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CHAPTER 5

Combined markers of tumor cell proliferation and apoptosis are clinically prognostic in stage II colon cancer patients

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ABSTRACT

Background: TNM staging methods fall short identifying stage II colon cancer patients at high risk of distant metastasis who might benefit from adjuvant treatment. The addition of combined biomarkers representing important hallmarks of cancer, disruption of apoptosis and proliferation, to current staging criteria may provide a window for improvement.

Methods: The results of biochemical assays of enzymatic activity of caspase-3 and cyclin dependent kinase 1, representing the level of apoptosis and proliferation in tumor resection specimens of 217 stage II colon cancer patients, were combined into one parameter and correlated to tumor microsatellite status and patient outcome.

Results: Low levels of the proliferation marker tumor cell CKD1 activity and high levels of the apoptosis marker caspase-3 activity were independent predictors of better disease free survival (CDK1, hazard ratio 9.6, p=0.029; caspase-3 activity, HR 0.5, p=0.03). Within the tumor microsatellite stable (MSS) patient cohort, based on the combined results of these assays, 87.5% of the stage II patients that developed distant metastasis were correctly classified as high-risk.

Conclusion: Combined analysis of the level of proliferation and apoptosis was proven to be a reliable parameter to identify high-risk patients within stage II, MSS colon cancer patients. These results warrant clinical application after validation.

INTRODUCTION

Patient outcome in colon cancer highly depends on disease stage at time of diagnosis. This stage is determined using the TNM (Tumor Node Metastasis) criteria (1). Traditionally, the TNM stage is used to determine whether a patient is recommended to receive (neo-) adjuvant treatment. Nowadays, a disparity has risen between therapeutic decision-making and TNM staging. The statistics on colon cancer point out that within the stage II cohort a patient population can be identified with a high risk of distant metastasis, a risk comparable to patients diagnosed with stage III disease (2–4). Therefore, adjuvant chemotherapy is recommended for high risk patients with stage II colon cancer (5). Unfortunately, this 'high-risk' population cannot be identified using the TNM staging criteria alone. The clinical decision-making process therefore needs to be 'updated' with the addition of biomarkers to the traditional TNM criteria in order to make the identification of this high risk group possible.

Tumor development and progression partly depends on the disruption of the balance between cell proliferation and apoptosis, which under normal circumstances is responsible for tissue homeostasis (6,7). It has recently been shown that clinical outcome of colon cancer patients is related to the balance between cell proliferation and apoptosis at tumor level (manuscript submitted for publication) (8). Combined analysis of these markers might therefore be useful to detect the high-risk stage II patient population. In the current study we determined both the level of tumor proliferation as well as the level of apoptosis using a biochemical assay. The advantages of the use of biochemical assays are their highly accurate performance on only a minimal amount of fresh frozen tissue and furthermore their results are less influenced by inter-observer variety than for example immunohistochemistry (IHC) (9). More importantly, biochemical assays provide information on the functional activity of the enzyme *versus* solely expression levels of the protein, determined using IHC. To determine the level of tumor cell proliferation we used the Cyclin Dependent Kinase 1 Specific Activity (CDK1 SA) assay and to determine the level of apoptosis we used a caspase-3 activity assay. Both have been previously successfully tested for their applicability on colon cancer tissue. CDK1 SA for example has been shown to significantly predict distant metastasis in colon cancer patients and was significantly elevated in microsatellite-stable tumors. (10–12). The aim of this study was to combine the results of the biochemical analyses of apoptosis and proliferation in order to determine both the prognostic quality of this combined analysis as well as the discriminative power of these assays in the identification of high-risk stage II colon cancer patients.

PATIENTS AND METHODS

Patients

The study population consisted of 217 stage II colon cancer patients. All patients underwent surgical resection of their tumors between 1987 and 2006 at the Klinikum rechts der Isar, Technische Universität in München, Germany. None of the patients underwent any (neo-) adjuvant treatment. Of all patients, fresh frozen samples of their colon tumor specimens were collected. The tumor tissue was immediately dissected after surgery by an experienced pathologist and stored at -80^{0}C. The median follow -up time was 7.9 years. The specimen collected were with informed consent, according to the vote of the Ethics committee of the Klinikum rechts der Isar (1927/2007).

Cyclin Dependent Kinase 1 specific activity assay

To determine the Cyclin Dependent Kinase 1 specific activity (CDK1 SA) an assay called C2P ('cell-cycle profiling'), which has been described previously, was used (12). In brief, from all patients 10-20 sections of 100 µm thickness were cut, analyzed for their tumor cell content and subjected to CDK1 SA analysis. CDK1 SA was calculated as cyclin dependent kinase (CDK) activity units (aU µl^{-1} lysate) divided by its corresponding CDK expression units (eU µl^{-1} lysate). Further details with respect to the CDK1 SA determinations in this specific cohort have been described previously (11).

Preparation of lysates and protein quantification

To determine the enzymatic activity of caspase-3 of each tumor sample 10 slides of 10 µm were cut and put in a 2.0 ml tube with 500 µl of lysis buffer (10 mM HEPES pH 7, 40 mM β-glycerophospate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA). Subsequently the samples were homogenized with a Polytron homogenisator (PT MR 2100, Kinematica AG, Lucerne, Switzerland) for 10 seconds and subjected to four freeze-thaw cycles that included storage in liquid nitrogen until samples were frozen followed by storage at 37_0C until the samples were fully thawed. Until further processing, samples were stored at -80_0C. Quantification of the total amount of protein in the lysates was performed using a Bradford assay (13). 10 µl of each sample was pipetted in a well of a 96 wells plate. Next, 300 µl of Coomassie Plus Reagent (Coomassie Blue Assay kit, Thermo Scientific, USA) was added to each well. The reagents were mixed in a plate shaker for 30 seconds and incubated for 10 minutes at room temperature. The absorbance levels were measured at 595 nm using a Benchmark Microplate Reader (Biorad, UK). Diluted bovine serum albumin (BSA, standard provided with Coomassie Blue Assay Kit, Thermo Scientific, USA) was used to create a calibration curve at protein concentrations ranging from 2000 µg/ml to 25 µg/ml. A control sample containing no BSA was used to serve as a blank measurement. The absorbance level of this sample was subtracted from all measured absorbance levels to correct for background absorbance. All measurements were performed in duplicate.

Caspase-3 enzymatic activity determination

To determine the activity of the caspase-3 enzyme, a caspase-3-specific fluorometric assay was performed using Ac-DEVD-AFC (Sigma-Aldrich, USA) as substrate. For this assay an AFC calibration curve was prepared by diluting 1 mM of AFC (Sigma-Aldrich, USA) in reaction buffer (100 mM HEPES buffer pH 7.25, containing 10% (w/v) sucrose, 0.1% (v/v) Nonidet-P40 and 10 mM dithiothreitol (DTT, Sigma –Aldrich, USA) to reach a final AFC concentration ranging from 5000 nM to 2,5 nM. The emission at 505 nm levels of the calibration curve and tissue samples were analyzed in a 96-well microplate using a fluorometer (Victor^{3}V 1420 Multilabel Counter, Perkin Elmer, USA) at excitation wavelength of 400 nm. Plotting the AFC emission levels against their concentrations in nM created the calibration curve. As a positive control of caspase-3 activation, a colorectal cancer cell line (SW480) was treated with 100 µM Cisplatin. Lysates from the treated cells were prepared as described above. Each well to compose the calibration curve contained 100 µl AFC standard sample, or for the detection of capase-3 activity 50 µl tumor sample lysate and 50 µl of the reaction buffer. 5 µl (1 mM) of the Ac-DEVD-AFC (Sigma-Aldrich, USA) substrate solution was added to each well and the plate was incubated for 2 hours at 37 $_0$C after which emission levels were measured. The emission level of a control sample containing 205 µl of reaction buffer only was used for background correction. All measurements were performed in duplicate.

Caspase-3 enzymatic activity calculations

The AFC and the Bradford calibration curves both showed a correlation coefficient of 0.99. For each sample the protein concentration and the activity level of caspase-3 were determined by plotting the absorbance and fluorescence measurements in the calibration curves, making it possible to read out the corresponding protein concentration and caspase-3 activity. The enzymatic activity was then calculated as a ratio of the total amount of protein in each sample by dividing the determined AFC values by the protein concentration of the samples. This yielded an activity value expressed in pmol AFC/min/mg protein. The Bradford assay as well as the AFC value measurements were performed in duplicate. Samples were excluded from analysis when the difference between the two measurements was more than two times the standard deviation of the entire group of measurements. Additionally, samples that contained too little material for both measurements were excluded from analysis.

Microsatellite status determination

Five slides of 10 µm of the fresh frozen tumor samples were cut for DNA isolation and subsequent determination of the microsatellite status. Of 191 patients tissue was available for this assay. DNA isolation was performed with the QIAampDNA™Mini Kit (QIAGEN Benelux, Venlo, The Netherlands) according to the manufacture's protocol. The DNA concentration and quality was checked with the ND-1000 NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The microsatellite status was determined with Promega MSI Analysis System, Version 1.2, which is

a fluorescence and multiplex PCR-based assay. In this assay 5 mononucleotide repeat markers, BAT-25, BAT-26, NR-21, NR-24, and MONO-27, were co-amplified to determine the microsatellite status. The results of this assay have been previously compared with the Bethesda panel markers and proven highly sensitive for microsatellite status determination (14). The results were checked and approved by an experienced pathologist.

Statistical analysis

The statistical analyses were conducted using the SPSS statistical analysis package version 17 UK. The One-Way Anova test and the Student's t-test were used to evaluate associations between the clinico-pathological variables, CDK1 SA and the caspase-3 activity levels. Survival analysis was performed using the standard Kaplan-Meier method to calculate survival probabilities and the log-rank test was applied to compare the survival curves. Disease Free Survival was defined as the time from date of surgery until disease recurrence (distant recurrence) or tumor-related death. Cox proportional hazards analysis was used for uni- and multivariate analysis. Test sensitivity was defined as the percentage of patients that developed a distant recurrence (DR) during follow-up correctly defined as high-risk. Test specificity was defined as the percentage of patients that did not develop a DR during follow-up correctly defined as low-risk. All statistical tests were two-tailed with a 0.05 significance level.

RESULTS

Patient characteristics, caspase-3 activity levels and CDK1 SA analysis

Tumor caspase-3 activity level could be successfully determined in 199 out of 217 patients. Eighteen patients (8.3%) were excluded from analysis because the measurements of the Bradford assay or the AFC value did not meet the quality control standards mentioned in the material and methods section. The characteristics of the successfully measured patients are summarized in table 1 and did not statistically significantly differ from the entire 217 patients included in this study. The mean caspase-3 activity level was 46.4 pmol AFC/min/mg protein with a range between 0 and 404.5 pmol AFC/min/mg protein. In concordance with previously published data, the dispersion of our results was skewed (15) Therefore, the median level of activity (24.4 pmol AFC/min/mg protein) was used as the cut-off value for group division into above-median caspase-3 activity (high) and below-median caspase-3 activity (low). The clinicopathological characteristics listed in table 1 were also analyzed for their association with the level of caspase-3 activity using this cut-off value. None of these showed a significant relation with the level of caspase-3 activity except for tumor microsatellite status. The tumor microsatellite stability status could be determined in 181 out of the 199 patients (91%), in the other cases to little tumor material was available for analysis. The percentage of patients with microsatellite stable (MSS) tumors was statistically significantly higher in the

Table 1

baseline characteristics of the stage II colon cancer patient population related to the caspase-3 activity assay

Patient characteristics	Number of patients (%) N=199	Caspase-3 activity mean	p-value
Gender			0.525
Male	133 (57)	48.7	
Female	86 (43)	43.5	
Age (median: 64 years)			0.279
Below median	100(50)	42.0	
Above median	99(50)	50.9	
Location primary			0.163
Colon Ascendens	53 (27)	55.7	
Colon Transversum	21 (10)	55	
Colon Descendens	24 (12)	32.7	
Coecum	30 (15)	58.4	
Sigmoid	71 (36)	36.6	
Tumor diameter (median: 5 cm)			0.122
Below median	101 (51)	40.2	
Above median	98 (49)	53	
T-stage			0.95
T3	173 (87)	46.5	
T4	26 (13)	45.8	
Differentiation grade			0.138
Well	1 (0,5)	9.7	
Moderate	134 (67)	41.3	
Poor	59 (30)	61.8	
Undifferentiated	2 (1)	30.4	
Missing	3 (1,5)		
Mucinous differentiation			0.573
Yes	21 (11)	44.3	
No	130 (65)	52.4	
Missing	48 (24)		
Tumor microsatellite status			**0.000**
MSS	131 (66)	33	
MSI	50 (25)	80	
Missing	18 (9)		
CDK1 SA (cut off: 11)			0.373
Below cut off	88 (44)	50	
Above cut off	111 (55)	43	

Abbreviations: Cm, centimeters; MSS, Micro Satellite Stable; MSI, Micro Satellite Instable; CDK1 SA, Cyclin-Dependent Kinase 1 Specific Activity.

This table shows the association of clinico-pathological characteristics with caspase-3 activity of the 199 primary stage II colon cancer patients. Caspase-3 activity was measured in pmol AFC/min/mg protein, CDK1 SA was measured in milli-activity unit per expression unit, maU eU^{-1}. The clinico-pathological characteristic did not differ significantly from the entire patient population of 217 patients.

low caspase-3 patient population compared to the high caspase-3 group (91.2% vs. 53.3%, P-value <0.001).
CDK1 SA measurements were available for all but 1 patient, in this case to little tumor material was available for analysis. The cut off level of high and low CDK1 SA was set at 11 (milli-activity unit per expression unit, maU eU^{-1}), based on a previously published study in which these activity level has been determined to be the optimal cut off level using bootstrap analysis (11). CDK1 SA was not significantly related to caspase-3 activity levels. In concordance with the previous published results CDK1 SA was not significantly related in direct comparison to the tumor microsatellite status (11).

The prognostic value of caspase-3 activity and CDK1 SA for disease free survival

The level of caspase-3 activity in tumor showed a significant relation to the patients' disease free survival (DFS). Patients with high levels of activity had a mean DFS time of 14.2 years compared to 13.3 years in the low activity level group (p-value: 0.012). The same was observed for the CDK1 SA. Patients with CDK1 SA below the cut off value had a mean DFS time of 14.8 years compared to a mean DFS time of 13 years in the group with CDK1 SA above the cut off value (p-value: 0.001, figure 1). When the survival analysis was stratified based on tumor microsatellite status this relation was only present in the MSS patient population. In this cohort, again high caspase-3 activity levels (p-value: 0.029) and low CDK1 SA levels (p-value: 0.005) were significantly related to a better DFS; this was not the case in the MSI population.
Table 2 shows the results of the Cox proportional univariate analysis for DFS. Both the caspase-3 activity level and the CDK1 SA were statistically significant prognostic indicators of DFS in univariate analyses. To determine the independent prognostic value of both caspase-3 activity level and CDK1 SA, multivariate analysis was performed taking into account known prognostic factors in colon cancer such as tumor microsatellite stability status. Both caspase-3 activity and CDK1 SA remained statistically significant, prognostic parameters in multivariate analysis, with a HR of 0.513 with a p-value of 0.03 and a HR of 9.6 with a p-value of 0.029, respectively. Additional multivariate analysis was performed to investigate the additional effect of microsatellite status through the implementation of an interaction term. This analysis showed no statistical significance. Therefore, the effects of CDK1 SA and caspase-3 activity on patient outcome are not influenced by the tumor microsatellite status.

The combined analysis of tumor proliferation and apoptosis level to identify high-risk stage II colon cancer patients

The goal of this study was to prove that the combined analysis of the level of tumor apoptosis and proliferation improves our ability to identify high-risk stage II colon cancer patients. To study this hypothesis we combined the results of both of our assays into one descriptive variable creating four groups. A double-positive

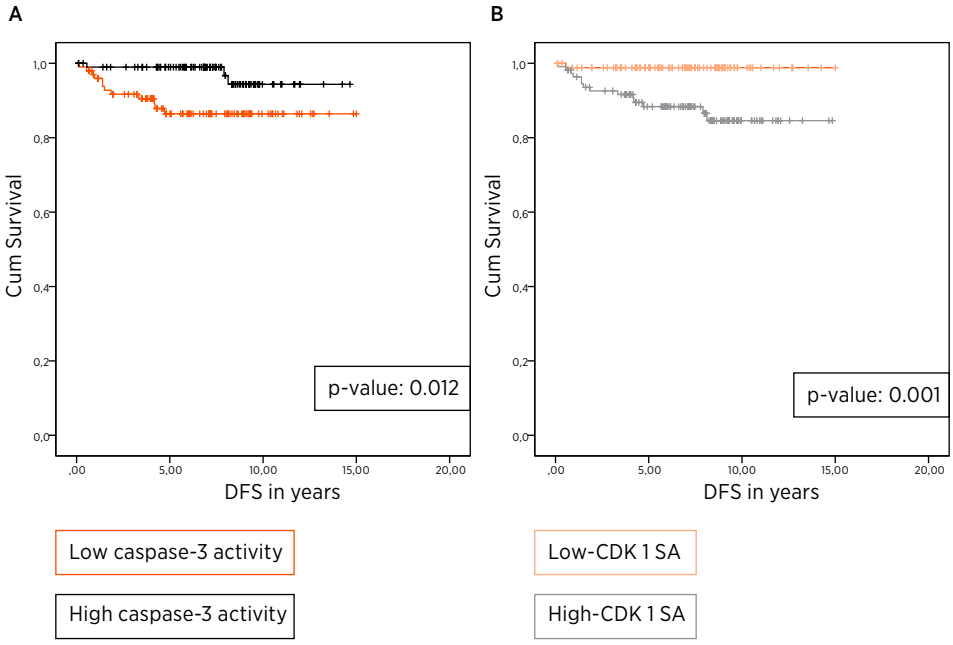

Figure 1

Abbreviations: DFS, disease-free survival; CDK1 SA, cyclin-dependent kinase 1 specific activity. Kaplan Meier curves for DFS in 199 stage II colon cancer patients according to level of (A) caspase-3 activity: high level of tumor caspase-3 activity is significantly related to a better DFS cancer patients; and (B) CDK1 SA: low level of tumor CDK1 SA is significantly related to a better DFS.

group, consisting of patients with tumors showing high level of apoptosis as well as proliferation; a double-negative patient group, showing low level of both apoptosis and proliferation; and two groups of patients of which one the tumor samples showed high level of proliferation and low level of apoptosis and one *vice versa*. We performed the same statistical analysis with this combination variable as previously described for both the assays separately. These results pointed out that patients who showed low levels of apoptosis but high levels of proliferation in their tumor samples had the worst outcome perspectives regarding DFS ($p < 0.001$) compared to all the other groups (figure 2). We performed the same analysis stratified for tumor microsatellite status (figure 2). From this analysis we concluded that our combination variable can be used to significantly determine outcome in the tumor MSS stage II patient cohort. We classified the low-caspase-3 activity and high-CDK1 SA population consisting of 53 (40%) patients of the entire MSS population as high-risk population, the remaining 78 (60%) as the low-risk population.

In 2004 the American Society of Clinical Oncology (ASCO) listed a number of criteria that clinicians can use to identify the high-risk stage II colon cancer patients who might benefit from adjuvant chemotherapy (5). Although these criteria are only considered to be recommendations, some countries, like the Netherlands and Germany have adopted them in their national guidelines of the treatment of colon cancer patients. Our clinico-pathological dataset contained information on two of

Table 2

The Cox univariate analysis for disease-free survival.

Variables	Number of patients (%) N=199	Hazard Ratio	p-value
Gender			0.418
Male	133 (57)	1	
Female	86 (43)	0.641	
Age (median:64)			0.636
Below median	100(50)	1	
Above median	99(50)	0.779	
Location primary			0.862
Colon Ascendens	53 (27)	1	
Colon Transversum	21 (10)	0.644	
Colon Descendens	24 (12)	0.581	
Coecum	30 (15)	0.648	
Sigmoid	71 (36)	0.729	
Tumor diameter (median: 5cm)			0.849
Below median	101 (51)	1	
Above median	98 (49)	0.906	
T-stage			0.995
T3	173 (87)	1	
T4	26 (13)	1.005	
Differentiation grade			0.862
Well/Moderate	135 (68)	1	
Poor/Undifferentiated	61(30,5)	2.315	
Missing	3 (1,5)		
Mucinous differentiation			0.194
No	130(65)		
Yes	21(11)	1	
Missing	48 (24)	6.282	
Microsatellite status			0.166
MSS	131 (66)	1	
MSI	50 (25)	0.235	
Missing	18 (9)		
CDK1SA (cut off; 11)			**0.021**
Below cut off	88 (44)	1	
Above cut off	111 (56)	10.906	
Caspase-3 Activity (median 24,4)			
Below median	100(50)	1	
Above Median	99(50)	0.477	**0.022**

Abbreviation: N, total number of patients included in the analysis; HR, Hazard Ratio; P, p-value; MSS, Micro Satellite Stable; MSI, Micro Satellite Instable; CDK1SA, Cyclin Dependent Kinase 1 Specific Activity.

Cox univariate analysis for disease free survival. The significant p-values are marked bold. Caspase-3 activity was measured in pmol/AFC/min/mg protein, CDK1 SA was measured in milli-activity unit per expression unit, maU eU^{-1}.

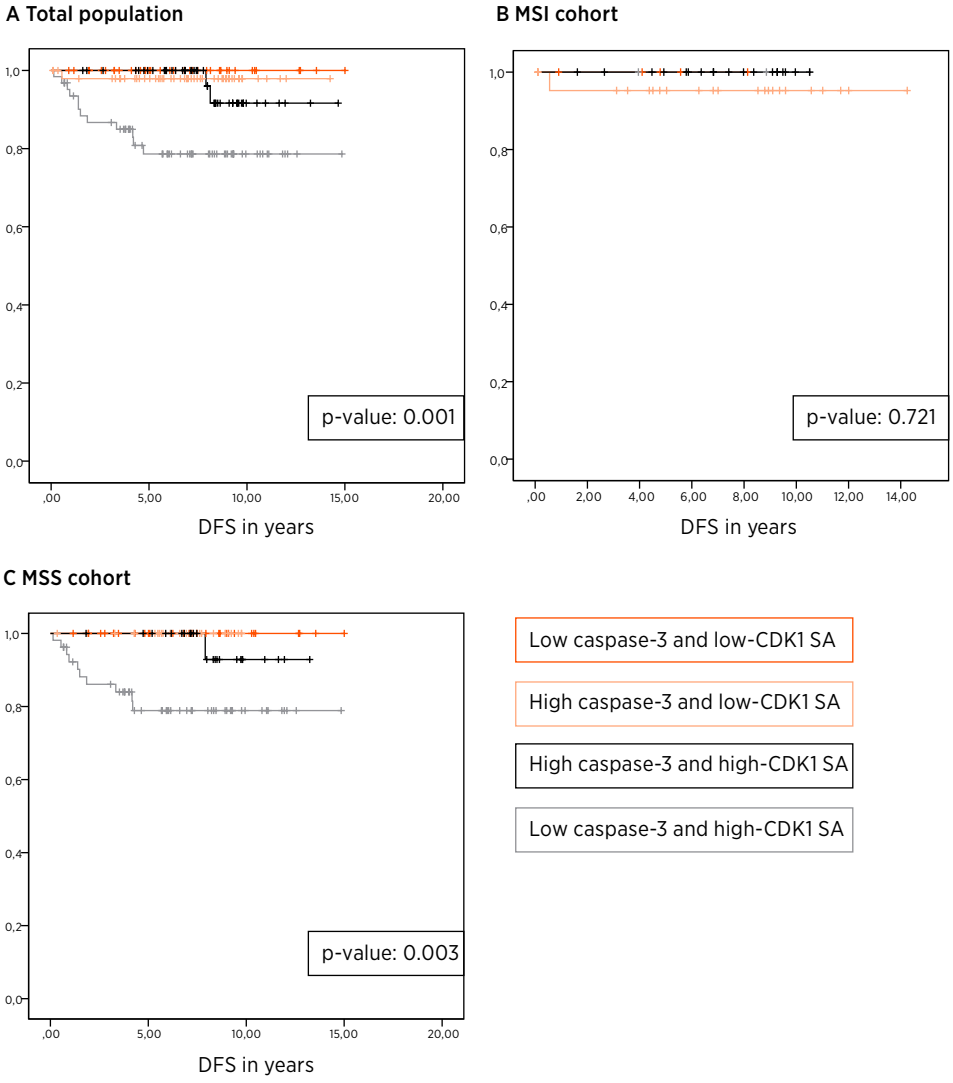

Figure 2

Abbreviation; DFS, disease free survival; CDK1 SA, cyclin dependent kinase 1 specific activity; MSI, microsatellite instability; MSS, microsatellite stability.

Kaplan Meier curves for DFS in years for the combined caspase-3 & CDK1 SA parameter in (A) the total study population, and separately analyzed stratified for tumor microsatellite status in (B) for the MSI cohort and in (C) for the MSS cohort.

the criteria mentioned in the ASCO recommendations: T-stage and the number of lymph nodes resected. Based on these two variables we were able to classify 109 of the 131 MSS stage II colon cancer patients analyzed as low-risk (83%) and the remaining 22 as high-risk (17%). Of these 131 colon cancer patients, 16 patients actually developed a distant recurrence (DR) during follow-up. Based on our combined variable of caspase-3 activity and CDK1 SA levels we identified 14 out of these 16 patients correctly as high-risk, hence only two patients (12.5% of the high-risk patients) were incorrectly classified as low-risk. Using the ASCO recommendations, 11 out of these 16 patients were correctly classified, and 5 (31% of the high-risk patients) patients were incorrectly classified as low-risk. Based on these numbers the sensitivity of our combined variable was 88% with a specificity of 66%. Application of two the ASCO recommendations showed a lower sensitivity of 68%, but at a higher specificity of 85%.

DISCUSSION

This study underlines the clinical importance of analyzing both tumor apoptosis and proliferation for the identification of a patient cohort at high risk of distant metastasis within a stage II colon cancer population. We showed that a variable, based on the combined measurements of the level of tumor apoptosis measured with a biochemical caspase-3 activity assay and the level of tumor proliferation measured with a CDK1 SA assay, has substantial discriminating power to indentify high risk stage II MSS colon cancer patients that might benefit from adjuvant therapy. Our variable performed equally to a subset of ASCO criteria designed to identify this patient selection at risk of distant metastasis in our MSS stage II colon cancer patient population (5).

Several countries, including the Netherlands, in their national guidelines, have adapted the ASCO criteria. Recent data from the Dutch Surgical Colorectal Audit (DSCA) pointed out that in the Netherlands only 18% of the stage II patients indicated as high-risk based on these recommendations actually received adjuvant treatment (16). From these data it can be concluded that the recommendations are not yet standard of care in the Netherlands, even though they are mentioned in the national guidelines. Furthermore, the criteria listed by the ASCO are all clinical and/or pathological parameters, no biomarkers were recommended for clinical use. It is our opinion that to better identify high-risk stage II colon patients the implementation of both clinical and pathological parameters as well as biomarkers should be applied. In other fields of medical care, such as in cardiovascular medicine, a positive effect on high-risk patient identification by combining biomarkers with established clinico-pathological parameters has already been proven (17). Until now both the ASCO's Tumor Markers Expert Panel and its European counterpart; The European Group on Tumor Markers, based on extensive reviews of the available literature, have not found any single biomarker to be of sufficient prognostic or predictive value for clinical application (18–20). We have recently proven that

clinical outcome of colon cancer patients greatly depends on the balance between cell proliferation and apoptosis at tumor level determined with IHC (manuscript submitted for publication). Both disruption of the pathway of apoptosis and the pathway of proliferation are important hallmarks of the highly regulated process of tumorigenesis (7,21). This current study validates the result of our previous study, only now using biochemical assays, proving the clinical significance of a combined parameter reflecting both tumor apoptosis and proliferation levels on patient outcome. Because we were able to validate the prognostic value of our parameter in the independent cohort in this current study and because our criteria showed to perform equally to a subset of the already clinically approved and applied ASCO criteria, future studies in independent cohorts will hopefully confirm that the use of our parameter together with the ASCO criteria will provide a synergistic effect in high-risk patient identification.

Interestingly, our parameter performed the best in high-risk patient identification in the MSS patient population. Microsatellite instability is predominantly found in right-sided tumors. These tumors display a phenotype that has been found to cause a substantial immune reaction resulting in higher levels of apoptosis and, moreover, tumor microsatellite instability has been significantly related to better patient outcome (22–24). Furthermore, a lack of benefit in DFS and OS has been shown by, amongst others, Ribic *et al.* with fluorouracil-based adjuvant chemotherapy in MSI patients (25,26). We feel that the results of these studies justify the application of our parameter in only a MSS stage II colon cancer patient cohort. Because they point out that the role of MSI status as a predictive marker for chemotherapy efficacy needs prospective validation and administration of chemotherapy in MSI Stage II CRC patients is controversially.Therefore it will be mainly within the MSS cohort that high risk patient that might benefit from adjuvant therapy.

In conclusion, current staging criteria have shown to be inadequate to determine a patient's need for adjuvant therapy especially those with a high risk of DR in stage II colon cancer. To improve these criteria, they should be re-evaluated which will implicate the addition of new biomarkers. This study indicates that the combination of the tumor apoptotic status, determined with a caspase-3 activity assay, and the determination of the tumor proliferation level, determined with a CDK1 SA assay, in especially MSS patients is a valuable tool in predicting distant recurrence in stage II colon cancer patients. Future validation of these assays for clinical application will enforce the reliability and clinical practicability.

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