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

Elza C. de Bruin,1,3 Cornelis J.H. van de Velde,2 Simone van de Pas,1 Iris D. Nagtegaal,5 J. Han J.M. van Krieken,5 Marleen J.E. M. Gosens,5 Lucy T.C. Peltenburg,1 Jan Paul Medema,1,3 and Corrie A.M. Marijnen1,4

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/mm² 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...
Radiotherapy Redundant in High-Apoptotic Rectal Tumors

Prognostic value of 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.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 section. 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 k = 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>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median age (y)</td>
<td>66</td>
<td>65</td>
</tr>
<tr>
<td>Males (%)</td>
<td>63</td>
<td>65</td>
</tr>
<tr>
<td>Primary tumor</td>
<td></td>
<td></td>
</tr>
<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>28</td>
<td>28</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>
</tr>
</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 M30-positive cells/mm² 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 (<8 apoptotic cells/mm² 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
Intrinsic apoptosis seemed to predict local failure; in difference in patients: rectal versus colorectal cancer patients. Therefore, we believe that investigations of the predictive value of intrinsic apoptosis was statistically significant for TNM stage II and III tumors. Of clinical importance, the low local recurrence rate of nonirradiated patients with high levels of apoptosis suggests that radiotherapy was redundant in these intrinsically high-apoptotic tumors.

To our knowledge, there is very little data on intrinsic apoptosis in early-stage rectal cancer. Moreover, these data are inconclusive, as Adell et al. (7) reported similar prognostic values for intrinsic apoptosis in a study of 162 irradiated and nonirradiated patients, and Schwandner et al. (8) did not establish a prognostic value in 160 patients. Our apoptosis data extend the observations of Marijnen et al. (11), who quantified a selection of tumors of the TME trial but thereby precluded extrapolation toward the general population of rectal cancer patients. For colorectal cancer patients, studies have been published that could not confirm a relation between intrinsic apoptosis and survival (9) or even found the opposite: a better prognosis for low-apoptotic tumors (10, 12).

Several reasons might explain these inconsistent findings. For instance, suboptimal surgery can obscure the prognostic value of apoptosis. In rectal cancer, suboptimal surgery leads to a high local recurrence risk mainly due to a positive CRM (14–16). The fact that suboptimal surgery can disturb the predictive value of apoptosis is also suggested by our observation that inclusion of CRM-positive tumors decreased the difference in local recurrences between high- and low-apoptotic tumors. Therefore, we believe that investigations of the predictive value of biological tumor characteristics (such as intrinsic apoptosis) are only of value when adequate surgery has been done. The standardized pathologic evaluation in our study enabled us to select patients without postoperative CRM involvement.

Another reason for the inconsistency in literature can be the difference in patients: rectal versus colorectal cancer patients. Low intrinsic apoptosis seemed to predict local failure; in colon cancer patients, recurrence-free survival depends on distant metastases rather than local control. Furthermore, patients’ outcome can be influenced by adjuvant treatment. In the study of Schwandner et al. (8), high-risk patients received chemoradiotherapy after surgery, influencing both local and distant recurrences and therefore disturbing the prognostic value of intrinsic apoptosis.

An increased number of apoptotic cells was observed when patients were irradiated. The intriguing observation that the level of apoptosis was quite stable during several days suggested that the induction of apoptosis is an ongoing process. Therefore, radiotherapy can result in a better local control without clear induction of caspase-dependent cell death as detected with the M30 antibody. Probably, other types of cell death were induced as well, such as caspase-independent cell death or mitotic catastrophe, which both result in reduced clonogenic survival (reviewed by ref. 17).

Because the M30 antibody does not detect these types of cell death, M30 staining likely underestimates the total amount of cell death induced by radiotherapy.

Although radiotherapy reduces the local recurrence rate, it is clear that not every rectal cancer patient needs radiotherapy. A recent meta-analysis of the Colorectal Cancer Collaborative Group showed a 57% reduction in local recurrences by preoperative radiotherapy (BED2\(z_{30}\); ref. 18). For patients with intrinsically high-apoptotic tumors, displaying a local recurrence risk of 6%, the absolute reduction will therefore only be 2% to 3%. This reduction does not counterbalance the negative side effects of radiotherapy as decreased sexual function, impaired daily activity, and increased long-term bowel dysfunction (3, 4) and shows that preoperative selection of patients is an absolute must.

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.

**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 (2.1-34.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>2.0 (1.0-4.1)</td>
<td>0.05</td>
</tr>
<tr>
<td>Distance from anal verge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5 cm</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5-10 cm</td>
<td>2.0 (0.8-4.7)</td>
<td>0.12</td>
</tr>
<tr>
<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


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

Elza C. de Bruin, Cornelis J.H. van de Velde, Simone van de Pas, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/21/6432

Cited articles
This article cites 26 articles, 5 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/12/21/6432.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/12/21/6432.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.