Preoperative radiotherapy for rectal cancer can significantly improve patient survival and reduce local recurrence rates versus postoperative radiation or surgery alone (1–4). Additionally, high-dose-rate preoperative conformal endorectal brachytherapy, a novel therapeutic approach to the treatment of invasive rectal cancer, may result in more frequent tumor down-staging or complete tumor regression, leading to a greater number of sphincter-sparing procedures (5, 6). The ability to predict tumor response before treatment may significantly impact the selection of patients for preoperative radiotherapy as well as potentially modify postoperative treatment plans.

It is now recognized that the differential expression of genes governing cell cycle arrest and apoptosis is an important determinant of radioresponse (7, 8). In normal cells, the p53 tumor suppressor gene mediates both cell cycle arrest and apoptosis through the transcriptional activation of p21, BCL-2, and BAX among others (9). In response to DNA damage, p53 enhances the transcription of p21, a cyclin-dependent kinase inhibitor that delays the progression of cells from G1 to S phase of the cell cycle, thereby preventing the replication of damaged DNA (10). p21 has been associated with radiosensitivity and improved outcome in rectal tumors following preoperative radiotherapy (11–13).

Mutations of p53 in rectal cancer have been linked to decreased survival and aggressive malignant behavior (14, 15). Kandioler et al. (16) showed, by DNA sequencing, that p53 mutations were predictive of lower survival rates and decreased response to preoperative radiotherapy. Similar studies using immunohistochemistry to detect p53 protein yield contradicting results (17–20).

p53 may alter angiogenesis by activating vascular endothelial growth factor (VEGF), a potent mediator of new blood vessel formation in tumorogenesis (21, 22). Expression of VEGF is induced by other factors as well, most notably hypoxia (23). In situ hybridization studies have found that transcription of VEGF mRNA in rectal tumors is up-regulated during the progression from adenoma to carcinoma (21, 22, 24, 25). Anti-VEGF therapy, in combination with chemotherapy and/or radiotherapy for rectal cancer, is an area of active investigation (26, 27).

Disruption of mitochondrial function and release of cytochrome c are early events in the apoptotic cascade (28). In the cytoplasm, cytochrome c associates with APAF-1, initiating the downstream cleavage of caspases and eventually resulting in cell death (28, 29). Although little is known about APAF-1 function, loss or mutation of APAF-1 has been associated with radioresistance in several tumor types (30).
Bcl-2, an antiapoptotic protein inhibiting release of cytchrome c and activation of APAF-1, is induced by VEGF and may play a role in determining radioreponse (28–30).

In this study, VEGF, Bcl-2, p21, p53, and APAF-1 in pretreatment rectal biopsies from patients undergoing preoperative conformal high-dose rate brachytherapy (5) were evaluated by immunohistochemistry. Classification and regression tree (CART) methods were then used to assess the value of each protein in predicting tumor response.

**Patients and Methods**

This study was approved by the Research Ethics Committee of the McGill University Health Center and informed written consent was obtained from 62 patients with rectal adenocarcinoma. Clinical staging according to the International Union against Cancer classification was carried out by both endorectal ultrasonography and magnetic resonance imaging. On the occasion of a disagreement between methods, the highest stage was assigned. Patients with abdominal nodal disease were excluded from the study as were patients with distant metastases. Three patients had cT4 tumors, one had cT4a, and 58 were cT4b. Radiation was delivered preoperatively with an eight-channel endorectal catheter using a high-dose rate remote after-loading system. A daily fraction of 6.5 Gy was administered over 4 consecutive days to a total of 26 Gy. Each patient was planned using a computed tomography simulator to obtain optimal conformal dosimetry. The dose was prescribed to a clinical target volume that included the gross tumor volume and any intramesorectal deposits visible at magnetic resonance imaging. Patients underwent cancer-directed surgery 4 to 8 weeks after brachytherapy regardless of tumor response.

Tumors were classified as responsive (complete or partial response) or nonresponsive to brachytherapy based on the pathologic evaluation of the specimen postoperatively. Complete response was defined as histologic evidence of residual viable carcinoma (ypT0). Partial response was characterized by the presence of at least one microfoci of residual carcinoma. Microfoci ranged from 0.3 to 0.9 cm in diameter. Nonresponsive tumors consisted of larger areas of residual carcinoma, rather than microfoci, that could be identified macroscopically and ranged in size from 2 to 6 cm.

**Immunohistochemistry.** Immunohistochemistry was used to detect p53, p21, Bcl-2, VEGF, and APAF-1 from pretreatment tumor biopsies. Formalin-fixed, paraffin-embedded serial sections were cut at 3 μm and dried at 37°C overnight. Immunohistochemistry was done using the avidin-biotin complex procedure, including heat-induced epitope retrieval and enzymatic antigen retrieval procedures. Incubation was carried out overnight at 4°C for p21 (clone SX118, 1:100; DAKO, Glostrup, Denmark), Bcl-2 (clone 124, 1:100; DAKO), and VEGF (VEGF-A20, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and in a moist chamber at 37°C for 1 hour for p53 (clone DO-7, 1:100; DAKO) and APAF-1 (NCL-APAF-1, 1:100; Novocastra, Newcastle, United Kingdom). Negative controls were treated identically with primary antibodies omitted. Positive controls consisted of tissue known to contain the protein of interest. Immunostaining was scored as a percentage of positive tumor cells by two independent observers.

**Statistical model.** CART methods were used to determine which proteins best predicted response to treatment (31). The CART trees were fit using the R statistical software tree library package (R Foundation for Statistical Computing, 2004, Vienna, Austria). The best tree fit to the full data has eight terminal nodes (tree not shown) with an overall misclassification rate of 16% (10 of 62).

To assess the amount of overfitting, we did 1,000 10-fold cross-validation experiments (32). In each of those 1,000 experiments, the data set was randomly split into 10 smaller data sets and a pruning method was used to choose the best number of nodes for the original tree pruned with respect to 90% of the data according to the misclassification rate for the other 10% of the data. Although the best average misclassification rate across 1,000 simulations was for five terminal nodes, the difference between five terminal nodes and one terminal node was very small (<1%). With further exploration, we found that average classification rate for one terminal node is primarily due to high variance resampling the small number of patients with zero traces of VEGF in the biopsy. With the reasonably large percentage of responsive tumors in the data set, many resampled data sets consisted primarily of responsive tumors (which made trees with one terminal node competitive with five terminal nodes in terms of misclassification rates).

To resolve the uncertainty in assessing the optimal number of terminal nodes for the full data set, we conducted a two-tailed Fisher’s exact test (33) to test for a relationship between the absence/presence of VEGF and response/nonresponse to treatment (Table 1). The P value for the Fisher’s exact test was <0.03, indicating a significant relationship between absence/presence of VEGF and response/nonresponse to treatment. Because of the instability of the full cross-validation due to the large effect of VEGF but the small number of subjects with negligible VEGF, we removed those 10 observations from the subsequent CART analyses. We fit a new classification tree with the remaining 52 observations and, using 100 10-fold cross-validation experiments, obtained an optimal tree with four terminal nodes. An average cross-validated 22% misclassification rate on the four-node subtree was observed conditioning on positive VEGF levels. We want to emphasize that the best number of terminal nodes for full data set is five and that our subanalysis using Fisher’s exact test is merely to confirm that there is strong evidence that VEGF can be used to predict responsiveness to tumors and moderately strong evidence that the remainder of the splits in our five-node tree can improve classification rates beyond that first split.

**Results**

Postoperative pathologic evaluation of the irradiated tumor bed gave rise to 43 responsive tumors (20 with complete response and 23 with partial response) and 19 nonresponsive tumors. The tumor stage distribution before and after brachytherapy may be found in Table 2. Cytoplasmic immunoreactivity for VEGF, APAF-1, and Bcl-2 ranged from 0% to 100% tumor cell staining. Nuclear immunoreactivity for p53 and p21 varied from 0% to 100% and from 0% to 40% tumor cell staining, respectively.

Of the five proteins initially selected for their potential predictive value, only VEGF, Bcl-2, and p21 contributed to the classification of responsive and nonresponsive tumors (Fig. 1). All 10 tumors with no VEGF immunoreactivity were completely responsive to therapy (ypT0). Those with >25% VEGF expression were further subdivided by the percentage of positive tumor cell staining for Bcl-2 and p21. A high classification rate was reached for tumors with no Bcl-2 and <92.5% immunostaining for VEGF. Such tumors were responsive to therapy in over 85% of cases.

<table>
<thead>
<tr>
<th>VEGF above 0</th>
<th>Response to treatment</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Yes</td>
<td>19</td>
<td>33</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>43</td>
</tr>
</tbody>
</table>

**NOTE:** P value for Fisher’s exact test of independence < 0.03.
whereas those with greater VEGF levels were largely nonresponsive (71%). Less efficient discrimination was observed in Bcl-2–positive tumors. Of the 10 Bcl-2–positive tumors, 8 had <1.5% tumor cell staining for p21.

Discussion

As tumors grow, their requirement for oxygen and nutrients expands beyond the limit of oxygen diffusion provided by the host vasculature (34). This creates a microenvironment of hypoxia in the central region of the tumor resulting in apoptosis in cells susceptible to low oxygen tension (35). Persistent hypoxic conditions lead to the production of VEGF (29). This cytokine serves as a mitogen for endothelial cells and activates proteolytic enzymes involved in the degradation of the basement membrane as well as the extracellular matrix (27). These processes ultimately result in the growth of a tumor vasculature. The new blood vessels are characterized by increased permeability, causing less efficient delivery of chemotherapeutic agents and decreasing response to radiotherapy (27, 29). Several studies have investigated serum VEGF levels as a prognostic marker in patients with colorectal cancer. A significant association between elevated serum VEGF and increased disease progression has been reported (36–38).

VEGF has also been shown to act on tumor cells by inducing Bcl-2 (27, 39). Early in the colorectal adenoma-carcinoma sequence, both VEGF and Bcl-2 seem to be up-regulated (25). In invasive cancer, VEGF levels increase whereas Bcl-2 expression may be significantly reduced (25, 40). Bcl-2 could, therefore, be important primarily in sustaining cell survival under initial hypoxic conditions until oxygen and nutrients can be reached via diffusion from newly formed tumor vessels. The presence of VEGF is likely an indirect reflection of the hypoxic state of the tumor.

Of the 10 tumors in this study that had no VEGF, all (100%) were responsive to radiotherapy. Absence of the protein may signify a well-oxygenated tumor that has not yet acquired the need for additional tumor vessels. Vascular permeability and partial oxygen pressure are maintained, thereby enhancing tumor response. Bcl-2–negative tumors with low levels of VEGF may not only be retaining their vascular permeability but might also be more susceptible to radiotherapy due to a lessened antiapoptotic signal. In this study, 85% of tumors with no Bcl-2 and with VEGF <92.5% were responsive to therapy. Nonresponsive Bcl-2–negative tumors with nearly all cells positive for VEGF may no longer require the survival advantage of Bcl-2 provided angiogenesis has already occurred.

Several studies have described both proliferation- and apoptosis-inhibiting roles for p21 (41). Others have reported an association between p21 in pretreatment rectal tumor biopsies and sensitivity to preoperative radiotherapy (11). In our study, p21-negative/bcl-2–positive tumors were largely nonresponsive to treatment (73%); p21-positive/bcl-2–negative tumors were generally associated with responsiveness (71%). However, due to the small number of tumors in our sample, it may be imprudent to draw a conclusion regarding p21 from these data.

There may be several factors confounding the results of this study. First, misclassification of clinical stages using magnetic resonance imaging for rectal cancer has recently been reported as high as 15% for pT3 tumors (42). More than 95% of patients included in this study were staged by magnetic resonance imaging as cT3. This may be an overestimation of the true number of T3 tumors in our sample. The results of this study may prove to be stage dependent. Second, protein expression in biopsies may not be representative of the entire tumor. p21-positive nuclei, for example, cluster and are typically concentrated in the upper one third of the colorectal mucosa. This may possibly be contributing to the inconclusive results involving p21 (43). Third, it is reasonable to assume

| Table 2. Tumor stage distribution before and after brachytherapy |
| T0 | T1 | T2 | T3 | T4 | Total |
| Pretreatment clinical stage (cT) | — | — | 3 | 58 | 1 | 62 |
| Postoperative pathologic stage (ypT) | 20 | 11 | 17 | 14 | 0 | 62 |

![Fig. 1. Optimal tree chosen by cross-validation after preliminary step classifying by the absence or presence of VEGF. The two sets of numbers underneath each terminal node are (number of observed nonresponsive subjects, number of observed responsive subjects) and (proportion of observed nonresponsive subjects, proportion of observed responsive subjects), respectively, for each terminal node.](image_url)
that the time delay between preoperative brachytherapy and surgery varies between patients. This difference may be affecting the pathologic diagnosis of response/nonresponse in these tumors postoperatively.

Despite these limitations, the results of this study suggest that VEGF and Bcl-2 status in pretreatment biopsies are important in predicting response of invasive rectal tumors to preoperative brachytherapy. Tumors absent for VEGF were associated with complete response to therapy. Those negative for Bcl-2 and with less than maximum immunoreactivity for VEGF were most frequently responsive to radiotherapy (85%).

Whether these results may be upheld across other treatment regimens, such as neoadjuvant radiochemotherapy, remains to be seen. There is evidence to suggest that VEGF, Bcl-2, and p21 may play a role in predicting tumor response to this therapy (44–46). It may, however, be important to tailor the selection of proteins used in the CART to incorporate other potential predictive markers specific to this treatment.

In conclusion, VEGF and Bcl-2 status in pretreatment tumor biopsies may prove to be an additional tool in patient selection for preoperative high-dose rate endorectal brachytherapy. A large-scale prospective study is necessary to validate these preliminary findings.

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A Predictive Model of Rectal Tumor Response to Preoperative Radiotherapy Using Classification and Regression Tree Methods

Inti Zlobec, Russell Steele, Nilima Nigam, et al.


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