The Relative Distribution of Membranous and Cytoplasmic Met Is a Prognostic Indicator in Stage I and II Colon Cancer

Fiona Ginty,1 Sudeshna Adak,2 Ali Can,1 Michael Gerdes,1 Melinda Larsen,3 Harvey Cline,1 Robert Filkins,5 Zhengyu Pang,1 Qing Li,1 and Michael C. Montalto1

Abstract

Purpose: The association hepatocyte growth factor receptor (Met) tyrosine kinase with prognosis and survival in colon cancer is unclear, due in part to the limitation of detection methods used. In particular, conventional chromogenic immunohistochemistry (IHC) has several limitations including the inability to separate compartmental measurements. Measurement of membrane, cytoplasm, and nuclear levels of Met could offer a superior approach to traditional IHC.

Experimental Design: Fluorescent-based IHC for Met was done in 583 colon cancer patients in a tissue microarray format. Using curvature and intensity-based image analysis, the membrane, nuclear, and cytoplasm were segmented. Probability distributions of Met within each compartment were determined, and an automated scoring algorithm was generated. An optimal score cutoff was calculated using 500-fold crossvalidation of a training and test data set. For comparison with conventional IHC, a second array from the same tissue microarray block was 3,3′-diaminobenzidine immunostained for Met.

Results: In crossvalidated and univariate Cox analysis, the membrane relative to cytoplasm Met score was a significant predictor of survival in stage I (hazard ratio, 0.16; P = 0.006) and in stage II patients (hazard ratio, 0.34; P ≤ 0.0005). Similar results were found with multivariate analysis. Met in the membrane alone was not a significant predictor of outcome in all patients or within stage. In the 3,3′-diaminobenzidine–stained array, no associations were found with Met expression and survival.

Conclusions: These data indicate that the relative subcellular distribution of Met, as measured by novel automated image analysis, may be a valuable biomarker for estimating colon cancer prognosis.

Standard immunohistochemistry techniques are limited to semiquantitative analysis (i.e., 0, +1, +2, +3) and are typically confined to measuring the intensity of biomarker within a single compartment (i.e., nucleus or combined membrane/cytoplasm). Cell surface receptors, including Met, have complex signaling mechanisms that include translocation into endosomal compartments and proteolytic cleavages, both of which play an important role in downstream signaling pathways (14–17). Therefore, it is logical that the activation of cell surface receptors should result in a measurable distribution of membrane and cytoplasmic localizations that are indicative of the activation status of a given pathway. Such measures may be independent of total protein or mRNA expression patterns.

In this study, we describe a novel algorithm that measures cytoplasmic, as well as membrane and nuclear distribution patterns of Met. These algorithms take advantage of spatial and intensity interrelationships of nuclei and membrane biomarkers to both segment the cytoplasm and to separate the epithelial nuclei from stroma nuclei. This is an advancement on current segmentation algorithms that rely solely on the intensity of compartment markers (i.e., cytokeratin) to distinguish subcellular compartments (18, 19). Using this new method, we wished to determine if subcellular compartmental distribution of Met levels or relative differences among these compartments were predictors of colon cancer survival.

The hepatocyte growth factor receptor/Met tyrosine kinase is dysregulated in many human cancers and is believed to play an important role in influencing tumor progression (1). Several studies using standard chromogenic tissue-based immunohistochemistry have shown a clear association between Met overexpression and reduced survival time in a variety of epithelial cancers including breast, nasopharyngeal, esophageal, renal, cervical, and ovarian (2–7). In colon cancer, immunohistochemistry studies have not shown an association with outcome (8), despite several reports that link mRNA and global protein overexpression patterns to cancer progression (9–13).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Michael C. Montalto, GE Global Research Center, One Research Circle, Niskayuna, New York; 2GE John F Welch Technology Centre, Bangalore, India; and 3Department of Biological Sciences, University at Albany, State University of New York, Albany, New York.

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The authors’ affiliations are: 1Department of Biological Sciences, University at Albany, State University of New York, Albany, New York; 2GE Global Research Center, One Research Circle, Niskayuna, New York; 3GE John F Welch Technology Centre, Bangalore, India; and 4Department of Biological Sciences, University at Albany, State University of New York, Albany, New York.

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We show that the relative distribution of membrane and cytoplasmic Met has clinical utility in predicting outcome. This has applications for other cell surface effector molecules with distributed activation patterns, specifically in the cytoplasm, and could tremendously improve our understanding of disease pathology.

Materials and Methods

Patient details and tissue microarrays
Colon Cancer tissue microarrays (YTMA8) were from the Yale Tissue Microarray Facility. Of the 684 patients included on the arrays, follow-up data were available for 600 patients and usable images available for 583 of those. The median follow-up was 4.5 y; with 34% of cases having >10 y of follow-up and 24% with >15 y of follow-up. Of the 583 patients, 485 (83%) had died and 264 of those were classified as dying from colon cancer. Ninety eight (17%) patients were alive as of their last follow-up. The primary tumor site was the colon in 437 (73%) patients and the rectum or other sites in the remaining 146 (27%) patients. Ninety percent of patient tumors were adenocarcinomas and 70% of tumors were well- or moderately differentiated. All stages of colon cancer were represented in this cohort: 122 (21%) were stage I patients, 145 (25%) were stage II, 224 (38%) were stage III, and 59 (10%) were stage IV. Treatment information was not available for any patients.

The tissue microarrays were constructed with histospots (0.6 mm in diameter) spaced 0.8 mm apart in a grid layout using a manual tissue microarrayer (Beecher Instruments). The resulting tissue microarray blocks were cut to 5-μm sections with a microtome, the sections were placed on slides with an adhesive tape-transfer method (Instrumedics, Inc.) and were UV crosslinked for subsequent use in antibody optimization protocols.

Specificity of Met antibody
Specificity of a commercial antibody directed against the NH$_2$-terminal extracellular domain of Met [clone DO-24; Upstate Biotechnology (now part of Millipore)], was evaluated using HeLa cells that were transfected with a negative siRNA control or siRNA targeting Met. After confirming knockdown by Western (knockdown was ~90%; data not shown), cells were fixed, cast in an agarose plug, embedded in paraffin, and immunostained with antibody DO-24 and 4',6-diamidino-2-phenylindole (DAPI), whereas parallel samples were labeled with rabbit IgG (Jackson ImmunoResearch) and DAPI. To quantitify Met knockdown, average Met pixel intensity in seven independent images was measured using an in-house image analysis software, background was subtracted, and Met was normalized to DAPI staining from another channel or IgG staining from a parallel sample. Percent differences in Met intensity in control cells compared with knockdown cells were determined.

Met antibody optimization on tissue arrays
Met antibody optimization and tissue staining was carried out externally by HistoRx. The DO-24 anti-Met antibody was optimized for fluorescent immunohistochemistry in human tissue using formalin-fixed paraffin-embedded tissues (including colon, breast, stomach, prostate, kidney, muscle, liver, brain, skin, and lymph) from the archives of the Yale University. A series of serial dilutions was evaluated on test tissue arrays, each containing 10 to 20 histospots, to identify the optimal concentration of DO-24 that provided a wide dynamic range on test tissue arrays, each containing 10 to 20 histospots, to identify the optimal concentration of DO-24 that provided a wide dynamic range. After rinsing briefly in 1× TBS, a 30-min incubation with 2.5% hydrogen peroxide/methanol block was used to block endogenous peroxidases, followed by incubation with 10% goat serum (Life Technologies) for 1 h at room temperature. The anti-Met antibody, DO-24, was incubated overnight at 4°C, at a dilution of 1:2,000. The anti-Met antibody was detected with Envision anti-mouse labeled polymer horseradish peroxidase (DAKO), followed by Cy5 tyramide (1:50; PerkinElmer). All slides were then incubated overnight at 4°C with an anti–pan-cadherin antibody (1:1,000; Abcam). Pan-cadherin was detected by incubation with biotinylated goat anti-tau (1:200; Jackson ImmunoResearch) for 60 min, followed by Cy-2 conjugated streptavidin (1:200; Jackson ImmunoResearch) for 30 min. DAPI, contained within the mounting medium (Prolong Gold Antifade w/DAPI mounting gel; Molecular Probes) was used to identify cell nuclei. Images were captured on the PM-2000 microscope platform (HistoRx) at ×20 magnification with an exposure time for the Cy5 channel of 400 ms. To compare traditional DAB staining to fluorescence based detection of Met, a second tissue microarray from the same block (YTMA8) was processed as above, except after application of horseradish peroxidase–conjugated secondary donkey anti-mouse (Jackson ImmunoResearch) to the slide, the bound antibody was detected using 3,3′-diaminobenzidine (DAB) substrate for 10 min (LabVision).

Immunohistochemical staining
Tissue microarrays were deparaffinized first by heating at 60°C, then by two xylene rinses followed by two rinses with 100% ethanol and a rinse in water. Antigen retrieval was done in a Tris-EDTA buffer at a pH of 9.0 in the PT Module device (LabVision). After rinsing briefly in 1× TBS, a 30-min incubation with 2.5% hydrogen peroxide/methanol block was used to block endogenous peroxidases, followed by incubation with 10% goat serum (Life Technologies) for 1 h at room temperature. The anti-Met antibody, DO-24, was incubated overnight at 4°C, at a dilution of 1:2,000. The anti-Met antibody was detected with Envision anti-mouse labeled polymer horseradish peroxidase (DAKO), followed by Cy5 tyramide (1:50; PerkinElmer). All slides were then incubated overnight at 4°C with an anti–pan-cadherin antibody (1:1,000; Abcam). Pan-cadherin was detected by incubation with biotinylated goat anti-tau (1:200; Jackson ImmunoResearch) for 60 min, followed by Cy-2 conjugated streptavidin (1:200; Jackson ImmunoResearch) for 30 min. DAPI, contained within the mounting medium (Prolong Gold Antifade w/DAPI mounting gel; Molecular Probes) was used to identify cell nuclei. Images were captured on the PM-2000 microscope platform (HistoRx) at ×20 magnification with an exposure time for the Cy5 channel of 400 ms. To compare traditional DAB staining to fluorescence based detection of Met, a second tissue microarray from the same block (YTMA8) was processed as above, except after application of horseradish peroxidase–conjugated secondary donkey anti-mouse (Jackson ImmunoResearch) to the slide, the bound antibody was detected using 3,3′-diaminobenzidine (DAB) substrate for 10 min (LabVision).

Image processing steps and quantification of Met
Automated scoring of fluorescent images. The membrane compartment of tumor cells was identified by pan-cadherin staining, and staining with DAPI was used to identify nuclei as previously reported (18). A nonlinear nonparametric mapping function with multiple inputs, including the local geometry of the pixel distributions as well as the intensity values, maps each of these two channels to probability values indicating the likelihood of pan-cadherin pixels being membrane, and likelihood of DAPI pixels being nuclei. The details of the likelihood estimation algorithm will be described elsewhere. To define cytoplasm, a definite decision for each pixel was determined by thresholding the probability maps at 50% rate. For example, a 50% probability of a pixel on a DAPI image implies that the pixel is equally likely to be background or nucleus. Let $M(x, y)$ and $N(x, y)$ denote the thresholded nuclei and membrane sets, respectively. Cytoplasm, denoted by $C(x, y)$, is defined as the union of the set of small regions circumscribed by membrane alone or membrane and nuclei pixels. Only pixels that are not defined as $M(x, y)$ or $N(x, y)$ can be defined as $C(x, y)$. The morphologic difference between epithelial and stromal nuclei was explored by defining a superset of the nuclei, cytoplasm, and membrane set. Let $U(x, y)$ denote this union superset, defined as the union of the detected regions,

$$U(x, y) = C(x, y) \cup N(x, y)$$

Because the stromal nuclei are not connected through membrane structures, and are sparsely distributed, they can be detected by a connected component analysis of $U(x, y)$. An epithelial mask, $E(x, y)$, is generated as a union of large connected components of $U(x, y)$. For the sample images, any connected component larger than 800 pixels is accepted as a part of the epithelial mask. The nuclei set is then separated.
into epithelial nuclei \( \text{Ne}(x, y) \) and stromal nuclei \( \text{Ns}(x, y) \) by masking,

\[
\text{Ne}(x, y) = N(x, y)E(x, y)
\]

\[
\text{Ns}(x, y) = N(x, y)(1 - E(x, y))
\]

Figure 1D illustrates the quality of stroma and epithelial segmentations. The distribution of Met in each of the regions was represented by a probability distribution function. For example, the probability distribution function of Met on the membrane is the weighted empirical distribution of the Met, where the membrane probability map determines the weights. We denote the mean and the SD of Met distribution on each of the regions as \( \mu_R \) and \( \sigma_R \), respectively, where \( R \) can be any of the nuclei, membrane, cytoplasm, or extracellular matrix regions. Extracellular matrix is defined as all the nonbackground pixels not classified as nuclei, membrane, or cytoplasm. A translocation score is defined as the normalized mean difference between the Met distributions on different regions. For example the membrane to cytoplasm translocation (MCT) score is defined as,
where the mean and the SD of any region, \( R \), are defined using the Met probability distribution function, \( p_c^R \), on that region:

\[
\mu_R = \sum_c p_c^R c
\]

\[
\sigma_R = \sum_c (c - \mu_R)^2 p_c^R (c)
\]

For the five regions we detected (epithelial nuclei, stromal nuclei, cytoplasm, membrane, extracellular matrix), 10 different translocation scores (5 choose 2) were defined.

### Statistical analysis

For manual scores, a comparison of survival differences between the scores from the DAB-stained images was made for all patients and by stage. A log-rank test was used to assess the survival differences among the cases scored categories. For the automated continuous scores, a robust and crossvalidated method was used to define an optimal cutpoint for each score. The cutpoint was chosen to maximize the difference in survival in the resulting two groups of patients (those with scores below the cutpoint and those with scores above the cutpoint). The two groups defined by the cutpoint were then considered relative to each other as the low-risk and high-risk groups. A threshold that minimized the log-rank test \( P \) value between the two groups was used, with adjustment for multiple testing because the optimal cutpoint is determined by repeated testing at each possible cutpoint (20, 21). However, to make the cutpoint robust to variation across data sets, we use the following crossvalidation method:

- **Two-fold crossvalidation:** The data were divided into training and test data sets, with 50% of the cases (both censored and uncensored) being selected as training and the remaining as test.
- **Cutpoint selection:** For each training data set, an optimal cutpoint was selected by the minimizing the log-rank test \( P \) value, and the Hothorn and Lausen adjusted \( P \) value (for multiple testing) was also computed.
- **Crossvalidation of cutpoint:** The optimal cutpoint was applied to the corresponding test data set, and the log-rank \( P \) value for comparing the two groups was computed.
- **Computing a robust cutpoint:** The above three steps are repeated 500 times and the median of thresholds, hazard ratios (HR), and \( P \) values from the 500 crossvalidation runs was used. The final robust cutpoint was the median threshold.

Test of association between risk category and clinical/pathologic stage variables were done using \( \chi^2 \) test of association in a contingency table. The Kaplan-Meier method was used to estimate the restricted mean survival and survival rates in each risk group, and Cox regression was used to assess the prognostic value of the risk category in a multivariate analysis. Restricted mean was used for survival time as censoring precludes the estimation of mean survival time (22). In multivariate analysis, a stepwise selection procedure (SAS v9.0) was selected by the minimizing the log-rank test \( P \) value, and the \( P \) value for multiple testing was used, with adjustment for multiple testing because the optimal cutpoint is determined by repeated testing at each possible cutpoint (20, 21).

Validation of Met antibody. Hela cells transected with siRNA targeting Met or universal negative control siRNA were immunostained with an anti-Met antibody (DO-24) to evaluate specificity of this antibody. We chose to use an extracellular domain antibody to evaluate translocation events and not previously reported cleavage events of the intracellular domain (15). Average pixel intensities were measured for DO-24, DAPI staining, and IgG staining of parallel samples. Quantification of Met knockdown versus control cells indicated a 77% difference between the Met signal obtained from the siRNA knockdown cells compared with controls (\( P < 0.05 \)), demonstrating good to excellent specificity of DO-24 antibody (Supplementary Fig. S1; data not shown).

Chromogenic staining of Met. DAB-based Met staining of patient tissue in this study exhibited a combined membranous and cytoplasmic pattern, as well as nuclear staining as others have reported (Supplementary Fig. S2; data not shown; refs. 8, 15). Staining intensity was classified by a pathologist as follows: negative, 0; weakly positive, +1; positive, +2; strongly positive +3 for staining on the membrane+cytoplasm. As expected, no association between survival and staining classification were observed, as has been reported previously using a categorical scoring method (8).

Image analysis and automated Met quantification. Figure 1A shows the automated image analysis of a single tissue histospot where the blue represents the nucleus (DAPI), red represents the membrane (pan-cadherin), and green represents Met. The probability maps computed for nuclei and membrane are shown in Fig. 1B and C, respectively. The brightness on these images represents the probability value; white representing the probability value of one, black representing the probability value of zero, and any shade of gray being proportional with the probability value. Figure 1D shows the computed different regions: red, membrane; blue, epithelial nuclei; gray, stromal nuclei; green, cytoplasm. Unconnected small membrane regions (denoted in pink) were excluded from quantification. Both the background and the extracellular matrix (i.e., non-epithelial tissue) are presented in black. The ability of the segmentation algorithm to define distinct membrane and cytoplasmic compartments are indicated by the delineation of cytoplasm in regions between nuclei and membrane borders or between membrane borders alone (Fig. 1D).

The distribution of Met in each of these regions was represented by a probability distribution function (Fig. 2). Met expression on a specific compartment is high when the mean intensity of the distribution is toward high Met intensity values relative to another compartment. For example, cytoplasmic Met expression for patient A is higher than the membrane Met expression (green plot is to the right of the red plot), compared with patient B, where the membrane expression is higher than cytoplasmic expression (green plot is on the left of the red plot).

Mean ± SE Met scores for each epithelial subcellular region were as follows: membrane, 25.93 ± 0.50; cytoplasm, 25.87 ± 0.50; stroma, 25.87 ± 0.50; epithelial nuclei, 25.87 ± 0.50; extracellular matrix, 25.87 ± 0.50.
0.47; nuclei, 25.05 ± 0.46; membrane-nuclei translocation, -0.01 ± 0.008; and membrane-cytoplasm translocation, 0.05 ± 0.005. No significant differences were found between stages for any of the scores (data not shown).

**Generation of Met score cutpoints.** Five hundred random crossvalidation runs were used to select robust cutpoints for Met membrane, cytoplasm, nuclei, and translocation scores. The results of the 500 crossvalidation runs for the test and training data set for patients are summarized in Table 1 for the membrane, cytoplasm, and MCT scores (results for nuclei scores not included were not significant). Although cutoffs were generated for membrane and cytoplasm scores that indicated significant separation using the training set data, these cutoffs did not maintain robust significant separations in the test sets. For the Met MCT score, the robust crossvalidated cutoffs were -0.07 for all patients, 0.12 for stage I patients, -0.07 for stage III patients, and -0.02 for stage III patients. These cutoffs were significant in training sets and had a similar trend for significance in validation sets (Table 1). The 5-year survival rates across the entire patient population were 48%.

**Table 1. Results of 2-fold crossvalidation of cutpoint selection on the membrane, cytoplasm and MCT scores with survival**

<table>
<thead>
<tr>
<th>Met membrane score</th>
<th>Overall (n = 583)</th>
<th>Stage I (n = 122)</th>
<th>Stage II (n = 145)</th>
<th>Stage III (n = 224)</th>
<th>Stage IV (n = 59)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal cutpoint: median</td>
<td>39 (18, 40.5)</td>
<td>26 (18, 40.5)</td>
<td>26 (16, 39)</td>
<td>25 (19, 32)</td>
<td>22 (16, 36)</td>
</tr>
<tr>
<td>Training sets: 5-y survival rate*</td>
<td>58%; 74%</td>
<td>79%; 84%</td>
<td>47%; 72%</td>
<td>48%; 32%</td>
<td>69%; 58%</td>
</tr>
<tr>
<td>Training sets: HR (P value)</td>
<td>0.53 (0.04)</td>
<td>0.445 (0.09)</td>
<td>0.42 (0.04)</td>
<td>1.64 (0.05)</td>
<td>1.81 (0.15)</td>
</tr>
<tr>
<td>Test sets: 5-y survival rate</td>
<td>59%; 67%</td>
<td>85%; 80%</td>
<td>59%; 69%</td>
<td>45%; 39%</td>
<td>63%; 65%</td>
</tr>
<tr>
<td>Test sets: HR (P value)</td>
<td>0.75 (0.25)</td>
<td>0.93 (0.64)</td>
<td>0.83 (0.58)</td>
<td>1.18 (0.49)</td>
<td>0.9 (0.55)</td>
</tr>
<tr>
<td>Met cytoplasm score</td>
<td>24 (16, 38)</td>
<td>26 (18, 38)</td>
<td>25 (16, 33)</td>
<td>25 (22, 36)</td>
<td>21 (15, 31)</td>
</tr>
<tr>
<td>Optimal cutpoint: median</td>
<td>59%; 60%</td>
<td>85%; 80%</td>
<td>57%; 69%</td>
<td>45%; 38%</td>
<td>64%; 63%</td>
</tr>
<tr>
<td>Training sets: 5-y survival rate</td>
<td>60%; 60%</td>
<td>83%; 82%</td>
<td>45%; 71%</td>
<td>49%; 30%</td>
<td>73%; 55%</td>
</tr>
<tr>
<td>Training sets: HR (P value)</td>
<td>0.73 (0.06)</td>
<td>0.56 (0.1)</td>
<td>0.45 (0.05)</td>
<td>1.87 (0.02)</td>
<td>2.205 (0.1)</td>
</tr>
<tr>
<td>Test sets: 5-y survival rate</td>
<td>59%</td>
<td>85%; 80%</td>
<td>57%; 69%</td>
<td>45%; 38%</td>
<td>64%; 63%</td>
</tr>
<tr>
<td>Test sets: HR (P value)</td>
<td>0.99 (0.69)</td>
<td>0.96 (0.53)</td>
<td>0.79 (0.52)</td>
<td>1.28 (0.36)</td>
<td>0.98 (0.66)</td>
</tr>
<tr>
<td>Met MCT score</td>
<td>-0.07 (-0.1, 0.0)</td>
<td>0.12 (0.1, 0.15)</td>
<td>-0.07 (-0.09, 0.17)</td>
<td>-0.02 (-0.09, 0.17)</td>
<td>0.01 (-0.1, 0.11)</td>
</tr>
<tr>
<td>Optimal cutpoint: median</td>
<td>44%; 63%</td>
<td>70%; 88%</td>
<td>19%; 70%</td>
<td>32%; 47%</td>
<td>75%; 50%</td>
</tr>
<tr>
<td>Training sets: 5-y survival rate</td>
<td>0.56 (0.006)</td>
<td>0.36 (0.035)</td>
<td>0.27 (0.002)</td>
<td>0.58 (0.04)</td>
<td>2.91 (0.05)</td>
</tr>
<tr>
<td>Training sets: HR (P value)</td>
<td>50%; 61%</td>
<td>82%; 84%</td>
<td>36%; 69%</td>
<td>39%; 43%</td>
<td>67%; 61%</td>
</tr>
<tr>
<td>Test sets: 5-y survival rate</td>
<td>0.67 (0.08)</td>
<td>0.64 (0.07)</td>
<td>0.41 (0.06)</td>
<td>0.83 (0.45)</td>
<td>1.2 (0.65)</td>
</tr>
<tr>
<td>Test sets: HR (P value)</td>
<td>44%; 63%</td>
<td>70%; 88%</td>
<td>19%; 70%</td>
<td>32%; 47%</td>
<td>75%; 50%</td>
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</table>

*5-y survival rates are for patients below and above the optimal cutpoints.
Results are shown in Table 3. In patients from all stages, significant predictors of survival included low Met MCT score (HR, 0.69; 95% CI, 0.51-0.94; \( P = 0.02 \)), age (HR, 1.58; 95% CI, 1.23-2.02; \( P < 0.001 \)), regional disease (HR, 0.21; 95% CI, 0.15-0.28; \( P < 0.001 \)), localized disease (HR, 0.09; 95% CI, 0.06-0.13; \( P < 0.0001 \)), stage N2 (HR, 1.55; 95% CI, 1.19-2.01; \( P < 0.001 \)), and tumor-node-metastasis stage III (HR, 1.49; 95% CI, 1.15-1.94; \( P < 0.001 \)).

In stage I patients, significant predictors were as follows: low MCT score (HR, 0.19; 95% CI, 0.04-0.79; \( P = 0.02 \)), regional disease (HR, 0.08; 95% CI, 0.02-0.3; \( P = 0.0002 \)), and localized disease (HR, 0.02; 95% CI, 0.01-0.07; \( P < 0.0001 \)). In stage II patients, low MCT score was the only significant predictor of survival (HR, 0.34; 95% CI, 0.18-0.64; \( P = 0.001 \)).

**Discussion**

Using curvature-based segmentation and compartmental probability distribution algorithms, we determined that a lower distribution of Met in membrane, relative to cytoplasm (low Met MCT score), is a predictor of outcome in stage I and stage II colon cancer patients. This is the first report of a significant association between Met protein expression and survival in colorectal cancer using an automated immunohistochemistry-based technique. In a direct comparison to conventional DAB staining with categorical manual scoring, we did not find an association between Met expression and survival on the same patient cohort, as has been reported (8). Despite associations of survival in several tumor types, associations between Met and
colon cancer have not been consistent. For example, Di Renzo et al. (9) did not find a correlation between Met overexpression and grade and stage of primary tumors using mRNA expression analysis, and in another study, no association between Met expression and prognosis was found in stage II colon cancer patients using standard immunohistochemistry methods (8).

Kammula et al. (12) showed that colon cancer patients with high Met, in conjunction with high hepatocyte growth factor mRNA expression, had poorer prognosis. Gene expression methods can only measure mRNA levels distributed across several cell types, and standard immunohistochemistry methods are limited to measuring the combined membrane/cytoplasmic or nuclear compartment expression patterns.

Our data are consistent with studies that note diffuse expression of Met in the cytoplasm of several tissue types (3, 8, 15), and some authors have suggested that activated intracellular Met may be biologically relevant. In cell culture, Met has enhanced and prolonged activation within the endosomal compartment (16, 17). Several studies have shown that the blockage of internalization of several receptor kinases after ligand binding significantly attenuates downstream signaling events, indicating that endocytic signaling may be essential to downstream pathway activation (23–26). The paradigmatic example of EGFR, which has as much as 70% to 80% of its protein localized to the endosome during steady-state activation, further highlights the biological significance of cytoplasmic translocation events in signal transduction pathways. Because Met has complex subcellular activation and regulatory mechanisms, it is possible that more sophisticated techniques that measure subtle activation patterns may be more relevant to study the associations of Met and cancer outcome.

One of the most critical factors in generating subcellular compartment biomarker data is the accurate segmentation of multichannel images into nuclear, cytoplasm, membrane, epithelial, and stromal compartments from complicated tissue architecture. Current methods that rely on biomarker signal intensity alone for segmentation may include staining artifacts as putative compartments, leading to false-positive segmentations (i.e., putative biomarker regions are included that are not truly biomarkers). In this study, segmentation was accomplished by computing the likelihood of a pixel belonging to a particular subcellular compartment. For example, instead of identifying membrane pixels, the probability of a pixel being a membrane was computed. Such probability maps were computed using the intensity and geometry information provided by each channel. Taking both shape and intensity

<table>
<thead>
<tr>
<th>Table 3. Cox Regression multivariate survival analysis</th>
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<tbody>
<tr>
<td>All stages</td>
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<tr>
<td>HR (95% CI)</td>
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<tr>
<td>-----------------------------------------------------</td>
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<tr>
<td>Met MCT score: low vs high</td>
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<tr>
<td>Age</td>
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<tr>
<td>Regional disease</td>
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<td>Localized disease</td>
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<td>Stage N2</td>
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<td>TNM Stage III</td>
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<tr>
<td>Localized disease</td>
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<td>Stage N2</td>
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</table>

Abbreviations: Poorly diff, poorly differentiated; undiff, undifferentiated; well-diff, well-differentiated; TNM, tumor-node-metastasis.

*Unadjusted P value when threshold is applied to entire data set.
into consideration creates a more accurate definition of a cellular compartment and may minimize false-positive segmentations. Although morphology constraints may favor false-negative segmentation (i.e., some true biomarker segmentations are not included in analysis), the large amount of pixels per image creates the likelihood that enough accurate segmentations are done to extract biologically relevant data from each image.

An additional advantage to the segmentation algorithms reported here is that the need to define the cytoplasm using a defined stain (such as cytokeratin) is circumvented. Instead, we define cytoplasm as the union of set of small regions circumscribed by membrane and nuclei pixels. The removal of one optical channel from the workflow provides an extra channel for future multiplexing applications. The power of analyzing multiple biomarkers using automated image analysis in sequential sections has been recently highlighted (27). However, the ability to analyze more than one target biomarker in a single tissue, as opposed to sequential sections, would greatly improve the workflow and accuracy of multiplexed analysis on tissue sections.

For biomarkers and other potential prognostic factors that have been measured on a continuous scale, it is a common practice to convert the continuous variable into two or more categories. The usual process is to define “optimal” cutpoints on the continuous prognostic variable (28). This allows the clinician to directly use these prognostic categories to assess patient risk. The usual method of selecting an optimal cutpoint is to search for a value that maximizes the survival differences between the resulting groups. For a maximally selected cutpoint, Lausen and Schumacher (28) have suggested a method for adjusting the α-level to account for multiple testing, whereas Farragi and Simon (29) have suggested using crossvalidation with a split sample approach. We have used a repeated crossvalidation approach, with the cutpoint selected being the median of cutpoints from 500 crossvalidation runs. This allows for a more robust cutpoint that can be applied generally. In this approach, the cutpoint is first selected based on a univariate analysis of the prognostic variable. Subsequently, the categorized factor is assessed in a multivariate analysis to determine its prognostic value. Mazumdar et al. (30) have proposed that it might be more appropriate to determine the cutpoint using the multivariate analyses rather than in the univariate analyses itself. Camp et al. (31) have extended the method to determine two cutpoints by a simultaneous search, allowing for the prognostic variable to be categorized as high, medium, and low risk. A limitation of all these models is that the hazard rate is constant when the biomarker is below a certain threshold and then jumps when the biomarker is above the threshold. A more flexible model with linearly increasing hazard rates within each risk category may be more suitable.

The average 5-year survival for stage 2 (Duke B) colon cancer patients is 80% to 90% depending on grade and/or classification approach (32). In our study, stage II and III patients had worse survival compared with average survival rates reported by O’Connell et al. (32); however, stage IV patients had better survival rates. This may be explained by demographics and standard of care differences. Nonetheless, the use of adjuvant therapy after first line treatment is controversial for patients with stage II colon cancer of any grade. (33–35). It has been suggested that stratifying patients based on molecular markers may provide the justification needed to perform adjuvant therapy (36). Stage II patients in this study with a low MCT score had similar survival statistics to stage III patients. Because it is generally accepted that adjuvant therapy can improve outcome in stage III patients, it is reasonable to suggest that adjuvant therapy may positively affect prognosis of stage II patients with low MCT scores. Additional validation of this hypothesis is justified. Met is also currently being pursued as a pharmaceutical target for neoadjuvant treatment (37–40). Thus, it is possible that selecting patients based on their Met activation status may better predict potential responders and increase the effectiveness of such therapies.

In summary, we have developed novel automated image analysis algorithms for measurement of subcellular biomarkers. We used this method to measure the relative distribution of Met in the membrane, nucleus, and cytoplasm in colon cancer patients and showed that the relative distribution of membrane to cytoplasm Met was a significant prognostic indicator in stage I and II colon cancer patients. This is the first report of an association with Met and colon cancer survival. This result was not found with conventional DAB staining and manually scored Met, demonstrating the power of automated image analysis and subcellular biomarker quantitation. Based on data reported here, it is tempting to speculate that blocking the internalization of c-met may attenuate the malignant potential of tumor cells. Although our data, and those generate by others (41), may support such a theory, it is not clear if the distribution of Met is causal or correlative. Further studies on cultured cells and animal models are warranted to shed light on this mechanism. Although the data reported is preliminary and validation is needed in additional patient groups, our approach may have potential value in the stratification of stage II colon cancer patients for adjuvant therapy. This technique also has broad applicability to measure the activation status of receptor kinases whose signaling is affected by cellular internalization.

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References
Imaging, Diagnosis, Prognosis


The Relative Distribution of Membranous and Cytoplasmic Met Is a Prognostic Indicator in Stage I and II Colon Cancer


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