Prognostic Value of Apoptosis in Rectal Cancer Patients of the Dutch Total Mesorectal Excision Trial: Radiotherapy Is Redundant in Intrinsically High-Apoptotic Tumors

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Abstract

Purpose: The combination of radiotherapy and good quality surgery reduces local recurrence rate for rectal cancer patients. This study assesses the prognostic value of both intrinsic and radiotherapy-induced apoptosis and evaluates the relevance of radiotherapy for outcome of rectal cancer patients.

Experimental Design: Tumor samples (1,198) were available from the Dutch Total Mesorectal Excision trial, in which rectal cancer patients were treated with standardized surgery and randomized for preoperative short-term radiotherapy or not. Tumor samples were obtained at time of surgery. Tissue microarrays were constructed and stained with the active caspase-specific M30 antibody to determine the amount of apoptotic epithelial tumor cells.

Results: Nonirradiated patients with a negative circumferential margin displaying lower than median levels of apoptosis developed more local recurrences (10.5% versus 6.1%; \( P = 0.06 \)) and more rapidly after surgery than patients with high intrinsic apoptosis in their tumors (median time to recurrence, 13.0 versus 21.3 months; \( P = 0.04 \)). In multivariate analysis, intrinsic apoptosis was an independent predictor for the development of local recurrences (hazard ratio, 2.0; \( P = 0.05 \)). Radiotherapy increased apoptosis level (11 versus 23 apoptotic cells/mm\(^2\) tumor epithelium; \( P < 0.001 \)), but this apoptosis did not influence patients’ prognosis.

Conclusions: Rectal cancer patients with low intrinsic apoptosis will benefit from radiotherapy with respect to the development of local recurrences. Because apoptosis is an inherent characteristic of tumors, patients who do not need radiotherapy may be selected based on the apoptotic index of the primary tumor.

Local recurrences are a serious problem in the treatment of rectal cancer. To improve local control, total mesorectal excision (TME) surgery has been introduced (1). In addition to TME, preoperative radiotherapy resulted in a further reduction of local recurrences (2) and has been accepted recently as standard treatment for rectal cancer patients in the Netherlands.

Although radiotherapy can prevent local recurrences in some patients, the majority of patients will not develop a recurrence without radiotherapy. Moreover, radiotherapy has several negative side effects (3, 4), emphasizing the need for prognostic indicators to select patients most likely benefiting from radiotherapy.

Because radiotherapy is known to induce DNA damage, which can result in apoptotic cell death, the better local control observed after radiotherapy might result from the induction of apoptosis. During tumor development, tumor cells can be triggered by lymphocytes of the patient’s immune system or other stress factors, such as growth factor deprivation, to undergo apoptosis (5, 6). However, tumor cells that acquired multiple antiapoptotic mutations fail to undergo apoptosis, resulting in selective outgrowth of these cells. The level of apoptosis, both intrinsic and after radiotherapy, might therefore have a prognostic value for the clinical outcome of rectal cancer patients.

Several publications have described the relevance of apoptosis for the clinical outcome of colorectal cancer patients (7–12). Whereas some studies reported a better prognosis for patients with high levels of intrinsic apoptosis, others could not find such a correlation or showed the opposite. Therefore, it is still not clear whether apoptosis has a prognostic value for rectal cancer patients. To obtain a conclusive answer, the
The two observers.

Intrinsic and radiotherapy-induced apoptosis has been evaluated in a large number of rectal cancer patients included in a prospective multicenter trial with standardized preoperative radiotherapy, TME surgery, and pathologic examination (2).

Patients and Methods

Patient population. Tumors were derived from the Dutch TME trial, in which patients were randomized to receive radiotherapy or not before surgery according to a standardized TME protocol (2). Radiotherapy consisted of five fractions of 5 Gy over 5 to 7 days followed by surgery within 1 week. Pathologic examination was done according to a standardized procedure as described by Quirke et al. (13).

Tissue microarray preparation. Tissue microarrays (TMA) were constructed with a tissue arrayer using a 2-mm-diameter punch (Beecher Instruments, Silver Spring, MD). Tumor sections were marked on H&E-stained slides of the primary tumors by two pathologists (I.D.N. and J.H.J.M.v.K.). Three punches from each tumor were sampled in the TMAs, resulting in an area identical to 10 high-power fields (10 mm²), shown previously to be sufficient to overcome heterogeneity (11).

Tumor selection. Paraffin-embedded tumor was obtained at time of surgery from 1,208 patients. After constructing TMAs, 10 samples did not contain tumor epithelium. The following patients were excluded: ineligible patients (n = 31), tumor-node-metastasis (TNM) stage IV tumors (n = 68), patients who received <5 × 5 Gy or >5 × 5 Gy (n = 28), or for whom the interval between radiotherapy and operation exceeded 7 days (n = 4), leaving 560 nonirradiated and 507 irradiated patients for the apoptosis quantification. For recurrence analyses, patients with a positive circumferential resection margin (CRM; ≤1 mm) were excluded, leaving 465 nonirradiated and 420 irradiated patients with a median follow-up of 65.0 months.

Immunohistochemistry. For quantification of apoptosis, 4-μm section of the TMAs were stained with the M30 antibody (Roche Diagnostics, Almere, the Netherlands) as described previously (11). This antibody recognizes a caspase-specific cleaved product of cytokeratin-18 and is thereby specific for apoptotic epithelial cells (Fig. 1). Apoptotic cells were counted per square millimeter tumor epithelium. The level of apoptosis was determined by two investigators (E.C.d.B. and S.v.d.P.). Because of the reproducibility in the first 10% of the samples (Spearman’s ρ = 0.90 for continuous data; Cohen’s κ = 0.75 after categorization into high and low apoptosis, indicating an agreement of 88.9%), the samples thereafter were scored by one of the two observers.

Statistical analysis. All analyses were done with SPSS statistical software (version 11.0 for Windows, SPSS, Inc., Chicago, IL). Mann-Whitney U tests were used to compare continuous variables. Kaplan-Meier analyses were done to calculate recurrence rates, with the time of surgery as entry date. To guarantee sufficient number of patients in both groups, dichotomization at the median level of apoptosis per treatment group has been used. Cox regression analyses were used to calculate hazard ratios with 95% confidence intervals.

Results

Patient characteristics. The characteristics of the patients are summarized in Table 1. Clinical data were equally distributed in both randomization groups. However, the 5-year local recurrence risk was significantly lower for the irradiated patients (5% versus 10%; P < 0.001). No difference in distant recurrence rates could be observed (P = 0.36).

Quantification of intrinsic and radiotherapy-induced apoptosis. The median number of M30-positive cells was 11 cells/mm² tumor epithelium in the nonirradiated tumors and 23 cells/mm² tumor epithelium in the irradiated tumors (P < 0.001), showing an induction of apoptosis by radiotherapy. After radiotherapy, more tumors displayed a relatively high level of apoptosis, but tumors with hardly any apoptotic cell were present as well (Fig. 2A).

Because the number of days between the last fraction of radiotherapy and surgery was not the same for all patients, the distribution of the level of apoptosis was evaluated for each interval separately. No significant change in the level of apoptosis could be observed over the intervals (Fig. 2B), excluding that the variation in time might interfere with the M30 scores of the irradiated tumors.

Correlation of intrinsic apoptosis with recurrences. In nonirradiated patients, tumors with intrinsically low apoptosis (≤11 M30-positive cells/mm² epithelium) tended to have a higher local recurrence risk than high-apoptotic tumors (5-year risks, 10.5% versus 6.1%; P = 0.06; Fig. 3A). This trend was

Table 1. Characteristics of nonirradiated and irradiated patients

<table>
<thead>
<tr>
<th></th>
<th>RT− (n = 560)</th>
<th>RT+ (n = 507)</th>
</tr>
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<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
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<tr>
<td>Median age (y)</td>
<td>66</td>
<td>65</td>
</tr>
<tr>
<td>Males (%)</td>
<td>63</td>
<td>65</td>
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<tr>
<td>Primary tumor</td>
<td></td>
<td></td>
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<tr>
<td>TNM stage (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td>II</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td>III</td>
<td>39</td>
<td>36</td>
</tr>
<tr>
<td>Negative circumferential margin (%)</td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>Distant from anal verge (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥10 cm</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>40</td>
<td>42</td>
</tr>
<tr>
<td>&lt;5 cm</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td>Operation type (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low anterior resection</td>
<td>66</td>
<td>64</td>
</tr>
<tr>
<td>Abdominoperineal resection</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Hartmann</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Recurrences, % at 5 y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Distant</td>
<td>29</td>
<td>25</td>
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</tbody>
</table>

Abbreviations: RT−, nonirradiated patients; RT+, irradiated patients.
confirmed by the difference in time to recurrence: the median
time to develop a local recurrence was 13.0 months for patients
with low-apoptotic tumors and 21.3 months for patients with
high-apoptotic tumors \( (P = 0.04) \). In addition, the correlation
between intrinsic apoptosis and the local recurrence risk was
stronger for TNM stage II and III tumors (5-year risks, 16.2% versus
8.8% for low-apoptotic tumors versus high-apoptotic
tumors; \( P = 0.03 \)). For these local recurrence analyses, patients
with positive CRM were excluded because positive CRMs
mainly result of suboptimal surgery with local failure as
consequence (14, 15). Inclusion of these patients diminished
the differences in local recurrence rates between low- and high-
apoptotic tumors (5-year risks, 11.9 versus 8.9%; \( P = 0.22 \)).

The level of intrinsic apoptosis was not correlated with
distant recurrences (5-year risks, 26.9 versus 21.5; \( P = 0.31 \)) or
overall recurrences (5-year risks, 29.7 versus 22.4; \( P = 0.11 \)),
although a positive trend was observed for high-apoptotic
tumors with regard to the overall recurrence rate (Fig. 3B).

A multivariate Cox regression analysis was done with the
level of intrinsic apoptosis, distance to anal verge, and TNM
classification as input variables. For nonirradiated patients,
both the level of apoptosis and TNM classification were
independent predictors for the development of local recurrences
(hazard ratio, 2.0; \( P = 0.05 \); Table 2).

**Correlation of radiotherapy-induced apoptosis with recurrences.**
Patients with high levels of apoptosis after radiotherapy
\( (>23 \text{ M30-positive cells/mm}^2 \text{ tumor epithelium}) \) had the same
local recurrence risk as patients with lower levels of apoptosis
(5-year risks, 3.7% versus 3.6%; \( P = 0.85 \); Fig. 3C). Even when
the data were dichotomized at the lowest quartile of apoptosis
\( (\leq 8 \text{ apoptotic cells/mm}^2 \text{ tumor epithelium}) \), there was no
association between apoptosis and local recurrences. The level
of radiotherapy-induced apoptosis was also not predictive for
distant recurrences (5-year risks, 20.9% versus 18.0%; \( P = 0.60 \))
or overall recurrences (5-year risks, 22.3% versus 19.3%; \( P = 0.58 \), Fig. 3D).

**Discussion**

In this study, we determined the prognostic value of intrinsic
apoptosis as well as apoptosis after radiotherapy in a large

![Fig. 2. Intrinsic apoptosis and apoptosis after radiotherapy. Box plots of the
levels of apoptosis in nonirradiated (RT−) and irradiated (RT+) patients (A) and the
level of apoptosis during several days after the last fraction of radiotherapy (B).
Because of non-Gaussian distribution, data are presented in box plots: lines, lowest
and highest quartiles (outliers were excluded from the figure); gray box, interquartile
range (25-75%); black bar, median. The N below the plots indicates the absolute
number of patients.](image1)

![Fig. 3. Recurrence rates since TME surgery for low-apoptotic tumors (black)
and high-apoptotic tumors (gray) of both nonirradiated (A, B) and irradiated (C, D)
patients. The N below the plots indicates the absolute number of patients at risk.](image2)
distant metastases rather than local control. Furthermore, colon cancer patients, recurrence-free survival depends on the difference in patients: rectal versus colorectal cancer patients. The standardized pathologic evaluation in our study enabled us to select patients without positive CRM involvement. The quantification of apoptosis in our study was done in tumors obtained at time of surgery. For clinical use, the correlation between preoperatively taken biopsies and operation specimen needs to be established. Lack of availability of such biopsies from patients of the TME trial hindered this comparison. Measuring M30-positive cells in patients’ serum can be an alternative method for apoptosis quantification (19, 20). Prospective validation of the predictive value of intrinsic apoptosis should therefore be combined with the challenge to assess the best method for preoperative quantification of apoptosis.

Currently, several tumor characteristics are known risk factors for local recurrences: positive CRMs, lymph node metastases, and tumors located within 10 cm from anal verge (14, 21, 22). Recent advantages in preoperative imaging techniques allow more adequate staging of the tumor, enabling preoperative selection of patients at high risk of a positive CRM for whom extensive radiotherapy is necessary (23, 24), and furthermore improved the quality of surgery (25, 26). These strategies will result in more patients with a negative CRM, in whom apart from TNM stage, other biological characteristics are important features for local recurrence risk. Therefore, identification of patients at high risk for local failure can be achieved by evaluation of the intrinsic apoptosis status of the primary tumor.

### Acknowledgments

We thank Paul Eilers (Department of Medical Statistics, Leiden University Medical Center, Leiden, the Netherlands) for advice and critical examination of the statistical analyses.

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### Table 2. Multivariate analysis of local recurrences in nonirradiated patients

<table>
<thead>
<tr>
<th>TNM stage</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>4.2 (1.1-16.0)</td>
<td>0.03</td>
</tr>
<tr>
<td>III</td>
<td>10.3 (3.1-34.3)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Distance from anal verge</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>&lt;5 cm</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-10 cm</td>
<td>2.0 (0.8-4.7)</td>
<td>0.12</td>
</tr>
<tr>
<td>Low</td>
<td>≥10 cm</td>
<td>0.9 (0.3-2.6)</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; 95% CI, 95 confidence interval.
References


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