# 5-ASA - colorectal cancer - cell death : an intriguing threesome

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# Chapter 2

# Clinical significance of stromal apoptosis in colorectal cancer

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**Abstract**

Both epithelial and stromal cells play an important role in colorectal cancer (CRC). We aimed to determine the prognostic significance of both epithelial and stromal cell apoptosis in CRC. Total apoptosis was determined by caspase-3 activity measurements in protein homogenates of CRC specimens and adjacent normal mucosa of 211 CRC patients. Epithelial apoptosis was determined by an ELISA specific for a caspase-3 degraded cytokeratin 18 product, the M30 antigen. Stromal apoptosis was determined from the ratio between total and epithelial apoptosis. Both epithelial and stromal apoptosis, as well as total apoptosis, were significantly higher in CRC compared with corresponding adjacent normal mucosa. Low total tumour apoptosis (≤median caspase-3 activity) was associated with a significantly worse disease recurrence [Hazard Ratio (HR), 95 % confidence interval (95% CI): 1.77 (1.05-3.01)], independent of clinico-pathological parameters. Epithelial apoptosis was not associated with clinical outcome. In contrast, low stromal apoptosis (≤median caspase-3/M30) was found to be an independent prognostic factor for overall survival, disease-free survival and disease recurrence, with HRs (95% CI) of 1.66 (1.17-2.35), 1.62 (1.15-2.29), and 1.69 (1.01-2.85) respectively. Therefore we conclude that stromal apoptosis, in contrast to epithelial apoptosis, is an important factor with respect to survival and disease-recurrence in CRC.

## Introduction

The classical normal mucosa-adenoma-carcinoma sequence of colorectal cancer (CRC) is associated with a resistance to apoptosis [1]. The use of apoptosis indices or measurements as a prognostic or predictive factor for CRC has been limited by the reliability of the detection techniques and time consuming evaluations, and have shown conflicting results [2]. Caspases, or death proteases, are cysteine-proteases that are responsible for the morphological changes within cells during apoptosis [3]. Caspase-3 is at the point of convergence of the two main apoptotic pathways and cleaves most of the cellular substrates in the apoptotic process [4]. Measurement of caspase-3 activity is therefore a major and the most reliable determinant of apoptosis [5]. Caspase-3-degraded proteins are also used to detect apoptosis, for instance by the M30 antibody that detects an epithelial specific caspase-3-degraded product of cytokeratin 18 (CK18), i.e., CK18-Asp396, and therefore specifically detects apoptotic epithelial cells [6].

In recent years it has become clear that stromal cells within the tumour, and their interactions with the tumour cells, are important in the initiation and progression of cancer [7], illustrated by the prognostic relevance of tumour stromal-epithelial ratios for CRC patients [8]. Apoptosis of stromal cells might therefore also be of prognostic importance for the patients. To investigate the prognostic impact of stromal apoptosis we determined total cellular apoptosis, by caspase-3 activity measurement, and epithelial apoptosis, by an ELISA for caspase-3-degraded CK18 (M30 antigen), in a large series of CRC specimens. Correction of the total apoptosis for the epithelial apoptosis (caspase-3/M30) revealed the apoptosis of non-epithelial cells, i.e., stromal apoptosis, which was found to be of major clinical relevance in the CRC patients.

## Material&Methods

*Patients*

The study population consisted of 211 CRC patients that were admitted to the Leiden University Medical Centre for tumour resection between December 1983 - September 1991, as previously described [9]. Fresh tissue was collected from the non-irradiated surgical specimen immediately after resection and attention was paid to collect material from the non-necrotic mid-central part of the tumour. Normal mucosa samples were obtained whenever possible at a distance of approximately 10 cm from the tumor. Tissue samples were frozen and stored at -70 °C until use. Clinical data and follow-up information were available for a period of at least 5 years after surgical intervention. Macroscopic (diameter and localization

of the tumour) as well as microscopic data were assessed, including Dukes' stage and differentiation (and ulceration) grade of the tumour according to the WHO classification. Colonic cancers were classified as being proximal or distal to, and including, the splenic flexure. The study was performed according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Centre and in compliance with the Helsinki Declaration.

*Tissue homogenization and protein determination*

Frozen tissue specimens were weighed and homogenized on ice for 2 minutes in 1 ml Tris-HCl, 0.1% Tween 80, pH 7.5 per 60 mg tissue using a Potter device (B Braun, Germany), and centrifuged twice at 8000 x *g* for 2.5 min at 4°C, as described before [9]. Protein content was measured according to Lowry *et al.* and standardized by bovine serum albumin [10].

*Caspase-3 activity measurement*

For measurement of caspase-3 enzymatic activity a colorimetric assay was used as described before [11]. Samples were incubated with a saturating concentratation of 25 μM specific enzyme substrate Ac-Aps-Glu-Val-Asp-AMC (Ac-DEVD-AMC, Bachem, Heidelberg, Germany) in a total of 100 μl 100-mM HEPES buffer, pH 7.25, containing 10% (w/v) sucrose, 0.1% (v/v) Nonidet-P40, and 10 mM dithiothreitol. During incubation at 37°C, fluorescent AMC release by active caspase-3 was monitored at an excitation of 360 nm and an emission of 460 nm using a Fluostar Optima plate reader (BMG Labtech GMBH, Offenburg, Germany). Calibration curves were constructed using free AMC. Caspase-3 activity was indicated in pmol AMC/min/mg protein. The intra- and inter-assay variabilities of the caspase-3 activity assay were <10%

*M30 and CK18 antigen level detection*

M30 antigen and total CK18 antigen levels were detected by M30 and M65 ELISAs, according to the manufacturer's protocol (Peviva BV, Bromma, Sweden), and expressed as antigen units per mg protein (U/mg). Both ELISAs had intra- and inter-assay variabilities <10%, in line with the manufacterer's specifications.

*Western blotting for caspase-3*

Tumour protein samples were separated on a 12 % polyacrylamide SDS gel and blotted to a nitrocellulose membrane (Whatman, Dassel, Germany). After blocking with 2.5 % milkpowder (Bio-Rad laboratories, Hercules, CA, USA) in 0.05 % Tween 20 in phosphate buffered saline (PBST) for 2 hours at room temperature (RT) blots were subsequently incubated overnight at 4˚C with a rabbit-anti-total-caspase-3 antibody (1:500, Cell Signaling Technology, Danvers, MA, USA) in 0.5 % BSA/PBST and Horseradish-peroxidase (HRP)-labelled mouse-anti-rabbit secondary antibody (1:1000, Dako, Glostrup, Denmark) for 2 hours at RT. The signal was developed with Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA), and detected by an LCD camera (Bio-Rad).

*Immunohistochemistry for active caspase-3, M30 antigen and cytokeratin*

Paraffin tissue sections (5 µm) were deparaffinised, blocked in 0.3 % $H_2O_2$ in methanol for 30 minutes at RT and rehydrated through graded ethanol to phosphate buffered saline (PBS). Antigen retrieval was performed by boiling the slides in 10 mM Citrate buffer (pH 6.0) for 10 minutes in a microwave oven. After cooling down to RT and being rinsed with PBS for three times the slides were blocked with 5 % goat serum (Dako) in 1% bovine serum albumin (BSA) in PBS for 20 minutes at RT. Afterwards the sections were incubated with the primary antibody in 1% BSA/PBS overnight at 4˚C: mouse-anti-pan-cytokeratin (1:5000, clone C11, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), mouse M30 Cytodeath antibody (1:400, Roche Applied Science, Penzberg, Germany) and rabbit-anti-active-caspase-3 (1:200, Cell Signaling Technology). After washings with PBS the slides were incubated with biotinylated goat-anti-mouse or goat-anti-rabbit secondary antibodies (both 1:200, Dako) in 1% BSA/PBS for 45 minutes. After thorough washings with PBS the slides were incubated with streptavidin-avidin-biotin complex/HRP (Dako) for 45 minutes at RT. The signal was developed with 0.015 % $H_2O_2$ (Merck, Darmstadt, Germany) in 0.05 % diaminobenzidine-tetrahydrochloride (Sigma) in 0.01 M Tris-HCL pH 7.6 for 10 minutes resulting in a brown staining product. Sections were counterstained with Mayers' haematoxylin (Merck), dehydrated and mounted in entallan (Merck). Slides without primary antibody incubations were included as negative controls. Photomicrographs of representative sections were taken with a Nikon Elipse E800 microscope equipped with a Nikon DXM 200 camera.

*Statistical Analysis*

Statistical analysis was performed with Statistical Package for Social Sciences (SPSS) statistical software (version 12.0 for Windows, SPSS Inc, Chicago, IL). Wilcoxon Signed Rank and Spearman Rho tests were used to compare paired observations that did not follow a normal distribution. Kruskall-Wallis and Mann-Whitney U tests were used to compare two or more groups that do not follow a normal distribution. The entry data for survival analysis was the time of surgery for the primary tumour. Biomarker expressions were dichotomized and overall survival, disease recurrence and disease-free survival were evaluated using Kaplan-Meier methodology with death, local and distant recurrence, or both, as events, including Log Rank tests. Univariate Cox proportional hazard models were used to explore the association of markers with clinical outcome. Variables with a $P \leq 0.1$ in the univariate analyses were subjected to multivariate analyses. Statistical tests were two-sided and $P<0.05$ was considered statistically significant. Graphs were made with Graphpad Prism (version 4.0, Graphpad Prism Inc., La Jolla, CA, USA) software.

## Results

*Total, epithelial and stromal apoptosis in colorectal cancer*

The caspase-3 activity, M30 antigen and CK18 levels were determined in colorectal carcinoma tissue of all 211 patients, whereas due to tissue/homogenate availability these parameters could only be determined in adjacent normal tissue from 177 patients. M30 antigen, CK18 and caspase-3 activity levels in the colorectal tumour tissue samples were significantly higher than in the corresponding normal tissue samples in these 177 patients (*Table 1*).

Table 1: Caspase-3 activity, M30 antigen and CK18 levels in CRC.

| Parameter / Tissue assessed | Tumour median (IQR) | Normal mucosa median (IQR) | P-value |
|---|---|---|---|
| Caspase-3 activity (pmol AMC/mg/min) | 6.74 (2.96-16.65) | 1.28 (0.66-2.20) | <0.0001 |
| M30 antigen (U/mg) | 7.58 (3.13-13.77) | 3.20 (1.13-5.66) | <0.0001 |
| CK18 (U/mg) | 209.6 (116.9-273.1) | 178.5 (129.6-132.5) | 0.008 |
| M30/CK18 x 100 (epithelial apoptosis) | 3.72 (2.52-5.78) | 1.83 (0.86-3.16) | <0.0001 |
| Caspase-3/M30 (stromal apoptosis) | 0.84 (0.44-2.97) | 0.38 (0.18-1.18) | <0.0001 |

*Caspase-3 activity, M30 antigen and CK18 levels in tumour tissue and normal adjacent mucosa pairs from 177 CRC patients. Epithelial apoptosis (M30/CK18 x 100) and stromal apoptosis (Caspase-3/ M30) were calculated from caspase-3, M30 antigen and CK18 levels. P-values were determined with Mann-Whitney U test. IQR=inter-quartile range.*

Immunoblot analysis confirmed that tumours with a high caspase-3 activity had high protein levels of active caspase-3 compared with low caspase-3 activity tumours (*Figure 1A*). Overall there was a weak but significant correlation between tumour caspase-3 activity and tumour M30 antigen levels, as expected because the M30 antigen is generated by active caspase-3 (*Figure 1B*). Some tumours had really high caspase-3 activity levels, without high M30 antigen levels, indicating high stromal apoptosis.

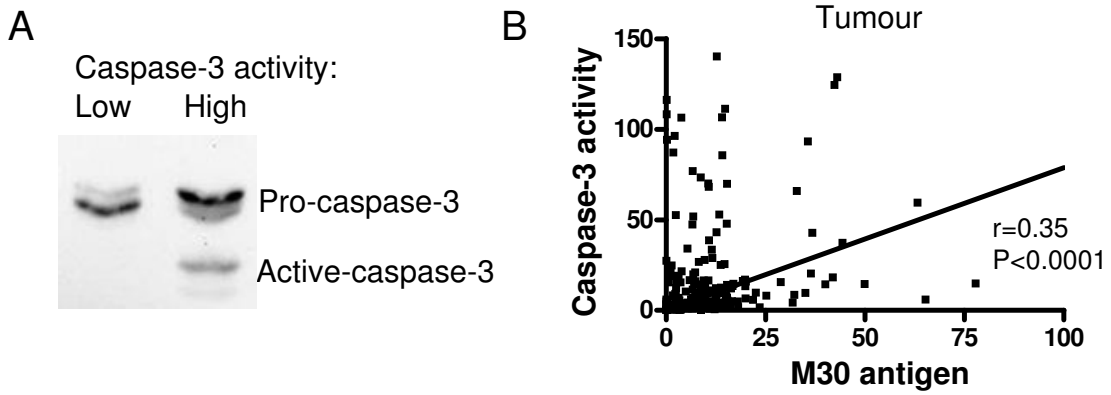


*Figure1: Caspase-3 activity in CRC.*

*CRC specimens with high caspase-3 activity show levels of the active form of caspase-3, while low caspase-3 activity CRC specimens only show inactive (pro-)caspase-3 as determined by immunoblotting for total caspase-3 (A). Caspase-3 activity and M30 antigen in CRC show a weak but significant correlation (B). Some tumours show high caspase-3 activity without high levels of M30 antigen, suggesting stromal apoptosis.*

Immunohistochemical analysis confirmed that in these tumours active caspase-3 was indeed mainly expressed by non-epithelial cells and were almost M30 antigen negative (*Figure 2*, represented by tumour 1), compared with other tumours in which epithelial cells were expressing most of the active caspase-3 and therefore M30 antigen positive (represented by tumour 2). Our measurements enabled us to further differentiate in cell type origin of apoptosis by calculating the percentage of apoptotic epithelial cells through the formula M30/CK18 x 100 and for stromal apoptosis through the formula caspase-3/M30. This epithelial as well as stromal apoptosis was also significantly higher in tumour tissue compared with corresponding normal tissue (*Table 1*).

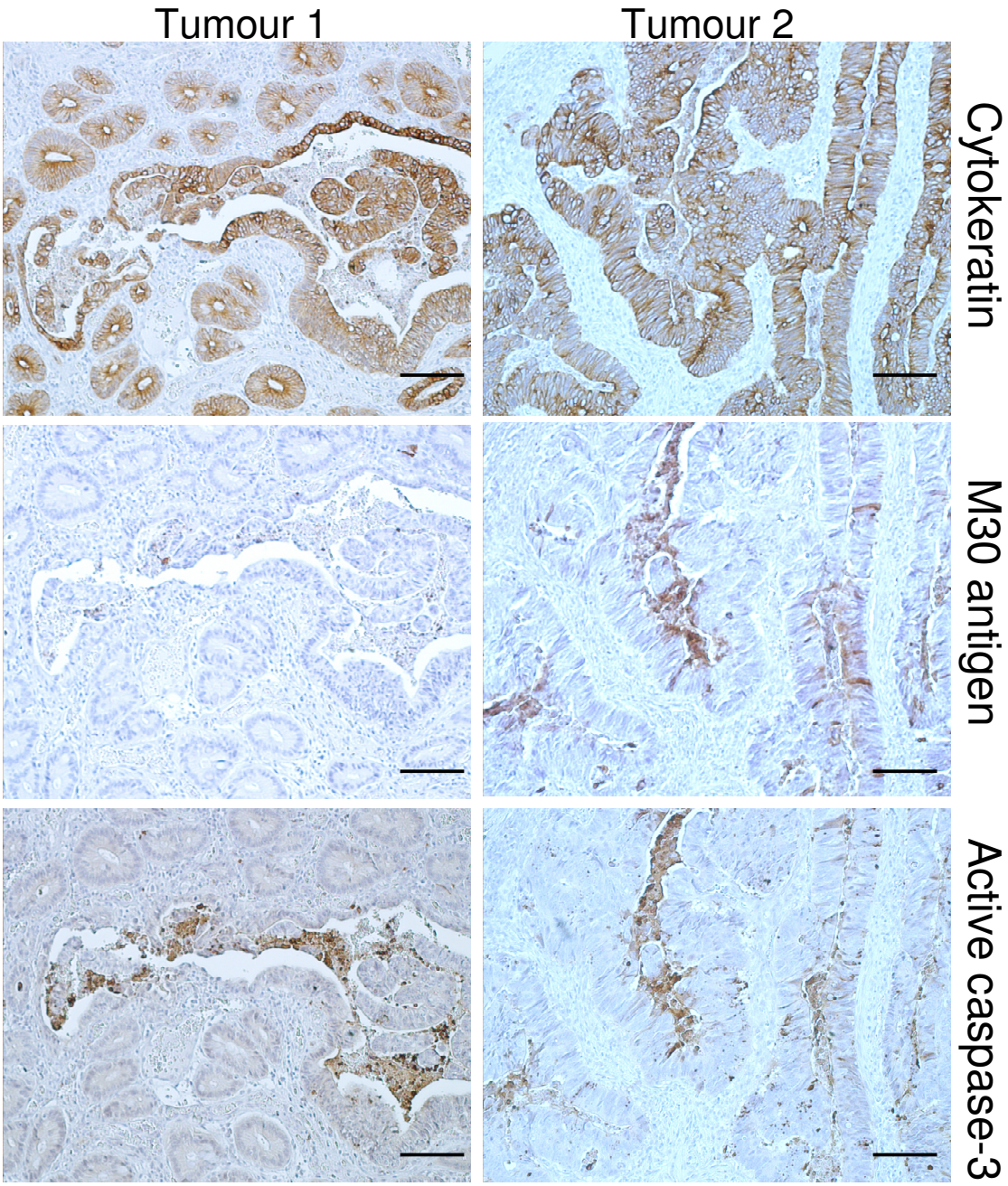


*Figure 2: Caspase-3 activity in CRC.*

*Photomicrographs of immunohistochemical stainings for active caspase-3, M30 and pan-cytokeratin showing tumours with stromal cells expressing active caspase-3 (as represented by Tumour 1, left panel), and tumours with mainly epithelial cells expressing active caspase-3, correlating with M30 staining (represented by Tumour 2, right panel). Scale bars= 100 µm. Full-colour on page 182.*

*Relation between total, epithelial and stromal apoptosis and clinical variables*

The association between the clinico-pathological characteristics of the 211 colorectal cancer patients and the tissue caspase-3 activity, as measure of total apoptosis, is summarized in *Table 2*. Caspase-3 activity level in the tumours correlated with gender, tumour location

and Dukes' stage, i.e., significantly higher in females (P=0.006) and in tumours located in the proximal colon (P=0.02), and decreased with increasing Dukes' stage (P=0.01). Caspase-3 activity in normal mucosa was also found to be higher in females (P=0.04) and in the proximal colon (P=0.04), but did not correlate with Dukes' stage.

Table 2: Caspase-3 activity in relation to patient and tumour characteristics.

| Tissue | Tumour | | | Normal mucosa | | |
|---|---|---|---|---|---|---|
| Patient and tumour characteristics | No. pts (%) Total n=211 | Caspase-3 activity Median (IQR) | P-value | No. pts (%) Total n=177 | Caspase-3 activity Median (IQR) | P-value |
| Gender | | | **0.006** | | | **0.04** |
| Male | 118 (56) | 5.93 (1.96-14.55) | | 102 (58) | 1.07 (0.59-2.19) | |
| Female | 93 (44) | 9.77 (3.89-26.00) | | 75 (42) | 1.40 (0.78-2.21) | |
| Age [#] | | | 0.48 | | | 0.60 |
| < median | 105 (50) | 7.33 (2.76-16.10) | | 88 (50) | 1.23 (0.75-2.05) | |
| ≥ median | 106 (50) | 7.10 (3.39-19.34) | | 89 (50) | 1.37 (0.62-2.38) | |
| Location | | | **0.02** | | | **0.04** |
| Right or transverse | 73 (35) | 10.79 (3.43-47.72) | | 62 (35) | 1.40 (0.80-2.67) | |
| Left colon or sigmoid | 72 (34) | 5.75 (1.83-16.76) | | 59 (33) | 1.37 (0.70-2.10) | |
| Rectum | 66 (31) | 5.25 (2.94-14.47) | | 56 (32) | 0.99 (0.52-1.93) | |
| Dukes stage | | | **0.01** | | | 0.66 |
| A/B | 122 (58) | 9.00 (3.51-20.35) | | 106 (60) | 1.25 (0.66-2.21) | |
| C | 57 (27) | 6.19 (2.36-15.27) | | 45 (25) | 1.18 (0.61-2.08) | |
| D | 32 (15) | 3.80 (1.24-13.16) | | 26 (15) | 1.38 (0.70-2.34) | |
| Tumour Ø [$] | | | 0.47 | | | 0.98 |
| < median | 93 (44) | 5.98 (3.37-15.64) | | 79 (45) | 1.31 (0.72-2.00) | |
| ≥ median | 118 (56) | 8.38 (2.89-19.36) | | 98 (55) | 1.21 (0.63-2.27) | |
| Grade of differentiation * | | | 0.10 | | | 0.36 |
| Poor | 25 (12) | 8.23 (3.76-14.77) | | 21 (12) | 2.00 (0.75-2.97) | |
| Moderate | 143 (68) | 13.46 (4.02-27.33) | | 122 (69) | 1.25 (0.64-2.14) | |
| Well | 40 (19) | 7.01 (3.79-40.51) | | 31 (18) | 1.28 (0.59-2.19) | |
| WHO classification * | | | 0.29 | | | 0.31 |
| Adenocarcinoma | 157 (75) | 7.65 (3.36-17.60) | | 137 (77) | 1.23 (0.64-2.09) | |
| Mucinous carcinoma | 52 (25) | 6.03 (1.75-15.92) | | 39 (22) | 1.39 (0.72-2.62) | |
| Ulceration* | | | 0.82 | | | 0.59 |
| No | 48 (27) | 6.73 (3.23-25.65) | | 44 (25) | 1.25 (0.74-2.26) | |
| Yes | 139 (73) | 7.33 (2.94-16.59) | | 118 (66) | 1.30 (0.62-2.17) | |

*[#] Median 69 years, range 31-90. [$] Median 5 cm, range 2-12.* Some values were missing. P-values were calculated with Kruskall-Wallis and Mann-Whitney U tests, and P-values ≤ 0.05 were considered statistically significant, shown in bold. IQR=inter-quartile range.*

Tumour M30 antigen levels correlated with grade of differentiation, well differentiated tumours having significantly higher levels (*Table 3*, P=0.02). The total amount of epithelial cells (CK18/mg protein) in the tumour samples correlated with the tumour diameter, larger tumours having significantly lower amounts of epithelial cells compared with smaller tumours (P=0.007). Epithelial cell apoptosis (M30/CK18 x 100) was significantly higher in tumours with ulceration (P=0.02). Stromal apoptosis (caspase-3/M30 ratio) was related to

gender and tumour location, i.e., significantly higher in females (P=0.02) and proximal colon tumours (P<0.001).

Table 3: Tumour M30 antigen, CK18, M30/CK18 x 100 and caspase-3/M30 ratio in relation to patient and tumour characteristics of CRC patients.

| Parameter<br>Patient and tumour characteristics | Tumour M30 (U/mg) Median (IQR) | P-value | Tumour CK18(U/mg) Median (IQR) | P-value | Tumour M30/CK18 x 100 Median (IQR) | P-value | Tumour Caspase-3/M30 Median (IQR) | P-value |
|---|---|---|---|---|---|---|---|---|
| Gender | | 0.34 | | 0.72 | | 0.53 | | **0.02** |
| Male | 8.7 (3.6-15.2) | | 218.7 (128.6-287.9) | | 3.9 (2.5-6.7) | | 0.8 (0.3-2.2) | |
| Female | 8.1 (3.2-13.7) | | 204.8 (124.4-291.8) | | 3.8 (2.3-5.4) | | 1.0 (0.5-4.9) | |
| Age | | 0.94 | | 0.38 | | 0.22 | | 0.43 |
| < median | 8.7 (3.1-15.2) | | 224.5 (127.6-296.7) | | 3.6 (2.2-5.9) | | 0.8 (0.4-4.0) | |
| ≥ median | 8.4 (3.9-13.8) | | 214.6 (125-7-263.7) | | 4.0 (2.9-5.8) | | 0.9 (0.4-2.9) | |
| Location | | 0.06 | | 0.17 | | 0.24 | | **<0.001** |
| Right colon | 6.8 (2.4-12.7) | | 189.0 (124.8-257.8) | | 3.6 (1.8-6.3) | | 2.1 (0.6-7.6) | |
| Left colon | 9.2 (3.5-15.5) | | 208.6 (113.4-288.8) | | 3.8 (2.7-5.8) | | 0.8 (0.4-2.2) | |
| Rectum | 9.4 (5.9-15.3) | | 224.6 (164.9-302.4) | | 3.9 (3.0-5.7) | | 0.7 (0.3-1.1) | |
| Dukes stage | | 0.54 | | 0.93 | | 0.38 | | 0.21 |
| A/B | 8.6 (3.5-15.2) | | 215.3 (128.7-292.5) | | 3.8 (2.7-6.6) | | 0.9 (0.5-3.2) | |
| C | 8.8 (3.4-14.9) | | 224.5 (137.9-278.6) | | 4.0 (2.5-5.7) | | 0.8 (0.3-2.8) | |
| D | 6.8 (2.8-12.6) | | 206.2 (104.0-291.2) | | 3.5 (2.1-5.0) | | 0.5 (0.3-2.3) | |
| Tumour Ø | | 0.14 | | | | 0.42 | | 0.15 |
| < median | 9.6 (3.7-15.2) | | 234.8 (153.6-316.0) | **0.007** | 3.7 (2.3-5.8) | | 0.7 (0.4-2.4) | |
| ≥ median | 7.2 (3.2-13.7) | | 192.0 (116.0-262.0) | | 3.8 (2.7-6.9) | | 0.9 (0.4-3.3) | |
| Grade of differentiation | | **0.02** | | 0.10 | | 0.17 | | 0.35 |
| Poor | 7.4 (1.6-15.4) | | 197.1 (78.1-274.8) | | 4.9 (2.6-7.0) | | 1.4 (0.4-5.0) | |
| Moderate | 8.0 (3.2-13.8) | | 215.1 (122.9-275.1) | | 3.6 (2.3-5.4) | | 0.9 (0.4-3.0) | |
| Well | 11.8(5.6-19.8) | | 226.7 (150.0-400.0) | | 4.0 (2.9-8.6) | | 0.8 (0.4-1.8) | |
| WHO* | | 0.35 | | 0.74 | | 0.29 | | 0.79 |
| Adeno | 8.7 (3.6-15.0) | | 214.5 (130.7-291.4) | | 3.8 (2.7-6.4) | | 0.8 (0.4-2.9) | |
| Mucinous | 7.7 (2.8-14.2) | | 223.6 (116.6-262.6) | | 3.7 (2.1-5.4) | | 0.9 (0.3-3.1) | |
| Ulceration | | 0.36 | | 0.85 | | **0.02** | | 0.07 |
| No | 5.5 (2.6-15.2) | | 210.4 (126.0-322.7) | | 3.1 (2.0-5.1) | | 1.3 (0.6-4.2) | |
| Yes | 8.6 (3.5-14.1) | | 217.4 (129.2-280.0) | | 3.8 (2.8-5.8) | | 0.8 (0.4-2.9) | |

**Classification according to the WHO. P-values were calculated with Kruskall-Wallis and Mann-Whitney U tests, and P-values ≤0.05 were considered statistically significant, shown in bold. IQR=inter-quartile range.*

*Stromal apoptosis predicts overall survival, disease-free survival and disease recurrence*

High caspase-3 activity (>median) in the tumour was associated with better overall patient survival: median overall survival: 58 vs 34 months (*Figure 3A*). These patients with high tumour caspase-3 activity (>median) also had a significantly better disease-free survival with a median disease-free survival of 47.5 versus 27 months for patients with low tumour caspase-3 activity (≤median, *Figure 3B*). Low tumour caspase-3 activity (≤median) was also accompanied by a shorter time to recurrence compared to patients with a high tumour caspase-3 activity (*Figure 3C*), with 5-years recurrence rates of 46.1 % and 30.3 %, respectively.

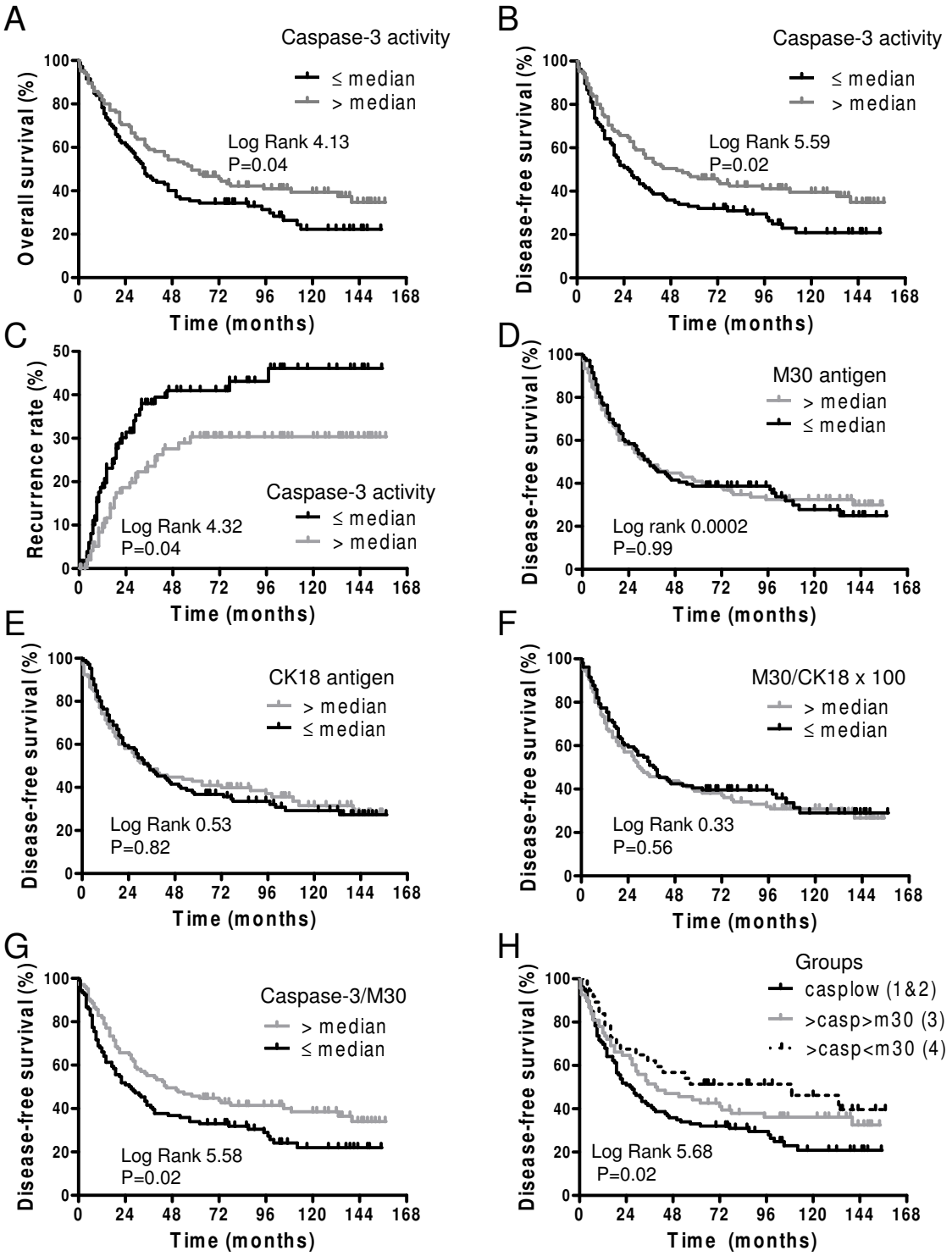


*Figure 3: Clinical outcome in relation to Caspase-3 activity, M30 and CK18 antigen levels.*

*Kaplan Meier overall survival (A), disease-free survival (B,D-H) and recurrence (C) curves of all CRC patients (n=211), groups divided upon median tumour caspase-3 activity (A-C), median M30 antigen (D), median CK18 (E), median M30/CK18 x 100 (F), median caspase-3/M30 (G) and both median caspase-3 and median M30 levels (H). Log Rank test were used to compare the curves.*

Caspase-3 activity in the normal adjacent mucosa was not associated with overall survival, disease-free survival or disease recurrence of the patients (not shown). Tumour M30 antigen, CK18 or M30/CK18 x 100 levels did not show any relation with disease-free survival (*Figure 3D*, *E* and *F*), overall survival or disease recurrence (not shown). In contrast, the caspase-3/M30 ratio of the tumours correlated very strongly with the disease-free survival of the patients (*Figure 3G*), with a high ratio (>median) having a significantly better median disease-free survival of 45 versus 26.5 months for the patients with tumours with a low ratio.

To underline the importance of stromal apoptosis to the clinical outcome of the CRC patients we divided our patient population in 4 groups based on median tumour M30 antigen and median tumour caspase-3 activity levels: 1) low caspase-3/M30 low (n=67), 2) low caspase-3/M30 high (n=39), 3) caspase-3 high/M30 high (n=68) and 4) caspase-3 high/M30 low (n=37). The M30 antigen levels did not have an additive effect in the 2 groups with low caspase-3 activity and therefore groups 1 and 2 were pooled. In the groups with high caspase-3 activity the disease-free survival was found to be the best for patients with a high tumour caspase-3 activity and low M30 antigen level (*Figure 3H*, group 4 versus group 1&2, Log Rank value: 5.68, P=0.02), which strengthened our notion that stromal apoptosis in the tumour is an important prognostic factor for the patients' disease-free survival.

The estimates of relative risk of the evaluated parameters to disease outcome, as calculated by hazard ratios in the univariate Cox regression analysis, are shown in *Table 4*. The clinico-pathological factors that were significantly associated with worse outcome were found to be old age and advanced Dukes' stage, as expected. With regard to the apoptosis parameters, a low total (caspase-3 activity) or low stromal (caspase-3/M30) apoptosis were consistently found to be associated with a worse overall and disease-free survival as well as with a higher risk of recurrence. Adjustment of the apoptosis parameters to the most relevant clinico-pathological parameters, i.e., gender, age, Dukes' stage, tumour localization and tumour diameter in the multivariate Cox proportional hazards analysis identified stromal apoptosis as an independent prognostic factor for overall survival, disease-free survival and disease recurrence. In contrast, total apoptosis was found to be an independent prognostic factor for disease recurrence only (*Table 4*).

Table 4: Univariate and multivariate Cox regression hazard analysis.

| Outcome<br>Patient and tumour characteristics | Overall survival HR (95 % CI) | P-value | Disease-free survival HR (95 % CI) | P-value | Risk of recurrence HR (95 % CI) | P-value |
|---|---|---|---|---|---|---|
| Univariate Analysis | | | | | | |
| Gender | | | | | | |
| Female | 1 (ref) | 0.08 | 1 (ref) | **0.03** | 1 (ref) | 0.16 |
| Male | 1.35 (0.96-1.89) | | 1.44(1.03-2.02) | | 1.43(0.87-2.36) | |
| Age | | | | | | |
| < median | 1 (ref) | **<0.001** | 1 (ref) | **0.001** | 1 (ref) | 0.26 |
| ≥ median | 1.83 (1.31-2.58) | | 1.78(1.27-2.49) | | 1.33(0.81-2.17) | |
| Location | | | | | | |
| Right or transverse | 1 (ref) | | 1 (ref) | | 1 (ref) | |
| Left colon or sigmoid | 1.07 (0.72-1.57) | 0.75 | 1.12 (0.76-1.64) | 0.58 | 1.13 (0.65-1.97) | 0.66 |
| Rectum | 0.69 (0.45-1.05) | 0.08 | 0.70 (0.46-1.06) | 0.09 | 0.80 (0.40-1.58) | 0.52 |
| Dukes stage | | | | | | |
| A/B | 1 (ref) | | 1 (ref) | | 1 (ref) | |
| C | 1.93 (1.31-2.84) | **0.001** | 2.00 (1.36-2.93) | **< 0.001** | 3.35 (2.04-5.52) | **< 0.001** |
| D | 7.54 (4.69-12.12) | **< 0.001** | 5.53 (3.50-8.73) | **< 0.001** | 0.31 (0.04-2.28) | 0.25 |
| Tumour Ø | | | | | | |
| < median | 1 (ref) | 0.14 | 1 (ref) | 0.09 | 1 (ref) | 0.12 |
| ≥ median | 1.29 (0.92-1.81) | | 1.34 (0.96-1.87) | | 1.50 (0.90-2.50) | |
| Grade of differentiation | | | | | | |
| Poor | 1 (ref) | 0.16 | 1 (ref) | 0.16 | 1 (ref) | 0.23 |
| Moderate/ Well | 1.42 (0.87-2.34) | | 1.30 (0.90-1.89) | | 0.62 (0.28-1.36) | |
| WHO classification | | | | | | |
| Adenocarcinoma | 1 (ref) | 0.94 | 1 (ref) | 0.93 | 1 (ref) | 0.73 |
| Mucinous carcinoma | 0.99(0.67-1.45) | | 0.98(0.67-1.44) | | 0.90 (0.51-1.61) | |
| Ulceration | | | | | | |
| No | 1 (ref) | 0.26 | 1 (ref) | 0.45 | 1 (ref) | 0.12 |
| Yes | 1.28 (0.83-1.98) | | 1.18(0.77-1.80) | | 1.57(0.82-3.02) | |
| Tumour Caspase-3 activity | | | | | | |
| > median | 1 (ref) | **0.04** | 1 (ref) | **0.02** | 1 (ref) | **0.04** |
| ≤ median | 1.41 (1.01-1.97) | | 1.48(1.07-2.06) | | 1.68 (1.02-2.78) | |
| Tumour M30 level | | | | | | |
| > median | 1 (ref) | 0.71 | 1 (ref) | 0.90 | 1 (ref) | 0.38 |
| ≤ median | 0.94 (0.67-1.31) | | 0.94 (0.71-1.36) | | 0.80 (0.49-1.31) | |
| Tumour CK18 level | | | | | | |
| > median | 1 (ref) | 0.99 | 1 (ref) | 0.82 | 1 (ref) | 0.67 |
| ≤ median | 1.00 (0.72-1.39) | | 1.04 (0.75-1.44) | | 0.90 (0.55-1.47) | |
| Tumour M30/CK18 x 100 level | | 0.52 | | 0.57 | | 0.83 |
| > median | 1 (ref) | | 1 (ref) | | 1 (ref) | |
| ≤ median | 0.90 (0.64-1.25) | | 0.91 (0.65-1.26) | | 0.95 (0.58-1.55) | |
| Tumour Caspase-3/ M30 level | | **0.02** | | **0.02** | | **0.05** |
| > median | 1 (ref) | | 1 (ref) | | 1 (ref) | |
| ≤ median | 1.51 (1.08-2.11) | | 1.48 (1.06-2.06) | | 1.64 (1.00-2.70) | |
| Multivariate Analysis | | | | | | |
| Low tumour caspase-3 activity | 1.25 (0.87-1.80) | 0.22 | 1.32 (0.92-1.88) | 0.13 | 1.77 (1.05-3.01) | **0.03** |
| Low tumour caspase-3/M30 level | 1.66 (1.17-2.35) | **0.004** | 1.62 (1.15-2.29) | **0.006** | 1.69 (1.01-2.85) | **0.04** |

*Univariate and multivariate Cox regression hazard analysis for overall and disease-free survival, and risk of recurrence including the clinico-pathological factors of all CRC patients (n=211) and the specified apoptosis assessments. In the multivariate analyses the apoptosis parameters were adjusted for gender, age, Dukes'stage, tumour diameter and tumour location. P-values ≤0.05 were considered statistically significant, shown in bold. CI=confidence interval.*

**Discussion**

The present study shows that stromal apoptosis in CRC is a major determinant for the clinical outcome of patients. The stromal compartment of colorectal carcinomas is populated by a wide variety of cell types, such as immune cells, fibroblasts, myofibroblasts and endothelial cells. Recent studies have demonstrated that genetic instability of both the stromal and epithelial compartment contributes to the genesis of CRC [12]. Important signalling pathways that are frequently disrupted in CRC, like the Transforming Growth Factor (TGF)-β [13], Bone Morphogenic Protein (BMP) [14] and Wnt pathway [15], play a role in both the cancer-associated stromal and epithelial compartment in the development of CRC. The presence of fibroblasts [16], myofibroblasts [17] and endothelial cells [18] in the cancer-associated stroma contributes to progression of CRC and a worse patient prognosis. Also, the expression of other proteins in the cancer-associated stroma, i.e., hypoxia regulated proteins [19], matrix degrading proteases [20] and cyclooxygenase-2 [21, 22] is correlated to CRC progression and disease prognosis. The importance of the cancer-associated stroma in CRC is most simply illustrated by the reported prognostic significance of the tumour-stroma ratio in CRC [8]. The cancer-associated stromal cells, and their interaction with the epithelial tumour cells, are therefore speculated to be important targets for future therapy [7].

Programmed cell death, i.e. apoptosis, is an important mechanism to maintain tissue homeostasis. Defects in the two main apoptotic pathways, the extrinsic and intrinsic pathway, lead to a resistance to apoptosis and increased cell survival. Epithelial apoptosis in CRC has been widely studied but still the significance for the prognosis of CRC patients, as well as the significance for the response to treatment like chemotherapy, is unclear [1, 2]. Because apoptosis in the cancer-associated stroma could also play an important role, and its induction could be relevant for treatment response, we investigated the contribution of stromal apoptosis, as well as epithelial apoptosis in the clinical outcome of CRC.

We showed that both epithelial and stromal apoptosis, as well as total apoptosis, were significantly higher in non-irradiated CRC than in normal adjacent mucosa, as found in other studies [1, 11, 23-25]. Because cancer cells usually have apoptotic defects and are more difficult to drive into apoptosis [26, 27], higher levels of apoptosis in cancer, compared with normal tissue, might seem to be somewhat unexpected. However, *the level of apoptosis* and *sensitivity to apoptotic signals* are two, although related, different things. Importantly, in the present study total CRC apoptosis decreased with increasing Dukes' stage, while no such correlation was found in adjacent normal mucosa, which indeed indicates that cells in the tumour become resistant to apoptotic signals during tumour progression resulting in a decreased caspase-3

activity in more advanced tumour stages. Moreover, total apoptosis in the tumour showed a strong association with risk of recurrence and disease-free survival. Cox multivariate hazards analysis identified low caspase-3 activity in the tumour as an independent risk factor of recurrence, with patients with tumour caspase-3 activity below the median value having a 1.77 times higher relative risk of recurrence, similar as reported by others for both colon and rectal cancer [11, 28]. Caspase-3 activity corrected for the percentage of epithelial cells, as determined on slides, showed similar results compared with the uncorrected caspase-3 activity in rectal cancer in the study by De Heer *et al.* [11], and therefore the authors concluded that caspase-3 activity is a prognostic factor for local recurrence in rectal cancer without the previous knowledge of epithelial-stromal ratios in the tumour. Correction of caspase-3 activity for the amount of epithelial cells (measured by CK18 antigen levels), caspase-3/CK18, in the present study indeed showed similar results with respect to clinco-pathological parameters and clinical outcome, as caspase-3 activity alone (not shown). However, this does not indicate the source of the caspase-3 activity, it could be that most of the caspase-3 activity is produced by apoptotic epithelial cells, but also apoptotic stromal cells could be an important source. To investigate the source we determined M30 antigen levels by ELISA in the same CRC homogenates, as a measurement of epithelial caspase-3 activity/apoptosis [6, 29]. If apoptotic epithelial cells were mainly responsible for the caspase-3 activity in the present study, M30 antigen levels should have shown a similar correlation with patient and tumour characteristics, which was clearly not the case. Our measurement of epithelial apoptosis, determined by M30/CK18 x 100 (%), with median values of 3.72 % for tumour tissue and 1.83 % for normal tissue, is very much in line with epithelial apoptosis found in other studies [1]. M30 antigen levels, either corrected for CK18 or uncorrected, did not correlate with overall survival, disease-free survival or disease recurrence, in contrast to caspase-3 activity, indicating the importance of non-epithelial caspase-3 activity, i.e., stromal apoptosis. This was confirmed by determining caspase-3/M30 ratios, as stromal apoptosis levels, which even showed a stronger association with overall survival, disease-free survival or disease recurrence. Low stromal apoptosis (≤median caspase-3/M30) was an independent prognostic factor for overall survival, disease-free survival and disease recurrence with a HR (95 % CI) of 1.66 (1.17-2.35), 1.62 (1.15-2.29), and 1.69 (1.01-2.85), respectively**.**

Epithelial tumour cell apoptosis has been extensively studied in CRC but still the relevance of the apoptotic index as a prognostic factor is a matter of debate [1, 2]. The detection technique is likely to influence the apoptotic levels, as some detection techniques are really specific for epithelial apoptotic cells, i.e., M30 immunohistochemistry and M30 ELISA [6, 29],

and others are (more or less) specific for apoptotic cells in general, i.e., TdT-mediated dUTP-biotin nick end-labeling (TUNEL) [30, 31], active caspase-3 immunohistochemistry or caspase-3 activity measurements [5], and also detect apoptotic stromal cells. The results presented here, i.e., stromal apoptosis as an important prognostic factor for CRC, suggests that discrepancies found in the literature might be explained by the different detection techniques that are used, i.e., "specific for epithelial apoptosis" versus "specific for apoptosis in general".

In conclusion, the present study confirms that caspase-3 activity within tumour tissue is an important denominator of disease recurrence and patient survival in CRC, with high levels of caspase-3 associated with good outcome. Remarkably, this caspase-3 activity was found to predominantly reside within the non-epithelial compartment of the tumours. These observations underline the importance of stromal cell apoptosis in CRC progression and identify the cancer-associated stroma as a potential therapeutic target.

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