Ki-ras Mutation and p53 Overexpression Predict the Clinical Behavior of Colorectal Cancer: A Southwest Oncology Group Study

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ABSTRACT

We assessed Ki-ras mutations by single-strand conformation polymorphism followed by DNA sequencing, p53 expression by immunohistochemistry, ploidy status, and S-phase fraction in 66 stage II and 163 stage III colon cancer patients enrolled on a randomized trial of surgery followed by observation or adjuvant levamisole or 5-fluorouracil (5FU) plus levamisole (Intergroup Trial 0035) to see whether these factors were independently associated with survival or with differential effects of adjuvant therapy. A Cox proportional hazards survival model was used to describe marker effects and therapy by marker interactions, with adjustment for the clinical covariates affecting survival. A Bonferroni adjustment was used to account for multiple testing. Mutation of the Ki-ras gene was found in 41% of the cancers and was associated with a poor prognosis in stage II but not stage III. In stage II, 7-year survival was 86% versus 58% in those with wild type versus Ki-ras mutations. After adjustment for treatment and clinical variables, the hazard ratio (HR) for death was 4.5; 95% confidence interval (CI), 1.7–12.1 (P = 0.012). Aneuploidy was more common in stage III than in stage II (66 versus 47%; P = 0.009) but was not independently related to survival. There was no benefit of adjuvant therapy in stage II nor in any of the stage II subgroups defined by mutational status. In stage III, adjuvant therapy with 5FU plus levamisole improved 7-year survival in patients with wild-type Ki-ras (76 versus 44%; HR, 0.4; 95% CI, 0.2–0.8) and in those without p53 overexpression (64 versus 26%; HR, 0.3; 95% CI, 0.1–0.7). Adjuvant therapy did not benefit those with Ki-ras mutations or p53 overexpression. The effects of adjuvant therapy did not differ according to ploidy status or proliferative rate. Ki-ras mutation is a significant risk factor for death in stage II, and the absence of p53 expression is a significant risk factor for death in stage III colon cancer after adjustment for treatment and clinical covariates. Exploratory analyses suggest that patients with stage III colon cancer with wild-type Ki-ras or no p53 expression benefit from adjuvant 5FU plus levamisole, whereas those with Ki-ras mutations or p53 overexpression do not. An independent study will be required to determine whether response to adjuvant therapy in colon cancer depends on mutational status.

INTRODUCTION

The clinical behavior of colorectal cancer is highly variable. The stage of the disease, as defined by the extent of invasion of the primary tumor and by the presence of metastases in lymph nodes or more distant sites, is the most important prognostic factor (1). Stage alone, however, accounts for only a part of the variability in prognosis of colorectal cancer. About 3 of patients with colon cancer and lymph node metastases (stage III) die of recurrent disease after surgical resection, whereas only 1 of patients without lymph node metastases (stage II) die of recurrent disease (1). Other features of the cancer, such as histological degree of differentiation, preoperative CEA level, size and location of the primary tumor, and Deleted in Colon Cancer (DCC) mutation have been reported in some series to confer independent prognostic information, but there still remains a great deal of unexplained stage-independent variability in the clinical behavior of the disease (1).

Adjuvant therapy with 5FU and levamisole after surgical resection of Dukes’ C colorectal cancer has been shown in prospective controlled trials to improve overall survival by up to 33%, but even with adjuvant therapy, about 40% of such patients die of recurrent disease (2, 3). It is not yet possible to reliably predict which patients are likely to benefit from adjuvant therapy, although it has been reported that thymidylate synthase activity might be useful in predicting the likelihood of benefit of 5FU therapy (4).

It is now widely accepted that colonic carcinogenesis is a multistep process driven by mutational events that ultimately give the cancer cells a growth advantage. In colorectal adenomas and carcinomas, various combinations of mutations of specific oncogenes (Ki-ras), tumor suppressor genes (APC, DCC, and p53), and DNA repair genes (MSH-2 and MLH-1), in addition to gross quantitative alterations in DNA content (aneuploidy), have been described (5–7). If mutational events are the driving force in the process of colonic carcinogenesis, one can hypothesize that the mutational profile of a cancer would predict its biological and clinical behavior. The purpose of this study was to test the hypothesis that different patterns of genetic alterations (ploidy status, Ki-ras mutation, p53 overexpression) and proliferative rate in colon cancers result in differences in the clinical behavior of the tumors as assessed by overall prognosis or by response to adjuvant therapy. The hypothesis was tested in a sample of patients enrolled by the Southwest Oncology Group (SWOG) in a randomized prospective trial of adjuvant therapy for patients with stage II or III colon cancer (Intergroup Trial 0035).

MATERIALS AND METHODS

Tissue Samples

Paraffin blocks were obtained from patients enrolled by the SWOG in Intergroup Trial (INT)-0035. SWOG, the North Central Cancer Treatment

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2To whom requests for reprints should be addressed, at Southwest Oncology Group (SWOG-9000), Operations Office, 14980 Omicron Drive, San Antonio, Texas 78245-3217.

3The abbreviations used are: 5FU, 5-fluorouracil; SSCP, single-strand conformation polymorphism; HR, hazard ratio; CI, confidence interval; SWOG, Southwest Oncology Group; INT, Intergroup.
Group, and the Eastern Cooperative Group registered a total of 1296 surgically resected patients to INT-0035, a prospective randomized controlled clinical trial to evaluate levamisole alone or levamisole plus 5FU as surgical adjuvant therapy for resectable colorectal cancer. Patients eligible for the Intergroup Trial had received potentially curative surgery for stage II (T1-2N0M0, T3), stage III (any T, N1-N3, M0) colorectal cancer and had no gross or microscopic evidence of disease present after surgery. After surgical resection, stage II patients were randomized to one of two treatment arms (observation or 5FU plus levamisole), whereas stage III patients were randomized to one of three treatment arms (observation, levamisole, or 5FU plus levamisole). At the time of entry into the trial, all patients were clinically and pathologically staged in a standard manner using the Gastrointestinal Tumor Study Group modification of the Duke’s staging system, in which the stage III tumors were separated into Duke’s C1 and C2 groups in which C1 designates tumors with 1–4 involved lymph nodes and C2 designates those with more than 4 nodes involved. All patients were also followed in a standard fashion for evidence of recurrence and survival. Further details of patient selection, treatment, and primary results for the therapeutic study have been described previously (2, 3, 8).

Representative H&E slides and paraffin blocks from the surgical resection, containing tissue from both the colorectal cancer and the normal mucosa at the resection margins, were requested after the therapeutic trial closed to accrual, and submission by the institutions was optional. Blocks were successfully obtained from 234 of the 502 patients enrolled into the Intergroup Trial by SWOG institutions.

**Kl-ras PCR/SSCP/DNA Sequencing Analysis**

**DNA Extraction.** Nine-μm sections of paraffin embedded tissue were cut, deparaffinized, stained for H&E, and rehydrated. The microtome blade was washed or replaced between samples to prevent cross-contamination between samples. Regions of malignant appearing epithelium was separated from the stroma by microdissection and transferred to TE buffer (1 mM EDTA, 50 mM Tris, pH 8.5, 0.5% Tween). A random sample of specimens of normal mucosa from the resection margins was also analyzed. The tissue samples were boiled for 10 min and centrifuged. The supernatant was removed, and the pellets were digested overnight at 37°C. Samples were then heated to 95°C for 8 min to inactivate proteinase K, and aliquots of DNA from each tissue sample were used for PCR-SSCP analysis of the Kl-ras gene.

**PCR Amplification.** PCR was performed in a laboratory area separate from the DNA extraction. A nested PCR amplification protocol was used. The first amplification used primers A (5′-TTT TAT AAG GCC TGC-3′) and B (3′-AAA GAA TGG TCC TGC ACC-5′). This PCR product was further amplified by a set of inner (nested) primers, primers C (5′-ATG ACT GAA TAT AAA CTT GT-3′) and D (3′-CTC TAT TGT TGG ATC ATA TT-5′). Primers A and B are located between -23 and +158 in the Kl-ras gene, yielding a 181-bp PCR product. Primers C and D are located between +1 and +111 in the Kl-ras gene, yielding a 111-bp PCR product that includes codons 1–37 of the Kl-ras gene. The amplification conditions of the first stage were as follows: 30 cycles at 94°C for 1 min, 52°C for 1 min, and 74°C for 45 s. The final reaction mixture contained approximately 10 ng of genomic DNA, with 0.4 mM of each primer, 70 mM of each of the four dNTPs, 10 mM Tris, pH 8.0, 50 mM KCl, 1.5 mM MgCl2, and 0.5 units of Taq polymerase in a total volume of 50 μl. The amplification conditions of the second stage using primers C and D were as follows: 35 cycles at 94°C for 1 min, 52°C for 1 min, and 74°C for 45 s, followed by a 7-min incubation at 74°C. The reaction mixture (100 μl) contained 0.5–1.0 μl of PCR product from the first stage, 0.5 mM each of primers C and D, 70 mM each of the four dNTPs, 10 mM Tris, pH 8.0, 50 mM KCl, 1.5 mM MgCl2, and 0.5 units of Taq polymerase. Following 35 cycles of amplification, 5 μl of the PCR product was run on a 2.5% (w/v) Nu-Sieve agarose gel containing 0.5% (w/v) ethidium bromide, and the DNA product bands were visualized under UV transillumination to check the efficiency and specificity of the amplification.

**SSCP Analysis.** SSCP was carried out by modification of the method described by Orita et al. (9). Primer D was labeled with [γ-32P]ATP by the T4 polynucleotide kinase end-labeling method (10), and 1 × 106 cpm of the labeled primer were added to each reaction mixture. After 30 cycles of amplification using the same conditions as described above, 1–2 μl of the PCR reaction mixture was added to 9 ml of stop solution (95% formamide, 10 mM NaOH, 0.05% bromphenol blue, and 0.05% xylene cyanol) and heated at 94°C for 5 min. The denatured DNA was placed on ice before being loaded onto 1% mutation detection enhancement gel. Samples were electrophoresed at 5 W overnight at room temperature. After electrophoresis, the gel was exposed to Kodak X-AR X-ray film for 6–48 h. DNA from known wild-type samples and mutated samples were run as negative and positive controls, respectively, with each set of experimental samples.

**Sequencing.** DNA sequencing was performed on all of the samples found to have an abnormal SSCP pattern and in 30 samples with a normal SSCP pattern using CircumVent Thermal Cycle Dideoxy DNA Sequencing kit (New England Biolabs) following the manufacturer’s protocol. Samples were electrophoresed in 8% polyacrylamide containing 8 mM urea for 2–3 h at 60–70 W. The gel was dried and exposed to Kodak X-AR X-ray film for 12–24 h.

**p53 Immunohistochemistry**

For immunohistochemical localization of p53, 5-μm sections were placed on positively charged slides (Fisher Scientific, Pittsburgh, PA), deparaffinized in xylene, rehydrated through a series of graded alcohols, and then heated in 0.01 M citrate buffer (pH 6.0) for 15 min in a microwave oven. Immunohistochemical staining was performed using the ABC method (Vector Laboratories) on a Ventana 320 automated immunostainer (Tucson, AZ) and counterstained with nuclear fast red. The p53 antibody (Oncogene Science, AB-6, clone DO7) was used at a dilution of 1:300. A tumor sample with known p53 overexpression was included as a positive control with each set of experimental samples. A negative control, in which the primary antibody was omitted from the staining procedure, was performed for each sample. A total of at least 500 cells were counted from each cancer sample, and sections were graded positive for p53 expression if >5% of the tumor cells showed nuclear staining and negative if <5% of the tumor cell nuclei were stained.

**Flow Cytometric Analysis of DNA Content**

Tumor blocks and a random sample of resection margins were prepared for flow cytometric analysis of DNA content using a modification of the method of Hedley et al. (10). A H&E slide from each paraffin block was examined to determine whether the block contained a representative portion of the tumor. For all adequate blocks, three 50-μm sections were cut, deparaffinized, and rehydrated. The tissue was then digested with 0.5% (w/v) pepsin (Sigma Chemical Co., St. Louis, MO; pH 1.5) for 2 h at 37°C, and the suspension was washed and filtered through a 60-mm nylon mesh to remove aggregates. The number of nuclei in the filtrate was estimated with a hemocytometer, and no more than 3 × 10^6 nuclei were added to each tube. Propidium iodide (46.1 mg/liter) with RNase A (10 mg/liter) was added to stain the nuclei overnight before flow cytometric analysis was conducted.

The relative DNA content of 10,000–20,000 nuclei was analyzed in a Coulter Epics XL (Hialeah, FL) flow cytometer with an argon ion laser operating at 500 mV at a wavelength of 488 nm. All tumor samples contained diploid inflammatory and stromal cells that served as an internal diploid standard. The data were analyzed using ModFit 5.02 (Verity Software House, Topsham, ME).

Samples were scored as containing aneuploidy cell populations if more than one discrete G0/G1 peak was identified in the histogram as described previously (11). The DNA index for aneuploid population was calculated as the quotient of the channel of the nondiploid peak divided by the channel of the diploid peak. If a peak was present at the diploid G0/G1 position (DNA index, 1.9–2.1), and represented greater than 10% of the total population and had a corresponding G0/G1 population, it was classified as tetraploid. The percentage of cells in S phase of the cell cycle was determined for each sample. For tumors showing aneuploid cell populations, this was recorded as the percentage of cells in the S-phase range of the aneuploid population. For diploid tumors, the percentage of S-phase cells in the entire diploid cell population was recorded.

**Statistics**

Survival was the primary study end point. Disease-free survival (the time from study randomization to recurrence or death) was also examined. Each of the four markers is presented as a dichotomized variable: Kl-ras (wild-type versus mutant), p53 (negative versus positive), ploidy status (diploid...
RESULTS

Patient Accrual and Follow-up

Results of the Intergroup therapeutic trial were recently reported separately for stage II and III patients. In the stage II study, a decreased relapse rate without a significant survival benefit was seen with adjuvant 5FU and levamisole (2, 3). In the stage III patients, a 33% reduction in mortality was found in the group treated with adjuvant 5FU and levamisole; no survival benefit was seen with levamisole alone.

A total of 234 patients (29% stage II and 71% stage III) were formally registered (i.e., tissue blocks were promised) to this biomarker substudy during the period from February 15, 1991, to September 15, 1993. Four of the registered patients were ineligible for the parent therapeutic trial because they were found postrandomization to have incompletely resected disease. Tissue blocks for a fifth patient were not forwarded to the laboratory. Data from these five patients have been excluded, leaving information on 66 stage II and 163 stage III patients. The clinical characteristics of the 229 patients enrolled in the biomarker study were comparable to those of the 1017 patients enrolled into INT-0035 but not registered to the biomarker study (Table 1). Survival information is as of July 15, 1996, at which time the median follow-up time was 8 years, the maximum was 11 years, and all but 1 patient had been followed 5 years or until death.

Marker Analyses: Quality Control and Distribution Among the Cancers Specimens

Kl-ras Mutations. Two hundred twenty of the 229 cancer samples (96%) and 100% of the 20 randomly selected resection margin samples were successfully analyzed by PCR-SSCP. Ninety of the 227

Table 1 Patient characteristics of patients in biomarker substudy and of all eligible intergroup patients not enrolled in biomarker study

<table>
<thead>
<tr>
<th></th>
<th>Stage II patients (%)</th>
<th>Stage III patients (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>In biomarker substudy</td>
<td>Not in biomarker substudy</td>
</tr>
<tr>
<td></td>
<td>(n = 66)</td>
<td>(n = 252)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>41</td>
<td>43</td>
</tr>
<tr>
<td>Male</td>
<td>59</td>
<td>57</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;61 yr</td>
<td>50</td>
<td>45</td>
</tr>
<tr>
<td>≥61 yr</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>Location of primary tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecum and right colon</td>
<td>32</td>
<td>27</td>
</tr>
<tr>
<td>Flexures and transverse colon</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>Left colon</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Sigmoid and rectosigmoid</td>
<td>29</td>
<td>35</td>
</tr>
<tr>
<td>Multiple sites</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Depth of invasion</td>
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<td></td>
</tr>
<tr>
<td>Submucosa or muscular layer</td>
<td>88</td>
<td>83</td>
</tr>
<tr>
<td>Serosa</td>
<td>84</td>
<td>86</td>
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<tr>
<td>Obstruction</td>
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<tr>
<td>No</td>
<td>88</td>
<td>83</td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>No. of nodes involved</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;4</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Histological differentiation</td>
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<tr>
<td>Well</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>Moderately well</td>
<td>80</td>
<td>73</td>
</tr>
<tr>
<td>Poorly</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observation</td>
<td>47</td>
<td>51</td>
</tr>
<tr>
<td>Levamisole only</td>
<td>53</td>
<td>49</td>
</tr>
<tr>
<td>Levamisole + 5FU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survival (7-yr rate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>74</td>
<td>73</td>
</tr>
<tr>
<td>Observation</td>
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<td>74</td>
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<tr>
<td>Levamisole only</td>
<td>74</td>
<td>73</td>
</tr>
<tr>
<td>Levamisole + 5FU</td>
<td></td>
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</tbody>
</table>

K-ras AND p53 IN COLORECTAL CANCER

versus aneuploid), and S phase (<10 versus ≥10%). Data for stage II and stage III patients were analyzed separately. All statistical analyses were carried out using the Statistical Analysis System (12, 13). Pairwise association among the markers was measured by the odds ratio (14). Survival curves were generated by the Kaplan-Meier method (15). The Cox proportional hazards model was used to explore whether marker status predicted survival and whether treatment by marker interactions occurred, i.e., if treatment effects comparing observation and adjuvant therapy arms differed by marker status (16). The multivariate survival analyses were adjusted for the clinical covariates found to have independent prognostic significance for survival in INT-0035 (2, 8). The first category listed below for each clinical variable is the reference group. In the stage II group, the model included covariates for site of primary tumor (sigmoid versus cecum and right colon versus transverse and flexures) and age (<61 versus ≥61 years). In the stage III group, the model included covariates for number of lymph nodes (1–4 versus >4), invasion of serosa (no versus yes), obstruction (no versus yes), invasion of adjacent organs (no versus yes), histological differentiation (moderately versus well versus poorly), and site of primary tumor (sigmoid versus cecum; right colon versus transverse and flexures).

Because blocks were not available for all patients or may not have contained sufficient tissue needed for all analyses, there was potential for selection bias favoring cases with bulky disease and excluding cases with small tumors. To determine whether selection bias was present, the pretreatment data for analyzable cases were compared to that of those in the larger group of patients for whom specimens were not provided.

Because four markers were measured in this study and multiple comparisons were made, the statistical analysis should be considered exploratory. The precision of some estimates (e.g., HRs in the treatment arms) is low due to the sample size. Except where noted, the Ps are unadjusted for multiple testing. A Bonferroni factor of 4 is recommended as an adjustment for Ps resulting from the Cox survival models (17) because four different biological markers were analyzed in this study. That is, an unadjusted P of 0.0125 is required to declare significance at the traditional 0.05 level.
cancer samples (40%) and 0 of 20 resection margin samples had an abnormal SSCP pattern. DNA sequencing of the abnormal SSCP patterns revealed that each of the 11 abnormal patterns was specific for a different point mutation. Among those with Ki-ras mutations, 68% were in codon 12, 29% were in codon 13, and single mutations were identified at codons 14, 15, and 19; 57% were G→A and 33% were G→T transversions, and 10% were G→C transitions.

Flow Cytometric Analysis of DNA Content. Interpretable DNA histograms for ploidy and S-phase analyses were obtained for 224 of the 229 tumors (98%). The mean coefficient of variation of the DNA histograms was 5.0 (range, 3.2-10.0). Nineteen of the tumors classified as aneuploid had tetraploid populations, and multiple DNA stem lines were detected in 10 cases.

p53 Immunohistochemistry. Interpretable immunostaining for p53 protein was obtained for 194 of the 229 cases. The other 35 cases were not analyzed because of inadequate tissue remaining on the block for the immunohistochemistry.

Distributions of Markers by Stage. Summary statistics for the markers as a function of stage are given in Table 2. Overall, 41% of the cancers had Ki-ras mutations, and 64% demonstrated overexpression of p53; there was no difference in the frequency of either marker between stage II and III cancers. Within the stage III group, Ki-ras mutation was significantly more common in those with more than four positive nodes than in those with 1–4 positive nodes (59 versus 32%; P = 0.002). Aneuploidy occurred more commonly in stage III than in stage II cancers (66 versus 47%; P = 0.009). A high proliferative rate (S-phase percentage, ≥10%) also tended to occur more commonly in stage III than in stage II cancers (55 versus 39%) but this difference did not reach statistical significance (P = 0.06).

There were no appreciable pairwise associations among the three genetic markers (Ki-ras mutation, p53 expression, and ploidy status) in either the stage II or III cancers (all P's exceeded 0.3). The S-phase fraction was lowest for diploid tumors (median, 5.6%), intermediate for aneuploid stage II tumors (median, 11.8%), and highest for aneuploid stage III tumors (median, 14.3%).

Marker Analysis and Survival

Univariate Analysis. The 7-year survival rates and the univariate HRs for death as a function of marker status are shown in Table 3. Ki-ras mutational status was a significant prognostic factor in patients with stage II cancers (HR, 3.8; P = 0.028 with Bonferroni correction) but not in the patients with stage III cancers. Expression of p53 and diploidy were associated with better 7-year survivals but were not statistically significant in the univariate analysis for either the stage II or III groups.

The survival curves of patients with Ki-ras wild-type and mutant cancers are shown in Fig. 1. In the stage II group, a persistent and progressively greater difference in the survival curves was present throughout the duration of the follow-up (P = 0.028 after Bonferroni correction). In the stage III group, patients with Ki-ras wild-type cancers also had a better survival throughout the follow-up period but the difference between the survival curves was not significant.

The survival curves for patients as a function of p53 protein expression are shown in Fig. 2. In both stage II and stage III, observed survival was better for patients with tumors that had overexpression of p53 protein, but the differences were not statistically significant in the univariate analysis.

Table 3 Survival rates and HRs by stage and marker status for patients with stage II and III colon cancers

<table>
<thead>
<tr>
<th>Factor</th>
<th>No.</th>
<th>7-yr survival (%)</th>
<th>Univariate model</th>
<th>Multivariate model</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>p*</td>
</tr>
<tr>
<td>Stage II</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ki-ras</td>
<td>Wild type</td>
<td>35</td>
<td>86</td>
<td>3.8 (1.4–10)</td>
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<tr>
<td>p53</td>
<td>Mutant</td>
<td>26</td>
<td>58</td>
<td>1.9 (0.7–5.1)</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>30</td>
<td>83</td>
<td>1.4 (0.6–3.4)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>24</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ploidy</td>
<td>35</td>
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<tr>
<td></td>
<td>Diploid</td>
<td>31</td>
<td>68</td>
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</tr>
<tr>
<td>S phase</td>
<td>&lt;10%</td>
<td>40</td>
<td>68</td>
<td>0.3 (0.1–1.01)</td>
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<tr>
<td></td>
<td>≥10%</td>
<td>26</td>
<td>85</td>
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</tr>
<tr>
<td>Stage III</td>
<td></td>
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<tr>
<td>Ki-ras</td>
<td>Wild type</td>
<td>96</td>
<td>59</td>
<td>1.5 (0.98–2.3)</td>
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<td>p53</td>
<td>Mutant</td>
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<td>Ploidy</td>
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<td></td>
<td>Diploid</td>
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<tr>
<td>S phase</td>
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<td>52</td>
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</tr>
<tr>
<td></td>
<td>≥10%</td>
<td>87</td>
<td>53</td>
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</tbody>
</table>

* A Bonferroni factor of 4 is recommended to correct for multiple testing (i.e., to declare significance at the 0.05 level when P ≤ 0.0125).

* From univariate Cox model, unadjusted for multiple comparisons.

* From multivariate Cox model with treatment and clinical covariates, unadjusted for multiple comparisons.
Analyses of disease-free survival curves were completely comparable to those found for overall survival (data not shown).

**Multivariate Analysis.** The multivariate analysis of the HR of death by marker as a function of treatment and the clinical covariates is shown in Table 3. In patients with stage II tumors, Ki-ras mutation was the only significant prognostic factor \((P = 0.012\) after Bonferroni correction). The other markers were not significantly related to survival.

In patients with stage III tumors, the presence of fewer than four positive lymph nodes and p53 protein expression were significant predictors of improved survival \((P < 0.00001\) and \(P = 0.012\), respectively, with Bonferroni correction). Ki-ras was not predictive of survival in the stage III group after multivariate adjustment for treatment and clinical covariates, due predominately to the positive association between Ki-ras mutation and nodal involvement.

Multivariate analysis combining stage II and stage III subjects using a COX model that included stage, grade, and either Ki-ras mutation or p53 protein expression revealed that Ki-ras and p53 were significant \((P = 0.001\) and 0.02, respectively) predictors of survival, independent of stage and grade.

**Marker Analysis and Adjuvant Therapy Effects**

The survival curves as a function of adjuvant therapy for patients with Ki-ras wild-type and mutant stage III colon cancers are shown in Fig. 3. In those with Ki-ras wild-type cancers, a persistent and progressively increasing survival advantage was present in the group treated with adjuvant 5FU plus levamisole therapy throughout the duration of the follow-up. No benefit of adjuvant levamisole was seen in the patients with Ki-ras wild-type, nor was any long-term benefit seen with either levamisole of 5FU plus levamisole in the patients with Ki-ras mutant, stage III tumors.

The survival curves as a function of adjuvant therapy for patients without and with p53 protein overexpressing stage III colon cancers are shown in Fig. 4. In those with cancers that did not express p53 protein, a persistent and progressively increasing survival advantage was seen in the group treated with adjuvant 5FU plus levamisole therapy throughout the duration of the follow-up. No benefit of adjuvant levamisole is seen in the patients without p53 expression nor with either adjuvant therapy regimen in the patients with p53-overexpressing stage III tumors. Analysis of disease-free survival gave results totally comparable to those found for overall survival.

The relationship between adjuvant therapy, survival, and marker status was investigated separately for stage II and stage III patient groups using survival rates and HRs for death for each treatment arm relative to the observation arm after adjustment for clinical covariates. In the 66 patients with stage II cancers, there was no improvement in survival as a result of adjuvant 5FU plus levamisole in either the entire group \((7\)-year survival was 74% in both treatment groups) or in any of the subsets defined by Ki-ras, p53 expression, ploidy status, or proliferative rate. In the patients with stage III cancers (Table 4), adjuvant therapy with levamisole alone was not associated with an improvement in survival in either the entire group or in any of the subsets defined by marker status. In the subset of patients with stage III tumors harboring a Ki-ras gene mutation, there was, however, an
K-ras AND p53 IN COLORECTAL CANCER

STAGE II

**p53 Positive (n=30)**

**p53 Negative (n=24)**

Survival Time in Years

10

8

6

4

2

0

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100 102 104 106 108 110 112 114 116 118 120

STAGE III

**p53 Positive (n=94)**

**p53 Negative (n=46)**

Survival Time in Years

10

8

6

4

2

0

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100 102 104 106 108 110 112 114 116 118 120

Fig. 2. Survival according to p53 protein expression. Overall survival of patients with stage II and III colon cancers as a function of the presence (p53 Positive) or absence (p53 Negative) of p53 protein expression in the primary cancer.

unexpectedly poor survival associated with levamisole treatment as compared to surgery alone. Adjuvant therapy with 5FU plus levamisole was associated with better survival compared to surgery alone in the patients with stage III cancers, but the difference was not statistically significant (Table 4). In the group of patients with wild-type K-ras tumors, there was improved survival associated with 5-FU plus levamisole therapy (76 versus 44%; HR, 0.4; 95% CI, 0.2–0.8), whereas no such improvement was observed in the group of patients harboring a K-ras-mutated tumor (52 versus 55%; HR, 1.1; 95% CI, 0.5–2.7). This differential treatment effect (interaction) between K-ras wild-type and mutant tumors was not significant (P = 0.28 with Bonferroni adjustment). That is, the therapy HRs for K-ras wild-type (HR, 0.4) and K-ras mutant type (HR, 1.1) did not differ significantly. In the group of patients with cancers that had no detectable p53 protein expression, adjuvant 5FU and levamisole was associated with an improved survival (64 versus 26%; HR, 0.3; 95% CI, 0.1–0.7), but no such improvement was seen in patients harboring a tumor with p53 overexpression (71 versus 53%; HR, 0.8; 95% CI, 0.4–1.6). This differential treatment effect between p53-expressing and p53-nonexpressing tumors was not significant (P = 0.28 after Bonferroni adjustment). Again, the therapy HRs for p53-negative (HR = 0.3) and p53-positive (HR = 0.8) tumors did not differ significantly. The effect of adjuvant 5-FU and levamisole was not different as a function of ploidy status or proliferative rate (Table 4).

DISCUSSION

This study has examined the relationship between a series of genetic (K-ras mutation, p53 protein expression, and ploidy status) and biological (proliferative rate) markers and the clinical behavior of colon cancer in the setting of a large prospective controlled trial of adjuvant therapy. This clinical setting offers several strengths for the design of the current study: all patients were staged in a standard manner, and the diagnoses and staging were confirmed centrally; all patients were followed on protocol at standard intervals for survival and disease recurrence; and follow-up is complete. Thus, the relationship between the genetic and biological profile of colon cancers and survival can be reliably assessed in a mature, prospectively followed cohort. Because adjuvant 5FU and levamisole therapy resulted in a 33% improvement in survival in the parent stage III Intergroup Trial (3), the current study design allowed us explore relationships between the marker profile of colon cancers and the survival benefits of adjuvant therapy. The interpretation of the results of the current study design is limited by the sample size and by the measurement of multiple genetic parameters in the same set of samples, which increases the probability of false declarations of statistical significance. For this reason, Ps have been adjusted for multiple comparisons using the Bonferroni method. Although this adjustment method could mask truly significant differences, its use seems prudent to avoid overinterpretation of the reported associations.

In general, the genetic profile of the colon cancers in this study is similar to previous reports. Our observations of mutations in codons 12 and 13 of the K-ras oncogene in 40% of the colon cancers examined is well within the range reported by others (4, 5, 18–25). Although our observation that the frequency of K-ras mutation is similar in the stage II and III cancers has been previously noted (18–25), we found a strong association between K-ras mutation and
the number of lymph nodes involved among the patients with stage III cancers. Ki-ras mutation was significantly less frequent in patients with 1–4 positive nodes than in those with more than 4 positive nodes cancers (32 versus 59%; $P = 0.008$ after Bonferroni adjustment). This association has not been noted in previous studies, probably because the number of positive lymph nodes is not routinely recorded in most colon cancer staging systems. Because the number of positive lymph nodes was the most significant single prognostic factor in the patients with stage III cancers in this study, the strong association between nodal status and Ki-ras mutation may explain some of the differences among previous reports of the relationship between Ki-ras mutation and survival (see below).

Conflicting results have been reported in previous studies of the relationship between survival and Ki-ras mutation in colon cancers; some have found an improved survival in patients with Ki-ras-mutated tumors (22, 25), whereas others have found no association (21). Our findings of the interrelationship between Ki-ras mutation, nodal status, and survival may explain these differences. In our study, having a Ki-ras mutant tumor was strongly associated with a worse prognosis in patients with stage II but not in those with stage III colon cancers. Within the stage II group, the survival in patients with Ki-ras mutant tumors (58% at 7 years) was not only substantially worse than those with wild-type Ki-ras tumors, it was similar to the overall survival in our patients with stage III cancers (52% at 7 years). Conversely, patients in this study with stage II/Ki-ras wild-type colon cancers had a survival (86% at 7 years) that is comparable to that reported for patients with $T_2$, $N_0$, $M_0$ (stage I) colon cancers (1). A trend toward an improved prognosis in patients with Ki-ras wild-type cancers was observed in the stage III group, but the survival difference (59 versus 43% at 7 years) was not statistically significant in either univariate or multivariate analysis. Most of the previous studies that have found an association between Ki-ras status and a poorer survival have been more heavily weighted toward patients with stage II cancers (22), whereas those that have failed to find an association have included more patients with stage III disease (21, 23). When Benhattar et al. (22) analyzed their retrospective study by stage, they found a pattern similar to our results. Ki-ras mutations were significantly more common in patients with recurrent than in those with nonrecurrent stage II tumors ($P < 0.0001$) but only a borderline significant relationship ($P = 0.049$) was found in patients with stage III disease. None of the previous reports that found an association between Ki-ras mutations and survival in stage III colon cancers included the number of positive nodes in their multivariate model.

The strong association between Ki-ras mutation and nodal involvement found in this series suggests the hypothesis that Ki-ras mutations in established colon cancers could reflect an enhanced capacity for lymphatic invasion. The findings of Miyahara et al. (24) that the incidence of lymphatic invasion and lymph node metastasis correlates with overexpression of the ras protein is also consistent with this hypothesis. If this is true, it could explain the association between Ki-ras mutation and poor prognosis in the stage II study, i.e., Ki-ras-mutated stage II tumors could have a higher rate of subclinical lymphatic involvement and thus a worse prognosis. Our data that Ki-ras mutation is associated with a poorer survival is similar to that
found in a recent meta-analysis of studies of the association between Ki-ras mutations and recurrence rate in colorectal cancer.4

Mutations in the p53 gene are frequently associated with stable overexpression of the mutant form of the p53 protein (26), and p53 overexpression detected by immunohistochemistry has been frequently used as a surrogate marker for p53 mutations in tissues (27–36). Our findings of p53 overexpression in 64% of the colon cancers examined and a similar frequency of p53 protein overexpression in stage II and III cancers confirm the observations of numerous previous reports (28–36). Most of the previous studies of p53 mutational status and colorectal cancer prognosis have found that patients with colorectal cancers harboring a p53 mutation have a worse prognosis than those with wild-type cancers (23, 37–39). In this light, our findings that p53 protein overexpression was associated with an improved survival in patients with stage III colon cancers was unexpected. In our patient population, an improved survival was observed in the p53-positive group in both the stage II and stage III groups, but not significantly so, by univariate analysis (Table 3 and Fig. 2). The association only became significant after adjustment for the other covariates (nodal status was the most significant) in the multivariate analysis. In no group was there any indication that p53 protein overexpression was associated with a worse prognosis.

The discordance between the results of previous studies examining the prognostic significance of p53 mutations assessed by direct mutational analyses and our results measuring overexpression of the p53 protein suggest that the two assays are not measuring the same thing. It is has been reported that only about 80% of p53 mutations result in overexpression of the mutant protein as detected by the DO7 anti-p53 antibody that we used and that nonmutational mechanisms, such as sequestration of the normal p53 protein by viral transforming proteins, can induce p53 protein overexpression (27). Variable p53 staining can also be seen with different antibodies (27), and the association between p53 overexpression and mutation may be higher than 80% with some of the other antibodies. It is likely that some of the differences between the p53 immunohistochemical reports are due to the use of antibodies with differing specificities. Yamaguchi et al. (30) used a polyclonal antibody (Pab1801) that detects both the wild-type and mutant p53 protein and observed a significantly improved survival in 55 patients with stage III colon cancers that were p53 negative compared to those that were positive by immunohistochemistry (5-year survival, 89 versus 59%). In contrast, Mulder et al. (36) used antibody DO7, the same one that we used in the current study, and found, as we did, a trend toward an improved survival in patients with cancers that expressed p53 immunoreactivity. These authors had only 27 stage III cancers in their series and did not include the number of nodes in their multivariate analysis. We have not directly measured p53 gene mutations in our series, so we cannot estimate the correlation between p53 protein expression and gene mutations. Our results do suggest, however, that the group of colon cancers that have p53 overexpression as assessed by immunohistochemistry with the AB6 clone DO7 anti-p53 antibody used in this study represents a subset that has a different clinical behavior than the overall group with p53 muta-

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4 H. J. N. Andreyev, personal communication.
tions. A dissociation between mutational and immunohistochemical assays of p53 and survival has also been reported in human lung cancers (37).

Our observations that 61% of the colon cancers examined were aneuploid and that aneuploidy is more common in stage III than in stage II colon cancers are consistent with most previous reports (40–47). In our series, ploidy status was not a significant prognostic factor in either the stage II or III groups (Tables 2 and 3). Conflicting data have been previously reported on this point in the literature, but most studies have found no effect or a weak association. Our data suggest that the interrelationships between ploidy status, stage, and other covariates may explain much of the variability in the previous literature about ploidy status and prognosis of colorectal cancer. The previous studies have not generally included the number of nodes in the multivariate analysis model. In contrast to some previous studies (41), no association was seen in this series between a high proliferative rate in the cancer and a poor prognosis in either stage II of stage III patients.

Patients with stage III colon cancers have been shown to have a survival benefit from adjuvant chemotherapy (2, 3), whereas studies in patients with stage II cancers have failed to find a survival benefit (8). One reason suggested for this apparent difference in response to adjuvant therapy in the two stages is that there are so few recurrences in the stage II group that a survival benefit of adjuvant therapy is missed. One reason to attempt to identify subsets of patients with stage II cancers that have a worse prognosis is that they might be more likely to benefit from adjuvant therapy. Our results in a small group (26 patients) of patients with stage II-Ki-ras mutant colon cancers suggest that this reasoning may be flawed. This group did have poor overall survival that was indeed similar to the stage III group, but there was no suggestion of a benefit from adjuvant therapy in the Ki-ras-mutated stage II group.

We speculate that the lack of benefit of adjuvant therapy in the presence of a low overall survival in patients with Ki-ras-mutated cancers may be due to a relative resistance to 5FU and levamisole induced by Ki-ras mutations. This possibility is supported by our observation that patients with Ki-ras-mutated stage III cancers failed to benefit from adjuvant therapy, whereas those with stage III-wild-type Ki-ras cancers did experience better survival with adjuvant 5FU plus levamisole than with surgery alone, although the differential treatment effect (interaction) was not significant.

In this series, patients with stage III cancers that had p53 overexpression failed to show a benefit from adjuvant therapy, whereas those without p53 expression showed better survival with adjuvant 5FU plus levamisole than with surgery alone, although the differential treatment effect was not significant. Previous uncontrolled series have suggested that p53 mutations might be associated with resistance to therapeutic agents (25), but this is the first prospective controlled evaluation of this question.

Our results provide no direct insight into any possible mechanism(s) for chemotherapeutic resistance of Ki-ras-mutated or p53-overexpressing colon cancers. It is known that at least part of 5FU cytotoxicity is due to a p53 dependent induction of apoptosis, so it is not surprising that cancers that do not have a normally functioning p53 gene would be less responsive to 5FU treatment. A similar resistance to cytotoxic therapy has been found in p53-overexpressing lung and bladder cancers. With respect to the Ki-ras-mutated cancers, one could hypothesize that part of the therapeutic effect of 5FU and levamisole is mediated by an upstream blockade of one or more of the Ki-ras mediated signaling pathways and mutations in Ki-ras activate the pathway downstream of the drug effect, but this is only speculation.

Regardless of the mechanisms involved, our results suggest that the relationship between Ki-ras mutations, p53 status, and response to 5FU in advanced colon cancer should also be evaluated.

On the basis of our results, we suggest that Ki-ras mutational status and p53 expression can be useful prognostic markers in colon cancer, and we question whether patients with stage III colon cancers that are shown to have either Ki-ras mutations or to express p53 protein should be routinely treated with the current standard adjuvant chemotherapy regimens. If our observations are confirmed, alternative regimens will need to be evaluated for these patients, and Ki-ras gene and p53 protein status will need to be included as a stratification or analysis criteria in future adjuvant trials.

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