Epidermal Growth Factor Receptor, c-MET, β-Catenin, and p53 Expression as Prognostic Indicators in Stage II Colon Cancer: A Tissue Microarray Study

Murray B. Resnick,1 Justin Routhier,1 Tamako Konkin,1 Edmund Sabo,3 and Victor E. Pricolo2
Departments of 1Pathology and 2Surgery, Rhode Island Hospital and Brown University School of Medicine, Providence, Rhode Island, and 3Department of Pathology, Carmel Medical Center, Haifa, Israel

ABSTRACT

Purpose: Through the use of molecular markers, it may be possible to identify aggressive tumor phenotypes and tailor therapies to treat them. This approach would be particularly useful for stage II colon cancer. The purpose of this study was to define the prognostic value of epidermal growth factor receptor (EGFR), c-MET, β-catenin, and p53 protein expression in TNM stage II colon cancer using tissue microarray technology.

Experimental Design: In this study, we retrospectively analyzed, resected, and otherwise untreated paraffin-embedded specimens from 134 consecutive patients with Tumor-Node-Metastasis stage II colonic carcinomas for EGFR, c-MET, β-catenin, and p53 protein expression by immunohistochemistry.

Results: Thirty-five percent, 77, and 65% of tumors exhibited strong (+2 and +3 immunopositivity) expression of EGFR, c-MET, and β-catenin, respectively. Fifty-four percent exhibited nuclear staining for p53 in >10% of the tumor cells. Univariate analysis revealed that increased nuclear p53 expression (P = 0.001), strong membranous EGFR expression (P = 0.04), and lymphovascular invasion (P = 0.01) were significantly related to disease recurrence and that p53 (P = 0.02) and EGFR (P = 0.01) expression were both associated with poor survival.

Conclusions: This retrospective tumor microarray study, restricted to Tumor-Node-Metastasis stage II colon cancer patients who did not undergo adjuvant therapy, supports the use of EGFR and p53 as biological markers, which may assist in predicting disease recurrence and survival.

INTRODUCTION

Colorectal carcinoma is the second most common type of cancer in the United States with >55,000 patients dying every year from the disease (1). Currently, the primary method for identifying prognostic differences among patients with early-stage disease is the Tumor-Node-Metastasis (TNM) system (2). However, patients with a similar pathological disease stage can exhibit varying survival outcomes. This is especially true for stage II (Dukes’ B) colon cancer. Despite the fact that 60–65% of patients with stage II and III (Dukes’ B and C) colon cancer are cured with surgery alone and most patients with stage II disease are not treated with adjuvant chemotherapy, many patients continue to receive chemotherapy unnecessarily, whereas others, who may benefit from adjuvant therapy, are not treated (1). Therefore, it is critical to identify molecular markers that will identify more aggressive colorectal cancer phenotypes to tailor patient therapy accordingly.

A significant amount of research in the past several years suggests that a number of prognostic molecular markers might be useful in defining individual colorectal cancer patients’ risk after surgery and determining which patients might benefit most from adjuvant chemotherapy (2–4). Although many particular markers have been studied extensively, data for most markers remains inconclusive. In this study, we have chosen to examine the expression of several putative biological markers [epidermal growth factor receptor (EGFR), c-MET, β-catenin, and p53] that are strongly suspected to play a significant role in colorectal tumor proliferation and invasion using tissue microarray (TMA) technology. TMAs are especially suitable for the immunohistochemical examination of multiple markers (5). The simultaneous evaluation of multiple cases on each slide virtually eliminates slide-to-slide variation, which is an important factor contributing to variability in immunohistochemical studies.

The EGFR, a M170,000 transmembrane glycoprotein encoded by the c-erbB-1 proto-oncogene, was the first member of the tyrosine kinase receptor family to be identified (6). On binding of ligand, EGFR dimerization occurs, and the cytoplasmic catalytic function of tyrosine kinase is activated, which in turn promotes autophosphorylation (7). Activation of EGFR is the critical initiating event that results in the stimulation of an intracellular signal transduction cascade, which regulates cell...
adhesion, proliferation, differentiation, apoptosis, and metastasis (8). Overexpression of EGFR has been found to correlate with poor prognosis in several cancers, including colorectal cancers (9).

The c-MET proto-oncogene, which encodes a protein for hepatocyte growth factor receptor, is another tyrosine kinase associated with cancer progression (10). It also contains a tyrosine kinase domain that activates signaling pathways involved in cell proliferation, motility, adhesion, and invasion (10). Overexpression of c-MET has been correlated with poor prognosis in cancers such as breast, stomach, liver, endometrium, and nasopharynx (11–15). More recently, the c-MET expression level in primary colon cancer has been shown to be an important predictive marker for early-stage invasion and regional disease metastasis (16).

Deregulation of β-catenin levels occurring through mutation of the adenomatous polyposis gene is an important mechanism for colorectal tumorigenesis (17–19). β-Catenin activity is controlled by a large number of binding partners that affect its stability and localization and is, thereby, able to participate in processes such as gene expression and cell adhesion (20). Variations in the level and pattern of β-catenin expression have been shown to correlate with outcome in colorectal cancer (21–25).

p53 is a well-characterized tumor suppressor gene encoding a protein involved in cell cycle regulation, DNA replication, and apoptosis (26). The p53 mutational and expression status of stage II colon cancers has been studied extensively; however, no definitive conclusion has been reached as to its prognostic or predictive use (2–3).

We hypothesized that overexpression of one or more of the aforementioned molecular markers may prove to be a useful prognostic indicator in stage II colon cancer.

MATERIALS AND METHODS

Patients and Specimens. Archival cases of TNM stage II colon cancer (not including rectal cancer) from 134 consecutive patients were retrieved nonselectively from the archives of the Department of Pathology at the Rhode Island Hospital between the years of 1983 and 1994. Stage was defined according to American Joint Committee on Cancer criteria (27). None of these patients received adjuvant chemotherapy or radiotherapy before surgery or after the initial resection. Recurrence and survival data were ascertained through the Rhode Island Tumor Registry. This study was approved by the Institutional Review Board at the Rhode Island Hospital.

Tissue samples were formalin fixed and paraffin embedded. All corresponding H&E slides were reviewed for confirmation of diagnosis and adequacy of material by M. Resnick. Each case was classified by the pathologist (M. Resnick) according to grade (high or low), mucinous differentiation (>50% of the tumor cells), and presence or absence of lymphovascular invasion.

TMA Construction. Paraffin blocks containing areas consisting of pure invasive carcinoma were identified on corresponding H&E-stained sections. Areas of interest that represented the invasive front of the tumor, rich in nonneoplastic tumoral glands, were identified and marked on the source block. The source block was cored and a 1-mm core transferred to the recipient master block using the Beecher tissue microarrayer (Beecher Instruments, Silver Spring, MD). Three to six cores were arrayed/specimen. Representative sections of normal colon were taken from ∼30% of the cases.

Immunohistochemistry (IHC). IHC for each antigen (EGFR, c-MET, β-catenin, and p53) was performed on 5-μm paraffin sections of each colon cancer TMA section described above. Slides were stained for β-catenin (polyclonal rabbit 1:200; Cell Signaling Technology, Beverly, MA), c-MET (polyclonal rabbit 1:250; Zymed Laboratories, South San Francisco, CA), and p53 ( monoclonal mouse clone DO-7, 1:500; Dako, Copenhagen, Denmark) using the Ventana Discovery automated staining system with the DAB Map kit (Ventana, Tucson, AZ). Slides were stained for EGFR with the Dako pharmDx kit (Dako) as per manufacturer’s instructions. All slides were counterstained with hematoxylin, dehydrated, cleared, and mounted. Negative controls included replacement of the primary antibody with nonreacting antibodies of the same species. A multitumor array consisting of a wide array of solid tumors was used as a positive control for all antibodies.

IHC Assessment. EGFR- and β-catenin-positive cells stained primarily at the cell membrane and occasionally in the cytoplasm, whereas c-MET-staining cells exhibited a combined membranous and cytoplasmic pattern. For EGFR and β-catenin, staining intensity was classified as the following: negative 0; +1 if the circumferential membrane pattern was incomplete; +2 circumferential staining with a weak or intermediate intensity; and +3 complete strong circumferential staining. c-MET staining was scored as negative 0, weak +1, moderate +2, or strong +3. For p53, arrays were scored as negative if <10% of nuclei were stained and positive if >10% of nuclei were stained. Similar criteria were used for determining nuclear positivity for β-catenin. The extent of staining (percentage of cells staining) was, for the most part, homogeneous within each 1-mm core and, therefore, was not included as a variable.

At least three cores were scored/case. The analysis of three cores/case has been shown to be comparable with the analysis of the whole section in a recent study (28) and in a pilot validation study in our lab (unpublished results). The vast majority of cases exhibited a uniform degree of staining between all cores, and in those that did not, an average score was determined. All sections were scored independently by J. Routhier and M. Resnick without knowledge of the clinicopathological features or clinical outcome. There was a high level of correlation between the two scorers, and in the few discrepant cases, a consensus was reached after joint review.

Statistical Analysis. Associations between categorical groups were evaluated using the χ² or the Fisher’s exact test when appropriate. The influence of prognostic factors on tumor-related recurrence as well as survival was assessed by Kaplan-Meier estimates, and subgroups were compared by the log-rank test for univariate analysis. The multivariate Cox’s proportional hazard model was applied using a stepwise forward method to detect independent predictors of recurrence/survival. Two-tailed Ps of ≤0.05 were considered to be statistically significant.
Clinicopathological Features. The mean age of the patients at initial surgery was 72 years (range, 31–96 years), and 62 males and 72 females were included in this study (Table 1). The mean duration of follow-up was 96 months (range, 1–216 months).

Sixty-three of the tumors were right-sided, and 71 were left-sided with the reference point being the splenic flexure. No rectal or rectosigmoid cases were included in this study. The degree of tumor differentiation as defined by the American Joint Committee on Cancer (27) was as follows: 22 high grade; 112 low grade; 26 mucinous; and 108 nonmucinous. Evidence of lymphatic or vascular invasion (lymphovascular invasion) was present in 13 cases.

Table 1 Clinicopathological characteristics of 134 colonic cancer patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Age at surgery (yrs)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>72.7 ± 10.3</td>
</tr>
<tr>
<td>Range</td>
<td>31–96</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>62</td>
</tr>
<tr>
<td>Female</td>
<td>72</td>
</tr>
<tr>
<td>Tumor location&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>63</td>
</tr>
<tr>
<td>Distal</td>
<td>71</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
</tr>
<tr>
<td>High grade</td>
<td>22</td>
</tr>
<tr>
<td>Low grade</td>
<td>112</td>
</tr>
<tr>
<td>Nonmucinous</td>
<td>108</td>
</tr>
<tr>
<td>Mucinous</td>
<td>26</td>
</tr>
<tr>
<td>Lymphovascular invasion</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>13</td>
</tr>
<tr>
<td>Absent</td>
<td>12</td>
</tr>
<tr>
<td>Colon cancer relapse</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>96</td>
</tr>
<tr>
<td>Local</td>
<td>8</td>
</tr>
<tr>
<td>Distal</td>
<td>16</td>
</tr>
<tr>
<td>Information unavailable</td>
<td>14</td>
</tr>
<tr>
<td>Vital statistics</td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>42</td>
</tr>
<tr>
<td>Dead—all causes</td>
<td>92</td>
</tr>
<tr>
<td>Dead—unrelated</td>
<td>56</td>
</tr>
<tr>
<td>Dead—colon cancer</td>
<td>22</td>
</tr>
<tr>
<td>Information unavailable</td>
<td>14</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reference point being the splenic flexure.

RESULTS

Clinicopathological Features. The mean age of the patients at initial surgery was 72 years (range, 31–96 years), and 62 males and 72 females were included in this study (Table 1). The mean duration of follow-up was 96 months (range, 1–216 months).

Sixty-three of the tumors were right-sided, and 71 were left-sided with the reference point being the splenic flexure. No rectal or rectosigmoid cases were included in this study. The degree of tumor differentiation as defined by the American Joint Committee on Cancer (27) was as follows: 22 high grade; 112 low grade; 26 mucinous; and 108 nonmucinous. Evidence of lymphatic or vascular invasion (lymphovascular invasion) was present in 13 cases.

The vital status of the patients and incidence of recurrent disease is also described in Table 1. Twenty-four patients had recurrent disease (8 local and 16 distal metastases). Ninety-two patients died during the follow-up period, 22 died of colon cancer, and 56 of unrelated causes, whereas the cause of death for 14 patients was unclear.

Frequency of Expression of EGFR, c-MET, β-catenin, and p53 in Colonic Tumors. Normal colonic epithelium showed weak membranous staining with EGFR and β-catenin and a negative to weak staining pattern for c-MET. No p53 staining was observed in normal colonic mucosa.

The frequency of expression of the biological markers evaluated is described in Table 2. Fig. 1A–D illustrates examples of TMA immunostains for these markers. When stratifying the frequency of expression into two groups—low (0 and +1 immunopositivity) and high (+2 and +3 immunopositivity)—35, 77, and 65% of the tumors exhibited high expression levels of EGFR, c-MET, and β-catenin, respectively. Fifty-four percent exhibited nuclear staining for p53 in >10% of the tumor cells, and 21% of the tumors exhibited >10% nuclear positivity for β-catenin.

Clinical Outcome. Analysis of Kaplan-Meier recurrence curves (Fig. 2A–F) revealed that lymphovascular invasion (P = 0.01), strong EGFR expression (+2 and +3 immunopositivity; P = 0.04), and p53 nuclear positivity (P = 0.001) were all significant risk factors for recurrence. A trend (P = 0.083) was noted between tumor grade and recurrence whereas evidence of mucinifer differentiation showed no correlation with recurrence (not shown). A strong trend (P = 0.065) was detected between the loss of membranous β-catenin expression and recurrence. No association was found between membranous/cytoplasmic c-MET expression or nuclear β-catenin expression and disease recurrence. Univariate analysis revealed that only p53 (P = 0.002) and EGFR (P = 0.05) were able to predict survival at 10 years.

Multivariate Cox analysis revealed that lymphovascular invasion, p53, and EGFR were associated with recurrent disease (P = 0.03, 0.04, and 0.05, respectively) and that p53 and EGFR were negative predictors of survival (P = 0.02 and 0.01, respectively) (Table 3). On multivariate analysis, the trend between loss of membranous β-catenin expression was greater (P = 0.056) than that detected by univariate analysis.

The association between the expression of the biomarkers examined and the presence of distal metastasis (8 patients), as well as high tumor grade (22 patients), was also analyzed. The control group consisted of all patients with low-grade, nonmeta-

Table 2 Frequency of different levels of biological marker expression

<table>
<thead>
<tr>
<th>Marker</th>
<th>0 (%)</th>
<th>1+ (%)</th>
<th>2+ (%)</th>
<th>3+ (%)</th>
<th>L&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>H&lt;sup&gt;a&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-MET</td>
<td>1 (1)</td>
<td>29 (23)</td>
<td>65 (50)</td>
<td>34 (26)</td>
<td>30 (23)</td>
<td>99 (77)</td>
</tr>
<tr>
<td>Epidermal growth factor receptor</td>
<td>25 (20)</td>
<td>57 (45)</td>
<td>23 (18)</td>
<td>21 (17)</td>
<td>82 (65)</td>
<td>44 (35)</td>
</tr>
<tr>
<td>β-catenin (nuclear)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11 (8)</td>
<td>35 (27)</td>
<td>49 (38)</td>
<td>35 (27)</td>
<td>46 (35)</td>
<td>84 (65)</td>
</tr>
<tr>
<td>p53 (nuclear)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>103 (79)</td>
<td>27 (21)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Low includes scores of 0 and +1; high scores of +2 and +3.
<sup>b</sup> Low nuclear staining, <10% positive nuclei; high, >10% positive staining nuclei.
static tumors who were alive for at least 5 years after initial resection (63 patients). There was a strong correlation between p53 expression and distal metastasis (87.5% of patients with distal metastasis were p53$^+$ as opposed to 47% of patients without distal metastasis; $P = 0.027$). No association was found between p53 expression and tumor grade. There was a strong association ($P = 0.001$) between loss of membranous $\beta$-catenin expression and high-grade tumors and a weak trend ($P = 0.21$) between loss of $\beta$-catenin expression and the presence of distal metastasis. Finally, there was no statistically significant association between increased EGFR or c-MET expression and tumor grade or the presence of distal metastasis.

**DISCUSSION**

A molecular marker screening process could provide a basis for more economical and precise decisions regarding prognosis and appropriate adjuvant therapy for patients with colon cancer in general and those with stage II colon cancer specifically. For the most part, studies searching for a biological prognostic marker for colon cancer have produced inconclusive results (2–4). This is, in part, related to the study of nonhomogeneous populations as relating to tumor stage, tumor site, or treatment protocols, as well as numerous technical aspects primarily concerning IHC technique and assessment of results. In this regard, our study has many advantages. The colonic cancer patient population studied did not include patients with rectal tumors, and perhaps, more importantly, none of the patients received preoperative or adjuvant chemotherapy or radiation therapy at the time of diagnosis. Technically, this was a TMA study, which allows for simultaneous evaluation of multiple cases on each slide. This method virtually eliminates slide-to-slide variation, which is an important factor contributing to variability in immunohistochemical studies (24, 28).

In this study, increased EGFR expression was found to be a significant uni- and multivariate indicator of disease recurrence and patient survival, although it was not significantly associated with the presence of distal metastasis. EGFR has been shown to be a strong prognostic indicator in certain cancer types (9); however, its role as an independent prognostic marker in colon cancer has not been clearly defined yet. In certain studies, increased EGFR expression in colon cancer was associated with tumor stage, metastatic potential, relapse-free, and
Fig. 2  Kaplan Meier disease-free analysis curves in colonic cancer. A, a strong trend was detected between high-grade tumors and disease recurrence. B, lymphatic/vascular invasion was associated with disease recurrence. C, moderate (+2) to strong (+3) epidermal growth factor receptor (EGFR) expression was associated with disease recurrence. D, no association was found between c-MET expression and disease recurrence. E, a strong trend was detected between loss of β-catenin membrane staining and disease recurrence. F, a strong association was seen between p53 nuclear staining and disease recurrence.
EGFR, c-MET Expression in Stage II Colon Cancer

In Goldstein's study, the highest percentage of strongly staining EGFR-positive cells was found at the invasive front of the tumor, which appeared to show the strongest EGFR staining correlated with length of survival. Determination of EGFR status may also have implications for therapeutic interventions because EGFR is currently the focus of many anticancer agents under investigation. Some with promising results, although the correlation between EGFR expression and response to therapy remains unclear.

Overexpression of c-MET has been shown to be associated with poor prognosis in a number of solid tumors. More recently, c-MET expression levels as determined by reverse transcription-PCR and IHC were found to correlate directly with colon cancer stage. In this study, moderate to strong c-MET expression was present in 77% of the tumors studied; however, no correlation was found with disease recurrence or patient survival. This study, as well as the others described above, provides additional evidence for the importance of c-MET expression in the early stages of carcinoma progression and suggests that c-MET may be a viable target for preventative treatment of early-stage disease.

In many, but not all, studies, the pattern of β-catenin expression in colorectal cancer has been shown to correlate with disease stage and prognosis. Loss of membranous and increased nuclear β-catenin expression has been associated with short survival in colorectal tumors. In this study, a strong trend was detected between loss of membranous β-catenin expression and patient prognosis, and a weaker correlation was found between loss of membranous β-catenin expression and the presence of distal metastasis. In contrast to the study by Hugh et al., no association was found between loss of membranous β-catenin expression and increased nuclear expression or between increased nuclear staining and patient outcome. Finally, in this study, high-grade tumors exhibited decreased membranous β-catenin expression. A similar trend was also described by Hugh et al.

One of the most extensively studied molecular prognostic markers for stage II colon cancers is p53. As summarized in recent review articles, the prognostic use of a tumor’s p53 status is still somewhat controversial. Indeed, conflicting results were obtained in the two largest recent studies that included 291 and 293 patients, respectively, and explored the role of p53 in stage II colon cancers. This may be related in part to the different technical approach used for p53 analysis because Soong et al. performed a mutational analysis, whereas Allegra et al. used IHC to detect abnormal nuclear accumulation. In our study, p53 was a strong univariate and multivariate predictor of disease recurrence, distal metastasis, and patient survival. Albeit, the cohort we studied was somewhat smaller than the groups studied by Allegra et al. (1) and Soong et al. (42), our cohort was more homogeneous in that none of the patients received perioperative chemo- or radiotherapy, and no rectal carcinomas were included.

In conclusion, in this cohort of untreated stage II colon cancer patients, immunohistochemical staining on a TMA revealed that expression of EGFR and p53 are both prognostic indicators of disease recurrence and survival. Additional larger studies will be needed to determine whether EGFR and p53 expression also predict response to adjuvant chemotherapy or targeted anti-EGFR therapy.

ACKNOWLEDGMENTS

We thank Patricia Meitner, Ph.D., Letitia Henry, and Jacqueline Fanion for their technical assistance and Laura Gantt for her clerical assistance.

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