Apoptotic and Mitotic Indices Predict Survival Rates in Lymph Node-negative Colon Carcinomas

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ABSTRACT

An imbalance between apoptosis and mitosis is believed to underlie colon cancer development and progression. These processes regulate the growth of normal and neoplastic epithelia, and in tumors, may confer prognostic information. To test this hypothesis, we determined apoptotic and mitotic indices (AI, MI) by morphology in H&E sections of 154 lymph node-negative, sporadic colon carcinomas. The relationship of these indices to genetic (p53 and Bcl-2) and biological features (DNA ploidy and cell kinetics) and patient survival rates was determined. Tumor features were compared in proximal and distal tumors, given postulated differences in their pathogenesis. Bcl-2 and p53 proteins were examined using immunohistochemistry and DNA ploidy and proliferative indices (PIs) by flow cytometry. Tumor features were dichotomized for analysis of relapse-free survival and overall survival (OS) rates using a Cox proportional hazards model. Median patient follow-up was 8.8 years. The median AI and MI were 1.2% (0–7.6) and 0.40% (0–1.8), respectively, and did not differ by tumor site. AI correlated with histological grade (P = 0.03); MI correlated with PI (P = 0.02) and inversely with Bcl-2 in distal tumors (P = 0.02), p53 and Bcl-2 expression were detected in 52 and 53% of tumors, respectively. Distal tumor site was associated with aneuploidy (P = 0.001), p53 (P = 0.001), and PI > 15% (P = 0.002). In a univariate analysis, colon cancers with high MIs (> 0.5%) had a poor prognosis (P = 0.04). Bcl-2 overexpression (> 20% + tumor cells) was associated with more favorable OS (P = 0.04). The association of ploidy and PI with outcome was of borderline significance for all tumors; however, diploidy predicted better survival in proximal cancers. In distal cancers, low AIs (< 0.25%) and high MIs (> 0.5%) were adverse prognostic markers. After adjustment for other variables, an increased MI predicted shorter OS with a hazard ratio (HR) for death of 2.70; 95% confidence interval (CI) was 1.23–5.91 (P = 0.01). Expression of Bcl-2 was associated with more favorable OS (HR, 0.46; 95% CI, 0.21–1.0; P = 0.06). In proximal cancers, Bcl-2 expression was the most important predictor of OS (HR, 0.17; 95% CI, 0.03–0.85; P = 0.03). In distal tumors, low AIs (HR, 3.33; 95% CI, 1.27–9.09; P = 0.01) and high MIs predicted poor survival. In conclusion, increased mitosis and low or absent Bcl-2 expression are significant risk factors for death in node-negative colon cancers, as are low rates of apoptosis in distal tumors. If validated prospectively, our results may identify patient subsets than can benefit from adjuvant chemotherapy.

INTRODUCTION

Colon carcinogenesis is characterized by a sequence of molecular genetic alterations that dysregulate mitosis and apoptosis (1). Epithelial homeostasis is maintained by a balance between these processes that when disturbed, can result in tumor development (2). Inhibition of apoptosis confers a growth advantage and blocks the elimination of genetically damaged cells, both of which contribute to tumor development and progression (3). We (4) and others (5) have shown that premalignant colorectal adenomas have an inverted gradient of apoptosis compared with normal mucosa, and that attenuated apoptosis accompanies adenoma to carcinoma progression (6). Furthermore, primary CRCs3 that had metastasized were found to have reduced AIs compared with those that did not (7). These studies demonstrate that dysregulated apoptosis is an important event during CRC development and progression. To date, a detailed analysis of the extent of spontaneous apoptosis and its relationship to mitosis and clinical outcome in human CRC has not been performed. Studies indicate that tumor stage is the most important prognostic variable in CRC (8). Despite this fact, considerable stage-independent variability in clinical outcome is observed that may be a consequence of altered rates of apoptosis and cell proliferation. Several reports indicate that tumor cell kinetics, as determined by flow cytometry, are an important prognostic

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3 The abbreviations used are: CRC, colorectal cancer; AI, apoptotic index; MI, mitotic index; PI, proliferative index; wt, wild type; mAb, monoclonal antibody; D.I., DNA index; RFS, relapse-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; IHC, immunohistochemistry; MSI, microsatellite instability; HNPPC, hereditary nonpolyposis colorectal cancer.
variable in CRC (9–11). Cell kinetics have generally been determined concurrent with analysis of DNA content. Alterations in DNA content, i.e., aneuploidy, have been shown in some, but not all, reports to predict adverse outcome in CRC (9–14). The MI is a measure of cellular proliferation that is distinct from the PI (S-phase + G2M) and can be determined in tissue sections at the time of quantifying apoptotic cells. Studics in breast cancer indicate that MIs yield results comparable with S-phase fraction (15). Furthermore, the MI has been shown in several solid tumors to be an independent prognostic variable (16–19).

Differences in the epidemiology and molecular genetics of proximal versus distal colon carcinomas have been reported. An age-dependent, proximal shift in the anatomical site of colon carcinomas has occurred over the past two decades (20, 21). Furthermore, the frequency of mutations in the p53 and K-ras genes were found to differ depending upon the site of the primary colon cancer (22–25). p53 mutation or protein overexpression has been detected in 45–75% of CRCs and in some primary colon cancer (22–25).

Bcl-2 expression with and to a lesser extent in carcinomas. To date, studies correlating apoptotic rates and with clinical outcome in colon carcinomas have produced conflicting results (33, 35–39).

We chose to study sporadic lymph node-negative colon carcinomas, given that approximately one-third of stage II patients will relapse and die of their disease (40). Studies have failed to demonstrate a survival advantage for stage II patients administered adjuvant chemotherapy (40). However, prognostic variables may identify a subset of these patients who can benefit from such treatment. We hypothesize that rates of apoptosis and mitosis determine tumor growth rates and may, therefore, be important prognostic variables. To address this issue, we determined the extent of spontaneous apoptosis and mitosis in untreated colon carcinomas and their relationship to genetic (p53 and Bcl-2) and biological features (DNA ploidy and cell kinetics), and patient survival rates.

**MATERIALS AND METHODS**

**Patient Materials.** The study population included 154 patients with primary colonic adenocarcinomas who were treated surgically at The University of Chicago Medical Center and The University of Texas M. D. Anderson Cancer Center. All tumors were formalin fixed and paraffin embedded, and 4–6 μm-thick sections were cut from tumor blocks for subsequent analysis. All tumors were optimally staged according to the American Joint Commission on Cancer and were stage I [invasion of the muscularis propria (T1N0M0; n = 11)] or stage II [serosal extension/penetration (T2N0M0; n = 143)] (41). None of the patients received postoperative adjuvant chemotherapy. Patients known to have a familial colon cancer syndrome were excluded, but all cases were otherwise unselected. Specimens were examined from 78 men and 76 women, including 97 Caucasian and 57 African-Americans. Mean age of the patients at surgery was 65.2 ± 11.5 (range, 26–89). There were 64 proximal and 82 distal colon carcinomas with the reference point being the splenic flexure. Marker analysis was performed in tumor sections whenever tissue was available.

**Histopathology.** H&E-stained slides for each patient were reviewed to confirm the diagnosis and histological grade of the tumor. The presence or absence of vessel and/or lymphatic microinvasion, defined as the presence of tumor cell(s) within an endothelial-lined space, was also determined on H&E-stained sections. Histopathological features were determined independently of immunostaining results.

**Clinical Follow-Up.** The median postoperative follow-up in this study was 105.5 months (range, 2–281) or 8.8 years. The disease status of each patient was determined at the date of last follow-up. Follow-up data were gathered by chart review and prior yearly contacts with the patient or treating physician. Patients were classified as having: (a) no evidence of disease; (b) local/regional recurrence; or (c) metastatic disease, and their vital status was recorded. Disease relapse was defined as either local recurrence or the development of distant metastasis and was determined by clinical and radiographic studies or by surgical means in all cases. The vital status of the patients was also determined at the date of last follow-up and if deceased, the cause of death was ascertained from the medical record and/or death certificate. Cause of death was classified as secondary to or unrelated to colon carcinoma. Only colon cancer deaths were considered as outcome events, given the extended patient follow-up time.

**Immunohistochemistry.** Sections were deparaffinized, and endogenous peroxidase activity was blocked by incubation in 3% H2O2 for 30 min. Antigen retrieval was performed by microwave treatment of the slides in PBS (pH 7.4) for 4 min. Bcl-2 and p53 immunoreactivity were detected by the ABC method (Vectastain Elite ABC kit; Vector Labs) as described previously (33). The primary anti-Bcl-2 (clone 124; Dako, Carpinteria, CA) is a murine anti-human mAb, subclass IgG1, that recognizes a cytoplasmic epitope of Bcl-2. The anti-p53 mAb D01 (Ab-6; Oncogene Science, Uniondale, NY) is murine anti-human mAb, subclass IgG2A, that recognizes an NH2-terminal epitope of the human p53 molecule. Slides were incubated overnight at room temperature with the primary Bcl-2 (1:20) or p53 (1:100) mAbs. Slides were subsequently incubated with a biotinylated secondary IgG antibody for 30 min. Avidin conjugated to horseradish peroxidase (ABC reagent) was then applied for 45 min. IHC staining was developed by immersing slides in 3,3’-diaminobenzidine and then counterstained with hematoxylin and mounted using an aqueous medium. Normal lymph node served as a positive control for Bcl-2 in all slide runs. A colon carcinomas known to contain a p53 mutation and to be immunoreactive for p53 served as a positive control. Negative control slides were processed with each slide run and excluded the primary antibody but included all other steps of the proce-
Table 1  Tumor characteristics stratified by site of the primary node-negative colon carcinoma

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>All patients (n = 154)</th>
<th>Proximal (n = 64)</th>
<th>Distal (n = 82)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Mean 65.2 ± 11.5</td>
<td>(26–89)</td>
<td>66.0 ± 11.8</td>
<td>64.6 ± 10.7</td>
</tr>
<tr>
<td>Age Range</td>
<td>64.6 ± 10.7</td>
<td>(31–83)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>Male 78</td>
<td>28</td>
<td>46</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>Female 76</td>
<td>36</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td>Caucasian 97</td>
<td>42</td>
<td>50</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>African-American 57</td>
<td>22</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>I; T N M 11</td>
<td>3</td>
<td>8</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>II; T N M 143</td>
<td>61</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td>Well 32</td>
<td>15</td>
<td>17</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Moderate 108</td>
<td>42</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poor 10</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Lymphatic or vascular invasion</td>
<td>Absent 130</td>
<td>56</td>
<td>67</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>Present 24</td>
<td>8</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>AI</td>
<td>≤0.25% 26</td>
<td>11</td>
<td>15</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>&gt;0.25% 128</td>
<td>53</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Mitotic index</td>
<td>≤0.5% 96</td>
<td>41</td>
<td>51</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>&gt;0.5% 56</td>
<td>22</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>≤15% 83</td>
<td>47</td>
<td>36</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>&gt;15% 61</td>
<td>16</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>DNA ploidy</td>
<td>Diploid 72</td>
<td>42</td>
<td>29</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Aneuploid 78</td>
<td>21</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Bcl-2 expression</td>
<td>Low 66</td>
<td>26</td>
<td>40</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>High 71</td>
<td>33</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>p53 expression</td>
<td>Low 82</td>
<td>44</td>
<td>38</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>High 62</td>
<td>19</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Median follow-up months</td>
<td>105.5</td>
<td>(2–281)</td>
<td>115.3</td>
<td>(4–281)</td>
</tr>
<tr>
<td>Disease relapse</td>
<td>None 120</td>
<td>52</td>
<td>62</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Local recurrence 15</td>
<td>5</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td>19</td>
<td>7</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Vital status</td>
<td>Alive 62</td>
<td>31</td>
<td>28</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Deaths, all cause 61</td>
<td>22</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Death colon cancer</td>
<td>31</td>
<td>11</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

* Relative to splenic flexure; tumor site unknown (n = 8).
* S-phase + G0/M.
* Low, ≤20% + tumor cells; high, >20%.

dure. These slides did not exhibit immunostaining. At light microscopy, the percentage of tumor cells expressing p53 or Bcl-2 proteins was determined. The data were then dichotomized into two groups: <20% versus >20% immunoreactive tumor cells. For a tumor to be regarded as immunopositive, the intensity of staining had to be clearly increased over background. Tumor specimens were scored independently by two examiners, who were blinded to clinical outcome. When a discrepancy occurred, a consensus opinion was achieved by both examiners at a dual-headed microscope.

Quantification of Apoptosis and Mitosis. Mitotic nuclei and apoptotic cells and bodies were identified in histological sections stained with H&E using established morphological criteria (1, 2, 15). By light microscopy, spontaneous AIs and MIs were calculated by counting the number of apoptotic or mitotic nuclei per 100 cells in each of five high power fields/slide at ×400, with the result expressed as a percentage (4). The AI or MI is not an absolute extent of apoptosis or mitosis but represents an index (4, 42). Apoptotic cells and bodies detected in colon carcinoma sections by morphology have been shown to contain DNA strand breaks using terminal deoxynucleotidyl transferase-mediated nick end labeling assay (4). Quantification of AIs and MIs was performed by a single observer who was blinded to clinical outcome.

Flow Cytometry. DNA content and PIs were determined by laboratories at the University of Chicago (n = 126) and M. D. Anderson Cancer Center (n = 28). Fifty-μm sections were cut from paraffin tumor blocks for analysis by flow cytometry. Blocks were selected that contained at least 75% tumor tissue. Nuclear suspensions were prepared using a modified version of the method of Hedley et al. (43) and were incubated overnight in propidium iodide solution. Cell proliferation measurements were performed on DNA histograms. The PI was calculated as the sum of the percentages of cells in S-phase and G0/M phases of the cell cycle, per the method of Basich et al. (44). The D.I. was calculated as the ratio of the modal value of the DNA histogram of the tumor sample to that of the reference cells. By definition, diploid tumors have a D.I. of 1.0, and tumors with a single G0-G1 peak at 2°C were regarded as diploid. Samples with a second peak distinct from the diploid 2°C peak and with a corresponding 4°C peak were considered to be aneuploid. In practice, tumors with a distinct G0-G1 population and with a D.I. >1.18 were classified as aneuploid (45); those with a D.I. of 1.9–2.1 were tetraploid; however, they were analyzed as aneuploid. Cell kinetic measurements were performed on combined populations of diploid and aneuploid cells.

Statistical Analysis. The frequency distributions of AI and MI data were plotted, and cutoffs were selected to allow grouping of the data to evaluate prognostic effect. For the AI and MI, cutoffs of 0.25 and 0.50, respectively, were found to best separate patients for survival analysis. The percentage of immunoreactive cells for Bcl-2 and p53 were dichotomized into two groups, ≤20% or >20%+ tumor cells. The cutoff of 20% was chosen based upon studies of p53 expression in colon cancer, demonstrating that this cutoff is clinically significant (25). Furthermore, a cutoff of 30% Bcl-2+ cells was found to best separate breast carcinoma patients into groups at low versus high risk of disease relapse and death (46). With regard to PI, we found that a cutoff of 15% best separated patients into subgroups at high versus low risk (≤15%) of relapse and colon-specific death. The associations between tumor features were analyzed using the χ2 test. The primary statistical outcomes were RFS and OS rates measured from the date of surgery. Both the time to relapse and to colon-specific death were analyzed using the Cox proportional hazards model for univariate and multivariate analyses (47). All potential prognostic factors were
entered into a stepwise regression model from which significance levels were determined. All prognostic factors found to be important in a univariate analysis were evaluated in multivariate models to assess their predictive effects on RFS and OS rates. Additionally, the HRs between prognostic groups and their 95% CIs were computed. RFS and OS data were plotted using the Kaplan-Meier method. Statistical significance was defined as a two-sided \( P \leq 0.05 \).

## RESULTS

### Patient Characteristics

We analyzed 154 node-negative colon carcinomas from patients that did not receive adjuvant chemotherapy. Median follow-up time was 8.8 years. Details of the patient population are outlined in Table 1, and clinicopathological features and tumor marker analyzes are shown stratified by tumor site.

### Tumor Site

We found that p53 expression \((P = 0.007)\), DNA ploidy \((P = 0.001)\), and the PI (S-phase + G2M; \(P = 0.0003\)) were strongly correlated with tumor site (Table 1). No differences were found for AI, MI, or Bcl-2 expression when stratified by anatomical site. Similarly, neither patient age, gender, race, histological grade, nor lymphatic or vascular invasion correlated with tumor site.

### AIs and MIs

Apoptotic cells displayed condensed chromatin, which appeared deeply basophilic on H&E staining, and nuclear collapse, which produced a characteristic surrounding halo (4). Apoptotic cells tended to have smaller nuclei and condensed cytoplasm. Apoptotic bodies resulting from nuclear fragmentation were observed frequently and usually appeared in clusters of two or three fragments. The median and mean AIs were 1.2 and 1.61 \(\pm\) 1.52, respectively, with a range from 0 to 7.6% (Fig. 1A). This range of AIs reflects heterogeneity in the extent of spontaneous apoptosis. Mitotic nuclei were identified by their characteristic morphology. The median and mean MIs were 0.4 and 0.44 \(\pm\) 0.37, respectively, and a relatively narrow range of values (0.4–1.8) was detected (Fig. 1B). Given that apoptosis and mitosis are counterbalancing processes, we examined their association in this series of tumors. We failed to detect a signifi-

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**Table 2** Univariate analysis of long-term RFS and OS rates in patients with node-negative colon carcinomas

<table>
<thead>
<tr>
<th></th>
<th>RFS</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio</td>
<td>(95%) CI</td>
</tr>
<tr>
<td>AI</td>
<td>1.74 (0.81–3.72)</td>
<td>0.16</td>
</tr>
<tr>
<td>MI</td>
<td>2.19 (1.11–4.29)</td>
<td>0.02</td>
</tr>
<tr>
<td>Bcl-2 expression</td>
<td>0.44 (0.21–0.91)</td>
<td>0.03</td>
</tr>
<tr>
<td>PI</td>
<td>1.93 (0.98–3.82)</td>
<td>0.06</td>
</tr>
<tr>
<td>DNA ploidy</td>
<td>1.99 (0.96–4.10)</td>
<td>0.06</td>
</tr>
<tr>
<td>p53 expression</td>
<td>0.99 (0.49–1.97)</td>
<td>0.97</td>
</tr>
<tr>
<td>Lymphovascular invasion</td>
<td>2.05 (0.93–4.54)</td>
<td>0.08</td>
</tr>
<tr>
<td>Race</td>
<td>1.83 (0.93–3.58)</td>
<td>0.08</td>
</tr>
<tr>
<td>Grade (well + moderate vs poor)</td>
<td>1.57 (0.48–5.13)</td>
<td>0.46</td>
</tr>
<tr>
<td>Tumor site</td>
<td>1.33 (0.67–2.63)</td>
<td>0.41</td>
</tr>
</tbody>
</table>
cant correlation between AI and MI ($P = 0.27$) or AI and PI ($P = 0.27$) in all tumors or when stratified by tumor site. This finding demonstrates an imbalance between these processes within established colon carcinomas. AIs and MIs did not differ depending upon tumor site (Table 1). The AI:MI ratio in proximal versus distal carcinomas was also examined but did not differ significantly. Although increased AIs ($>0.25$) were associated with poor and moderate versus well differentiation ($P = 0.03$), only 10 tumors had poor differentiation. Increased AIs have been associated with high-grade tumors of the breast (16), ovary (17), bladder (19), and kidney (48). The dichotomized AI did not correlate with the expression of Bcl-2 ($P = 0.19$), p53 ($P = 0.50$), or ploidy ($P = 0.84$). In proximal tumors, p53 expression was associated with lower AIs ($\leq 0.25$; $P = 0.07$).

The MI strongly correlated with the PI ($P = 0.02$), consistent with the fact that both are measures of cell proliferation. MI was related to DNA ploidy in that high MIs ($>0.5$) were associated with aneuploid tumors ($P = 0.067$). Although the correlation between MI and Bcl-2 did not achieve significance in all tumors ($P = 0.10$), an inverse relationship was found in distal carcinomas, where lower MIs ($\leq 0.5$) were detected in tumors overexpressing Bcl-2 ($P = 0.02$).

p53 Expression

Increased p53 protein expression was detected in 62 of 144 (43%) tumors examined (Table 1). p53 expression was confined to the nucleus, and its staining pattern was generally diffuse as opposed to focal (4, 33). Increased p53 was less frequent in proximal versus distal colon cancers (69% versus 31%; $P = 0.007$). Aneuploid tumors ($P = 0.001$) and those with a high PI ($P = 0.027$) were likely to overexpress p53. The association of p53 and aneuploidy is consistent with other reports in colon carcinomas, where p53 was analyzed by IHC or loss of het-

erozygosity at chromosome 17p (49, 50). An association between p53 and reduced AI ($=0.25$) was seen in proximal cancers ($P = 0.07$).

Bcl-2 Expression

Bcl-2 overexpression was detected in 71 of 137 (52%) colon carcinomas (33, 38). Staining was confined to the cytoplasm of tumor cells and was frequently expressed in infiltrating lymphocytes within the stroma (33, 38). Heterogeneity in Bcl-2 expression within tumor sections was observed, and focal staining was detected more frequently than was seen for p53. An inverse correlation between Bcl-2 and the MI was suggested in all tumors ($P = 0.10$), and this relationship became statistically significant in distal carcinomas ($P = 0.02$). Bcl-2 staining did not correlate with AI, PI, or p53 and did not differ significantly when stratified by tumor site (Table 1).

DNA Ploidy and PIs

DNA content and PI data were available in 144 (94%) cases that included a nearly equal number of diploid (49%) and aneuploid (51%) tumors. Aneuploidy was more common in distal versus proximal cancers (71% versus 29%; $P = 0.001$; Table 1). Aneuploid tumors were found to have higher PIs ($P = 0.001$) and MIs ($P = 0.06$) relative to diploid tumors. Ploidy was associated with p53 but not with Bcl-2 expression. High PI ($>15\%$) was associated with distal tumor site ($P = 0.0003$). PI and MI were correlated ($P = 0.02$).

Relationship of Tumor Markers to Survival

At a median follow-up of 8.8 years, there were 34 (of 154; 22%) relapses and 31 (34%) colon cancer-related deaths (Table
The number of patients with disease relapse and colon-specific death were similar in patients with proximal versus distal colon carcinomas, respectively (Table 1). By univariate analysis, MI was found to be a significant predictor of RFS (HR, 2.2; P = 0.02) and OS (HR, 2.1; P = 0.04; Table 2). Specifically, carcinomas with MIs exceeding the median (>0.5%) behaved more aggressively than did those with lower MIs (Fig. 2). Patients whose tumors had very low spontaneous AIs (≤0.25%) had reduced survival rates compared with those with higher AIs, although this result achieved statistical significance only for distal cancers (Table 2; Fig. 3). We postulated that the AI:MI ratio may be more informative than either index alone. We, therefore, analyzed the relationship between AI/MI and survival, but no association was found (P = 0.27).

Patients with aneuploid carcinomas had reduced survival compared with those with diploid tumors (RFS: HR, 2.0; P = 0.06; OS: HR, 2.1; P = 0.06; Fig. 4). Similarly, patients whose tumors had high PIs had worse survival rates compared with those with low PIs (RFS: HR, 1.9; P = 0.06; OS: HR, 1.9; P = 0.07; Table 2). Tumor site was not, by itself, a prognostic variable. However, stratification of variables by tumor site yielded additional prognostic information as discussed below. Histological grade did not predict clinical outcome (Table 2). A trend toward shorter RFS was observed for patients whose tumors had lymphatic or vascular invasion (P = 0.08) and for African-American versus Caucasian patients (P = 0.08).

p53 expression failed to predict clinical outcome in the entire series of tumors (Fig. 5). Bcl-2 expression was a significant predictor of RFS (HR, 0.44; P = 0.03) and OS (HR, 0.45; P = 0.04) rates (Table 2; Fig. 6). Patients whose tumors overexpressed Bcl-2 (>20% Bcl-2 + tumor cells) had better survival compared with tumors with low or undetectable staining. The association of Bcl-2 with better survival is consistent with other reports in colon cancer (35, 36) and studies in breast carcinoma (46, 51, 52).

Analysis of only stage II colon cancer patients, thereby omitting the 11 stage I cases, did not significantly alter any of the results. After adjustment for covariates, the MI was found to independently predict RFS (HR, 2.8; P = 0.007) and OS (HR, 2.7; P = 0.01) and was the strongest prognostic variable (Table 3). Overexpression of Bcl-2 predicted better RFS (HR, 0.45; P = 0.04), and its association with OS (HR, 0.46; P = 0.06) was of borderline statistical significance. Neither the AI nor DNA ploidy was found to predict clinical outcome in this model.

Stratification by Tumor Site

**Proximal Colon Carcinomas.** In a univariate analysis, DNA ploidy achieved statistical significance as a predictor of RFS (HR, 3.8; P = 0.03) and OS (HR, 3.5; P = 0.05; Table 4). Patients with diploid tumors had better survival than did aneuploid cancers (Fig. 4). p53 overexpression predicted shorter OS (HR, 3.2; P = 0.05) but not RFS (P = 0.31; Fig. 5). As was found in all tumors, Bcl-2 expression predicted better RFS (HR, 0.28; P = 0.05) and OS (HR, 0.19; P = 0.03) rates (Fig. 6). Neither MI (Fig. 2), PI, nor AI (Fig. 3) was a significant predictor of patient survival within this tumor subset (Table 4). After adjustment for other variables, only Bcl-2 expression was an independent predictor of better survival in proximal tumors (RFS: HR, 0.23; P = 0.04; OS: HR, 0.17; P = 0.03; Table 5).

**Distal Colon Carcinomas.** Increased MIs (>0.5%) predicted worse RFS (HR, 2.9; P = 0.02) and OS (HR, 3.2; P = 0.02) rates (Table 4; Fig. 2), as it did in all colon tumors. Distal tumors with very low AIs (≤0.25) had worse RFS (HR, 0.44; P = 0.07) and OS (HR, 0.39; P = 0.05) rates (Table 4; Fig. 3). Overlapping survival curves were found for diploid and aneuploid cancers of the distal colon (Fig. 4). Neither p53 nor Bcl-2

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**Overall Survival by Apoptotic Index**

![Graph showing overall survival by apoptotic index for proximal, distal, and all cancers.](graph_url)

**Fig. 3 Probability of OS in patients with node-negative colon carcinomas as a function of the AI. AI is dichotomized into low (≤0.25) and high (>0.25) groups. Shown are data stratified by tumor site (left, proximal; middle, distal) and results for all tumors (right). Ps result from univariate analysis.**
expression was prognostic in distal cancers (Figs. 5 and 6). Tumors overexpressing p53 had worse survival in the proximal colon (Table 4; Fig. 5). In a multivariate analysis, low AIs (RFS: HR, 0.33; P = 0.04; OS: HR, 0.30; P = 0.02) and high MIs (RFS: HR, 3.4; P = 0.02; OS: HR, 3.5, P = 0.02) were independent predictors of adverse outcome (Table 5).

DISCUSSION

We performed a retrospective analysis of the relationship of genetic and biological markers to tumor site and clinical outcome in node-negative colon carcinomas. The strengths of this study include the fact that nearly all patients had stage II tumors (all were Dukes’ stage B), all had lengthy clinical follow-up for recurrence and death, and disease-specific survival was the primary endpoint. We found that the MI was the most important prognostic variable. Increased MI independently predicted reduced survival in all tumors and in the subset of distal colon carcinomas. This result is consistent with some (9–11) but not other (53, 54) studies examining proliferative activity in colon carcinomas. The MI has also been shown to predict outcome in patients with breast (16), ovarian (17), prostate (18), and bladder (19) cancers. Although the MI was strongly correlated with the PI, MI was a stronger prognostic variable, indicating that these proliferation markers are not interchangeable. An advantage of the MI is that counts can be determined in H&E sections at low cost, and AIs can be determined concurrently. To our knowledge, this is the first report demonstrating the prognostic significance of the MI in colon carcinomas.

Although the AI was not prognostic in all colon carcinomas, stratification by tumor site revealed that the AI was a significant independent predictor of survival in distal tumors. The association of very low AIs with adverse outcome is consistent with the observation that attenuated apoptosis accompanies CRC development and progression (5–7). Furthermore, metastases from colon carcinomas were reported to have reduced AIs relative to the primary tumor (7). An association of reduced apoptosis with adverse outcome was also reported in patients <45 years of age at CRC diagnosis (53). The AI did not significantly correlate with the MI or PI, reflecting loss of their coordinate regulation. An imbalance between apoptosis and cell proliferation (measured by proliferating cell nuclear antigen or Ki-67 expression) was shown in colorectal adenomas and carcinomas by ourselves (4) and others (5). Our findings suggest that cancers of the distal versus proximal colon may respond differently to chemotherapy and/or irradiation. We emphasize that none of the patients in our series received adjuvant chemotherapy. Rich et al. (55) found that increased AIs in rectal carcinoma biopsies predicted a more favorable pathological response to preoperative chemoradiation. This observation is supported by studies in murine tumor models, where spontaneous AIs have been shown to predict the peak apoptotic response to chemotherapy (56) and radiation (57), as well as tumor growth delay, a measure of treatment efficacy.
Primary tumor site was not prognostic in this series of patients. This result is consistent with an analysis of over 500 resectable colon cancers, where tumor site was not a significant prognostic variable (58). Genetic and biological heterogeneity between tumors is likely to explain this lack of association. However, the association of certain tumor markers with survival may be dependent upon tumor site, as shown for AI and ploidy. The better survival of patients with diploid tumors of the proximal colon suggests that the weak or lack of association of ploidy with survival found in several studies in colon cancer may be a consequence of its dependence upon tumor site (14).

This has been shown for the K-ras gene, where mutations were prognostic in distal, but not proximal, colon cancers (24).

The association of p53 overexpression with aneuploidy is consistent with other reports (12, 39, 50) and suggests that intact p53 function may be required to maintain diploidy (59, 60). Furthermore, wt p53 can prevent replication of damaged DNA as well as DNA re-replication that can lead to aneuploidy (61). p53 expression was not prognostic in the entire group of node-negative colon carcinomas. p53 did predict adverse outcome in proximal cancers, but this finding was not maintained after adjustment for covariates. The lack of an independent

### Table 4  Univariate analysis of RFS and OS in proximal and distal node-negative colon carcinomas

<table>
<thead>
<tr>
<th>Marker</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RFS</td>
<td></td>
<td>OS</td>
<td></td>
</tr>
<tr>
<td><strong>Proximal colon carcinomas</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AI</td>
<td>0.93 (0.20–4.27)</td>
<td>0.93</td>
<td>0.47 (0.06–3.71)</td>
<td>0.48</td>
</tr>
<tr>
<td>MI</td>
<td>2.2 (0.72–6.89)</td>
<td>0.17</td>
<td>1.84 (0.56–6.04)</td>
<td>0.31</td>
</tr>
<tr>
<td>Bcl-2 expression</td>
<td>0.28 (0.08–1.03)</td>
<td>0.05</td>
<td>0.19 (0.04–0.88)</td>
<td>0.03</td>
</tr>
<tr>
<td>PI</td>
<td>2.22 (0.70–6.99)</td>
<td>0.17</td>
<td>1.84 (0.539–6.29)</td>
<td>0.33</td>
</tr>
<tr>
<td>DNA ploidy</td>
<td>3.75 (1.10–12.82)</td>
<td>0.03</td>
<td>3.46 (0.98–12.27)</td>
<td>0.05</td>
</tr>
<tr>
<td>p53 expression</td>
<td>1.8 (0.57–5.69)</td>
<td>0.31</td>
<td>3.23 (0.98–10.61)</td>
<td>0.05</td>
</tr>
<tr>
<td>Grade</td>
<td>1.90 (0.41–8.69)</td>
<td>0.41</td>
<td>2.29 (0.49–10.64)</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>Distal colon carcinomas</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AI</td>
<td>2.30 (0.92–5.77)</td>
<td>0.07</td>
<td>2.58 (0.99–6.65)</td>
<td>0.05</td>
</tr>
<tr>
<td>MI</td>
<td>2.92 (1.19–7.12)</td>
<td>0.02</td>
<td>3.15 (1.23–8.09)</td>
<td>0.02</td>
</tr>
<tr>
<td>Bcl-2 expression</td>
<td>0.55 (0.22–1.39)</td>
<td>0.21</td>
<td>0.68 (0.26–1.76)</td>
<td>0.43</td>
</tr>
<tr>
<td>PI</td>
<td>1.50 (0.60–3.76)</td>
<td>0.39</td>
<td>1.68 (0.63–4.49)</td>
<td>0.03</td>
</tr>
<tr>
<td>DNA ploidy</td>
<td>1.07 (0.43–2.69)</td>
<td>0.88</td>
<td>1.19 (0.45–3.17)</td>
<td>0.73</td>
</tr>
<tr>
<td>p53 expression</td>
<td>0.61 (0.25–1.49)</td>
<td>0.27</td>
<td>0.58 (0.22–1.50)</td>
<td>0.26</td>
</tr>
<tr>
<td>Grade</td>
<td>1.34 (0.18–10.08)</td>
<td>0.77</td>
<td>1.71 (0.22–13.01)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Overall Survival by p53 Expression

**Fig. 5** Probability of OS in patients with node-negative colon carcinomas as a function of p53 protein expression. p53 is dichotomized into low (≤20% p53+ tumor cells) and high (≥20% p53+ tumor cells) groups. Shown are data for stratified by tumor site (left, proximal; middle, distal) and results for all tumors (right). P values result from univariate analysis.
The effect of p53 on survival agrees with other reports using IHC (27, 62). Dependence upon tumor stage was reported by Ahnen et al. (25), where p53 staining was prognostic in stage III, but not stage II, colon carcinomas. In contrast to IHC, p53 mutation has, in general, been associated with shortened survival in CRC (23). Potential explanations for this disparity include the fact that only 70% of p53 mutations were shown to result in the accumulation of mutant proteins detectable by IHC (63). False-positive p53 staining can result from stabilization of wt p53 proteins in the absence of p53 mutation (64). In addition, the concordance between IHC and mutational analysis has been shown to depend upon the anti-p53 antibody used (65).

We found that proximal colon cancers had a reduced frequency of p53 expression and were more frequently diploid. These features have been shown to correlate with the presence of MSI. MSI has been detected in up to 15% of sporadic colon cancers that display reduced loss of heterozygosity at chromosomes 5q, 17p, and 18q (sites of APC, p53, and DCC genes; Refs. 66 and 67). These same tumor features are also found in MSI+ sporadic colon cancers and HNPCCs have more favorable survival rates compared to stage-matched controls (67, 68, 70). Potentially, the better survival of diploid tumors of the proximal colon, as seen in this study, may be related to MSI and/or favorable prognostic features including Bcl-2.

Bcl-2 overexpression was a favorable prognostic variable and was the most important prognostic factor in proximal tumors. The association of Bcl-2 with better survival rates is consistent with studies in breast cancers (51, 52) and some (35–38) but not other reports in CRCs (39). The association of Bcl-2 with better survival in a study in breast cancers was attributed to its inverse relationship with p53 (46). However, our Bcl-2 data in colon cancer, and that of others (35–37), do not reveal an inverse relationship with p53, although such an association was reported by Watson et al. (71).

We found a trend toward an inverse relationship between Bcl-2 and S-phase fraction has been reported in non-Hodgkin’s lymphomas (72). Furthermore, recent in vitro and in vivo studies demonstrate that Bcl-2 can reduce cell proliferation by delaying cell cycle entry, at least in certain cell types (73–75). Overexpression of the Bcl-2 transgene in colon carcinoma cell lines was reported to significantly inhibit its growth in a colony formation assay (76). However, our attempt to replicate these
experiments revealed no such effect of Bcl-2 upon cell growth.\textsuperscript{4} To date, the mechanism of the association of Bcl-2 and favorable clinical outcome remains unknown. Bcl-2 is a member of an ever-expanding family of cytoplasmic proteins that regulate apoptosis (31). Understanding the influence of Bcl-2 upon clinical behavior may, therefore, depend upon its association with other Bcl-2 family members and/or from cellular effects that are independent of inhibition of apoptosis. In this regard, the antiapoptotic function of Bcl-2 has been shown to be mechanistically distinct from its inhibitory influence on cell cycle entry (77).

In conclusion, MI and Bcl-2 expression were independent predictors of patient survival rates in node-negative colon carcinomas. Stratification of variables according to primary tumor site was shown to provide additional prognostic information. Specifically, DNA ploidy and AI were important prognostic variables in proximal and distal colon carcinomas, respectively. If validated prospectively, these variables may identify patients at higher risk of disease relapse and death for whom adjuvant chemotherapy may be beneficial.

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REFERENCES


Apoptotic and Mitotic Indices Predict Survival Rates in Lymph Node-negative Colon Carcinomas


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