1	7 (38.9)	6 (13.3)	9 (60.0)	18 (40.9)
N2	7 (38.9)	1 (2.2)	4 (26.7)	4 (9.1)
Stage				
I	2 (11.1)	8 (17.8)	0 (0)	16 (36.4)
IIA	2 (11.1)	24 (53.3)	2 (13.3)	6 (13.6)
IIB	0 (0)	6 (13.3)	0 (0)	0 (0)
IIIA–C	13 (72.2)	7 (15.6)	13 (86.7)	22 (50.0)
Unknown	1 (5.6)	0 (0)	0 (0)	0 (0)
Grading (differentiation)				
Well	5 (27.8)	9 (20.0)	1 (6.7)	9 (20.5)
Moderate	10 (55.6)	27 (60.0)	6 (40.0)	22 (50.0)
Poor	2 (11.1)	4 (8.9)	8 (53.3)	10 (22.7)
Unknown	1 (5.6)	5 (11.1)	0 (0)	3 (6.8)
MSI				
MSS	16 (88.9)	34 (75.6)	15 (100)	34 (77.3)
MSI-H left sided	0 (0)	0 (0)	0 (0)	1 (2.3)
MSI-H right sided	2 (11.1)	11 (24.4)	0 (0)	9 (20.4)

* Original series: mean age defined as period from birth until diagnosis. Validation series: mean age defined as period from birth until resection.
** Difference statistically not significant in the original and validation series.
All tumors were radically resected (R0).

of morphological information (for clarity reasons we only give carcinoma percentages but complementary will give the stromal percentage; e.g. CP 70% implies a stromal percentage of 30%). In case of tumor heterogeneity, areas with the lowest CP were considered decisive as is performed in routine pathology to determine tumor differentiation. Percentages were scored ranging from 20 to 90%. Percentages of 10 and 100% were not seen. Shortly the protocol: H&E sections of the tumor with the most invasive part of the primary tumor were chosen. Using a 2.5× or a 5× objective the invasive area with the desmoplastic stroma was selected. Subsequently, using a 10× objective only the fields were scored where the stroma was infiltrated with small tumor nests within all sides of the image field. The tumorpercentage was estimated (per tenfold: 10, 20, 30% etc.) per image-field. The lowest scored percentage was considered decisive. In some cases of necrosis or mucus forming tumors, scoring of the stroma percentage was more difficult and sometimes caused over- or underscoring.

For the identification of MSI-H (MSI-high) patients, 5 μm slides were immunohistochemically stained for the markers MLH1 and PMS2 [14,15].

2.3. Statistics

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. Disease Free Survival (DFS) was defined 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 (irrespective of site) or the date of a second primary tumor whatever occurs first. If no recurrence or second primary tumor occurred DFS was calculated as the time period until date of last follow-up. To calculate Disease Specific (Overall) Survival (DS-OS) and Disease Specific (Disease Free) Survival (DS-DFS) death was restricted to death due to colon cancer.

Tumor status, lymph node status and status of present metastases were applied according to AJCC/TNM guidelines.

Right sided tumors were defined as follows: coecum, colon ascendens, flexura hepatica, colon transversum and for left sided: flexura lienalis, colon descendens, colon sigmoideum, rectosigmoideum.

Carcinoma percentage (CP) was defined as CP-low: $<$50% including the values 20, 30 and 40% tumor and CP-high: $\geqslant$50% including the values 50, 60, 70, 80 and 90%.

Analysis of the survival curves was performed using Kaplan–Meier Survival Analysis and differences in equality of survival distributions were tested with the Log Rank Statistics. The Cox proportional hazards model was used to determine the Relative Risk (RR) or Hazard Ratio (HZ) of explanatory variables on OS and DFS.

Differences in OS and DFS between in the original series of 63 patients (original series) and the validation series of 59 patients (validation series) were tested by Kaplan–Meier Survival Analysis.

3. Results

3.1. Patient demographics

The original study (training set) consisted of 34 men (54%) and 29 women (46%), with a mean age of 68.2 years (SD 13.1; range 21.7–91.4 years). From sixty-three primary tumors 20 (32%) were located left sided and 43 (68%) right sided.

For the validation study 38 men (64.4%) and 21 women (35.6%) were included with a mean age of 66.4 (SD 12.5; range 30.1–85.0 years). Twenty-eight (47.5%) were located left sided and 31 (52.5%) right sided.

Right sided tumors included were: coecum ($n = 36$), colon ascendens ($n = 17$), flexura hepatica ($n = 8$) and colon transversum ($n = 13$) and for left sided: flexura lienalis ($n = 2$), colon descendens ($n = 1$), colon sigmoideum ($n = 32$) and rectosigmoideum ($n = 13$). For all patients tumors were radically resected (R0). For detailed TNM patient characteristics see Table 1.

3.2. Determination of the cut-off level for carcinoma percentage

We determined the optimal threshold level of CP on the basis of a maximum discriminating power for OS and DFS in the original study (training set) (see Table 2). This approach resulted in a cut-off point for CP at the 50% level for further analysis. Consecutively we applied this cut-off level for the validation series and the combined series. Results of the last two series were in line with those obtained for the original study.

3.3. Histopathology

Routine H&E stained slides from the most invasive part of the tumor were microscopically analyzed for the presence of stromal involvement using a 5$\times$ and a 10$\times$ objective. This desmoplastic stroma was not related to the total tumor size. We observed areas with

Table 2
Determination of the CP 50% cut-off value of the original series

Carcinoma percentage	Original series	
	OS	DFS
$<$40	2.77	1.53
$\geqslant$40	4.46	3.86
	$p = 0.236$	$p = 0.085$
$<$50	1.40	1.36
$\geqslant$50	5.40	4.82
	$p = 0.001$	$p = 0.0000$
$<$60	3.27	2.26
$\geqslant$60	5.51	5.31
	$p = 0.016$	$p = 0.001$
$<$70	3.66	2.76
$\geqslant$70	5.73	5.55
	$p = 0.047$	$p = 0.006$
$<$80	2.85	3.08
$\geqslant$80	3.14	5.26
	$p = 0.235$	$p = 0.058$

abundant stroma (CP-low) with a size as large as one microscopic field (100× total magnification), but also larger areas matching 2–4 fields were seen or even more, independent from the size of the tumor.

In general the CP was estimated on one single representative section from the primary tumor only. From 38 patients our archive contained multiple H&E stained slides from different areas of the same primary tumor, which allowed us to investigate how the scored CP percentage depended on the sampling. We noticed some heterogeneity in the CP percentage throughout the tumor. However, areas with the highest infiltration depth (T stage) had the lowest CP percentage whereas at the borders of the tumor, in case heterogeneity was found, the CP was higher. For clinical use of the CP percentage we therefore recommend the evaluation of sections taken from areas of the primary tumor with the highest T stage, which is common clinical practice.

Preliminary information of a new study by our group (to be published) shows a high agreement in the scoring for CP-low versus CP-high between three pathologists ($p < 0.0001$). Within the 27 discrepancies found for the three observers, 6 (22%) were within the 40–50% decision range.

Examples of images of H&E stained slides from the primary tumor from patients with a low CP (30%) and a high CP (80%) are given in Fig. 1. Incidentally, slides from the same tissue were differentially stained for tu-

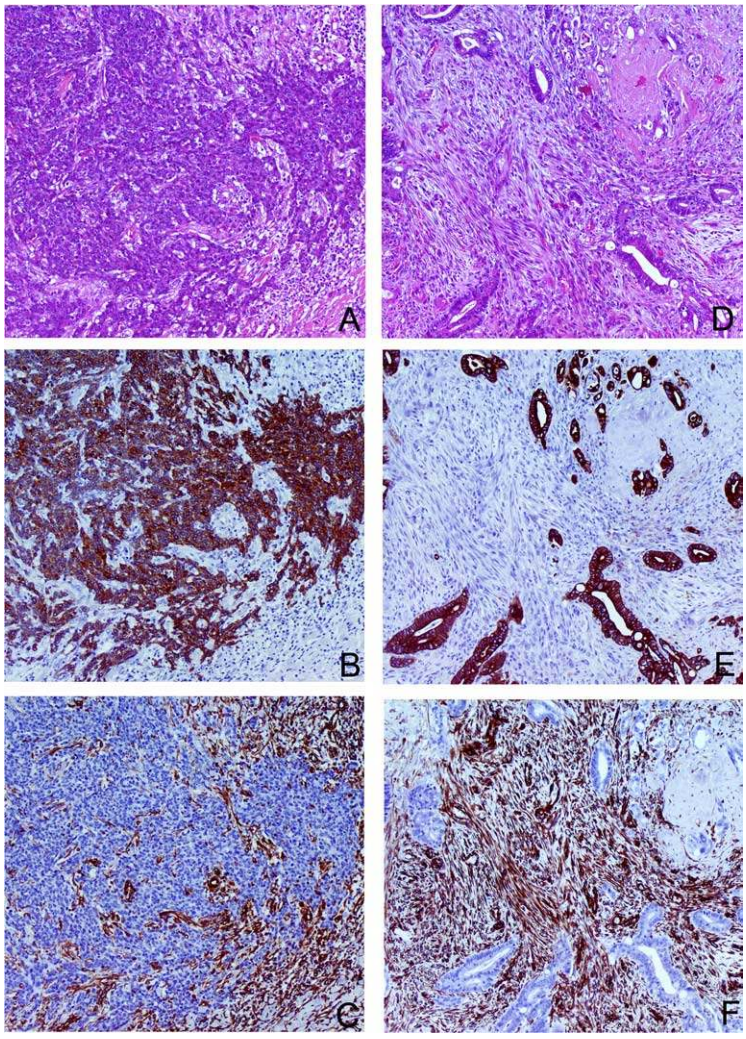


Fig. 1. H&E stained 5 μm paraffin sections of primary colon tumors. Carcinoma percentage estimated as 80% in a patient with long OS/DFS: (a) H&E staining; (b) cytokeratin staining for carcinoma cells; (c) vimentin staining of stromal compartments. Carcinoma percentage estimated as more than 30% in patient with short OS/DFS: (d) H&E staining; (e) cytokeratin staining for carcinoma cells; (f) vimentin staining of stromal compartment.

mor cells and stromal cells using antibodies specific for cytokeratin and vimentin respectively in order to check the status of the carcinoma-stromal proportion. This immunohistochemical method proved that the morphological judgment of the CP as used here was adequate.

3.4. Correlation with prognosis

3.4.1. Original series

From 63 patients analyzed 18 (28.6%) had a low CP and 45 (71.4%) a high CP. The mean OS for patients with CP-low was 1.40 years and 5.40 years for CP-high ($p < 0.0001$, HZ 4.31) (DFS $p < 0.0001$, HZ 4.53). Five year survival rates for OS and DFS for CP-low compared to CP-high patients were respectively 16.7%/11.1% and 77.8%/68.9%.

CP was compared to LN status, tumor status and stage. Significant differences of OS and DFS were found, respectively for LN status and staging. Tumor status did not show significant difference. For detailed data see Tables 2, 3, 4, 5 and Fig. 2.

3.4.2. Validation series

From 59 patients analyzed 15 (25.4%) had a low CP and 44 (74.6%) a high CP. The mean OS for patients with CP-low was 1.82 years and 8.64 years for CP-high ($p = 0.0001$, HZ 3.45) (DFS $p < 0.0001$, HZ 3.91). Five year survival rates for OS and DFS for CP-low compared to CP-high patients were respectively 13.3%/13.3% and 68.2%/65.9%.

With respect to the TNM parameters significant differences of OS and DFS were found, respectively for LN status and for tumor status, but not for stage. For detailed data see Tables 2, 3, 4, 5 and Fig. 3.

Both series (original and validation) were selected on basis of the same selection criteria. Since there was no significant difference between both series for OS and DFS (OS $p = 0.96$, DFS $p = 0.52$) it was decided to combine the two sets and analyze them as one series.

In this combined series of 122 patients the OS for patients with CP-low was 2.13 years and 7.36 years for CP-high ($p < 0.0001$, HZ 3.74) (DFS $p < 0.0001$, HZ 4.18). See Tables 2, 3, 5 and Fig. 4a, b.

Table 3
P values (univariate) for CP and TNM parameters

	Combined series			Original series			Validation series		
	Total $n = 122$	Left $n = 48$	Right $n = 74$	Total $n = 63$	Left $n = 20$	Right $n = 43$	Total $n = 59$	Left $n = 28$	Right $n = 31$
CP									
OS	<0.0001	0.0764	<0.0001	<0.0001	0.2512	<0.0001	0.0001	0.1594	0.0001
DFS	<0.0001	0.0095	<0.0001	<0.0001	0.0811	<0.0001	<0.0001	0.0598	<0.0001
DSS/OS**		0.0061			0.3157*			0.0056	
DSS/DFS		0.0015			0.1942*			0.0038	
LN status									
OS	<0.0001	0.2446	<0.0001	<0.0001	0.0855	0.0002	0.0477	0.5223	0.0034
DFS	<0.0001	0.1857	<0.0001	<0.0001	0.0072	0.0002	0.0347	0.5207	0.0034
DSS/OS		0.0035			0.0472			0.0435	
DSS/DFS		0.0015			0.0042			0.0402	
Tumor status									
OS	0.0091	0.2245	0.1905	0.1865	0.2458	0.1073	0.0003	0.0511	0.0071
DFS	0.0060	0.0378	0.0297	0.1405	0.0260	0.1881	0.0007	0.0475	0.0167
DSS/OS		0.0054			0.0675			0.0031	
DSS/DFS		0.0003			0.0104			0.0018	
Stage									
OS	<0.0001	0.1905	0.0001	0.0001	0.1198	0.0006	0.0836	0.5427	0.0204
DFS	<0.0001	0.0297	<0.0001	<0.0001	0.0015	0.0010	0.0518	0.2761	0.0206
DSS/OS		0.0028			0.0510			0.0540	
DSS/DFS		0.0001			0.0018			0.0169	

* Discrepancy caused by one patient outlier; low CP, long survival.
** DSS: Disease specific survival.

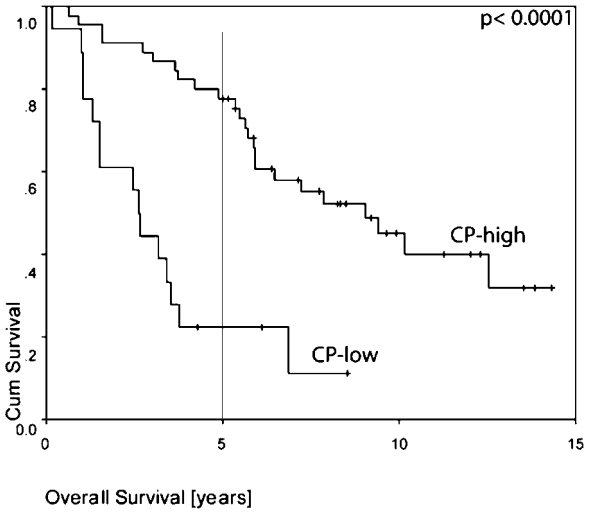
Table 4
Percentage of patients alive 5 years after operation for overall and disease free survival

	Combined series			Original series			Validation series		
	Total	Left	Right	Total	Left	Right	Total	Left	Right
CP									
Low	15.2/12.1*	28.6/21.4	5.3/5.3	16.7/11.1	33.3/16.7	8.3/8.3	13.3/13.3	25.0/25.0	0/0
High	73.0/67.4	64.7/55.9	78.2/74.5	77.8/68.9	71.4/57.1	80.6/74.2	68.2/65.9	60.0/55.0	75.0/75.0
LN status									
N0	78.8/71.2	70.8/58.3	83.3/78.6	78.6/69.0	71.4/57.1	82.1/75.0	79.2/75.0	70.0/60.0	85.7/85.7
N1	40.0/37.5	42.1/36.8	38.1/3801	30.8/23.1	40.0/20.0	25.0/25.0	44.4/44.4	42.9/42.9	46.2/46.2
N2	12.5/12.5	20.0/20.0	9.1/9.1	12.5/12.5	0/0	14.3/14.3	12.5/12.5	25.0/25.0	0/0
Tumor status									
T1	75.0/75.0	50.0/50.0	100/100	75.0/75.0	50.0/50.0	100/100	**	**	**
T2	81.3/78.1	78.9/73.7	84.6/84.6	87.5/87.5	83.3/83.3	100/100	79.2/75.0	76.9/69.2	81.8/81.8
T3	52.2/44.9	39.1/26.1	58.7/54.3	59.0/46.2	45.5/18.2	64.3/57.1	43.3/43.3	33.3/33.3	50.0/50.0
T4	29.4/29.4	25.0/25.0	30.8/30.8	41.7/41.7	100/100	36.4/36.4	0/0	0/0	0/0
Stage									
I	84.6/80.8	75.0/68.8	100/100	80.0/80.0	71.4/71.4	100/100	87.5/81.3	77.8/66.7	100/100
IIA	76.6/64.7	57.1/28.6	81.5/74.1	80.8/65.4	66.7/33.3	85.0/75.0	62.5/62.5	0/0	71.4/71.4
IIB	66.7/66.7	100/100	60.0/60.0	66.7/66.7	100/100	60.0/60.0	**	**	**
IIIA–C	32.7/30.9	37.5/33.3	29.0/29.0	25.0/20.0	33.3/17.7	21.4/21.4	37.1/37.1	38.9/38.9	35.3/35.3

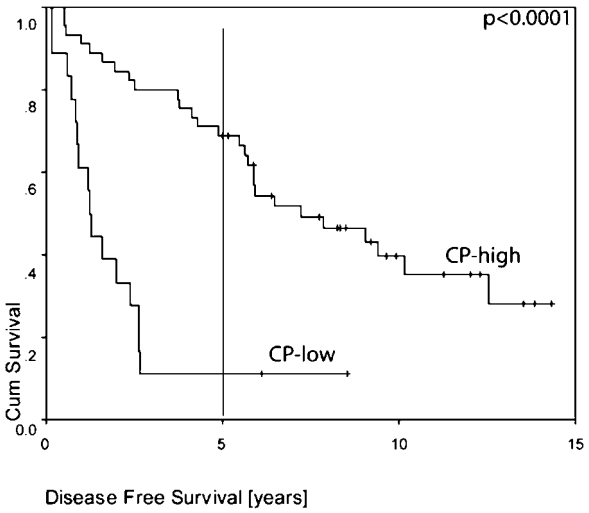
* OS/DFS.
** no patients with this classification in series.
Note: for 5 year and 10 year survival comparative data were observed.

Table 5
Cox proportional Hazards regression (univariate)

	n	Topography	OS or DFS	Hazard ratio	95% CI
Combined series	122	Total colon	OS	3.74	2.32–6.01
			DFS	4.18	2.63–6.65
	48	Left sided	OS	1.98	0.92–4.27
			DFS	2.51	1.22–5.17
	74	Right sided	OS	9.56	4.70–19.48
			DFS	9.14	4.55–18.38
Original series	63	Total colon	OS	4.31	2.15–8.66
			DFS	4.53	2.31–8.90
	20	Left sided	OS	2.07	0.58–7.40
			DFS	2.75	0.84–8.95
	43	Right sided	OS	7.50	3.09–18.22
			DFS	6.15	2.62–14.44
Validation series	59	Total colon	OS	3.45	1.77–6.74
			DFS	3.91	2.03–7.51
	28	Left sided	OS	1.99	0.75–5.27
			DFS	2.38	0.94–6.03
	31	Right sided	OS	16.93	4.60–62.27
			DFS	21.06	5.03–88.14

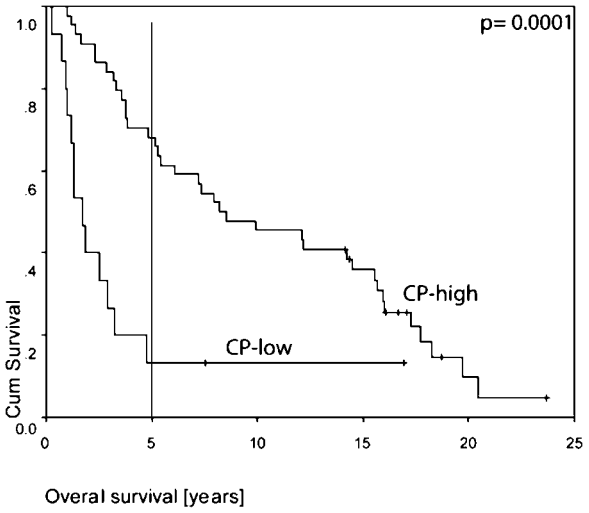


(a)

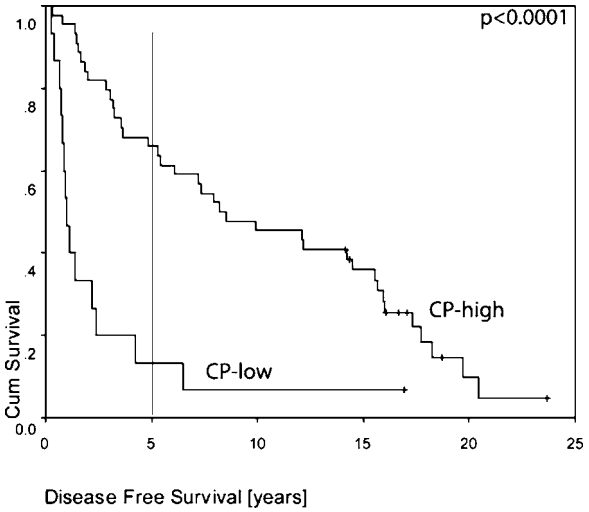


(b)

Fig. 2. Kaplan–Meier curves of the *original series* for CP-low and CP-high patients: (a) OS and (b) DFS. The dashed line indicates the 5-year survival time.



(a)



(b)

Fig. 3. Kaplan–Meier curves of the *validation series* for CP-low and CP-high patients: (a) OS and (b) DFS. The dashed line indicates the 5-year survival time.

In a multivariate Cox regression analysis, CP remained an independent variable when corrected for either stage (OS $p < 0.001$, HZ 0.39, 95% CI 0.22–0.71) (DFS $p < 0.0001$, HZ 0.34, 95% CI 0.19–0.60) or for tumor status and LN status (OS $p < 0.001$, HZ 0.37, 95% CI 0.20–0.68) (DFS $p < 0.0001$, HZ 0.34, 95% CI 0.19–0.61).

A large difference was observed between 5 year survival rates for both CP groups. A comparison with the conventional TNM parameters is given in Table 4.

3.5. Topography and the MSI status

We have investigated the topography (left and right sided) and the MSI status separately, known to be parameters that have impact on prognosis.

3.5.1. Left sided and right sided tumors

The combined series consists of 122 patients of which in 39% ($n = 48$) of the cases the tumor was located left sided (a) in the colon and in 61% ($n = 74$) right sided (b).

(a) Sixteen (33.3%) of the left sided tumors had a low CP and 32 (66.7%) a high CP (OS $p = 0.0764$, HZ

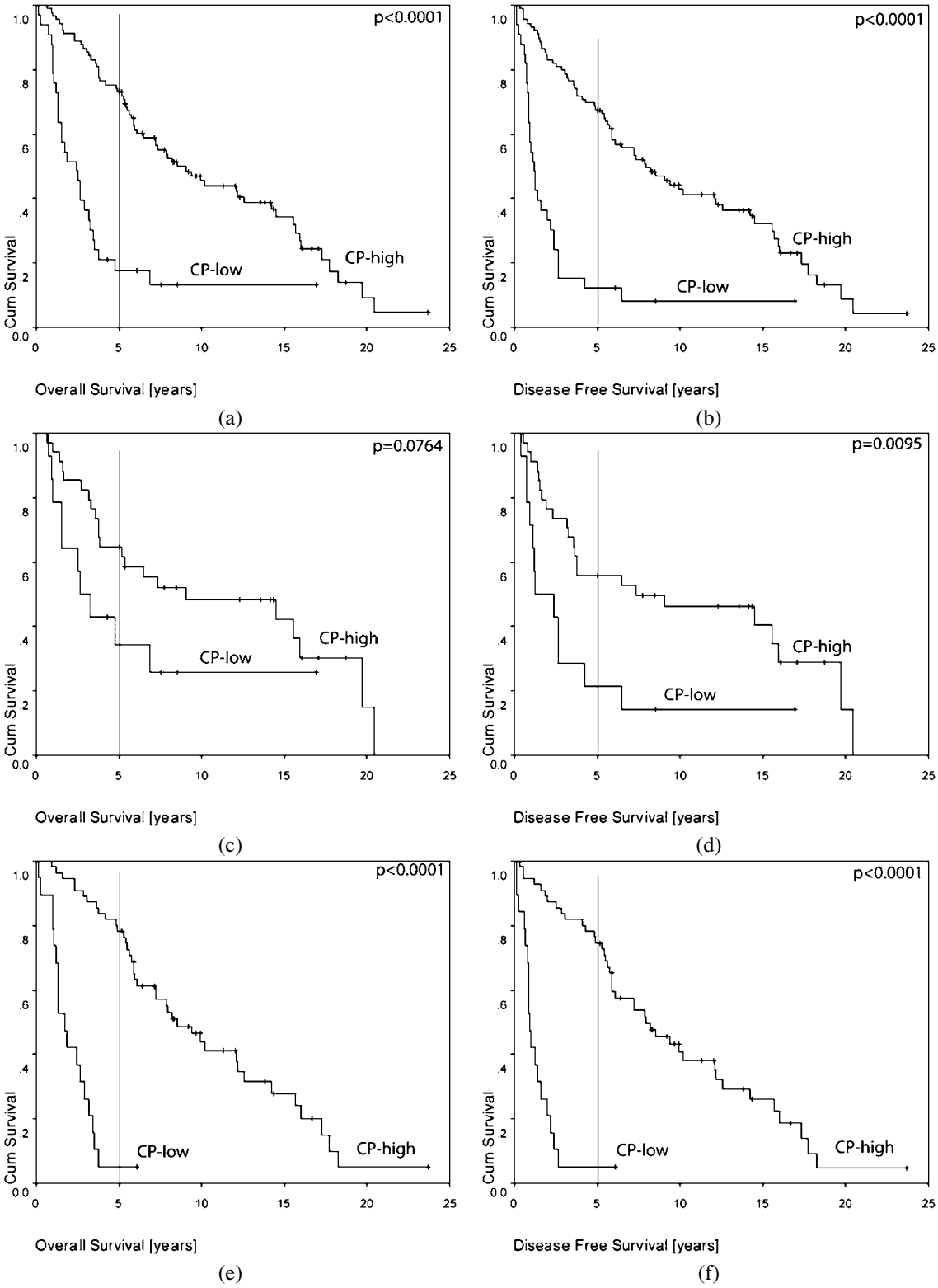


Fig. 4. Kaplan–Meier curves of the *combined series* for CP-low and CP-high patients: (a) OS and (b) DFS of the complete set of patients, (c, d) represent the OS and DFS of the left sided tumors and (e, f) of the right sided tumor. The dashed line indicates the 5-year survival time.

1.98; DFS $p = 0.0095$, HZ 2.51). OS and DFS were not significantly different for LN status but for DFS tumor status and stage differed significantly. However disease specific survival (DSS) did show significant values for all parameters (Table 3).

(b) Eighteen (24%) of the right sided patients had a low CP and 56 (76%) a high CP. Survival analysis using Kaplan–Meyer showed highly significant values for OS and DFS (OS $p < 0.0001$, HZ 9.56; DFS $p < 0.0001$, HZ 9.14). Five year survival rates (OS/DFS) for CP-low compared to CP-high patients were respectively 5.3%/5.3% and 78.2%/74.5%. Significant differences for the TNM parameters were found, respectively for LN status and for stage, but not for DFS for tumor status. See Tables 3, 4 and Fig. 4c–f. We conclude that CP is of prognostic value for patients with either a left or right sided tumor, although for patients with a right sided tumor this is more evident.

3.5.2. MSI status

Twenty-three (18.9%) out of 122 patients showed abrogation of MLH1 and PMS2 and were MSI-H. One MSI-H patient had a colon carcinoma located left sided and 22 patients right sided.

Two patients with right sided tumors had a low CP. For MSI-H the five year survival rates for OS and DFS for CP-low patients compared to CP-high were respectively 0%/0% and 81.5%/76.6%.

Excluding the MSI-H tumors from analysis resulted in identical data for OS and DFS (OS $p < 0.0001$, DFS $p < 0.0001$ for both series). Since both series were not significantly different with respect to CP values ($p = 0.3$), OS ($p = 0.3$) and DFS ($p = 0.3$) we can conclude that these results indicate that the prognostic power of CP remained independent of MSI status.

3.6. Relation with tumor stage

Twenty-six patients were classified as stage I, 34 stage IIA, 6 stage IIB and respectively 8, 31 and 16 stage IIIA, B or C (Table 1).

The mean OS for CP-low versus CP-high for stage I and II patients was 3.96 years (range 1.30–6.62) and 10.33 years (range 8.80–11.86) ($p = 0.026$). For DFS this was 3.74 years (range 1.93–5.56) and 9.93 years (range 9.64–12.92) ($p = 0.0007$).

The mean OS for CP-low versus CP-high for stage IIIA–C patients was 3.85 years (range 1.12–6.58) and 9.61 years (range 8.04–11.19) ($p = 0.076$). For DFS this was 2.13 years (range 0.88–3.38) and 9.73 years (range 6.67–12.79) ($p < 0.0001$).

These results indicate that CP can be a discriminative parameter for as well low as high staged patients.

4. Discussion

The carcinoma-stromal composition is an important prognostic parameter as is proven in the presented studies in patients with stage I–III colon cancer. The determined carcinoma percentage (CP) classification can easily be applied in routine pathology in addition to the TNM classification to select patients with increased risk for recurrence of disease. Although statistical analysis of two independent series proved that CP is an independent parameter, we realize that the series that were analyzed are relatively small.

The use of adjuvant therapy for stage II patients remains controversial, and the identification of reliable prognostic factors may aid therapeutic decision-making. In our study we noticed a high number of patients with a low carcinoma percentage (CP-low) depending on stage, from 7.7% in stage I to 68.7% in stage IIIC patients. For stage I, II patients OS and DFS was significantly lower for patients with CP-low compared to patients with CP-high; 3.96/3.74 years versus 10.33/9.93 years (OS $p = 0.0255$, DFS $p = 0.0007$).

Three out of 4 (75%) stage IIa patients with CP-low died within 5 years due to their disease and 5 out of 30 (17%) patients with CP-high died within 5 years (sensitivity 37.5%, specificity 96.2%). For stage III patients, 22 out of 26 (96%) with CP-low died within 5 years due to their disease and 14 out of 29 (64%) patients with CP-high died within 5 years (sensitivity 61%, specificity 70%). Although the sensitivity is quite low, the specificity is very high and therefore CP-low in stage II patients could be indicative for adjuvant therapy or better-individualized treatment for an additional group of patient. In contrast, for stage III patients the sensitivity is too low and would result in undertreatment of patients.

Notably, in Northern European countries for stage II patients standard treatment does not include adjuvant treatment with chemotherapy, although for high risk patients the ESMO (European Society for Medical Oncology) recommends adjuvant treatment. In a recent study treatment with FOLFOX resulted in a relative reduction on risk of recurrence of 28% for high risk patients [16,17].

Our results for stage II patients are encouraging, nevertheless we should confirm our results in a much larger patient set. Our future research is directed to this goal.

Furthermore we observed that for the patients with a low CP the T stage is of less importance and that there-

fore these tumors might have a different mechanism for metastasizing.

Invasion and metastasis of colorectal cancers include various steps, such as proteolysis, adhesion, angiogenesis and cell growth, for which many genes have been identified [18]. In the proteolysis step, proteinases, which are produced by cancer cells but also by fibroblasts, degrade extracellular matrix (ECM) components and enable cancer cells to detach from the primary site [19]. In our study an increase of stromal cells in the primary tumor correlated significantly with poor prognosis. Malignancy emerges from a tumor-host microenvironment in which the host participates in the induction, selection and expansion of the neoplastic cells [20]. The stromal matrix has been shown to influence epithelial cell function in both malignant behavior and nonmalignant differentiation [21]. Stromal cell activation may be reflected in modifications of the adjacent ECM that are favorable to the microinvasion of cancer cells. This phenomenon could explain our findings.

A variety of cell types populate the stromal compartment, such as lymphocytes, granulocytes, fibroblasts and endothelial cells. The relative abundance of each cell type may change at the local site of tumor cell invasion [22,23].

Cancer cells expressing adhesion molecules are more likely to adhere to the ECM, leading to subsequent invasion and metastases. A prominent example is the epithelial-to-mesenchymal transition (EMT) during the process of wound healing in which cells loosen their intimate cell–cell contacts and acquire mesenchymal properties which means that epithelial cells can be converted into fibroblast-like cells. Cancer cells undergoing EMT develop invasive and migratory abilities. EMT of cancer cells is increasingly being recognized as an important determinant of tumor progression but also fibroblasts are implicated to play a role in metastasis [12,24]. A prominent factor to induce EMT is the transforming growth factor-β (TGF-β), which mediates fibroblast activation during wound healing [25]. For microarray analysis of gene expression patterns a wound-response signature is already known for breast cancer patients showing improved risk stratification for a poor prognosis independently of known clinicopathologic features [13].

Data for microsatellite instability (MSI) and chromosomal instability (CIN) have demonstrated that these groups are characterized by a different clinical outcome; tumors originating from the right colon have a better prognosis than tumors from the left part due to a high percentage of MSI-H lesions. In a publication by Gervaz et al. it was even stated that clinical decision making regarding adjuvant therapy might be stratified in the future according to MSI status of cancer [26]. Tumors with MSI-H rarely metastasize, neither locally, nor distant, have a more favorable stage and have been repeatedly reported as a favorable prognostic marker [27,28]. In our study we have excluded HNPCC patients, therefore patients were only tested for sporadic MSI-H using immunohistochemical staining for MLH1 and PMS2, this combination confirms the abrogation of the MLH1 protein for all MSI-H sporadic tumors. For MSI-H patients we found significant differences in OS and DFS when CP was added as additional parameter: 0%/0% versus 81.5%/76.6%.

We observed a difference between left and right sided tumors. For tumors located right sided in the colon, significant differences were found for CP, but also for LN status and stage but less for tumor status. For the left sided tumors, CP was a significant prognostic factor. All other TNM parameters did not reach significance for OS, only DFS for tumor status and stage were significantly different. However, disease specific survival (DSS) did show significance for all parameters.

As far as we know, no data are published about the influence of the carcinoma-stromal proportion on outcome in primary colon tumors. Although many pathologists will recognize the feature, the impact on prognosis was not known by now. Our study describes a candidate parameter that after proper training could be used in routine diagnosis, in addition to the TNM classification, to further stratify in more individual risk.

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References

[1] P. Boyle, H. Vainio, R. Smith, R. Benamouzig, W.C. Lee, N. Segnan et al., Workgroup I: Criteria for screening, *Ann. Oncol.* **16**(1) (2005), 25–30. UICC International Workshop on Facilitating Screening for Colorectal Cancer, Oslo, Norway (29 and 30 June 2002).

[2] L.H. Sobin and I.D. Fleming, *TNM Classification of Malignant Tumors*, 5th edn, Union Internationale Contre le Cancer and the American Joint Committee on Cancer, 1997. (*Cancer* **80**(9) (1997), 1803–1804.)

[3] G. Cserni, Nodal staging of colorectal carcinomas and sentinel nodes, *J. Clin. Pathol.* **56**(5) (2003), 327–335.

[4] J.B. O'Connell, M.A. Maggard and C.Y. Ko, Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging, *J. Natl. Cancer Inst.* **96**(19) (2004), 1420–1425.

[5] International Multicentre Pooled Analysis of B2 Colon Cancer Trials (IMPACT B2) Investigators, Efficacy of adjuvant fluorouracil and folinic acid in B2 colon cancer. *J. Clin. Oncol.* **17**(5) (1999), 1356–1363.

[6] A. Figueredo, M.L. Charette, J. Maroun, M.C. Brouwers and L. Zuraw, Adjuvant therapy for stage II colon cancer: A systematic review from the Cancer Care Ontario Program in evidence-based care's gastrointestinal cancer disease site group, *J. Clin. Oncol.* **22**(16) (2004), 3395–3407.

[7] S. Gill, C.L. Loprinzi, D.J. Sargent, S.D. Thome, S.R. Alberts, D.G. Haller et al., Pooled analysis of fluorouracil-based adjuvant therapy for stage II and III colon cancer: Who benefits and by how much?, *J. Clin. Oncol.* **22**(10) (2004), 1797–1806.

[8] E. Mamounas, S. Wieand, N. Wolmark, H.D. Bear, J.N. Atkins, K. Song et al., Comparative efficacy of adjuvant chemotherapy in patients with Dukes' B versus Dukes' C colon cancer: Results from four National Surgical Adjuvant Breast and Bowel Project adjuvant studies (C-01, C-02, C-03, and C-04), *J. Clin. Oncol.* **17**(5) (1999), 1349–1355.

[9] J. Sakamoto, Y. Ohashi, C. Hamada, M. Buyse, T. Burzykowski and P. Piedbois, Efficacy of oral adjuvant therapy after resection of colorectal cancer: 5-year results from three randomized trials, *J. Clin. Oncol.* **22**(3) (2004), 484–492.

[10] A. Barrier, F. Roser, P.Y. Boelle, B. Franc, C. Tse, D. Brault et al., Prognosis of stage II colon cancer by non-neoplastic mucosa gene expression profiling, *Oncogene* 2006.

[11] M.Y. Kim, S.H. Yim, M.S. Kwon, T.M. Kim, S.H. Shin, H.M. Kang et al., Recurrent genomic alterations with impact on survival in colorectal cancer identified by genome-wide array comparative genomic hybridization, *Gastroenterology* **131**(6) (2006), 1913–1924.

[12] R. Kalluri and M. Zeisberg, Fibroblasts in cancer, *Nat. Rev. Cancer* **6**(5) (2006), 392–401.

[13] H.Y. Chang, D.S. Nuyten, J.B. Sneddon, T. Hastie, R. Tibshirani, T. Sorlie et al., Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival, *Proc. Natl. Acad. Sci. USA* **102**(10) (2005), 3738–3743.

[14] J. Young, L.A. Simms, K.G. Biden, C. Wynter, V. Whitehall, R. Karamatic et al., Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: Parallel pathways of tumorigenesis, *Am. J. Pathol.* **159**(6) (2001), 2107–2116.

[15] J.W. Dierssen, N.F. de Miranda, S. Ferrone, M. van Puijenbroek, C.J. Cornelisse, G.J. Fleuren et al., HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression, *BMC Cancer* **7** (2007), 33.

[16] T. Andre, C. Boni, L. Mounedji-Boudiaf, M. Navarro, J. Tabernero, T. Hickish et al., Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer, *N. Engl. J. Med.* **350**(23) (2004), 2343–2351.

[17] E.J. van Cutsem and V.V. Kataja, ESMO Minimum Clinical Recommendations for diagnosis, adjuvant treatment and follow-up of colon cancer, *Ann. Oncol.* **16**(Suppl. 1) (2005), i16–i17.

[18] T. Takayama, K. Miyanishi, T. Hayashi, Y. Sato and Y. Niitsu, Colorectal cancer: Genetics of development and metastasis, *J. Gastroenterol.* **41**(3) (2006), 185–192.

[19] T. Ishikawa, Y. Ichikawa, M. Mitsuhashi, N. Momiyama, T. Chishima, K. Tanaka et al., Matrilysin is associated with progression of colorectal tumor, *Cancer Lett.* **107**(1) (1996), 5–10.

[20] L.A. Liotta and E.C. Kohn, The microenvironment of the tumour-host interface, *Nature* **411**(6835) (2001), 375–379.

[21] C.C. Park, M.J. Bissell and M.H. Barcellos-Hoff, The influence of the microenvironment on the malignant phenotype, *Mol. Med. Today* **6**(8) (2000), 324–329.

[22] A.H. Lee, E.A. Dublin and L.G. Bobrow, Angiogenesis and expression of thymidine phosphorylase by inflammatory and carcinoma cells in ductal carcinoma in situ of the breast, *J. Pathol.* **187**(3) (1999), 285–290.

[23] J. Galon, A. Costes, F. Sanchez-Cabo, A. Kirilovsky, B. Mlecnik, C. Lagorce-Pages et al., Type, density, and location of immune cells within human colorectal tumors predict clinical outcome, *Science* **313**(5795) (2006), 1960–1964.

[24] J.P. Thiery, Epithelial-mesenchymal transitions in tumour progression, *Nat. Rev. Cancer* **2**(6) (2002), 442–454.

[25] P.M. Siegel and J. Massague, Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer, *Nat. Rev. Cancer* **3**(11) (2003), 807–821.

[26] P. Gervaz, P. Bucher and P. Morel, Two colons-two cancers: paradigm shift and clinical implications, *J. Surg. Oncol.* **88**(4) (2004), 261–266.

[27] H. Kim, J. Jen, B. Vogelstein and S.R. Hamilton, Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences, *Am. J. Pathol.* **145**(1) (1994), 148–156.

[28] W.S. Samowitz, K. Curtin, K.N. Ma, D. Schaffer, L.W. Coleman, M. Leppert et al., Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level, *Cancer Epidemiol. Biomarkers Prev.* **10**(9) (2001), 917–923.