Durable Complete Response of Metastatic Gastric Cancer with Anti-Met Therapy Followed by Resistance at Recurrence

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ABSTRACT

A 48-year-old woman with chemorefractory metastatic gastric cancer to the liver was treated in a phase I clinical trial with MetMAb, a monoclonal antibody targeting the Met tyrosine kinase receptor. The primary tumor had high MET gene polysomy and evidence for an autocrine production of hepatocyte growth factor, the growth factor ligand of Met. A complete response was obtained that lasted 2 years; the cancer recurred as a peritoneal deposit invading into the transverse colon and a gastrohepatic ligament node. Compassionate use of MetMAb therapy at recurrence achieved a mixed response: a partial response of the two initial lesions but with development of multiple new foci of carcinomatosis. Tissue and serum studies to evaluate the Met signaling pathway correlated with MetMAb treatment response initially and at the time of recurrence.

SIGNIFICANCE: This research brief is the first to describe a durable complete response obtained with a molecularly targeted monoclonal antibody, MetMAb, to the receptor tyrosine kinase, Met, in a patient with chemorefractory metastatic gastric cancer. It is also the first to report biomarkers that predicted therapeutic response to Met inhibition. Cancer Discovery; 1(7); OF1–OF7. © 2011 AACR.

INTRODUCTION

A 48-year-old woman with a remote history of stage I, right-sided, estrogen receptor/progesterone receptor–positive, HER2-negative, infiltrating ductal breast cancer who was treated with mastectomy and axillary lymph node dissection followed by 4 cycles of cyclophosphamide and doxorubicin in 2000 presented with upper gastrointestinal bleeding in February 2007. A biopsy of an ulcerated lesion located at the gastric incisure was identified as poorly differentiated adenocarcinoma with signet ring features. A computed tomographic (CT) scan on March 5, 2007, revealed marked thickening along the lesser curvature of the stomach with no evidence of metastatic spread (Fig. 1A and B).

The patient underwent a total gastrectomy with D2 lymphadenectomy and cholecystectomy in April 2007. The primary tumor arose in a background of atrrophic antral gastritis with intestinal metaplasia consistent with a primary gastric cancer. Four of 21 perigastric lymph nodes were involved. Intraoperatively, a serosal nodule was palpated in the gallbladder that was consistent with metastatic disease. The final pathology report was pT3, N1, M1 (Fig. 2A). The margins were free of tumor. Postoperatively, the patient had no evidence of residual disease (Fig. 1C).

Systemic chemotherapy with 5-fluorouracil/leucovorin and oxaliplatin (FOLFOX) was initiated. Because of the patient's previous exposure to anthracyclines, she did not receive perioperative epirubicin (MAGIC regimen, i.e., epirubicin, cisplatin, and 5-fluorouracil) (1), nor did she receive adjuvant chemo-radiotherapy (MacDonald regimen) (2), given the existence of metastasis to the gallbladder.

After 6 cycles of biweekly FOLFOX, imaging revealed a new hypodense lesion in the right hepatic lobe measuring 8.7 mm × 12.4 mm that was consistent with progressive disease.
Figure 1. Radiologic assessment. A and B, CT scan, March 5, 2007. Primary gastric tumor (white arrows) on the lesser curvature in the axial (A) and frontal (B) views. C, CT scan, May 26, 2007. Postoperative image showing no radiographic evidence of disease. D, CT scan, August 28, 2007. Radiographic appearance of a new liver hypodensity (arrow) after 3 months of adjuvant chemotherapy with FOLFOX. E, radiographic evidence of partial response to treatment with the Met/VEGFR2 inhibitor after 2 cycles in the liver lesion (arrow). F, CT scan, October 4, 2007. Growth of the liver lesion after 6 months of treatment with the Met/VEGFR2 inhibitor (arrow). G, CT scan, June 12, 2008. Interval disappearance of the liver lesion after 4 cycles of MetMAb monoclonal antibody therapy (arrow). H and I, CT scan, October 22, 2010. Recurrence of metastatic gastric cancer on routine surveillance at the transverse colon (H; arrow) measuring 54.1 mm in longest dimension (straight line), and in the gastrohepatic ligament node abutting the left lobe of the liver (I; arrow). J-L, CT scan, February 12, 2011. Partial regression of the transverse colon lesion (J; arrow) and the gastrohepatic ligament node (K; thick arrow) after rechallenging with 3 cycles of MetMAb. However, there were multiple new foci of disease visualized in the peritoneum, some represented in panels K (thin arrow) and L (arrow). M and N, Positron emission tomographic imaging before rechallenge with MetMAb (M, November 12, 2010) and after 3 cycles of MetMAb (N, January 20, 2011). However, identified uptake of the transverse colon lesion (M; arrow) had regressed after MetMAb therapy (N; arrow) despite progression of disease elsewhere.
Figure 2. Tissue correlates with immunohistochemistry and FISH. A, immunohistochemistry. First column, hematoxylin and eosin (H&E) stain; second column, hepatocyte growth factor (HGF); third column, Met; fourth column, activated Met (p-Met). First row, adjacent paraneoplastic normal mucosa in the proximal stomach (Adj Ni); second row, primary tumor (PT) at low power (5×); third row, primary tumor (PT) at medium power (15×); fourth row, primary tumor (PT) at high power (40×); fifth row, metastatic tumor involvement in regional lymph node (LN) from surgical specimen analyzed in 2007 with gland-forming cells; sixth row, H&E (15×) and metastatic serosal deposit on the gallbladder (GB) focusing (40×) on a gland-forming differentiating region (diff); seventh row, H&E (5×) and metastatic serosal deposit on the gallbladder (GB) focusing (40×) on the signet ring (SR) component; eighth (last) row, recurrent metastatic lesion invading the colon (Col inv); p-Met and MSP were not conducted because of insufficient tissue remaining from the biopsy. Blue arrows, poorly differentiated cells; black arrows, signet ring cells. B, FISH gene copy number analysis of the primary tumor (left) and the metastatic colon deposit (right) for MET in nonsignet ring (differentiated cells, left column) compared with signet ring component (right column).

(Fig. 1D). The patient was enrolled in an open-label, phase II, nonrandomized trial in which investigators were evaluating an investigational small-molecule receptor tyrosine kinase (RTK) inhibitor. This investigational agent was reported to inhibit Met (a receptor tyrosine kinase involved in survival, proliferation, migration, and metastasis), vascular endothelial growth factor receptor 2 (VEGFR2), and many other tyrosine kinases. The patient received 4 cycles of this therapy.

An unconfirmed partial response was observed after the first 2 cycles (Fig. 1E); however, the lesion progressed as evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) after the next 2 cycles (Fig. 1F). After a 4-week washout period, the patient was enrolled in a phase I trial to evaluate the safety of MetMAb (3, 4), a monoclonal, monovalent (one-armed) antibody that binds to the extracellular component of the Met transmembrane receptor. The rationale for subsequent Met inhibition was multifactorial. Despite the progression by RECIST criteria, the tumor size was considered to be marginal to the treating clinicians, and there was lack of evidence of new lesions elsewhere, suggesting partial benefit from the RTK inhibitor.

It also appeared that cytotoxic therapy would not be urgently necessary for disease control. In addition, this patient had previously progressed on oxaliplatin-based chemotherapy.

Analysis for MET gene copy number from the primary gastric tumor (as well as metastatic lymph nodes and gallbladder deposit) revealed high polosity, and Met protein expression was detectable by immunohistochemistry (Fig. 2A and B). This last point, combined with the question of specificity for Met versus VEGFR2 inhibition versus other tyrosine kinase domains with the initial RTK inhibitor, suggested the possibility that isolated Met inhibition, by an antibody approach, may be active.

The patient was enrolled into the 20 mg/kg cohort in the phase I study (OAM4224g) that tested MetMAb monotherapy in patients with solid tumors refractory to standard treatment (final manuscript in preparation; refs. 4 and 5). MetMAb was administered intravenously every 3 weeks beginning March 2008 for 10 doses. Complete response (CR) was observed in June 2008, after the patient had received 4 doses of MetMAb (Fig. 1G), and was confirmed by MRI in September 2008. Toxicities reported included grade 2 anasarca and grade 2 hypoalbuminemia. No
other patient enrolled into the phase I study had a response to single-agent MetMAb (5). In November 2008, despite a sustained CR, the patient discontinued MetMAb because of drug-related side effects and treatment fatigue combined with an ongoing CR, calling into question the benefit from additional infusions. The hypalbuminemia and anasarca resolved within 4 weeks after cessation of MetMAb, and the patient underwent serial surveillance imaging and physician visits every 3 months for approximately 2 years. In October 2010, an asymptomatic lesion on the transverse colon was found (Fig. 1H), along with a new metastatic deposit at the gastrohepatic ligament (Fig. 1I). Biopsy of the colon confirmed poorly differentiated adenocarcinoma along with signet ring cell type and HER2 negativity by immunohistochemistry, consistent with the original gastric cancer pathology (Fig 2A).

Given the previous CR to MetMAb, a single-patient Investigational New Drug application for compassionate use was approved by the Food and Drug Administration and the study sponsor (Investigational New Drug application 105303). MetMAb was given at a dose of 15 mg/kg, which is the recommended phase II dose found to be active in patients with non-small cell lung carcinoma (6). After 3 cycles, CT imaging in December 2010 revealed that the transverse colonic metastatic lesion partially responded to therapy, decreasing from 54.1 mm \( \times \) 37.4 mm to 38.7 mm \( \times \) 35.9 mm (Fig. 1J). The gastrohepatic ligament node also decreased, measuring 21.9 mm \( \times \) 13.3 mm, from 24.5 mm \( \times \) 25.1 mm previously (Fig. 1K, thick arrow). Unfortunately, multiple new foci of carcinomatosis developed (Fig. 1K, thin arrow, and Fig. 1L). Given this mixed response, MetMAb was discontinued, and the patient was treated with salvage irinotecan chemotherapy; the carcinomatosis progressed after 4 cycles.

**RESULTS**

**Tissue and Serum Correlates**

**Primary Tumor (before Anti-Met Therapy, 2007)**

To further explain the durable complete radiologic response observed after treatment with MetMAb, additional correlative studies were conducted. Analysis of MET gene copy number by FISH was performed as previously described (7) on the primary tumor, metastatic lymph node, and metastatic gallbladder of the surgical specimen obtained in 2007. There was no evidence for high-level, focal MET gene amplification, which has been identified in approximately 5% to 10% of gastroesophageal adenocarcinomas (8, 9). However, in these samples, the poorly differentiated nonsignet ring cells showed \( \geq 4 \) MET copies per cell in 60% of the tumor cells, consistent with high polysomy/FISH positive as scored by the Go method (10) or high polysomy/FISH negative, as scored by the Cappuozzo method (11), and FISH negative by MET/CEP7 ratio, which was 1.04 when the cutoff of \( > 2 \) was used (Fig. 2B; Table 1). The mean copy of MET per cell was 3.7. The most common cell type was 4 MET/CEP7, which was found in 41.67% of nuclei. The increased gene copy correlated with moderate to high expression (2') of Met observed by immunohistochemistry (Fig. 2A). These gene copy number scores were similar in the signet ring cells as well as both the lymph node and gallbladder metastases (Table 1). Sequencing of the MET open reading frame did not reveal a coding mutation, nor was there evidence for the expression of the MET exon 14 oncogenic splice variant.

**Table 1. FISH analysis of MET and KRAS gene copy for the primary tumor and metastatic deposit at recurrence in the nonsignet ring and signet ring cells**

<table>
<thead>
<tr>
<th>Primary tumor, nonsignet ring morphology</th>
<th>MET</th>
<th>CEP7</th>
<th>MET copy number</th>
<th>MET/CEP7 ratio</th>
<th>Most common cell type</th>
<th>4 MET: 4 CEP7 found in</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET/CEP7 copy number ( \geq 4 )</td>
<td>60%</td>
<td>Ratio MET/CEP7 ( = 2 )</td>
<td>0.74</td>
<td>4 MET/CEP7</td>
<td>41.67%</td>
<td>of nuclei.</td>
</tr>
<tr>
<td>KRAS/CEP7: 2:27</td>
<td>2.17</td>
<td>KRAS copy number ( \geq 4 )</td>
<td>0.0%</td>
<td>KRAS/CEP7: 1.05</td>
<td>KRAS/CEP7: 2:0%</td>
<td>Most common cell type is 2 KRAS: 2 CEP12 found in 46.66% of nuclei.</td>
</tr>
</tbody>
</table>

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<tr>
<th>Transverse colon metastasis, nonsignet ring morphology</th>
<th>MET</th>
<th>CEP7</th>
<th>MET copy number</th>
<th>MET/CEP7 ratio</th>
<th>Most common cell type</th>
<th>4 MET: 4 CEP7 found in</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET/CEP7 copy number ( \geq 4 )</td>
<td>42%</td>
<td>Ratio MET/CEP7 ( = 2 )</td>
<td>0.74</td>
<td>MET/CEP7</td>
<td>28%</td>
<td>of nuclei.</td>
</tr>
<tr>
<td>KRAS/CEP7: 2:52</td>
<td>2.53</td>
<td>KRAS copy number ( \geq 4 )</td>
<td>2%</td>
<td>KRAS/CEP7: 1.00</td>
<td>KRAS/CEP7: 2:0%</td>
<td>Most common cell type is 3 KRAS: 3 CEP12 found in 59.00% of nuclei.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primary tumor, signet ring morphology</th>
<th>MET</th>
<th>CEP7</th>
<th>MET copy number</th>
<th>MET/CEP7 ratio</th>
<th>Most common cell type</th>
<th>4 MET: 4 CEP7 found in</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET/CEP7 copy number ( \geq 4 )</td>
<td>63.33%</td>
<td>Ratio MET/CEP7 ( = 2 )</td>
<td>0.74</td>
<td>MET/CEP7</td>
<td>40%</td>
<td>of nuclei.</td>
</tr>
<tr>
<td>KRAS/CEP7: 2:60</td>
<td>2.43</td>
<td>KRAS copy number ( \geq 4 )</td>
<td>5.00%</td>
<td>KRAS/CEP7: 1.07</td>
<td>KRAS/CEP7: 2:0%</td>
<td>Most common cell type is 3 KRAS: 3 CEP12 found in 40.00% of nuclei.</td>
</tr>
</tbody>
</table>

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<tr>
<th>Transverse colon metastasis, signet ring morphology</th>
<th>MET</th>
<th>CEP7</th>
<th>MET copy number</th>
<th>MET/CEP7 ratio</th>
<th>Most common cell type</th>
<th>4 MET: 4 CEP7 found in</th>
</tr>
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<tbody>
<tr>
<td>MET/CEP7 copy number ( \geq 4 )</td>
<td>36.66%</td>
<td>Ratio MET/CEP7 ( = 2 )</td>
<td>0.74</td>
<td>MET/CEP7</td>
<td>16.66%</td>
<td>of nuclei.</td>
</tr>
<tr>
<td>KRAS/CEP7: 2:21</td>
<td>2.26</td>
<td>KRAS copy number ( \geq 4 )</td>
<td>1.00%</td>
<td>KRAS/CEP7: 0.98</td>
<td>KRAS/CEP7: 2:0%</td>
<td>Most common cell type is 2 KRAS: 2 CEP12 found in 47.00% of nuclei.</td>
</tr>
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Complete Response of Gastric Cancer with Anti-Met Therapy

To determine a possible autocrine/paracrine phenomenon, evaluation of hepatocyte growth factor, the sole ligand for Met, was observed to be expressed in the cytoplasm of tumor cells by immunohistochemistry (3°) and not localized solely to the cell membrane, consistent with an autocrine loop (Fig. 2A) (5).

Transverse Colon Metastasis (at Tumor Recurrence, 2010)

Tissue from the transverse colonic metastasis was analyzed for potential resistant mechanisms to Met-directed therapy, including MET gene copy by FISH, as well as Met and hepatocyte growth factor expression (Fig. 2, Table 1). MET FISH analysis revealed a similar scoring pattern with a slightly lower mean MET copies per cell of 2.99 in the nonsignet ring cells (Fig. 2B). The most common cell type was now 2 MET2 CEP7, found in 28% of nuclei, and the second most common was 4 MET+ CEP7, found in 22% of nuclei. Met expression was determined to be slightly greater in this specimen (3°) when compared with expression from the 2007 specimens (Fig 2A), whereas hepatocyte growth factor expression was significantly lower than in the earlier tissue samples (0–1°). Unfortunately, it was not possible to obtain tissue representing the multiple new foci of carcinomatosis observed after the 3 recent doses of MetMab, nor of the original transverse colon lesion after partial response to rechallenge with MetMab treatment.

Serum Hepatocyte Growth Factor

Serum hepatocyte growth factor levels were evaluated in all phase I patients at baseline and throughout treatment at regular intervals. This patient exhibited one of the highest baseline plasma hepatocyte growth factor levels (3441 ± 301.8 pg/mL; Fig. 3). Moreover, this was the only patient on the phase I study to experience a posttreatment rapid and sustained decrease in serum hepatocyte growth factor (526.8 ± 91.98 pg/mL). Hepatocyte growth factor serum levels at recurrence were similar to levels post-MetMab initially (346 pg/mL ± 30.08 pg/mL; Fig. 3) (5).

DISCUSSION

We present an intriguing case of chemotherapy-refractory gastric cancer with metastasis to the liver for which the patient achieved a durable CR to MetMab, a monovalent antibody that inhibits the receptor Met. Salient points of the original case include the following: (i) the primary gastric tumor resected in 2007 was determined to have MET gene high polysomy; (ii) both Met and hepatocyte growth factor were expressed by immunohistochemistry in the primary tumor, and expression increased with histologic progression to the gallbladder metastasis; and (iii) the hepatocyte growth factor serum level was extremely high before treatment with MetMab, precipitously decreased immediately after drug exposure, and remained low, even at the time of widespread recurrence of disease.

The detection of a strong Met and hepatocyte growth factor expression restricted to the tumor epithelium in the primary tumor is particularly relevant and in preclinical models is consistent with autocrine Met signaling. In addition, our patient had one of the highest baseline hepatocyte growth factor levels in the phase I trial (median = 1104 pg/mL, n = 29) (5). Of importance, this was the only patient on the phase I study to experience a posttreatment rapid decrease that persisted throughout the remaining evaluated time points. The decrease in hepatocyte growth factor levels is difficult to explain, given that interruption of a presumed dependence on a hepatocyte growth factor/Met autocrine loop may be expected to lead to a reflexive increase in circulating hepatocyte growth factor.

A confounding aspect of this case was the previous treatment with the RTK inhibitor for 4 months, leading to a transient partial response after 2 months, and then ensuing disease progression after the next 2 months. One may speculate that the effect observed after MetMab therapy was attributable to an essential concomitant “dual” or synergistic “vertical” Met inhibition with an intracellular tyrosine kinase inhibitor (TKI) and an extracellular monoclonal antibody blockade. Evidence against this possibility is that a 4-week washout period of the RTK inhibitor (which generally has a t½ clearance on the order of hours to days) was required before commencing MetMab.

A similar concept, albeit reciprocal to our case, of “vertical” inhibition of RTKs has been described with the efficacy
of RTK inhibitor lapatinib (targeting Her1/Her2) in patients who progressed after treatment with trastuzumab, a Her2-directed antibody (12, 13). It is also a possibility that the RTK inhibitor “primed” the tumor in an undetermined manner, which resulted in extreme sensitivity to subsequent MetMab. An alternate hypothesis is that a transient response caused by inhibition of non-Met tyrosine kinases cannot be ruled out. In fact, the phase II RTK inhibitor potently inhibits VEGFR2 with much more affinity than Met. Regardless, the addition of MetMab seemed to overcome an acquired resistance that occurred while the patient was receiving the RTK inhibitor. Given that the clearance of MetMab is in the range of 6.7 to 8.3 mL/kg/day, it is not likely that the duration of response of 2 years resulted as a function of the lower clearance rate as compared with a small-molecule TKI.

Two years after the CR from MetMab therapy, the patient had a recurrence of her tumor at two locations seen on radiographic imaging. Salient points of this most recent treatment course with MetMab were (i) that the transverse colon tumor exhibited greater Met expression; (ii) that hepatocyte growth factor levels were relatively low in the tumor and the serum at recurrence compared with intratumoral levels at diagnosis and serum levels just prior to treatment with MetMab originally; and (iii) that the colonic lesion and the gastrohepatic ligament node both had partial responses to repeat MetMab therapy, whereas multiple new peritoneal lesions led to an overall mixed response and therefore overall disease progression by RECIST criteria.

The partial response of the transverse colon lesion could potentially be explained by the high levels of Met protein expression, greater than in the 2007 surgical specimen. It appeared that this colonic metastasis, and presumably the gastrohepatic ligament node that also partially responded, became “hepatocyte growth factor” independent as the result of Met overexpression and thus no longer depended on hepatocyte growth factor autocrine signaling. A shift in disease biology is supported by the low serum and tumor hepatocyte growth factor levels at the time of recurrence versus serum and tumor hepatocyte growth factor levels before original MetMab treatment.

In an attempt to determine a mechanism of the observed greater Met expression, MET FISH was conducted to evaluate for clonal evolution by selection of a greater gene copy number population. However, MET copy number was similar to the 2007 samples (Table 1). Regardless, MetMab maintained a partial inhibitory effect on this lesion, suggesting the tumor may have retained some responsiveness to hepatocyte growth factor ligand, or more likely, that MetMab may partially abrogate Met activation in Met overexpressed cells by interfering with homodimerization, despite lower levels of hepatocyte growth factor (14, 15). However, given that there was not a complete response on this second treatment cycle, this finding suggests that a population of the cells in these 2 recurrent lesions were resistant to the MetMab therapy.

Given that tumor recurrence occurred long after the original MetMab treatment was discontinued, one might expect that the recurrent tumor would have retained complete sensitivity when rechallenged with MetMab. However, after 3 doses, there were multiple new peritoneal lesions, and the original recurrent lesions had only partially responded (the majority of tumor remained). It has been reported that treatment of hepatocyte growth factor/Met-driven tumors with a low concentration of Met inhibitors leads to selection of higher MET gene amplified clones in vitro and in vivo as a mechanism of resistance (16). In this case we discovered that “resistance” after original MetMab was not through clonal evolution to greater MET gene amplification. However, one may speculate that this patient’s tumor evolved an increased Met expression, to sustain the addiction to the MET signaling pathway, through mechanisms other than gene amplification (such as increased transcription and/or increased mRNA and protein stability) (17) as a resistance mechanism to be exposing to anti-Met therapies.

Of importance, Cepero and colleagues (16) also reported that greater concentrations of Met inhibitors can lead to KRAS gene amplification and overexpression of the protein. However, we evaluated KRAS copy number by FISH and found a mean copy number of 2.52 per cell and only 2% of cells with >4 copies per cell in the recurrent metastatic lesion, which was similar to the primary tumor (Table 1). This finding suggests that clonal selection or evolution to KRAS gene amplification was not present at the time of recurrence and did not play a significant role in the acquired anti-Met resistance of the 2010 tumor biopsied in this case.

However, it is possible that KRAS became gene amplified in a small subset of cells to overcome the initial MetMab treatment in 2008 and then reverted back to non-gene-amplified status during the long period between CR and recurrence. This theory is plausible because Cepero and colleagues (16) demonstrated the phenomenon of transient KRAS amplification during active exposure to a Met inhibitor, which was reversible to non-KRAS-amplified status after withdrawal of the drug. Tumor tissue was not obtained immediately after MetMab exposure originally in 2008 (i.e., there was nothing to biopsy) nor after rechallenge in 2010, and therefore we cannot confirm or refute this hypothesis. Further investigation is underway to determine novel biomarkers that may be predictive of resistance to Met targeted therapy, such as RON (MST1R), the other member of the Met RTK family (7).

Gastroesophageal adenocarcinoma remains a challenging problem in oncology. In 2010, there were 21,000 new cases of gastric cancer and 10,570 deaths in the United States (18). There is an estimated 350% increase in esophageal/gastroesophageal junction adenocarcinoma in the United States in the past 3 decades (18). Taken together, gastroesophageal adenocarcinoma is the third most frequent cancer worldwide, accounting for more than 1 million deaths per year (19). This case is the first reported of a durable CR of metastatic gastric cancer to anti-Met therapy. Laboratory correlatives evaluating the Met pathways assisted in explaining clinical response initially as well as predicting outcomes at the time of recurrence. Further investigation of Met inhibitors in clinical trials for gastroesophageal adenocarcinoma with well-designed laboratory correlatives is warranted (20).

**METHODS**

Immunohistochemistry and FISH were conducted as described in our previous work (7).
Hepatocyte Growth Factor ELISA

Wells of NUNC MaxiSorp microtiter plates were coated (ON, 4°C) with 0.5 µg/mL of affinity-purified Goat antihuman hepatocyte growth factor polyclonal antibody in 100 µL of coating buffer (0.05M sodium carbonate buffer, pH 9.6) and were then blocked with 0.5% bovine serum albumin (BSA) in assay buffer (PBS, 0.5% BSA, 0.05% P 20, 0.25% CHAPS, 0.35% NaCl, 5mM EDTA, 10 ppm Proclin300, pH 7.4) for 1 hour at room temperature. Diluted human hepatocyte growth factor controls and plasma samples (100 µL) in assay buffer were loaded in duplicates and incubated for 2 hours at room temperature, followed by the addition of 100 µL of affinity-purified goat antihuman hepatocyte growth factor biotin (150 ng/mL) for an additional 1 hour at room temperature. Avidin-conjugated horseradish peroxidase (40 ng/mL) in PBS, 0.5% BSA, 0.05% P 20, 10ppm Proclin300, pH 7.4, was added (1 hour, room temperature), and the reaction was visualized by the addition of 100 µL of chromogenic substrate (TMB) for 15 minutes. The reaction was stopped with 1M phosphoric acid and absorbance at 450 nm was measured with reduction at 630 nm with an ELISA plate reader. Plates were washed 3 times with washing buffer (0.05% Tween 20/ PBS) after each step. As a reference for quantification, a standard curve was established by a serial dilution of human hepatocyte growth factor (Citrus CR67, 2000–15.625 µg/mL).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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