The Use of Molecular Profiling of Early Colorectal Cancer to Predict Micrometastases

Anton J. Bilchik, MD, PhD; Dean T. Nora, MD; Sukamal Saha, MD; Roderick Turner, MD; David Wiese, MD; Christine Kuo, BS; Xing Ye, BS; Donald L. Morton, MD; Dave S. B. Hoon, PhD

Background: Approximately one third of node-negative colorectal cancers (CRCs) recur, suggesting the failure to detect occult disease. Lymphatic mapping followed by focused analysis of the sentinel node is highly accurate in identifying micrometastases.

Hypothesis: Because aberrant genetic changes occur early in tumor progression and are associated with lymphatic metastases, we hypothesized that the molecular profiling of specific tumor markers in the primary tumor might predict that tumor’s metastatic potential.

Design: A prospective patient series.

Patients and Interventions: Forty consecutive patients with early CRC underwent lymphatic mapping after subserosal injection of 1 mL of isosulfan blue dye. All lymph nodes were examined by hematoxylin-eosin (HE) staining, and multiple sections of each sentinel node were examined by HE and cytokeratin immunohistochemistry (CK-IHC) staining. Primary tumors were analyzed for p53 expression using IHC staining and for β-human chorionic gonadotropin (β-hCG), hepatocyte growth factor receptor (c-Met), and universal melanoma antigen (uMAGE) messenger RNA expression using reverse-transcriptase polymerase chain reaction and electrochemiluminescence.

Results: Nine patients (23%) had positive nodes by routine HE staining. Of the remaining 31 patients with negative nodes on HE staining, 8 tumors (26%) were upstaged by CK-IHC identification of occult micrometastases. There was a direct correlation between the number of markers and the T stage ($P = .001$). The expression of p53, β-hCG, c-Met, and uMAGE in primary tumors was significantly higher in the presence of nodal micrometastases vs no metastases ($P = .03$).

Conclusions: Sentinel lymphatic mapping is an accurate method of detecting micrometastases in CRC. Molecular profiling of primary CRC tumors, similar to that used for breast cancer, may be important in predicting metastatic potential and determining which patients may benefit from adjuvant therapy.

Arch Surg. 2002;137:1377-1383

Original Article

From the Departments of Molecular Oncology and Gastrointestinal Research, John Wayne Cancer Institute, Santa Monica, Calif (Drs Bilchik, Nora, Turner, Morton, and Hoon and Mss Kuo and Ye), and Michigan State University, Flint (Drs Saha and Wiese).

©2002 American Medical Association. All rights reserved.
bryonic antigen revealed micrometastasis in 54% of patients. Five-year survival was only 50% in the RT-PCR node-positive group compared with 91% in the absence of RT-PCR micrometastases. Similarly, Hayashi et al10 demonstrated decreased survival in patients with p53 or K-ras mutations in colon lymph nodes.

There is little doubt, therefore, that sensitive tests, such as IHC analysis and RT-PCR, can enhance tumor detection. It is, however, neither practical nor cost-effective to perform these tests on numerous lymph nodes. Intraoperative lymphatic mapping as first described by Morton et al9 can identify the initial node that drains a focus of micrometastasis.10,11

Figure 1. Colon sentinel node (blue with suture) identified 60 seconds after subserosal injection of 1 mL of isosulfan blue dye.

METHODS

Between August 3, 1999, and August 28, 2001, 40 consecutive patients with localized CRC underwent lymphatic mapping of the SN at the time of curative surgical resection. Informed consent was obtained preoperatively from the patients in accordance with the institutional review boards of Saint John’s Health Center, Santa Monica, Calif, and McLaren Regional Medical Center, Flint, Mich.

ASSESSMENT OF REGIONAL NODES

All lymph node sections were analyzed by conventional hematoxylin-eosin (HE) staining. Each SN was then specifically examined using a focused technique originally described for breast carcinoma.29 Each SN was bisected and sectioned at 2- to 3-mm intervals. Serial frozen sections were cut 6 µm thick. Paraffin-embedded sections, each approximately 4 µm thick, were cut at 2 levels separated by 200 µm. One section from each level was stained with HE and another section was stained with CK-IHC. Tumor cells were considered micrometastatic if negative by HE staining but positive by CK-IHC staining. Paraffin sections for CK-IHC staining were placed on charged slides (Superfrost Plus M6416-plus; Becton Diagnostics Inc, McGaw Park, Ill). An automated immunostainer (Ventana ES; Ventana Medical Systems Inc, Tucson, Ariz) was used.

©2002 American Medical Association. All rights reserved.
were interpreted according to predetermined criteria combining pathologic and immunoreactive features.

**ASSESSMENT OF PRIMARY TUMORS**

The primary tumors were then assessed for expression of p53 by IHC staining and for expression of β-hCG, c-Met, and uMAGE mRNA by RT-PCR. Molecular markers were previously examined for a similar cohort, but the information was updated to include IHC analysis and correlations with SN findings.

**IHC ANALYSIS**

The IHC analysis of p53 was performed according to previously published methods. Tissue sections 5 μm thick of paraffin-embedded tumor specimens were cut and mounted on poly-l-lysine slides (VWR Superfrost Plus; Baxter Diagnostics Inc). The tissues were deparaffinized in Histoclear for 15 minutes. Specimens were rehydrated by sequential washing in progressively increasing aqueous ethanol solutions. Endogenous peroxidase activity was inhibited by a 10-minute incubation in 3% hydrogen peroxide (Peroxide Block; BioGenex Laboratories Inc, San Ramon, Calif). Slides were then washed in phosphate-buffered saline. Nonspecific protein binding was abolished by treating the slides with normal goat serum Protein Block (BioGenex Laboratories Inc) for 10 minutes, followed by application of the primary antibody. Paraffin-embedded MCF-40F cells were used as a positive control for p53. Control cell line expression was confirmed by Western blot analysis. Each specimen had a negative control using nonimmune IgG. Slides were exposed to 0.5 mg/ml of mouse monoclonal anti-p53 antibody clone DO-1 (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) for 1 hour.

Tissue sections were scored for intensity and percentage of tumor cells staining positively for p53 by light microscopy and image analysis. The intensity of staining was scored according to the following scale: negative when less than 5% of tumor cells displayed stain, 1+ for mild intensity, 2+ for moderate intensity, 3+ for intensity equal to positive controls, and 4+ for staining greater than positive controls.

**RT-PCR ANALYSIS**

Expression of mRNA tumor markers β-hCG, c-Met, and uMAGE mRNA was evaluated in the primary tumor from a prior study. These markers were analyzed in CRC primary tumors, CRC cell lines, donor peripheral blood lymphocytes (PBLs) from healthy volunteers, and nonmalignant lymph nodes from 5 patients undergoing colon operations for noncancerous conditions. Colon cancer cell lines HT-29 and SW480 were obtained from American Type Culture Collection (Rockville, Md). JAR choriocarcinoma cell line was obtained from American Type Culture Collection. Colorectal cancer cell lines JWCI-0044 (A), JWCI-0361, JWCI-0427, JWCI-0485, JWCI-1100, and JWCI-1203 were established and characterized at John Wayne Cancer Institute (JWCI), Santa Monica. Cells were cultured and prepared, and total RNA was extracted as previously described.

The RT was performed using Moloney murine leukemia virus RT (Promega Corporation, Madison, Wis). The same amount of RNA (1 μg) was used for all specimens and controls assayed. The PCR was then performed on the complementary DNA according to the following sequence: denaturing at 95°C for 5 minutes; 35 cycles at 95°C for 1 minute; annealing at 65°C for β-hCG, 55°C for c-Met, and 62°C for uMAGE for 1 minute; and final primer sequence extension incubation at 72°C for 10 minutes. The RT-PCR was performed using a Hybaid thermocycler (Hybaid, Middlesex, England).

The PCR products were evaluated by electrochemiluminescence detection analysis on the ORIgen analyzer (Igen International Inc, Gaithersburg, Md). The RT-PCR assay and electrochemiluminescence detection system (hereafter referred to as RT-PCR assay system) was designed so that primers of the individual markers spanned at least 1 intron region. All assays contained equal amounts of RNA, and RT was performed with olig d(T) primers. At least 2 positive controls (CRC lines), 4 negative controls (normal donor PBLs and normal lymph nodes), and reagent controls (reagents alone without RNA or complementary DNA) were included. Each assay contained its own set of controls for establishing background levels. Any sample electrochemiluminescence unit value above the established cutoff was considered a positive result in each assay. The RT-PCR analysis of tumor tissue was performed at least twice to verify the results.

The uMAGE assay was developed to assess multiple MAGE-A gene family markers. The MAGE-A genes are expressed in tumor cells, including carcinomas, and not normal cells, except testis and placenta. Colorectal cancer expresses several of the MAGE-A gene family members. To avoid performing multiple individual MAGE-A assays, we developed a primer and probe set to detect multiple MAGE-A genes in an individual reaction. Previously, we demonstrated the sensitivity and specificity of the assay. At the JWCI, we developed a novel multimarker RT-PCR assay for the detection of micrometastases. This multimarker system eliminates some of the inherent problems associated with single-marker techniques, such as tumor heterogeneity, clonal selection, and variable mRNA expression of individual genes. The 3 RT-PCR markers were assessed in established CRC lines: any individual marker was expressed in more than 50% of cell lines, and at least 1 marker was expressed in any given cell line (n=6) (data not shown). Overall, the assay’s detection sensitivity was from 1 to 5 tumor cells in 10 million PBLs. The sensitivity of the assay for individual markers ranged from 0.1 to 10 ng of mRNA. These studies demonstrated that the markers were frequently expressed and the detection sensitivity of the assay was high. The specificity of the assay was determined by analyzing healthy donor PBLs (n=25) and lymph nodes from patients undergoing surgery for benign conditions. None of the 3 markers was detected in healthy donors’ PBLs or CRC-negative lymph nodes.

**STATISTICAL ANALYSIS**

The McNemar test was used to compare patients with HE-positive SNs with those whose SNs were positive by either HE or CK-IHC staining (each T stage was evaluated independently and then combined). The Fisher exact test was conducted to compare percentages of patients who had nodal metastases confined to the SN among different T stages. The correlation between the number and type of tumor markers expressed by the primary tumor and the presence of micrometastases in the SN was determined by the Fisher exact test. The multivariate logistic regression of micrometastases was performed by exact methods. T stage, tumor markers, and number of markers were included in the model as explanatory variables.

**RESULTS**

There were 19 men and 21 women in our study, with an average age of 63 years (range, 38-85 years). Primary tumors were in the right colon (n=20), left colon (n=5),...
and sigmoid colon (n=15). Lymphatic mapping demonstrated at least 1 SN in all cases. Overall, an average of 1.7 SNs (range, 1-4) and 15 total nodes (range, 6-35) were harvested from each CRC specimen. The tumor status of the SN accurately reflected the status of the nodal basin in all patients.

Twenty-three patients had no nodal metastases. Of the 40 patients, 9 (23%) had nodal metastases by routine HE analysis (macrometastases). Of the 31 patients without nodal metastases by HE, 8 patients (26%) had micrometastatic foci in the SN only (Figure 2). Increasing T stage was related inversely to the probability of detecting isolated micrometastases in the SN (1/1 node-positive T1 tumors, 2/3 node-positive T2 tumors, 5/10 node-positive T3 tumors, and 0/3 node-positive T4 tumors) (Table 1).

The p53 phenotype was expressed in the primary tumors of 22 patients (55%), with node-positive tumors having the highest incidence (88% vs 30%; P<.001). All tumors with SN micrometastases expressed p53. In Table 2, the percentage of individual marker expression in the primary tumor relative to metastases in the sentinel node is shown. Of all the markers analyzed individually, only p53 was shown to be significantly different in node-positive (macrometastatic or micrometastatic) vs node-negative tumors (Table 2).

There was a significant correlation (P=.03; 2-tailed Fisher exact test) between the number of mRNA tumor markers in the primary tumor and the presence of nodal micrometastases vs no metastases. Essentially, more markers were expressed in tumors with micrometastatic disease than their node-negative counterparts (Table 3).

When analysis was performed using paired groupings of individual molecular markers, tumors that ex-
pressed β-hCG and c-Met or β-hCG and uMAGE mRNA had a significantly higher incidence of nodal micrometastases \( (P = .05) \) (Table 4). c-Met and uMAGE expression approached significance \( (P = .06) \). Using the Fisher exact test, there was a significant correlation between SN pathologic status and number of positive markers (molecular and IHC analysis) (Table 5). There was a significant correlation of 2 or more vs 0 to 1 markers in the primary tumor to the SN status. There was also a significant correlation \( (P = .03; \) Fisher exact test) between p53 expression and the number of mRNA tumor markers detected in the primary tumor (Table 6). Using multivariate analysis, p53 expression in the primary tumor significantly correlated with micrometastasis \( (P = .02) \) (Table 7). When we combined the 2 tests (molecular markers and p53 IHC staining), we found the combination to be extremely sensitive in detecting occult disease as determined by lymphatic mapping. The sensitivity was highest (94%) when both tests were performed compared with individual testing.

In patients with primary CRC tumors, lymph node analysis continues to be the most important prognostic factor and the best means of selecting candidates for adjuvant chemotherapy.\(^2\) Approximately one third of CRCs, however, are likely to be understaged because too few nodes are examined and/or occult micrometastases are not detected. Because it is impractical and expensive for the pathologist to perform multiple sectioning and IHC analysis on multiple lymph nodes, the SN procedure was developed to identify the first nodal site of regional metastasis. Studies in different solid malignancies, includ-

### Table 3. Correlation Between Marker Expression in Primary Tumor and Metastases in SN\(^*\)

<table>
<thead>
<tr>
<th>Tumor Status of SN</th>
<th>β-hCG</th>
<th>c-MET</th>
<th>uMAGE</th>
<th>No. of Positive Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macro vs micro vs node-negative marker</td>
<td>.67</td>
<td>.29</td>
<td>.90</td>
<td>.17</td>
</tr>
<tr>
<td>Micro vs node-negative marker</td>
<td>.42</td>
<td>.20</td>
<td>.67</td>
<td>.03</td>
</tr>
</tbody>
</table>

\(^*\)SN indicates sentinel node; β-hCG, β-human chorionic gonadotropin; c-MET, hepatocyte growth factor receptor; uMAGE, universal melanoma antigen; macro, metastases detected by hematoxylin-eosin staining; and micro, micrometastases detected by immunohistochemical staining. †Fisher exact test.

### Table 4. Correlation Between Marker Pairs in Primary Tumor and Histopathologic Status of SN\(^*\)

<table>
<thead>
<tr>
<th>Tumor Status of SN</th>
<th>β-hCG + c-MET</th>
<th>β-hCG + uMAGE</th>
<th>c-MET + uMAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macro vs micro vs node-negative marker</td>
<td>.20</td>
<td>.04</td>
<td>.24</td>
</tr>
<tr>
<td>Micro vs node-negative marker</td>
<td>.05</td>
<td>.05</td>
<td>.06</td>
</tr>
</tbody>
</table>

\(^*\)SN indicates sentinel node; β-hCG, β-human chorionic gonadotropin; c-MET, hepatocyte growth factor receptor; uMAGE, universal melanoma antigen; macro, metastases detected by hematoxylin-eosin staining; and micro, micrometastases detected by immunohistochemical staining. †Fisher exact test.

### Table 5. Correlation Between Number of Markers and SN Status\(^*\)

<table>
<thead>
<tr>
<th>Tumor Status of SN</th>
<th>≥2 vs 0-1</th>
<th>≥3 vs 0-2</th>
<th>≥4 vs 0-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macro vs micro vs node-negative marker</td>
<td>.03</td>
<td>.17</td>
<td>.99</td>
</tr>
<tr>
<td>Micro vs node-negative marker</td>
<td>.03</td>
<td>.10</td>
<td>.99</td>
</tr>
</tbody>
</table>

\(^*\)Markers expressed were p53 (detected by immunohistochemical [IHC] staining), β-human chorionic gonadotropin, hepatocyte growth factor, and universal melanoma antigen messenger RNA. SN indicates sentinel node; macro, metastases detected by hematoxylin-eosin staining; and micro, micrometastases detected by IHC staining. †Fisher exact test.

### Table 6. Correlation Between Expression of p53 and mRNA Markers in the Primary Tumor\(^*\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>p53</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE and IHC of SN</td>
<td>Positive</td>
<td>.12</td>
</tr>
<tr>
<td>Negative</td>
<td>.16</td>
<td>.007</td>
</tr>
<tr>
<td>β-hCG</td>
<td>Positive</td>
<td>.14</td>
</tr>
<tr>
<td>Negative</td>
<td>.08</td>
<td>.52</td>
</tr>
<tr>
<td>c-Met</td>
<td>Positive</td>
<td>.11</td>
</tr>
<tr>
<td>Negative</td>
<td>.13</td>
<td>.2</td>
</tr>
<tr>
<td>uMAGE</td>
<td>Positive</td>
<td>.08</td>
</tr>
<tr>
<td>Negative</td>
<td>.12</td>
<td>.99</td>
</tr>
<tr>
<td>No. of positive mRNA markers</td>
<td>0</td>
<td>.03</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^*\)mRNA indicates messenger RNA; HE, hematoxylin-eosin staining; IHC, immunohistochemical staining; SN, sentinel node; β-hCG, β-human chorionic gonadotropin; c-Met, hepatocyte growth factor receptor; and uMAGE, universal melanoma antigen. †Fisher exact test.

### Table 7. Multivariate Analysis of Association Between Marker Expression and Lymph Node Metastases\(^*\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-stage</td>
<td>2.884 (1.005-8.275)</td>
<td>.05</td>
</tr>
<tr>
<td>p53</td>
<td>9.467 (1.475-60.777)</td>
<td>.02</td>
</tr>
<tr>
<td>No. of positive markers (≥2 vs 0-1)</td>
<td>1.961 (0.237-15.457)</td>
<td>.54</td>
</tr>
</tbody>
</table>

\(^*\)Logistic regression, N = 40. OR indicates odds ratio; CI, confidence interval.

©2002 American Medical Association. All rights reserved.
ing CRC, demonstrate that if the SN is negative for metastasis, then the likelihood of skip metastasis is low (<4%).31,32 We have shown that meticulous and focused analysis of the SN can increase the identification of micrometastatic disease.11 In this study, SN evaluation was extremely accurate as an indicator of regional lymph node status, and using IHC analysis, micrometastases were identified in an additional 26% of patients. In the primary tumor, β-hCG was the most frequently expressed molecular marker, followed by c-MET and then uMAGE. There was an increase in the expression of the molecular markers in primary tumors with micrometastasis. This corresponded with p53 expression assessed by IHC analysis.

The pathologic role of the mRNA markers is still unknown. β-hCG may function as a suppressive factor of immune responses.27 c-Met may be important as a molecular phenotypic marker for metastasis and as a marker for early detection.23,24 c-Met, a proto-oncogene, encodes for the receptor that binds to the ligand hepatocyte growth factor/scatter factor, which can induce cell proliferation, invasion, and motility of CRC cells. Some organs, such as the liver, to which CRC frequently metastasizes, produce significant levels of hepatocyte growth factor. The expression of c-Met may enhance tumor cell survival at distant organ sites. The physiologic role of MAGE-A gene expression is also unclear, but both MAGE-A-1 and MAGE-A3/6 are immunogenic in humans and are potential targets for active-specific immunotherapy.26 The p53 tumor suppressor gene plays an important role in DNA repair, apoptosis, and cell cycle progression. The p53 nuclear protein expression assessed by IHC staining is a marker for p53 gene overexpression.15 The detection (genetic aberration) of p53 is common in advanced CRC tumors, but it is detected far less in early-stage tumors (adenomas). Therefore, the functional inactivation of p53 is considered a late event. Overexpression in early CRC is likely related to early events of genetic instability during tumor progression. It is, however, unclear whether p53 overexpression is correlated with poor survival13-15,17,18 or improved survival. Vermeulen et al.28 demonstrated that p53 gene overexpression may be associated with increased tumor vascularity. This is currently being investigated in our laboratory.

In the United States, many patients with node-negative colon cancer are treated with systemic chemotherapy with little or no benefit. This translates to both unnecessary toxic effects and expense. Molecular markers that accurately predict nodal metastasis are therefore needed. In this study, we demonstrate that molecular markers in combination with the IHC marker p53 can predict nodal metastasis with excellent sensitivity. Furthermore, the molecular profile of tumors with micrometastatic disease was not significantly different from the molecular profile of tumors with macrometastatic nodal involvement. It remains unclear whether tumors associated with micrometastatic disease to lymph nodes have the same prognosis as those with gross nodal involvement. However, they were significantly different from node-negative tumors, suggesting that high-risk, node-negative tumors have acquired the necessary molecular changes that allow them to metastasize. This was demonstrated by the discovery of micrometastasis in lymph nodes from initially node-negative tumors using lymphatic mapping technology.

The sequence of genetic alterations that lead to CRC progression was initially proposed by Vogelstein et al.33 Genetic alterations that seem to be important prognostic markers include loss of heterozygosity of chromosome 18q, K-ras mutations, changes in the level of certain gene products such as the DCC (deleted in colorectal cancer) protein, p53 protein and p27 protein, and increased expression of genes involved in fluoropyrimidine metabolism.10,20 In addition, high expression of microsatellite instability (MSI) in tumors has been associated with a better prognosis and improved response to chemotherapy.21 These markers can potentially be used to identify molecular fingerprints that help stratify high-risk patients best suited for more aggressive therapy following complete surgical resection.

To our knowledge, this is the first time changes in the molecular profile of the primary tumor have been associated with regional draining nodal micrometastasis. Although the prognostic value of nodal micrometastasis is unknown, the overexpression of molecular markers in the primary tumor would suggest that these patients are at higher risk for recurrence and therefore are better candidates for systemic chemotherapy than those with low expression and the absence of nodal micrometastasis. The identification of primary tumor molecular profiles at the very early stages of regional node metastasis may also be of importance in determining if any particular genes are likely to favor tumor spread.

Clearly, better staging methods are needed for patient stratification. To better elucidate the prognostic significance of phenotypic and genotypic characteristics of nodal micrometastases in CRC and to determine how these factors might play a part in a comprehensive staging schema, we have initiated a prospective trial focused on an in-depth analysis of SNs, including IHC analysis and multimarker molecular studies.

Accepted for publication July 6, 2002.

This study was supported in part by grant CA 090848 from the National Cancer Institute, Bethesda, Md, and funding from the Rogovin-Davidow Foundation, Los Angeles, Calif, and the Rod Fasone Memorial Cancer Fund, Indianapolis, Ind.

This study was presented at the 2001 Annual Meeting of the Western Surgical Association, San Antonio, Tex, November 12, 2001.

We thank Oncotech Inc, Tustin, Calif, for their assistance in the p53 analysis of this study.

Corresponding author and reprints: Anton J. Bilchik, MD, PhD, John Wayne Cancer Institute, 2200 Santa Monica Blvd, Santa Monica, CA 90404 (e-mail: bilchika@jwci.org).

REFERENCES


