CHAPTER 4

Apoptosis is a poor prognostic factor in colorectal cancer


Submitted for publication
Abstract

To determine parameters that predict prognosis of colorectal cancer patients, this study evaluated the correlation and prognostic value of apoptosis and microsatellite instability and colorectal cancer. One hundred and four patients with sporadic colorectal cancer with a median clinical follow-up of 5.9 years were evaluated immunohistochemically for presence of apoptotic tumor cells using a monoclonal antibody, M30 that recognizes a cleavage fragment of cytokeratin 18 (M30 neoantigen) specific for apoptotic epithelial cells. Eight patients were excluded from evaluation as they had received preoperative radiotherapy. The number of apoptotic tumor cells was associated with clinicopathological parameters including tumor microsatellite stability. The latter was immunohistochemically determined by evaluation of nuclear PMS2 and MHL-1 expression. A negative nuclear staining for either PMS2 or MLH-1 was used to indicate microsatellite instability (MSI), a positive staining to indicate microsatellite stable (MSS).

A relatively high number of apoptotic tumor cells was significantly associated with unfavorable prognosis (p=0.005). Of the patients evaluated, 14 tumors showed MSI, the other 82 MSS. Although MSI was not associated with an increase in number of apoptotic tumor cells (p=0.44), these patients showed better prognosis than MSS patients (p=0.05). Apoptosis proved to be an independent prognostic factor for disease free survival and tumor recurrence in the multivariate analysis (p=0.003, HR 2.2 CI: 1.32-3.94), and p=0.05, HR: 2.1 CI: 0.98-4.78 respectively).

Our data indicate that apoptosis and microsatellite instability are independent mechanisms that determine patient outcome in colorectal cancer.
Introduction

Apoptosis, or programmed cell death, is a subject of extensive research as it has been implicated in the development and progression of cancer. Abnormalities in apoptotic function contribute to both the pathogenesis of colorectal cancer, as to its resistance to chemotherapeutic drugs and radiotherapy. Apoptosis results from activation of several closely regulated pathways that ultimately lead to chromatin condensation, membrane blebbing, and DNA and protein degradation by downstream effector caspases. The clinical significance of apoptosis on patient survival in colorectal cancer has extensively been studied. However, its role remains contradictory as high levels of apoptosis of tumor cells has been associated with improved as well as impaired prognosis.

Tumors of the colorectum originate from two main genetic pathways. A subgroup of colorectal cancers are characterized by high levels of DNA microsatellite instability (MSI) and are associated with an improved prognosis and favorable prognostic characteristics as increased immune cell infiltration. In general, the mutational burden MSI tumors is suspected to be detrimental to tumor growth and development, however an association with apoptosis remains unclear.

One of the principal problems of determining the impact of apoptosis on patient prognosis in colorectal cancer is that large inconsistencies exist in evaluation methods. This complicates direct comparisons between studies and restricts analysis of the independent prognostic value of apoptosis. To ensure adequate characterization of tumor cell apoptosis in the current study we evaluated apoptosis by immunohistochemical staining with the monoclonal antibody M30 that recognizes a cleavage fragment of Cytokeratin 18 (M30 neoantigen) and is specific for apoptotic epithelial cells.

Material and methods

Tumor specimens

The study comprised a random series of 104 consecutive sporadic colorectal carcinomas obtained from the department of Pathology of the Leiden University Medical Center. Tumor samples were derived from patients who underwent curative surgery between 1980 and 1987 and from whom follow up information was available. Patients presenting with colon cancer received a hemicolectomy. Patients with a rectal cancer were operated by conventional blunt dissection of the cancer. Patient follow-up was completed until January 2003, with a median follow-up of 5.4 years (range, 0.1-18.6 years; SD, 5.2 years). Patients with stage II and III colorectal cancer (as defined by American Joint committee on Cancer and Union Internationale Contre le Cancer criteria) were selected for this study. Patient data were collected retrospectively from hospital records. Patients with another primary malignancy, or patients who deceased within 30 days after surgery were not eligible for this study. Colon tumors were defined as those originating 15 cm above the anal verge; rectal tumors were defined as those distal to this site. Right-sided tumors were defined as those originating proximal to the splenic flexure and left sided as those arising distal to splenic flexure.
**Immunohistochemistry**

Tissue sections were immunostained using antibodies against a caspase-cleaved product of cytokeratin 18, (clone M30, Roche diagnostics, Germany, 1:800)\(^1\). Staining of mismatch repair proteins was performed using anti-PMS2 (clone A16-4; 1:50; BD Biosciences) and MLH-1 antibodies (1:50, Zymed laboratories, San Francisco, USA). Paraffin sections (4 μm) were prepared on aminopropylethoxysilane (APES) coated slides, and dried overnight at 37°C. Tissue sections were deparaffinized in xylol and subsequently rinsed in ethanol. Endogenous peroxidase was blocked by 0.3% hydrogen peroxidase-methanol for 20 min. After being immersed in alcohol the sections were rehydrated. Antigen retrieval was performed by boiling the sections in 10mM citrate buffer (pH 6.0) for 10 min, after which the sections were allowed to cool down in this buffer for 2 hours at room temperature. Subsequently the sections were washed in demin water and phosphate buffered saline (PBS). Sections were incubated overnight at room temperature with the previously mentioned antibodies. All antibodies were diluted in PBS with 1% Bovine Serum Albumin (PBS/BSA). After three washing steps of 5 minutes in PBS, sections were incubated for 30 minutes with SWaR/biotin or RaM/ biotin (1:400; DAKO, Glustrup, Denmark). The sections were washed in PBS, followed by 30-minute incubation with Streptavidin-Biotin-Complex (SABC) (1:100; DAKO, Denmark). The sections were then washed in, rinsed in 0.05M Tris/HCl-buffer (pH 7.6) and developed in a 3.3 dianinobenzidine tetrahydrochloride (DAB) with 0.002% hydrogen-peroxide for 10 minutes. Sections were counterstained with haematoxylin, dehydrated with ethanol, cleared in xylene and mounted with pertex. A negative control was included for each tumor sample by using PBS/BSA instead of the before mentioned antibodies in the overnight incubations.

**Quantification of Immunohistochemistry**

PMS2 and MLH-1 immunohistochemical staining can be used to distinguish between MSI and MSS\(^2\). Nuclear PMS2 and MLH-1 analysis was done by a pathologist (H. M.). Tumor cells were scored as positive or negative for PMS2 MLH-1 on condition that the internal stromal control was positive. M30 immunoreactivity on all slides was evaluated by two independent observers (N.G.E. and R.I.J.M.A.) as previously described\(^3\). In brief, the apoptotic index was documented as the number of M30-positive cells per square millimeter of tumor cells and was counted in randomly chosen high-power fields (25 per tumor) at a 200x magnification. For survival analyses the median apoptotic index was used as a cut-off point for low and high number of apoptotic cells. All observers were blinded to clinical outcome. Two tumors were not assessable after immunohistochemical staining, due to technical failures, unavailability of tumor material or excessive tumor necrosis.

**Statistics**

Of the 104 primary tumor specimens 8 patients received preoperative radiotherapy and were excluded from this study. Kruskal-Wallis, Mann-Whitney’s U-test and Spearman’s rho analyses were used to analyse correlation between number of apoptotic cells, clinicopathological features, immunological parameters and mutational pathway. For survival analysis Kaplan-Meier analysis was used and differences between the survival curves were analysed using the log-rank
test. Events for time to recurrence, disease-free and overall survival were defined as follows: time from surgical resection to disease relapse, time from surgical resection to disease relapse or death, and time from surgical resection to death respectively. Cox’s stepwise proportional hazard regression models were used for multivariate analyses of apoptosis, MSI and clinicopathological features. The statistical package SPSS version 12.0 (SPSS Inc, Chicago, IL) was used to conduct statistical analyses. A p-value < 0.05 (two-tailed) was considered as indicating a statistical significant result.

Results

Patient and tumor characteristics

The patient and tumor characteristics are summarized in Table 1. Patients had a median follow-up of 5.9 years (range, 0.1-18.6 years; SD, 5.2 years). Tumors in this study were obtained from a randomly selected group of 96 non-irradiated patients presenting with stage II and III colorectal carcinoma. The average age of the patients in this study was 66.9 years (range, 26.0-85.0 years). In this study 14 of 96 were negative for PMS2 or MLH-1 and thus considered microsatellite instable (MSI) the remaining 90 were considered to be microsatellite stable (MSS).

Figure 1

Representative immunohistochemical staining of apoptotic tumor cells, and nuclear PMS2 expression in colorectal cancer specimens. Figure 1A Apoptotic tumor cells were detected with M30 monoclonal antibody. Arrows indicate examples of apoptotic epithelial cells. Arrowheads indicate apoptotic bodies, these were not counted. B MSI colon adenocarcinoma with loss of PMS2 expression. Nuclear staining of non-epithelial cells serves as internal positive control. C adenocarcinoma with normal nuclear PMS2 expression.
Immunohistochemical analysis of apoptosis in colorectal cancer specimens and its relation to clinicopathological variables and survival

Figure 1A shows a representative tumor specimen after immunohistochemical staining with the M30 monoclonal antibody. Cells from epithelial origin, positive for staining with the M30 monoclonal antibody were clearly recognizable in the tumor tissue. These positive cells represent cells in the first part of the degradation phase of apoptosis (Fig 1.). The median number of apoptotic cells per square mm tumor epithelial cells was 4.2 cells/mm².

To evaluate whether apoptosis and clinicopathological features were associated, the number of apoptotic cells was correlated to each feature. Number of apoptotic tumor cells was not associated with any of the clinicopathological variables (table 1).

Table 1  Patient characteristics and association with levels of apoptosis (94 patients)

<table>
<thead>
<tr>
<th></th>
<th>No. of patients (%)</th>
<th>Number of apoptotic cells (cells/mm²)</th>
<th>p-value</th>
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<tr>
<td></td>
<td></td>
<td>Median (IQR)</td>
<td></td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>56(60)</td>
<td>4.8 (1.9-8.7)</td>
<td>0.81*</td>
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<tr>
<td>Female</td>
<td>38(40)</td>
<td>3.9 (1.3-8.6)</td>
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<tr>
<td>Age (median [range])</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≤ median</td>
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<td></td>
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<tr>
<td>&gt; median</td>
<td>(67.5[26-85])</td>
<td>3.0 (1.6-8.8)</td>
<td>0.20*</td>
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<td></td>
<td>48 (50)</td>
<td>5.2 (2.6-8.6)</td>
<td></td>
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<tr>
<td>Tumor location</td>
<td></td>
<td></td>
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<tr>
<td>colon</td>
<td>61(66)</td>
<td>3.7 (1.6-9.6)</td>
<td>0.80*</td>
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<tr>
<td>rectum**</td>
<td>33(34)</td>
<td>5.5 (2.4-7.9)</td>
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<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>II</td>
<td>55(59)</td>
<td>4.7 (2.0-9.6)</td>
<td>0.36*</td>
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<tr>
<td>III</td>
<td>39(41)</td>
<td>4.6 (1.7-8.5)</td>
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<tr>
<td>Grade of differentiation</td>
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<tr>
<td>poor</td>
<td>54(57)</td>
<td>4.2 (1.7-8.6)</td>
<td>0.81†</td>
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<tr>
<td>moderate</td>
<td>21(23)</td>
<td>5.4 (1.3-8.4)</td>
<td></td>
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<tr>
<td>well</td>
<td>19(16)</td>
<td>5.1 (1.7-21.7)</td>
<td></td>
</tr>
<tr>
<td>MSI</td>
<td>13(15)</td>
<td>4.2 (2.7-8.4)</td>
<td>0.53*</td>
</tr>
<tr>
<td>MSI</td>
<td>81(85)</td>
<td>4.6 (1.6-8.7)</td>
<td></td>
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<td>MSS</td>
<td></td>
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*Mann-Whitney, †Kruscal-Wallis

Table 1  Characteristics of the patients 96 investigated in the present study. Apoptosis was defined as number of apoptotic cells/mm², 2 patients were not evaluable after immunohistochemistry and excluded from further analyses. Apoptosis levels were evenly distributed amongst patient characteristics.

Prognostic significance of tumor cell apoptosis and MSI status

We analysed the number of apoptotic tumor cells in relation with patient survival data. As is shown in figure 2, tumor specimens with a relatively high number of apoptotic cells were bor-
APOPTOSIS IS A POOR PROGNOSTIC FACTOR IN COLORECTAL CANCER

derline significantly associated with high recurrence rates (p=0.09, figure 2A) and significantly associated with a poor disease free survival (p=0.005, figure 2B). MSI tumors were associated with low recurrence rates (p=0.05, figure 2C); this did however not translate into a survival benefit (p=0.59, figure 2D).

When separately analysed in the colon (n=63) and rectal (n=33) tumors, high number of apoptotic tumor cells were associated with a poor DFS in both colon (p=0.04) as well as in rectal (p=0.05) tumors (data not shown).

Figure 2A Apoptosis, TTR

Figure 2B Apoptosis, DFS

Figure 2C MSI, TTR

Figure 2D MSI, DFS

Prognostic value of apoptosis and microsatellite instability on time to recurrence (TTR) and DFS in 96 colorectal cancer patients. The continuous line represents a relative low number of apoptotic cells per square millimeter (≤ median), the dotted line a relative high number (> median). 2A: High levels of apoptosis was associated with increased recurrence rates in colorectal cancer (p=0.09). 2B: High levels of apoptosis was associated with a poor DFS in colorectal cancer specimens (p=0.005). 2C: Microsatellite instability was associated with low recurrence rates in colorectal cancer (0.05). 2D Microsatellite instability did not have a significant impact of DFS in colorectal cancer (p=0.59)
All parameters with a p-value ≤0.10 in the univariate analysis were subjected to a multivariate analysis using a Cox proportional hazards model, to evaluate whether apoptosis showed prognostic significance for tumor recurrence, independent of other factors. Tumor stage, tumor cells apoptosis and microsatellite stability status were included in the analysis. A relatively high number of apoptotic cells (p=0.05, HR: 2.1 CI: 0.98-4.78) and advanced tumor stage (p=0.006, HR 3.0 CI: 1.37-6.63) retained their strength as independent prognostic variables for tumor recurrence (table 2). In addition apoptosis was an independent prognostic factor for disease free survival (p=0.003, HR 2.2 CI: 1.32-3.94). Therefore, these results indicate that the number of apoptotic cells in a tumor resection specimen is highly prognostic in colorectal cancer.

Discussion

We demonstrate in the current study that colorectal tumors with a high number of apoptotic tumor cells show a poor prognosis. This negative impact of high number of apoptotic cells on patient survival is confirmed by several other studies5-7. A large number of studies have focused on the prognostic significance of the number of apoptotic cells in colorectal cancer, however, the results of these studies are often contradictory13. The inconsistencies in observed impact of apoptosis on clinical outcome can partly be explained by the different methods for detecting apoptosis and partly by the differences between the evaluated patient populations. Several techniques are employed to detect apoptosis, including TUNEL assay14-16, flow cytometry6, pro- and anti-apoptotic protein activity5,17 or the measurement of caspase-3 activity in tumor lysates7. The limitation of most of these methods is that they reflect apoptosis of both stromal cells as well as tumor cells. The use of the monoclonal antibody M30 overcomes these limitations as the antibody is directed against the apoptosis-induced cleavage product of cytokeratin 18. This protein is only present in epithelial cells and allows a highly reproducible discrimination of apoptotic tumor cells from other cell types3,8. The evaluation of apoptosis with immunohistochemical M30 expression provided information about apoptosis on a cellular level and is sensitive to slight changes in expression levels, thus yielding an accurate evaluation3,8.

<table>
<thead>
<tr>
<th>Table 2  Cox proportional hazards model (first model) for Time to Recurrence (TTR)</th>
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<tbody>
<tr>
<td>Variables</td>
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<tr>
<td>----------------------------------</td>
</tr>
<tr>
<td>Microsatellite stability</td>
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<tr>
<td>Tumor stage</td>
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<tr>
<td>Apoptosis</td>
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The type of treatment, including preoperative therapy and type of surgical resection, may a point that should also be taken into account when discussing the impact of the number of apoptotic tumor cells in a resection specimen. Considering the different studies, the progno-
Apoptosis is a poor prognostic factor in colorectal cancer. Much of the published literature has evaluated apoptosis in resected colorectal tumor specimens, regardless of adjuvant treatment or type of surgery. However, the introduction of new surgical techniques as the Total Mesorectal Excision and neoadjuvant treatment has greatly improved the clinical outcome of colorectal cancer, but certainly will influence apoptosis in resected tumor material. Not only preoperative radiotherapy, but also surgical resection has been shown to induce apoptosis as levels of apoptosis were significantly higher in resected tumors than in preoperative biopsies. A recent study evaluated apoptosis in tumors of patients included in the Dutch TME trial and showed by M30 immunostaining that low numbers of apoptotic tumor cells in rectal cancer were associated with high local recurrence rates. Our study showed different clinical behavior for patients with high numbers of apoptotic tumor cells when operated with conventional surgery for rectal cancer as was used before TME surgery was introduced. In addition, the number of apoptotic cells after TME surgery was much higher than the number we found in the current study. Together, these results indicate that type of surgery highly influences tumor cell apoptosis. Therefore, the clinical impact of tumor cells apoptosis should only be considered in the context of patient's treatment.

The mechanism behind the negative impact of high number of apoptotic cells on DFS in the present study could be explained by disturbance in the structural cohesion of the tumor by the presence of apoptotic tumor cells. Disturbance in the structural cohesion may result in an increased dissemination of tumor cells, and thus more tumor cells in blood and lymphatic vessels and thus a higher change of developing distant metastases.

The favorable clinical outcome of MSI tumors as seen in the current study has been reported previously and has been attributed to increased immune cell infiltration in such cancers. MSI in the current study was not associated with increased tumor cell apoptosis. This lack of association was also apparent in a study evaluating apoptosis and tumor infiltrating lymphocytes in cancer specimens with defective MMR genes. Although MSI tumors in several studies were associated with increased numbers of tumor infiltrating lymphocytes, they also did not find increased numbers of apoptotic cells. These studies concluded that the level of apoptosis in MSI cancers is spontaneous in nature. Our study is in line with these data and shows that apoptosis reflects clinical tumor behavior.

In conclusion, the current study demonstrates that high numbers of apoptotic tumor cells in resection specimens is a poor prognostic factor for patient survival in colorectal cancer and also that apoptosis and MSI are independent mechanisms that determine patient outcome. Furthermore, tumor cell apoptosis should be considered in the context of the preoperative and surgical treatment of the patient.
References


