Development of a Novel c-MET-Based CTC Detection Platform

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Abstract

Amplification of the MET oncogene is associated with poor prognosis, metastatic dissemination, and drug resistance in many malignancies. We developed a method to capture and characterize circulating tumor cells (CTC) expressing c-MET using a ferromagnetic antibody. Immunofluorescence was used to characterize cells for c-MET, DAPI, and pan-CK, excluding CD45 \(^{+}\) leukocytes. The assay was validated using appropriate cell line controls spiked into peripheral blood collected from healthy volunteers (HV). In addition, peripheral blood was analyzed from patients with metastatic gastric, pancreatic, colorectal, bladder, renal, or prostate cancers. CTCs captured by c-MET were enumerated, and DNA FISH for c-MET were tested in replicate samples from 3 patients [gastric, colorectal, and renal cell carcinoma (RCC)] with 6% prevalence. CTC FISH demonstrated that MET amplification in both gastric and colorectal cancer patients and trisomy 7 with gain of MET gene copies in the RCC patient. The c-MET CTC assay is a rapid, noninvasive, sensitive, and specific method for detecting MET-amplified tumor cells. CTCs with MET amplification can be detected in patients with gastric, colorectal, and renal cancers.

Introduction

c-MET, the receptor for hepatocyte growth factor, is a receptor tyrosine kinase, which binds its ligand, dimerizes, autophosphorylates, and drives cellular proliferation through MAPK and AKT pathways (1). When mutated or amplified, c-MET acts as an oncogene in driving tumorigenesis and the metastatic potential of multiple solid tumors, including prostate (2), renal cell (3), particularly papillary (4), bladder (5), colorectal (6), gastric (7), pancreatic cancers (8), and non–small cell lung cancers (NSCLC; ref. 9). Importantly, c-MET overexpression is rare in primary cancers, but expression increases in metastatic tumors (10, 11). Overexpression of c-MET has been demonstrated in up to 50% of metastatic colorectal adenocarcinomas (10), approximately 25% of metastatic gastric cancers (7), and a high proportion of bony metastases in prostate cancer (2).

In addition, c-MET overexpression has been linked to hormonal therapy resistance in metastatic prostate cancer (12, 13), and VEGF resistance in metastatic renal cell carcinoma (RCC; refs. 14, 15). Furthermore, MET gene amplification appears to drive treatment resistance. Although best described in gastroesophageal tumors (16, 17), MET gene amplification can occur in several other malignancies, including NSCLC (18), ovarian cancer (19), and colorectal cancer (20), particularly as a mechanism of resistance to therapies targeting EGFR (e.g., cetuximab in colorectal cancer and gefitinib in NSCLC; refs. 20, 21). Indeed, it is estimated that 5% to 20% of NSCLC patients who develop resistance to EGFR inhibitors have MET amplification as a mechanism of resistance (22–24). In addition, MET amplification has also been observed in 20% of metastatic colorectal (6, 20) and up to 20% of gastric cancer patients (16, 25).

Therefore, MET amplification may be a biomarker for c-MET–directed therapies. Other than RAS mutations in colorectal cancer (26, 27) and HER2 expression in gastric cancer (28–30), there are few validated predictive biomarkers that are clinically useful in patients with metastatic prostate, renal cell, bladder, colorectal, gastric, and pancreatic cancers (26). To date, therapies directed against c-MET have been unsuccessful in unselected patients or in patients selected on the basis of tumor protein overexpression (31–33). However, there are promising response rates for c-MET–directed inhibitors in MET-amplified gastric cancer (34, 35) and NSCLC (36), thus necessitating the development of a predictive biomarker to target c-MET.

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

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Circulating tumor cells (CTC) represent cells which are shed from either primary or metastatic sites, and which migrate in the circulation in the process of tumor metastasis. The measurement of CTCs in the peripheral bloodstream represents one avenue to develop tumor-specific biomarkers with both prognostic and predictive utility (37–39).

The process of epithelial–mesenchymal transition in malignant cells has been demonstrated in a variety of solid tumors and is an important part of metastasis (40). Cells change from an epithelial phenotype, with tight adhesions and polarized layers, to a mesenchymal phenotype, without polarization. Cells can toggle between epithelial and mesenchymal phenotypes via transitions in what has been termed epithelial plasticity (EP); this important mechanism has been proposed to play an important role in tumorigenesis (41). Several translational studies have suggested a link between loss of epithelial markers, gain of mesenchymal markers, and the induction of signaling pathways that promote survival and cellular proliferation (42–44). These transitions between epithelial and mesenchymal phenotypes are also fluid; our group previously found that the majority of CTCs from patients with metastatic prostate cancer and breast cancer coexpress both epithelial [epithelial cell adhesion (EpCAM), E-cadherin, and cytokeratins (CK)] and mesenchymal (vimentin, NCadherin, and OB-cadherin) markers (45). These methods have also previously been used to detect mesenchymal CTCs (46). More recently, Yu and colleagues described CTCs from breast cancer patients displaying both epithelial and mesenchymal markers on RNA in situ hybridization (47). These studies and others suggest the existence of nonepithelial CTCs that have gained expression of mesenchymal markers and lost expression of epithelial markers (45, 47–50). Although c-MET has not been a canonical marker of EP or the mesenchymal phenotype, c-MET signaling is essential for the migration of myogenic stem cells into limb buds of embryos (51). Therefore, c-MET has a physiologic role for mesenchymal cells, and, as noted above, is overexpressed in metastatic disease.

Therefore, given the importance of c-MET in the process of metastasis, EP, and treatment resistance, we hypothesized that CTCs may overexpress c-MET during the process of metastasis and that these CTCs may be captured and represent distinct CTC phenotypes from standard EpCAM-captured CTCs (45, 48–50).

The goals of this current study were to develop a c-MET CTC assay and to perform a pilot study to capture and characterize the CTCs expressing c-MET in patients with metastatic prostate cancer and breast cancer.

**Materials and Methods**

**Development of novel c-MET CTC assay**

We used the CellSearch assay for the traditional EpCAM CTC capture, as described previously (52). For novel c-MET CTC capture, we designed an anti-c-MET ferrofluid for the immunomagnetic capture of CTCs. An antibody targeting extracellular c-MET (clone L6E7, Cell Signaling Technology) was used in conjugation with iron nanoparticles via a biotin–streptavidin interaction, similar to the CellSearch method (53). After capture and enhancement, fluorescent reagents were added for identification and enumeration of the target cells. These reagents included a confirmatory antibody targeting intracellular c-MET (clone 3D4, Invitrogen) conjugated to phycocerythrin, 4′,6-diamidino-2-phenylindole (DAPI), an anti-CD45 mAb (Veriﬁdx clone H130) conjugated to allophycocyanin (APC), and antibodies directed to cytokeratins 8, 18, and 19 conjugated to FITC. The processed reagent/sample mixture was dispensed by the CellTracks Auto-Rep System into a cartridge that was inserted into a MagNest device and processed on the CellTracks Analyzer II. CTCs were deﬁned as c-MET-positive and DAPI-positive nucleated and intact cells lacking CD45, without using cell size in the deﬁnition.

**Specimen characteristics**

All cell lines were obtained through the Duke Cell Culture Facility and passaged in the Garcia-Blanco laboratory for less than 6 months after attainment. Cell lines were grown to confluence in either DMEM or RPMI medium and harvested in PBS. The following cell lines were used for assay characterization: SNU5 cells (MET-ampliﬁed, EpCAM-positive gastric cancer cell line), A549 (c-MET-expressing, EpCAM-positive lung cancer cell line), SiHA (c-MET–expressing, EpCAM-positive cervical cancer cell line), PC3 (c-MET–expressing, EpCAM-positive prostate cancer cell line), HeLa cells (c-MET–expressing, EpCAM-negative cervical cancer cell line), BT549 (c-MET–expressing, EpCAM-negative breast adenocarcinoma cell line), and LnCAP (c-MET–negative, EpCAM-positive prostate cancer cell line). The assay was tested for sensitivity and specificity using these cell lines spiked in buffer as well as whole blood from healthy volunteers recruited at Duke University Medical Center through an Institutional review board (IRB)-approved protocol and after informed consent. Cells were counted and diluted, and between 30 and 10,000 cells were spiked in each sample tested. As the CellSearch EpCAM CTC assay has been fully characterized, the cell lines A549, SiHA, and PC3 were only tested once. Once the HeLa cell line was found to be fully c-MET positive and EpCAM negative, the BT549 cell line was also tested only once in the c-MET CTC assay.

**Immunofluorescence and flow cytometry**

Cells were harvested with cell dissociation buffer and ﬁxed with 1% paraformaldehyde (PFA). Cells were washed with PBS and then permeabilized with 0.1% Triton X-100. Two samples were made for each cell line. The first sample was stained with c-MET (external, Cell Signaling Technology 8741 with goat-anti-mouse IgG-488, A11001), and the second sample was stained with c-MET (internal, Life Technologies, 37-0100 antibody-labeled with ZS1202-A488) and EpCAM (from Veriﬁdx mouse antibody labeled with ZS2005-A647). After incubating and staining, the cells were washed with PBS and then divided equally for either ﬂow cytometry (BD FACS Canto II) or staining with DAPI. Final analysis was performed with ﬂuorescence microscopy.

**Patient selection**

All patients were enrolled at the Duke Cancer Institute (Durham, NC). We designed a prospective feasibility study to capture c-MET-expressing CTCs from patients with gastrointestinal (including gastroesophageal, pancreatic, and colorectal adenocarcinomas) and genitourinary (including prostate, RCC, and bladder urothelial carcinoma) malignancies. All patients were older than age 18 years, had histologically conﬁrmed malignancies as well as clinical or radiographic evidence of metastatic disease, and were enrolled before the initiation of a new systemic therapy. All patients had progression of disease on or following...
their most recent systemic therapy, with disease progression defined as radiographic progression or clinical progression of disease including cutaneous or palpable lesions, as well as new fluid accumulation such as pleural effusions or ascites. Patients with metastatic castration-resistant prostate cancer (CRPC) could have disease progression defined as two consecutive PSA levels greater than the PSA nadir achieved on androgen deprivation therapy and their most recent therapy. Because of the aggressive nature of pancreatic cancers as well as nonclear cell RCCs, these patients could be enrolled prior to their first systemic therapy. For clear cell RCCs, patients were eligible if they had disease progression within a year of starting a VEGF-targeting therapy.

Healthy volunteers were recruited from a separate healthy volunteer protocol, and samples were run on the c-MET CTC assay but not the EpCAM CTC assay as the EpCAM CTC assay is fully validated in previous studies (53).

All study subjects signed informed consent to participate in this Duke IRB-approved, single institution, investigator-initiated study. A single peripheral blood draw was performed. Subjects had peripheral whole blood collected into four 10-mL Cellsave vacutainers. Samples were stored at ambient room temperature and processed within 48 hours of collection. A total of 7.5 mL of whole blood was run per sample, and duplicate samples were processed for c-MET and EpCAM capture.

CTC enumeration for c-MET and EpCAM capture was performed as described above. When c-MET CTCs were present, DNA FISH was performed using a dual color FISH probe for c-MET and SE7. A repeat-free FISH probe for c-MET was prepared using BAC clones RP11-114O6 and CTD-2369N14 and labeled with PlatinumBright 550 (Leica Biosystems) as described previously (54). The chromosome 7 centromere probe (SE7) was labeled with Platinum Bright 415 and was obtained from Leica Biosystems. Methods for performing FISH on CTCs have been described previously (55).

Statistical analysis
Descriptive statistics were used to describe clinical parameters and CTC enumeration for c-MET and EpCAM capture. Prevalence of detectable CTCs using c-MET capture was calculated as the proportion of patients with at least one c-MET CTC that was validated by replicate. The Wilcoxon rank-sum test was used to assess the difference in the number of c-MET+/CD45−/CK− cells among cancer patients and healthy controls.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>c-MET/EpCAM status</th>
<th>% cells captured c-MET assay mean (SD)</th>
<th>% cells captured EpCAM assay mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNU5</td>
<td>c-MET amplified</td>
<td>65% (15)</td>
<td>70% (21)</td>
</tr>
<tr>
<td>A549</td>
<td>c-MET high-expressing, EpCAM positive</td>
<td>32% (17)</td>
<td>51% (N/A)</td>
</tr>
<tr>
<td>SiHA</td>
<td>c-MET low expressing, EpCAM positive</td>
<td>18% (8)</td>
<td>23% (N/A)</td>
</tr>
<tr>
<td>HeLa</td>
<td>c-MET high-expressing, EpCAM negative</td>
<td>66% (15)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>BT549</td>
<td>c-MET high-expressing, EpCAM negative</td>
<td>35% (N/A)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>PC3</td>
<td>c-MET high-expressing, EpCAM positive</td>
<td>40% (2)</td>
<td>88% (N/A)</td>
</tr>
<tr>
<td>LnCAP</td>
<td>c-MET negative</td>
<td>0% (0)</td>
<td>N/A</td>
</tr>
<tr>
<td>Healthy control</td>
<td>n/a</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
</tbody>
</table>

Figure 1.
Depiction of c-MET CTCs (A), captured with c-MET ferrofluid, staining positive for intracellular c-MET and DAPI for the nucleus, and negative for CD45. c-MET immunoblot of cell lines for c-MET expression (B). Spiking studies with percentage of cells captured for each cell line in c-MET and EpCAM assays (C). MET-amplified cell line: SNU5; c-MET high-expressing, EpCAM-negative cell lines: BT549, HeLa; c-MET high-expressing, EpCAM-positive cell lines: A549, A1549; c-MET low-expressing cell line: SiHA; c-MET negative: LnCAP.
### Table 1

<table>
<thead>
<tr>
<th>Disease site</th>
<th>No. of patients</th>
<th>Sex</th>
<th>Ethnicity</th>
<th>Prior Therapies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate (10)</td>
<td></td>
<td></td>
<td>W, AA</td>
<td>Surgical castration (1), abiraterone or enzalutamide (9), sipuleucel-T (5), docetaxel (9), cabazitaxel (4)</td>
</tr>
<tr>
<td>Bladder (8)</td>
<td></td>
<td></td>
<td>W, AA</td>
<td>Gemcitabine/cisplatin (6), carboplatin (10), radiation (3)</td>
</tr>
<tr>
<td>Gastric (7)</td>
<td></td>
<td></td>
<td>W, AA</td>
<td>5-FU/oxaliplatin (6), Trastuzumab (2), irinotecan (9), regorafenib (5), flibercept (3)</td>
</tr>
<tr>
<td>Rectal (10)</td>
<td></td>
<td></td>
<td>W</td>
<td>Carboplatin-paclitaxel (1), oxaliplatin (4), gemcitabine (5), irinotecan (1), ruxolitinib (1)</td>
</tr>
<tr>
<td>Pancreas (7)</td>
<td></td>
<td></td>
<td>W, AA</td>
<td>5-FU (6), oxaliplatin (4), gemcitabine (5), irinotecan (1), ruxolitinib (1)</td>
</tr>
<tr>
<td>Lung (2), Liver (4), LNs (4)</td>
<td></td>
<td></td>
<td>W</td>
<td>Carboplatin, radiation, gemcitabine, cisplatin, mitomycin C (2), radiation (2)</td>
</tr>
<tr>
<td>Renal cell (10)</td>
<td></td>
<td></td>
<td>W</td>
<td>5-FU/oxaliplatin (6), Trastuzumab (2), irinotecan (9), regorafenib (5), flibercept (3)</td>
</tr>
</tbody>
</table>

**Abbreviations:** AA, African American; CA 19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; 5-FU, 5-fluorouracil; LDH, lactate dehydrogenase; LN, lymph node; M, male; n/a, not applicable; W, white.

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**Results**

**c-MET–expressing cell lines can be captured with c-MET CTC assay**

We defined c-MET CTCs as nucleated intact cells captured using a ferromagnetic antibody directed against the c-MET extracellular domain, and for c-MET (intracellular domain) by immunofluorescence staining and DAPI, negative for the leukocyte marker CD45, and without any specific size criteria (Fig. 1A). As proof of the principle experiment to show that c-MET CTCs can be captured, cell lines were first characterized for c-MET by immunoblot (Fig. 1B). The MET-amplified SNU5 cell line was used as a positive control, and the c-MET-negative LnCAP cell line and donor leukocytes were used as negative controls, with cells in buffer or spiked into peripheral blood samples from healthy controls. Recovery of c-MET cells with the c-MET CTC assay ranged from 20% to 40% in c-MET–expressing cell lines to 60%–80% for the MET-amplified SNU5 and c-MET–overexpressing HeLa cell lines (Fig. 1C). None of the spiked LnCAP cells were captured with the c-MET CTC assay, either in buffer or in healthy control blood; therefore the specificity of the c-MET CTC assay was 100%.

We next compared the efficiency of c-MET capture to the current EpCAM-based capture methods. Most of the cell lines were captured with both c-MET capture as well as the EpCAM capture assay. However, to determine whether the c-MET assay can identify CTCs that have lost their epithelial phenotype and EpCAM expression, HeLa and BTS49 cell lines were tested, which are known to express c-MET and lack EpCAM. These cells were captured by the c-MET assay with a sensitivity of about 65% and 34.6%, respectively, but not captured by the EpCAM assay as expected. SNU5, HeLa, and SIHA cell lines were also characterized for EpCAM and c-MET expression by immunofluorescence confirming c-MET expression and lack of EpCAM (Supplementary Fig. S1).

**c-MET–expressing CTCs in patients can be captured with the c-MET CTC assay**

We next evaluated the c-MET CTC assay in a range of patients with metastatic gastrointestinal and genitourinary malignancies. Fifty-two patients with metastatic solid tumors were enrolled in the Duke Cancer Center clinics between March 10, 2014, and January 2, 2015. Patients were enrolled for each of the following: prostate adenocarcinoma (10 patients), RCC (10 patients), colorectal adenocarcinoma (10 patients), urothelial carcinoma (8 patients), gastroesophageal adenocarcinoma (7 patients), and pancreatic adenocarcinoma (7 patients). Efforts were made to enroll patients with resistant disease to VEGF (RCC) or EGFR (colon) inhibitors, or for men with bone metastatic CRPC (prostate) to enrich for c-MET expression (13, 14, 20). All patients had metastatic disease with predominantly lymph node, liver, lung, and bone metastases (Table 1). Most of the patients had undergone multiple lines of targeted and systemic chemotherapies. All of the prostate patients had received either combined androgen blockade or surgical castration, 9 had received either abiraterone or enzalutamide, 9 had received docetaxel, and all had bone metastases. Nine of the RCC patients (7 clear cell, 2 papillary, and 1 collecting duct) were refractory to VEGF-targeting therapies including sunitinib, axitinib, and pazopanib. All 8 of the bladder cancer patients were refractory to previous platinum-based chemotherapy. Of the 7 gastroesophageal cancer patients who were...
enrolled, 6 had received prior 5-fluorouracil (5-FU)/leucovorin (LV)/oxaliplatin, and 2 had received prior trastuzumab for HER2-positive disease. All of the colorectal cancer patients had been treated with 5-FU/LV/oxaliplatin, 9 had been treated with bevacizumab, and 6 had received prior EGFR-targeting therapy (either cetuximab or panitumumab). Of the 7 pancreatic cancer patients, 6 had received prior 5-FU, 5 had received prior gemcitabine, 4 had received prior oxaliplatin, and 2 had received prior nab-paclitaxel. Further details are provided in Table 1.

c-MET cells captured from four patients across four disease types

c-MET CTCs and EpCAM CTCs were enumerated in duplicate in all patients and summarized (Table 2). c-MET CTCs meeting the criteria described above (Fig. 1A) were found in 4 patients (8%), and at least one EpCAM CTC was identified in 23 patients (44%; Fig. 2). Of the 4 cases that had detectable c-MET CTCs, 3 cases were validated in replicate samples with MET amplification and trisomy 7 confirmed by DNA FISH, for a total cross-

Table 2. Cell types captured per disease site

<table>
<thead>
<tr>
<th>Disease site</th>
<th>c-MET CTCs</th>
<th>Subjects (N,%) with greater than 0 c-MET CTCs</th>
<th>c-MET capture CD45⁺/CK⁺ cells</th>
<th>EpCAM CTCs, median (range)</th>
<th>Subjects (N,%) with greater than 0 EpCAM CTCs</th>
<th>EpCAM capture CD45⁺/CK⁺ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate, n = 10</td>
<td>0 (0–0)</td>
<td>0 (0%)</td>
<td>0 (1–59)</td>
<td>28 (0–705)</td>
<td>9 (90%)</td>
<td>0 (0–2)</td>
</tr>
<tr>
<td>Renal cell, n = 10</td>
<td>0 (0–3)</td>
<td>1 (10%)</td>
<td>2 (0–16)</td>
<td>0 (0–9)</td>
<td>3 (30%)</td>
<td>3 (N/A)</td>
</tr>
<tr>
<td>Bladder, n = 8</td>
<td>0 (0–4)</td>
<td>1 (13%)</td>
<td>0 (0–15)</td>
<td>0 (0–2)</td>
<td>2 (25%)</td>
<td>12 (1–54)</td>
</tr>
<tr>
<td>Gastric, n = 7</td>
<td>0 (0–7)</td>
<td>1 (14%)</td>
<td>4 (0–24)</td>
<td>0 (0–20)</td>
<td>3 (43%)</td>
<td>0.5 (0–13)</td>
</tr>
<tr>
<td>Colon, n = 10</td>
<td>0 (0–7)</td>
<td>1 (10%)</td>
<td>0 (0–28)</td>
<td>0 (0–24)</td>
<td>5 (50%)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>Pancreas, n = 7</td>
<td>0 (0–0)</td>
<td>0 (0%)</td>
<td>1 (0–488)</td>
<td>0 (0–1)</td>
<td>1 (14%)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>Overall cancer, n = 52</td>
<td>0 (0–90)</td>
<td>4 (7.7%)</td>
<td>1 (0–488)</td>
<td>0 (0–705)</td>
<td>23 (44%)</td>
<td>0.5 (0–54)</td>
</tr>
<tr>
<td>Healthy control, n = 20</td>
<td>0 (0–0)</td>
<td>0 (0%)</td>
<td>0 (0–11)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Abbreviations: EpCAM, epithelial cell adhesion molecule; N, number; N/A, not applicable.

*EpCAM capture c-MET PE was performed for only 1 patient sample.

Figure 2.

Enumeration of CTCs captured either by c-MET (A) or EpCAM (B), separated by disease site.
sectional prevalence of 6% [95% confidence interval (CI), 1%–16%]. The subgroup cross-section point-estimated prevalence was 14% for gastric cancer, 10% for colorectal and RCC, and 13% for urothelial carcinoma. These cases are further described below to provide greater clinical context for the successful c-MET CTC capture events in this study.

Patient A was a 56-year-old Caucasian man with clear cell RCC, who had undergone nephrectomy but developed metastases in the liver, lungs, and pancreas. He had progression of disease after 8 months of pazopanib, was anemic, had an elevated lactate dehydrogenase (LDH), and low albumin. Patient A was found to have 1 and 3 c-MET CTCs in duplicate samples (Fig. 3A). He had 9 EpCAM CTCs in one of two samples (data not shown).

Patient B was a 65-year-old Caucasian man with metastatic urothelial carcinoma, with metastases in the left pelvis, and progression of disease in mediastinal lymph nodes and lung despite prior gemcitabine and cisplatin. Patient B was found to have 1 and 3 c-MET CTCs in duplicate samples (Fig. 3B). He had 9 EpCAM CTCs in one of two samples (data not shown).

Patient C was a 55-year-old Caucasian woman with metastatic rectal cancer with regional recurrence in the presacral space, as well as disseminated metastases with retroperitoneal and bilateral hilar lymphadenopathy, hepatic lesions, and pulmonary nodules. She had undergone several lines of chemotherapy including FOLFOX with bevacizumab, FOLFIRI, panitumumab, cetuximab, regorafenib, as well as ziv-afibercept. She was anemic, had an elevated LDH, low albumin and high CEA of 702.4 ng/mL. Patient C was found to have 7 and 2 c-MET CTCs in duplicate samples (Fig. 3C).

Patient D was a 64-year-old Caucasian man with adenocarcinoma at the gastroesophageal junction, with metastatic disease in lymph nodes, liver, and bone. His tumor was initially tested and found to be HER2 amplified, and he completed a course of therapy with 5-FU, LV, oxaliplatin (FOLFOX) and trastuzumab. Peripheral blood samples were taken upon disease progression on this chemotherapy regimen. He was found to have 52 and 90 c-MET CTCs in duplicate samples (Fig. 3D).

Single-cell DNA FISH shows chromosomal abnormalities in patients with detectable c-MET CTCs

We next evaluated the c-MET–captured CTCs by FISH to determine the presence of any chromosome 7 gains or focal amplification of the c-MET locus, which would thus suggest a malignant origin to these CTCs. c-MET CTCs isolated from patients A, C, and
20 healthy control samples, 7 healthy controls had c-MET rare cells, which were positive for both CD45 and pan-CK, and 35 (67% prevalence; 95% CI, 14%–56%) cancer patients enrolled on this study, 35 (67% prevalence; 95% CI, 14%–56%) had polysomy 7 and c-MET amplification (MET/CEP7 ratio >10) in all tested CTCs; Fig. 4B). Patient C with metastatic colorectal cancer also had polysomy 7 and MET amplification (MET/CEP7 ratio >10) in all tested CTCs; Fig. 4C). Leukocytes in each sample underwent DNA FISH as internal control cells and had diploid chromosome 7 and two copies of the MET gene (representative shown in Fig. 4D). These results suggest a malignant origin to the c-MET–captured CTCs.

c-MET+, CD45+, CK+ cells isolated from a subset of patients

During isolation of c-MET CTCs, we also isolated and identified rare cells, which were positive for both CD45 and pan-CK, and which had a careful morphology consistent with benign cells. In 20 healthy control samples, 7 healthy controls had c-MET+/CD45+/CK+ cells, with 6 of 7 samples containing only 1 or 2 cells and only one healthy control with a 11 c-MET+/CD45+/CK+ cells (median 0 cells; prevalence 35%; 95% CI, 14%–56%). In 52 cancer patients enrolled on this study, 35 (67% prevalence; 95% CI, 53%–80%) had detectable c-MET+/CD45+/CK+ cells, with a median of 1 cell (range 0 to 488; Supplementary Fig. S2B). Using a two-sided Wilcoxon rank-sum test between cancer patients and healthy volunteers, the difference was statistically significant (P = 0.013). These cells were smaller in morphology than c-MET CTCs described above, and some had bilobed nuclei (Supplementary Fig. S2C), suggestive of immature neutrophils.

The number of c-MET+/CD45+/CK+ cells did not correlate with absolute neutrophil count (Spearman ρ coefficient 0.10, P = 0.49). When tested by DNA FISH, all of the c-MET+, CD45+ cells identified in cancer patients were diploid (data not shown), indicating their likely benign nature. However, the presence of these cells almost exclusively in cancer patients versus healthy volunteers suggests that these c-MET circulating cells are highly associated with cancer.

Discussion

In this study, we have developed a novel, highly specific, and minimally invasive assay for c-MET–amplified CTCs based on c-MET capture and characterization of CTCs from the peripheral blood of multiple patients with metastatic carcinomas, including gastric, colorectal, and RCCs. Importantly, cancer cells that overexpress c-MET and lack EpCAM (like the HeLa and BT549 cell lines) can be captured with the c-MET CTC assay even when they are not detected by the CellSearch EpCAM assay, indicating loss of epithelial differentiation. The prevalence of a positive test (at least one detectable c-MET CTC) in our cohort was 6% to 14% (depending on disease site), similar to the expected prevalence of MET amplification in patients with metastatic cancer, and was not present in normal healthy volunteers. The prevalence of c-MET–positive CTCs ranged from 0% in men with metastatic CRPC to 14% in patients with metastatic gastric cancer, indicating the importance of tumor lineage and context for this assay.

c-MET–positive and MET amplification of the c-MET isolated and characterized from patients with various malignancies and therefore present a new potential biomarker for these patients, potentially enabling clinical studies that utilize MET amplification as a predictive biomarker. We have reproducibly isolated c-MET CTCs from patients with metastatic, treatment-refractory RCC, gastrointestinal, and colorectal adenocarcinomas. Patients were selected for high tumor burdens and treatment refractoriness to VEGF or EGF/HER2-based therapies, where c-MET may play a role in mediating resistance (14, 20). Surprisingly, c-MET CTCs were not detected in the majority of patients, indicating that this assay may not detect c-MET overexpressing, nonamplified CTCs. This may be either due to cleavage of the c-MET extracellular domain (i.e., shedding; ref. 56), altered conformation of the c-MET extracellular domain, or relatively low abundance of c-MET expression on the cell surface of CTCs in the absence of gene amplification.

Of note, in 3 of 4 cases, the presence of c-MET CTCs was linked to the presence of genomic changes in MET, with either trisomy 7 or MET amplification. Therefore, MET amplification and the degree of c-MET expression on the cell surface may be critical to the ability to capture these cells. Further studies are ongoing in selected patients with known MET amplification, or enriched for the context by which c-MET is commonly overexpressed, such as in NSCLC or in patients harboring known tumor-specific genomic amplifications in the MET locus, such as papillary RCC. This noninvasive technique for identifying c-MET CTCs may potentially be applied in clinical trials of c-MET inhibitors, such as the upcoming SWOG 1500 study of papillary RCC.

We identified a high prevalence of CD45+ leukocytes coexpressing both CK and c-MET in patients with metastatic solid tumors but rarely in healthy volunteers. These cells were diploid and had the appearance of band neutrophils, an immature and activated myeloid cell type. Previous work has suggested the expression of...
activated c-MET signaling in phagocytic immune cells (57), which may represent the population of leukocytes that we have found. Recent work by Finiguerra and colleagues has also demonstrated the importance of c-MET on neutrophils for chemotaxis and cytotoxic killing of cancer cells (58). Therefore, we theorize that this population of c-MET-positive leukocytes is a biomarker of immune activation in the setting of cancer. Further studies of the prevalence of this c-MET–based leukocyte assay in early, localized cancer are needed to evaluate its clinical relevance.

In conclusion, we have developed a novel method for the isolation and characterization of c-MET–expressing cells (CTCs and leukocytes) in patients with a diverse range of metastatic solid tumors, using a noninvasive and reproducible assay with high sensitivity and specificity. While the prevalence of a positive test in our cohort was low, this prevalence was consistent with the known prevalence of MET amplification in these patients, which is very rare in prostate cancer and relatively more common in treatment-refractory gastric and colorectal cancer. Given the association of c-MET CTCs with time in patients with Ongoing studies are currently evaluating this platform over high sensitivity and specific solid tumors, using a noninvasive and reproducible assay with isolation and characterization of c-MET–directed therapies. Ongoing studies are currently evaluating this platform over time in patients with MET amplification and in the context of c-MET–directed therapies.

Disclosure of Potential Conflicts of Interest

T. Zhang reports receiving a commercial research grant from Janssen Research and Development and Acerta Pharmaceuticals, is a consultant/advisory board member for G1 Therapeutics, and has ownership interest (including patents) in a pending patent. M. Garcia-Blanco has ownership interest in a patent. A.J. Armstrong reports receiving commercial research support from Medivation/Astellas and Janssen, is a consultant/advisory board member for Janssen and Medivation, and has ownership interest (including patent) in a pending patent. No potential conflicts of interest were disclosed by the other authors. A.J. Armstrong is supported by a grant from the Prostate Cancer Foundation.

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