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CHAPTER 4

Combined analysis of biomarkers of proliferation and apoptosis in colon cancer; an immunohistochemistry based study using tissue microarray

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ABSTRACT

Background

Disturbance of the balance between proliferation and apoptosis is an important hallmark of tumor development. The goal of this study was to develop a descriptive parameter that represents this imbalance and relate this parameter to clinical outcome in all four stages of colon cancer.

Methods

The study population consisted of 285 stage I-IV colon cancer patients of which a tumor tissue micro array (TMA) was available. TMA sections were immunohistochemically stained and quantified for presence of Ki67 and cleaved caspase-3 tumor expression. These results were used to develop the combined apoptosis proliferation (CAP) parameter and correlated to patient outcome.

Results

The CAP parameter was significantly related to clinical outcome; patients with CAP ++ (high level of both apoptosis and proliferation) showed the best outcome perspectives (Overall Survival (OS), p=0.004 and Disease Free Survival (DFS), p=0.009). The effect of the CAP parameter was related to tumor microsatellite status, and indirectly to tumor location, where left-sided tumors with CAP + - (high level of proliferation, low level of apoptosis) showed a worse prognosis (DFS p-value 0.02) and right-sided tumors with CAP + - had a better prognosis (DFS p-value 0.032). With stratified analyses, the CAP parameter remained significant in stage II tumors only.

Conclusions

The CAP parameter, representing outcome of the balance between the level of apoptosis and proliferation, can be used as a prognostic marker in colon cancer patients for both DFS and OS, particularly in left sided, microsatellite stable tumors when TNM stage is taken into account.

INTRODUCTION

A key factor in tissue homeostasis, especially of the intestinal mucosa, is the balance that exists between the level of cell death and the level of cell proliferation [1-3]. Two important hallmarks of the process of tumorigenesis are responsible for disturbance of this balance and therefore contribute to the initiation and maintenance of tumor growth and development [4;5]. These hallmarks are: deregulation of the proliferative signaling pathway and deregulation of the pathway of apoptosis [4]. Both result in either non- or malfunction of important enzymes or unrestricted release of growth-promoting signals that under normal circumstances are necessary to maintain tissue homeostasis [5-12]. The level of cell proliferation and apoptosis can be studied with immunohistochemistry (IHC) taking advantage of all of the benefits of this technique, such as speed, routine availability, low costs, and high level of automation. The level of apoptosis can be evaluated through staining specifically the activated, cleaved form of the pro-apoptotic enzyme caspase-3 in the tumor cell cytoplasm. Caspase-3 is the final enzyme to become activated in the caspase cascade, which is the common pathway in the execution of apoptosis after the intrinsic and extrinsic apoptosis induction pathways converge. Therefore, the expression level of activated or cleaved caspase-3 should give a reliable measure of the level of apoptosis [13]. The proliferation activity of a tumor can be estimated by determining the expression levels of specific cell cycle-related proteins also by using IHC. A widely used marker is the Ki67 antigen, which is expressed in nuclei during all cell cycle phases except during the G_0 phase [14]. Previous studies showed contradicting results with respect to the relation of the level of apoptosis or proliferation in tumor resection specimens and patient outcome in colon cancer [15-22]. We hypothesize that, because tissue homeostasis depends on the balance between cell death and proliferation levels, the level of disbalance between these processes indicate tumor aggressiveness. Therefore, combined and not separate analysis of these parameters might be of prognostic relevance in colon cancer patients.

In this study we determined both the level of tumor cell apoptosis and proliferation in resection specimens of a large cohort of colon cancer patients. We combined the results into one parameter and related this parameter to patient outcome data.

MATERIALS AND METHODS

Patients and tumors

The patient cohort consisted of 470 colorectal cancer patients treated with surgery for their primary tumor in the LUMC between 1991 and 2001. Clinico-pathological and follow-up data were collected retrospectively from hospital records and the hospital's

oncology database. This research was performed according to the code of conduct for responsible use.

Patient records information was anonymized and de-identified prior to analysis according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies). Patients with a history of cancer other than basal cell carcinoma or cervical carcinoma *in situ*, patients that received radio- and/or chemotherapy treatment prior to resection, patients with multiple synchronous colon tumors, and patients with rectal cancers were excluded from the analysis (n=185). The entire study cohort consisted of 285 patients. Right-sided tumors were defined as those originating proximal to the splenic flexure and left-sided as those originating distal to the splenic flexure.

Primary Antibodies

The following antibodies were used in the immunohistochemical stainings: Mouse monoclonal antibody anti-Ki67 (DAKO Glostrup Denmark Art.M7240 clone MIB-1) to determine the level of tumor cell proliferation and rabbit polyclonal antibody anti-ASP-175 (Cell signaling Danvers, USA, Art.9661) was used for cleaved caspase-3 identification to determine the level of apoptosis.

Immunohistochemistry

Qualified pathologist evaluated the tumor material from all patients included for histopathological characteristics according to current standards during the routine hospital diagnostic process. Formalin-fixed paraffin-embedded tumor blocks of the primary tumor were collected from the pathology department. Sections were cut for haematoxylin and eosin staining, and representative tumor regions based on histological assessment were used for preparation of tumor tissue microarray (TMA) blocks. From each donor block, three 0.6 mm tissue cores were punched from tumor areas and transferred into a recipient paraffin block using a custom-made precision instrument.

Immunohistochemistry (IHC) staining was performed on 4 µm sections that were cut from each receiver block and mounted on glass. For each primary antibody, all slides were stained simultaneously to avoid inter-assay variation. Tissue sections were deparaffinized and rehydrated. For antigen retrieval, 0.01 M EDTA buffer (pH 8) was used for 10 minutes at maximum power in a microwave oven for anti-Ki67. Citrate buffer 0.1M (pH 6) was used for anti-ASP-175. Endogenous peroxidase was blocked for 20 minutes in 0.3% hydrogen peroxide in methanol. Sections were incubated overnight with either anti-Ki67 or anti-ASP-175 at predetermined optimal dilutions. After 30 minutes of incubation with Envision anti-mouse (K4001; DAKO Cytomation, Glostrup, Denmark) or Envision anti-rabbit (K4003); DAKO Cytomation, Glostrup, Denmark), sections were visualized using diaminobenzidine solution. Tissue sections were counterstained with haematoxylin,

dehydrated and finally mounted in malinol. Sections with phosphate buffer saline (PBS) instead of primary antibody, which underwent the complete staining protocol served as negative controls.

Evaluation of immunohistochemistry

Microscopic analyses of Ki67 and cleaved caspase-3 expression was performed by two independent observers (M.S.R: 100% and T.C.A.: 30%) in a blinded manner. For Ki67, the percentage of tumor cells that showed nuclear staining was assessed. For determination of tumor cell apoptosis, the absolute number of caspase-3 expressing tumor cells in each tumor punch that showed cytoplasmatic and perinuclear staining was counted. The Cohen's Kappa for inter observer variability was 0.73 and 0.6 for Ki67 and cleaved caspase-3 respectively. Therefore, there was substantial agreement between the two observers and all scores were averaged. For analysis a cut-off at the median was chosen, dividing the samples in low (<27% positive tumor cells) or high nuclear Ki67 expression (≥27%). The use of this percentage of positive cells as a cut-off point is supported by Fluge *et al.* [18]. Cleaved caspase-3 was quantified into two categories of IHC cytoplasmatic tumor staining levels. Negative staining; implied no positive tumor cells in either of the three cores, in all other cases the staining was denoted as positive. Representative images of the Ki67 and caspase-3 staining are shown in Figure 1.

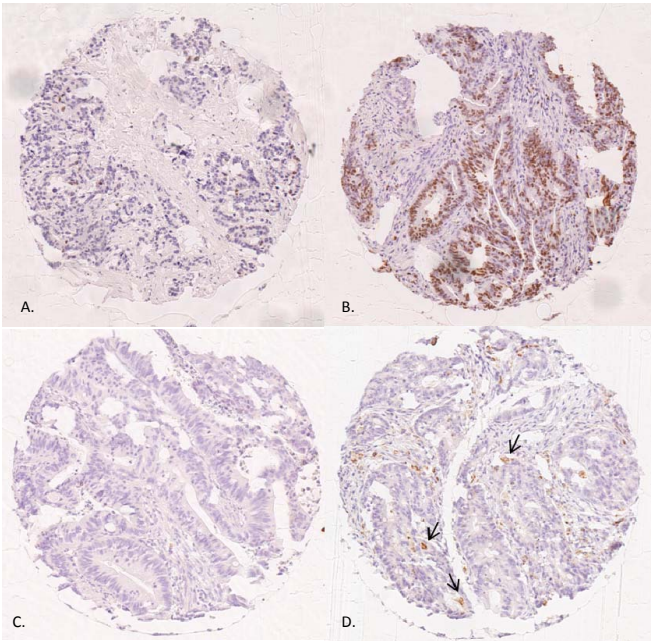


Figure 1: Representative images of Ki67 and cleaved caspase-3 immunohistochemical staining in colon cancer tissues.
A) Ki67 tumor staining with low expression; B) Ki67tumor staining with high expression; C) Tumor showing absence of cleaved Caspase-3 tumor cell expression and; D) Tumor showing presence of cleaved caspase-3 tumor cell expression, as indicated by the arrows. All magnifications x200.

Determination of microsatellite stability status

DNA was extracted from 2mm tumor-cores. Paraffin was dissolved in xylene, tissue was rehydrated in ethanol (100%/70%) and dried for 10 minutes at 37°C. Nucleospin 96 Tissue kit (Machery-Nagel, Düren, Germany) was used for DNA extraction according to the manufacturer's protocol.

Microsatellite stability status was tested using the MSI Analysis System Version 1.2 (Promega, Mannheim, Germany) and interpreted by an experienced pathologist, as described previously [23].

Statistical Analysis

Statistical analyses were performed using the statistical package SPSS (version 17.0 for Windows; SPSS, inc). The Student's T-test and the Chi-squared test were used to evaluate associations between Ki67 or cleaved caspase-3 and various clinico-pathological parameters. The Overall Survival (OS) was defined as time between primary tumor resection and time of death and Disease Free Survival (DFS) as time between primary tumor resection and time of death or relapse of disease, whichever came first. The Kaplan-Meier method was used for calculation of survival probabilities and the Log-rank test for comparison of survival curves. Cox regression was used for univariate and multivariable analysis for OS and DFS. Significant variables (in univariate analysis) were included in multivariable analysis. For all tests, a p-value <0.05 was considered to be statistical significant.

RESULTS

Patient characteristics, and cleaved caspase-3 and Ki67 expression levels

The study cohort consisted of 285 patients. In 41 cases for Ki67, and 38 for cleaved caspase-3 the results of the IHC could not be analyzed due to loss of the tumor material during IHC or due to staining artifacts. Representative images of the biomarkers and their staining categories are shown in Figure 1. The mean percentage of tumor cells expressing Ki67 in the tumor tissue cores was 29.2% with a median of 27.5%. For analysis we used the median as cutoff based on skewness of the data distribution. This resulted in 121 patients (49.6%) with tumors showing low expression level (below median) of Ki67 and ('low' tumor cell proliferation level) and 123 patients (50.4%) with high expression level (above median) of Ki67 ('high' tumor cell proliferation level). In 85 (34.4%) patients the tumor tissue cores showed no staining of cleaved caspase-3 and therefore no apoptotic activity of tumor cells. The remaining 65.6% of the samples showed positive staining and thus ongoing tumor cell apoptosis.

The clinico-pathological characteristics of the patient cohort and their relation to expression levels of the biomarkers are listed in Table I. Interestingly, tumor location

Table I: Patient Characteristics of the Total Colon Cancer Cohort and Stratified for Ki67 and cCaspase-3 expression

	Total Population (N=285)	Ki67 Absence N=121	Ki67 Presence N=123	cCaspase-3 Absence N=85	cCaspase-3 Presence N=162
Gender					
Male	137 (48.1%)	55 (45.5%)	65 (52.8%)	42 (49.4%)	80 (49.4%)
Female	148 (51.9%)	66 (54.5%)	58 (47.2%)	43(50.6%)	82 (50.6%)
Age (average)	65.7 (±13.3 SD)	67.3 (±11.6 SD)	64.2 (±13.6 SD)	66.5 (±12.6 SD)	65.6 (±12.9 SD)
TNM stage					
I	44 (15.4%)	14 (11.6%)	20 (16.3%)	16(18.8%)	18 (11.1%)
II	114 (40.0%)	43 (35.5%)	53 (43.1%)	31 (36.5%)	68 (42.0%)
III	74 (26.0%)	36 (29.8%)	30 (24.4%)	19 (22.4%)	47 (29.0%)
IV	48 (16.8%)	27 (22.3%)	19 (15.4%)	17 (20.0%)	29 (17.9%)
Unknown	5 (1.8%)	1 (0.8%)	1 (0.8%)	2 (2.4%)	0 (0.0%)
Grade			*p=0.004*		*p=0.017*
Moderate	145 (50.9%)	61 (50.4%)	63 (51.2%)	35(41.2%)	89 (54.9%)
Poor	23 (8.1%)	5 (4.1%)	16 (13.0%)	4 (4.7%)	18(11.1%)
Good	58 (20.4%)	32 (26.4%)	16 (13.0%)	22(25.9%)	26 (16.0%)
Unknown	59 (20.7%)	23 (19.0%)	28 (22.8%)	24 (28.2%)	29(17.9%)
MS Status					*p=0.004*
MSS	168 (58.9%)	77 (63.6%)	87 (70.7%)	61 (71.8%)	103 (63.6%)
MSI	30 (10.5%)	12 (9.9%)	14 (11.4%)	2 (2.4%)	27 (16.7%)
Unknown	87 (30.5%)	32 (26.4%)	22 (17.9%)	22 (25.9)	32 (19.8%)
Location			*p=0.023*		*p=0.011*
Right	110 (38.6%)	37 (30.6%)	53 (43.1%)	27 (31.8%)	64(39.5%)
Left	153 (53.7%)	78 (64.5%)	60 (48.8%)	57 (67.1%)	83(51,2%)
Unknown	22 (7.7%)	6 (5.0%)	10 (8.1%)	1 (1.2%)	15 (9.3%)

This table describes the baseline characteristics of the entire cohort of 285 patients in the first column. The Ki67 immunohistochemistry results could be analyzed in 244 cases and Ki67 expression (above the median of 27.5% expression level) was found to be present in 123 and absent in 121 patients. The second and third columns describe the relation of either Ki67 absence or presence in the tumor resection specimens to clinico-pathological parameters. The cleaved caspase 3 results were available for analysis in 247 patients. In this population 85 tumor samples showed no presence of cleaved caspase 3, expression was present in 162 tumor samples of patients. The fourth and fifth column describe the relation of either cleaved caspase 3 absence or presence to clinico-pathological parameters. Only significant ($p<0.05$) differences between marker expression as proven by χ^2 tests are displayed. Abbreviations: MS Status; Microsatellite Status, cCaspase3; cleaved caspase 3.

was significantly related to both cleaved caspase-3 expression level and Ki67 expression level. Microsatellite instability also showed statistical significance, but was only significantly related to cleaved caspase-3 expression and not to Ki67 expression. In the tumor samples without cleaved caspase-3 expression, 2.4% of the cases showed microsatellite instability *vs.* 16.7% in the tumors with expression of cleaved caspase-3 (*p*-value 0.004). Additional analysis showed in our patient cohort a strong, significant correlation between tumor location and microsatellite stability status with significantly more microsatellite instable tumors (MSI) located on the right side of the colon and the majority of the microsatellite stable (MSS) tumors located on the left side of the colon (70%), whereas this was only 8% in microsatellite instable tumors (MSI) (*p*=<0.001).

Relation of single marker expression with patient outcome

The level of tumor cell proliferation based on Ki67 expression level was significantly related to OS and DFS: high tumor expression level correlated significantly to a better patient OS and DFS (OS, Logrank *p*-value 0.002; DFS, Logrank *p*-value 0.003) (Figure 2). Tumor cell apoptotic level, as represented by cleaved caspase-3 expression, was not related to either OS or DFS (OS, Logrank *p*-value 0.83; DFS, Logrank *p*-value 0.73).

Combined analysis of tumor cell apoptosis and proliferation in relation to patient outcome

To analyze the effect of the balance between apoptosis and proliferation levels in the tumor resection specimens on patient outcome, the results of the Ki67 expression analysis were combined with those of the cleaved caspase-3 expression analysis in a combined apoptosis-proliferation (CAP) parameter (Table II). The CAP parameter was not significantly related to TNM stage (*p*-value 0.211), but was significantly related to the tumor microsatellite status (*p*-value 0.008). Tumors of both the CAP -+ (Ki67 below median, presence of cleaved caspase-3) and ++ (Ki67 above median, presence of cleaved caspase-3) patients showed significantly more often microsatellite stability compared to the CAP +- (Ki67 above median, absence of cleaved caspase-3) and CAP — (Ki67 below median, absence of cleaved caspase-3) patients (*p*-value 0.03). Patients with a CAP ++ tumor showed the best survival outcomes with respect to OS and DFS (Figure 3). In the entire cohort patients with a CAP -+ tumor had the worst outcome perspectives. Because tumor microsatellite status was significantly related to the presence of cleaved caspase-3, the next step would be to perform the survival analysis with the CAP parameters stratified for tumor microsatellite status. Unfortunately the number of MSI tumors that was successfully determined was too small to perform this analysis specifically for MSI within this population. We therefore used tumor location, which we previously showed to be highly correlated to tumor microsatellite status, as a surrogate marker in this analysis (Figure 4). These Kaplan Meier curves showed in left-sided tumors

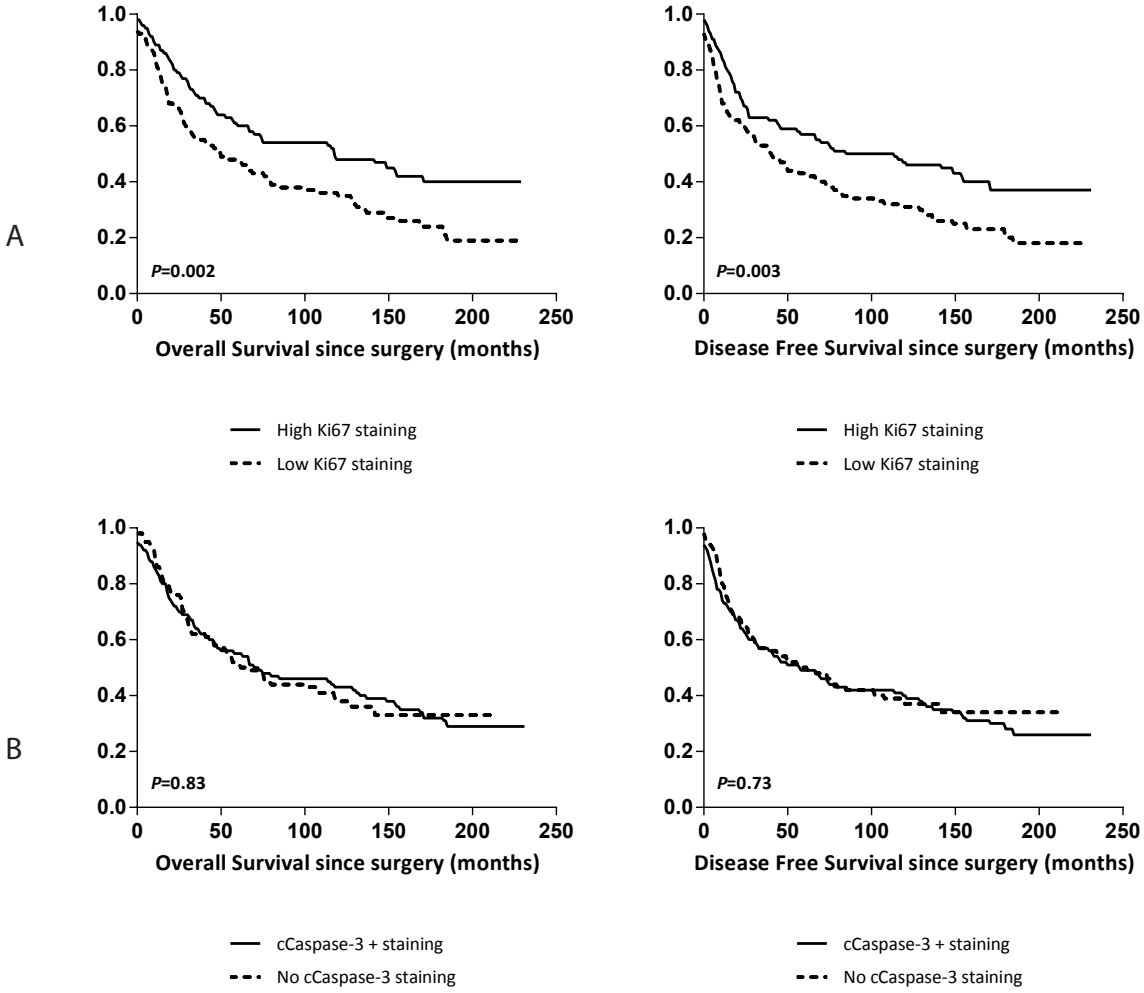


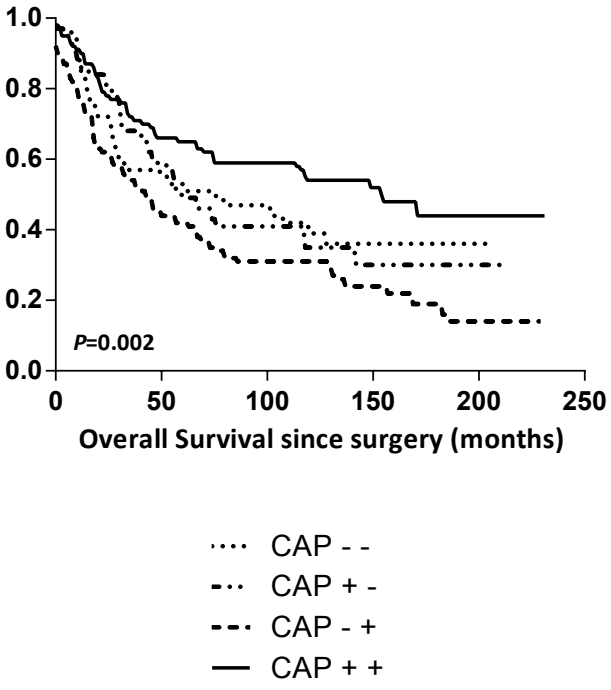
Figure 2: Survival curves stratified for Ki67 and cleaved caspase-3 tumor expression in colon cancer. A) Kaplan Meier curves for OS and DFS in the study population of 285 colon cancer patients stratified for Ki67 tumor expression. B) Kaplan Meier curve OS and DFS in the study population of 285 colon cancer patients stratified for cleaved caspase-3 expression in their tumor sections. Abbreviations: cCaspase3; cleaved caspase-3.

Table II: Description of the CAP (Combined Apoptosis and Proliferation) parameter.

CAP ++	Ki67 expression above the median and presence of cleaved caspase-3 IHC
CAP +-	Ki67 expression above the median and no presence of cleaved caspase-3 IHC
CAP -+	Ki67 expression below the median and presence of cleaved caspase-3 IHC
CAP —	Ki67 expression below the median and no presence of cleaved caspase-3 IHC

This table provides the definitions of the CAP parameter. This parameter resulted from data combination on the tumor cell apoptotic level based on the cleaved caspase-3 immunohistochemistry results with the data on the tumor cell proliferation level based on the Ki67 expression levels as determined with IHC. Abbreviations: IHC; immunohistochemistry.

A



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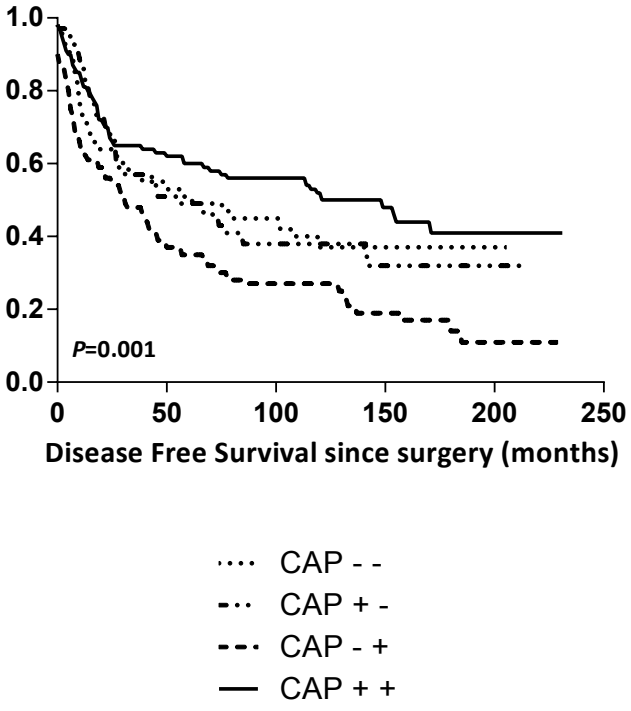

Figure 3: Survival curves stratified for combined tumor apoptosis-proliferation (CAP) expression in colon cancer.

Kaplan Meier curves for OS and DFS in the study population of 285 colon cancer patients stratified for combined tumor apoptosis-proliferation (CAP) expression. This parameter is described in detail in Table II and in the *Results* section.

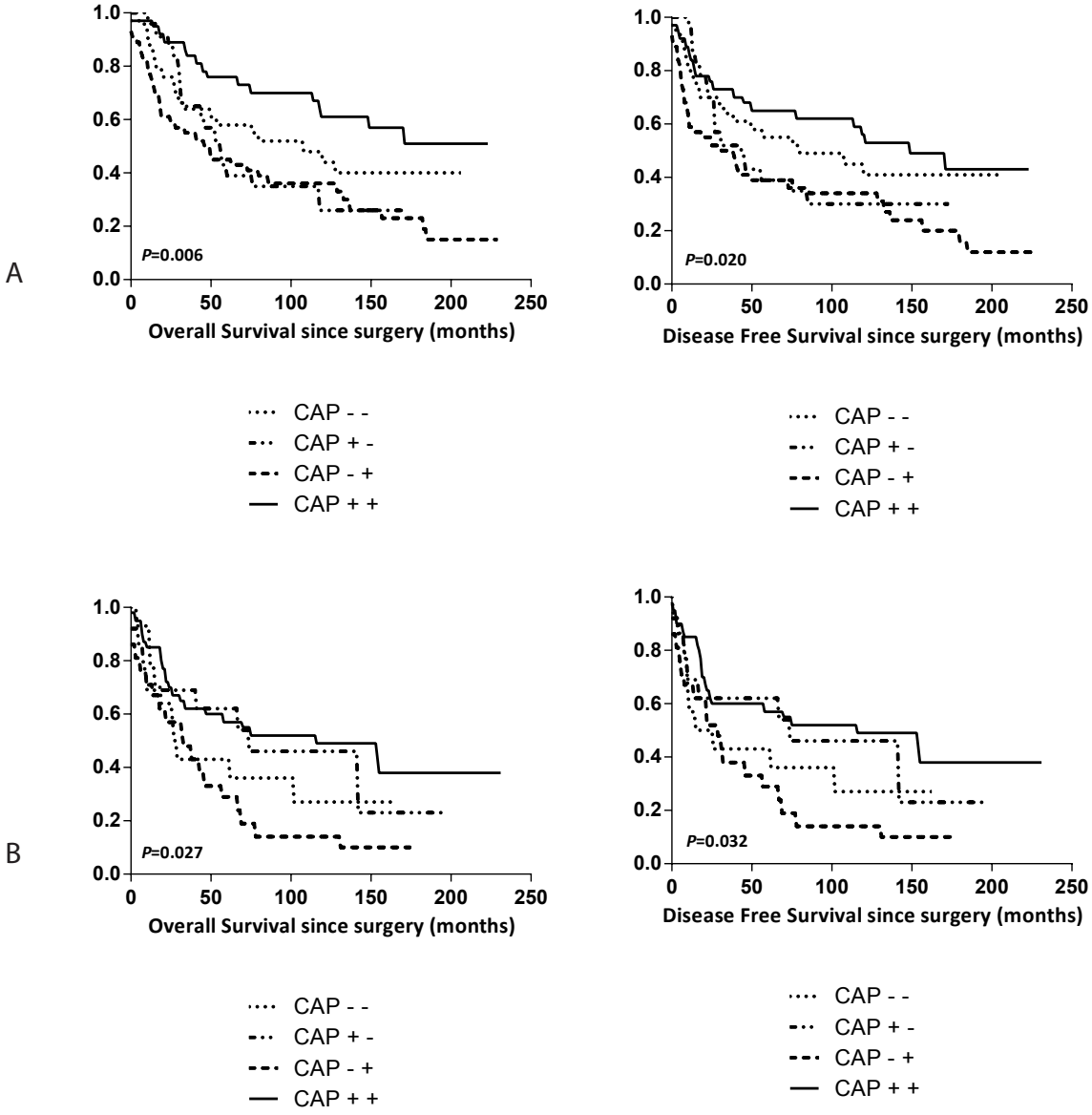

Figure 4: Survival curves stratified for combined tumor apoptosis-proliferation expression and for location of the colon tumor.

A) Kaplan Meier curves for OS and DFS stratified for the CAP parameter in patients with left sided colon tumors (originating distal to the splenic flexure). B) Kaplan Meier curves for OS and DFS in patients with right-sided tumors (originating proximal to the splenic flexure). The CAP parameter is described in detail in Table II and in the *Results* section.

comparable curves to those presented in Figure 3 of the total cohort, but the course of the curves changed in right-sided tumors. The CAP ++ and the CAP - - population within the cohort of left-sided tumors had the best outcome perspectives as opposed to the CAP -+ and +- population that had similar but worse outcome perspectives. The CAP + - actually had, within this left-sided cohort, the worst outcome perspectives (DFS *p*-value 0.02). In right-sided tumors, the CAP ++ and CAP +- population had the best outcome perspectives as opposed to the CAP — and CAP -+ population that had worse

outcome perspectives. We conclude based on these results that combined analysis of apoptosis and proliferation as described with the CAP parameter is related to survival in stage I-IV colon cancer patients. The impact of this parameter on patient outcome, however, varies with tumor location and therefore highly likely with tumor microsatellite status.

Univariate and multivariable analysis

Both for OS and DFS a multivariable analysis was performed including the variables; sex, age at time of operation, TNM stage, tumor grade, administration of adjuvant therapy, microsatellite status, tumor location and the CAP parameter. Age and TNM stage were found to be independent predictors of OS and DFS (Table III & IV). To test whether the effect of the CAP parameter on outcome differed between patients with left- and right-sided tumors, an interaction term was implemented that was borderline significant (*p*-value 0.06). Although the CAP parameter was not significantly related to TNM stage, stratified analyses for TNM stage showed that the effect of CAP on outcome only remained significant in the stage II patient population. Therefore analysis was again performed with an interaction term, and again this term was borderline significant (*p*-value 0.05).

Table III: Univariate and multivariable analyses of Overall Survival (OS)

	Univariate analysis			Multivariable analysis		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
TNM			<0.001			<0.001
1	1			1		
2	1.57	0.9-2.6		2.01	1.1-3.7	
3	2.17	1.3-3.6		2.61	1.4-4.8	
4	6.27	3.6-10.7		7.67	4.1-14.3	
Age	1.04	1.026-1.053	<0.0001	1.054	1.037-1.071	<0.001
CAP			0.006			0.28
—	1.00			1		
-+	1.42	0.9-2.4		1.10	0.6-1.9	
+-	0.71	0.5-1.0		1.27	0.8-2.0	
++	0.52	0.3-0.9		0.85	0.5-1.4	

This table provides the data for the univariate en multivariable analysis of OS. The univariate analysis included sex, age, tumor grade, adjuvant therapy administration, microsatellite status, TNM stage, the CAP parameter, microsatellite status and tumor location. The CAP parameter, age and TNM stage were all significant predictors of OS in univariate analysis. In multivariable analysis only TNM stage and the patient age at time of surgery retained significance. The CAP parameter is therefore not an independent predictor of overall survival in stage I-IV in this cohort of colon cancer patients.

Table IV: Univariate and multivariable analyses of Disease Free Survival (DFS)

	Univariate analysis			Multivariable analysis		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
TNM			<0.001			<0.001
1	1			1		
2	1.56	0.9-2.5		1.94	1.1-3.5	
3	2.25	1.4-3.7		2.52	1.4-4.6	
4	6.14	3.6-10.4		7.17	3.9-13.1	
Age	1.032	1.019-1.045	<0.001	1.043	1.028-1.059	<0.001
CAP			0.02			0.235
—	1			1		
+-	1.04	0.6-1.8		1.08	0.6-1.8	
-+	1.60	1.0-2.5		1.37	0.9-2.1	
++	0.75	0.5-1.2		0,91	0.6-1.5	

This table provides the data for the univariate en multivariable analysis of DFS The univariate analysis included sex, age, tumor grade, adjuvant therapy administration, microsatellite status, TNM stage, the CAP parameter, microsatellite status and tumor location. The CAP parameter, age and TNM stage were all significant predictors of DFS in univariate analysis. In multivariable analysis only TNM stage and the patient age at time of surgery retained significance. The CAP parameter is therefore not an independent predictor of disease free survival in this cohort of stage I-IV colon cancer patients.

Based on these results we conclude that although the CAP parameter is not a statistically independent prognostic indicator of survival in the total patient cohort, the CAP parameter, which is influenced by location, microsatellite stability status and TNM stage, does behold prognostic significance in certain subsets of patients populations such as in stage II, MSS patients.

DISCUSSION

Our study shows that a combined parameter, CAP, describing the level of tumor cell proliferation and apoptosis is significantly related to patient outcome in a stage I-IV colon cancer patient cohort with respect to DFS and OS. Although counterintuitively, patients with CAP ++ tumor, showing high levels of both proliferation and apoptosis, showed the best clinical outcome perspectives. The effect of the CAP parameter, however, varied with TNM stage and tumor location and was significantly related to tumor location and tumor microsatellite status. These results confirm our hypothesis that clinical outcome is dependent on both tumor cell proliferation and apoptosis.

The processes of both tumor cell proliferation and apoptosis both have been extensively studied with varying results in many types of cancer. In general, high tumor cell

proliferation levels were associated with aggressive tumor development and progression [20;24]. However, other studies reported on an inverse association between tumor cell proliferation level and clinical outcome [8;17;22;25]. These latter results are in line with what we have found: a better outcome perspective in colon cancer patients with high levels of tumor cell proliferation. In this study we were not able to establish a relationship between the level of apoptosis as a single marker and patient outcome in colon cancer patients. Although there are studies that describe a link between tumor cell apoptosis and clinical outcome, for example Jonges *et al.* who described cleaved caspase-3 expression as a prognostic marker in colon cancer patients [19], the majority of the studies have presented us with more ambiguous results [1;15;16;26;27].

The contradicting results derived from studies reporting on either proliferation or apoptosis in colon cancer strengthened our hypothesis that a balance between both these processes determines patient's clinical outcome. Michael-Robinson *et al.* previously reported on a cohort of 100 colorectal cancer patients in which they determined an Apoptotic Index: Proliferation Index (AI:PI) ratio [25]. This AI:PI ratio was based on M30 IHC for the apoptosis level and Ki67 IHC for the proliferation level. They were able to determine a relationship between the proliferation index and outcome comparable to our results: they also related their AI:PI index significantly to patient outcome. In previous studies the use of the apoptotic index has been criticized as researches found the use of the parameter to be accompanied with high amounts of interobserver variability [28]. Therefore we didn't use a continuous variable based on counted percentages, but developed a more descriptive parameter, the CAP, to determine the combined effect of apoptosis and proliferation within our patients tumor samples. The differences in outcome parameters and also patient selection make it difficult to perform a one-to-one correlation of the results of Michael-Robinson *et al.* and our results. Their conclusions though do affirm our hypothesis. Interestingly, the survival difference they found between a high and low AI:PI index was similar in both MSS and MSI patients. Our results showed that the effect of the CAP parameter differed between tumors emerging from colon proximal and colon distal to the splenic flexure. In the left-sided cohort the patients with CAP — and ++ tumors performed better with respect to outcome. In the right-sided cohort the CAP +- performed significantly better than the CAP — cohort. This is comparable to what we have previously found and described by Jonges *et al.* [19]. The effect of apoptosis on patient outcome is related to tumor location. Based on our results we hypothesize that it is either tumor microsatellite status as suggested by both Jonges and Michael-Robison, or tumor location which might influence the balance between tumor cell proliferation and apoptosis and therefore patient outcome [19;25]. The concept of the effect of tumor location is in accordance with what has recently been described by The Cancer Genome Atlas Network in their publication in Nature in 2012, who tested the hypothesis that differences between tumors originating from the left

or the right side of the colon is not based on their microsatellite status but it might be caused by the different embryonic origins of the right- and left-sided colon [29].

It is not unlikely that the tumor microsatellite status influences the balance between tumor cell proliferation and apoptosis. Microsatellite instable tumors are known to have high levels of proliferation and tend to accumulate gene mutations leading to increased production of abnormal peptides [30;31]. This phenotype has been hypothesized to cause an immune reaction resulting in higher levels of apoptosis, eventually resulting in better patient outcome [32].

In conclusion, the CAP variable described in this study reflects the balance between the apoptosis and proliferation in colon cancer tissue and showed to be related to patient outcome. These results confirm our hypothesis that apoptosis and proliferation together determine patient outcome in colon cancer and this relation is influenced by tumor location and/ or by tumor microsatellite instability. This was shown by the different effects of the CAP parameter on patient outcome in the left and right-sided colon cancer patients cohorts and the statically significant relation of the level of apoptosis with tumor microsatellite status, also described in previous studies [19;25] . Important steps have been taken towards the implementation of a CAP like parameter into clinical practice, such as the development of the CDK1 SA (Cyclin Dependent Kinase 1 Specific Activity Assay), a biochemical assay that can replace the Ki67 IHC, and the improvement of the existing biochemical assays to measure cleaved caspase-3 activity for easy clinical use [23]. Further studies should focus on the design of clinical tests combining both proliferation-based markers and apoptosis-based markers into one analysis to assure clinical applicability.

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