Development of a novel c-MET based CTC detection platform

Authors:

Tian Zhang, Rengasamy Boominathan, Brad Foulk, Chandra Rao, Gabor Kemeny, John H. Strickler, James L. Abbruzzese, Michael R. Harrison, David S. Hsu, Patrick Healy, Jing Li, Cinthia Pi, Katherine Prendergast, Carey Hobbs, Sarah Gemberling, Daniel J. George, Herbert I. Hurwitz, Mark Connelly, Mariano A. Garcia-Blanco, Andrew J. Armstrong*

Tian Zhang, MD
Medical Instructor
Division of Medical Oncology
Department of Medicine
Duke Cancer Institute
Duke University

*Correspondence to:

Andrew J. Armstrong, MD ScM FACP
Associate Professor of Medicine and Surgery
Associate Director for Clinical Research in Genitourinary Oncology
Duke Cancer Institute
Divisions of Medical Oncology and Urology
Duke University
Mail address: DUMC Box 103861, Durham NC 27710 USA
Deliveries: 905 La Salle Street, GSRB1 Room 3006, Durham, NC 27710
Phone: (919) 668-8797
Fax: (919) 660-0178
Email: andrew.armstrong@duke.edu

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Conflict of Interest

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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 (CTCs) 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 (HVs). In addition, peripheral blood was analyzed from patients (pts) with metastatic: gastric, pancreatic, colorectal, bladder, renal, or prostate cancers. CTCs captured by c-MET were enumerated, and DNA FISH for *MET* amplification was performed. The approach was highly sensitive (80%) for *MET*-amplified cells, sensitive (40-80%) for c-MET-overexpressed cells, and specific (100%) for both c-MET negative cells and in 20 HVs. Of 52 pts with metastatic carcinomas tested, c-MET CTCs were captured in replicate samples from 3 pts (gastric, colorectal, and renal cell carcinoma) with 6% prevalence. CTC FISH demonstrated *MET* amplification in both gastric and colorectal cancer pts and trisomy 7 with gain of *MET* gene copies in the RCC pt. The c-MET CTC assay is a rapid, non-invasive, 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.

Implications: This study developed a novel c-MET CTC assay for detecting c-MET CTCs in patients with *MET* amplification and warrants further investigation to determine its clinical applicability.

Keywords: Circulating tumor cells, c-MET, epithelial plasticity, gastrointestinal malignancies, *MET* amplification, genitourinary malignancies, renal cell carcinoma, gastric cancer, colorectal cancer

Introduction:

c-MET, the receptor for hepatocyte growth factor (HGF), is a receptor tyrosine kinase, which binds its ligand, dimerizes, auto-phosphorylates, and drives cellular proliferation through MAPK and AKT pathways¹. When mutated or amplified, c-MET acts as an oncogene in driving tumorigenesis and the metastatic potential of multiple solid tumors, including prostate², renal cell³ (particularly papillary⁴), bladder⁵, colorectal⁶, gastric⁷, pancreatic cancers⁸, and non-small cell lung cancers (NSCLC)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 adenocarcinomas¹⁰, approximately 25% of metastatic gastric cancers⁷, and a high proportion of bony metastases in prostate cancer². In addition, c-MET overexpression has been linked to hormonal therapy resistance in metastatic prostate cancer^{12,13}, and vascular endothelial growth factor resistance in metastatic renal cell carcinoma^{14,15}.

Furthermore, *MET* gene amplification appears to drive treatment resistance. Although best described in gastro-esophageal tumors^{16,17}, *MET* gene amplification can occur in several other malignancies, including non-small cell lung cancer (NSCLC)¹⁸, ovarian cancer¹⁹, and colorectal cancer²⁰, particularly as a mechanism of resistance to therapies targeting epidermal growth factor receptor (EGFR) (e.g., cetuximab in colorectal cancer and gefitinib in NSCLC)^{20,21}. Indeed, it is estimated that 5-20% of NSCLC patients who develop resistance to EGFR inhibitors have *MET* amplification as a mechanism of resistance²²⁻²⁴. 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^{28,29,30}, there are few validated predictive biomarkers that are clinically useful in patients with metastatic prostate,

renal cell, bladder, colorectal, gastric, and pancreatic cancers²⁶. To date, therapies directed against c-MET have been unsuccessful in unselected patients or in patients selected based on tumor protein overexpression³¹⁻³³. However, there are promising response rates for c-MET directed inhibitors in *MET* amplified gastric cancer^{34,35} and non-small cell lung cancer³⁶, thus necessitating the development of a predictive biomarker to target c-MET.

Circulating tumor cells (CTCs) 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 utility³⁷⁻³⁹.

The process of epithelial-mesenchymal transition (EMT) in malignant cells has been demonstrated in a variety of solid tumors and is an important part of metastasis⁴⁰. 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 tumorigenesis⁴¹. 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 proliferation⁴²⁻⁴⁴. 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 co-express both epithelial (EpCAM, E-cadherin, and cytokeratins (CK)) and mesenchymal (vimentin, N-cadherin, and OB-cadherin) markers⁴⁵. These methods have also previously been used to detect mesenchymal CTCs⁴⁶. More recently, Yu et al described CTCs from breast cancer patients displaying both epithelial and mesenchymal markers on RNA-*in situ*

hybridization (ISH)⁴⁷. These studies and others suggest the existence of non-epithelial 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 embryos⁵¹. 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 prevalence of c-MET expressing CTCs in patients with metastatic gastrointestinal and genitourinary cancers.

Materials and Methods:

Development of novel c-MET CTC assay

We used the CellSearch[®] assay for the traditional EpCAM CTC capture, as previously described⁵². 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 Technologies, Beverly, MA) was used in conjugation with iron nanoparticles via a biotin-streptavidin interaction, similar to the CellSearch[®] method⁵³. 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, Carlsbad, CA) conjugated to phycoerythrin, 4',6-diamidino-2-phenylindole (DAPI), an anti-CD45 monoclonal antibody (Veridex clone HI30) conjugated to allophycocyanin (APC), and antibodies directed to

cytokeratins 8, 18, and 19 conjugated to fluorescein isothiocyanate (FITC). The processed reagent/sample mixture is dispensed by the CellTracks® AutoPrep System into a cartridge that is inserted into a MagNest® device and processed on the CellTracks® Analyzer II. Circulating tumor cells 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 Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI) medium and harvested in phosphate buffer saline (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) were used for assay characterization. The assay was tested for sensitivity and specificity using these cell lines spiked in buffer, as well as spiked into whole blood from healthy volunteers recruited at Duke University Medical Center through an 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. Since 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% PFA. Cells were washed

with PBS and then permeabilized with 0.1% triton in PBS for 30 minutes at room temperature, then blocked with 10% goat-serum in PBS. For staining we adjusted the cell density to 1×10^6 cells/mL. Two samples were made for each cell line. The first sample was stained with c-MET (external, CellSignaling 8741 with goat-anti-mouse IgG-488, A11001), and the second sample was stained with c-MET (internal, LifeTechnologies, 37-0100 antibody- labeled with Z25102-A488) and EpCAM (from Veridex mouse-antibody labeled with Z25005-A647). After incubating and staining, the cells were washed with PBS and then divided equally for either flow cytometry (BD Canto II) or staining with DAPI. Final analysis was performed with fluorescence microscopy.

Patient selection:

All patients were enrolled at the Duke Cancer Institute in 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, renal cell carcinoma, and bladder urothelial carcinoma) malignancies. All patients were older than age 18, 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 could have disease progression defined as 2 consecutive PSA levels greater than the PSA nadir achieved on androgen deprivation therapy and their most recent therapy. Due to the aggressive nature of pancreatic cancers as well as non-clear cell renal cell carcinomas, these patients could be enrolled prior to their first systemic therapy. For clear cell renal cell carcinomas, 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 since the EpCAM CTC assay is fully validated in previous studies⁵³.

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 10mL Cellsave[®] vacutainers. Samples were stored at ambient room temperature and processed within 48 hours of collection. 7.5mL 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 fluorescent *in situ* hybridization (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 previously described⁵⁴. The chromosome 7 centromere probe (SE7) was labeled with Platinum Bright 415 and was obtained from Leica Biosystems. Methods for performing FISH on CTC have been previously described⁵⁵.

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.

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 (**Figure 1A**). As proof of the principal experiment to show that c-MET CTCs can be captured, cell lines were first characterized for c-MET by immunoblot (**Figure 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 20-40% in c-MET expressing cell lines to 60-80% for the *MET* amplified SNU5 and c-MET overexpressing HeLa cell lines (**Figure 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, in order to determine if the c-MET assay can identify CTC that have lost their epithelial phenotype and EpCAM expression, HeLa and BT549 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 (**Supplemental Figure 1**).

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 February 1, 2015. Patients were enrolled for each of the following: prostate adenocarcinoma (10 pts), renal cell carcinoma (RCC, 10 pts), colorectal adenocarcinoma (10 pts), urothelial carcinoma (8 pts), gastro-esophageal adenocarcinoma (7 pts), and pancreatic adenocarcinoma (7 pts). Efforts were made to enroll patients with resistant disease to VEGF (RCC) or EGFR (colon) inhibitors, or for men with bone metastatic CRPC (prostate) in order 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 gastro-esophageal cancer patients who were enrolled, 6 had received prior 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 (**Figure 1A**) were found in 4 patients

(8%), and at least one EpCAM CTC was identified in 23 patients (44%) (**Figure 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-sectional prevalence of 6% (95% 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 in order 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 LDH, and low albumin. Patient A was found to have 1 and 3 c-MET CTCs in duplicate samples (**Figure 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 4 and 0 c-MET CTCs in duplicate samples (**Figure 3B**). Of note, patient B's cells were smaller in size and more elongated when compared to the other c-MET CTCs that were isolated. Patient B did not have any EpCAM CTCs in duplicate samples. As this sample did not replicate, we did not include him in the overall prevalence estimate as we could not confirm this finding.

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-aflibercept. 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 (**Figure 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 (**Figure 3D**). He also had 20 and 69 EpCAM CTCs in duplicate samples. Once patient D was found to have a significant number of c-MET CTCs and *MET* amplification, he was treated with off-label crizotinib, a known c-MET tyrosine kinase inhibitor. He had rapid improvement of multiple areas of lymphadenopathy, with a 4-week clinical response, before experiencing disease progression and dying from metastatic disease.

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

We next evaluated the c-MET captured CTCs by FISH in order 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 D underwent DNA FISH analysis for chromosome 7 and the *MET* gene. Patient A with clear cell renal cell carcinoma had trisomy 7 and likely diffuse amplification of the *MET* gene (**Figure 4A**). Patient D with metastatic gastroesophageal adenocarcinoma had polysomy 7 and abundant *MET* gene amplification (MET/CEP7 ratio >10 in all tested CTCs) (**Figure 4B**). Patient C with metastatic colorectal cancer also had polysomy 7 and *MET* gene amplification (MET/CEP7 ratio >10 in all tested CTCs) (**Figure 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 **Figure 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-cytokeratin, and which had a nuclear 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 from 0 to 488) (**Supplemental Figure 2B**). 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 bi-lobed nuclei (**Supplemental Figure 2C**), 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 vs. 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 renal cell carcinomas. Importantly, cancer cells that over-express 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 castration resistant prostate cancer to 14% in patients with metastatic gastric cancer, indicating the importance of tumor lineage and context for this assay.

c-MET positive and *MET* amplified CTCs can be 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 renal cell carcinoma, gastroesophageal, and colorectal adenocarcinomas. Patients were selected for high tumor burdens and treatment refractoriness to VEGF or EGFR/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, non-amplified CTCs. This may be either due to cleavage of the c-MET extracellular domain (i.e. shedding)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 three out of four 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 non-small cell lung cancer or in patients harboring known tumor-specific genomic amplifications in the *MET* locus, such as papillary RCC. This non-invasive 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 co-expressing both cytokeratin 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 cells⁵⁷, which may represent the population of leukocytes that we have found. Recent work by Finisguerra et al. has also demonstrated the importance of c-MET on neutrophils for chemoattraction and cytotoxic killing of cancer cells⁵⁸. 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 non-invasive 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 *MET*

amplification, the presence of c-MET CTCs may be potentially useful as a predictive biomarker for 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.

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Tables and Figure Legends.

Table 1. Baseline characteristics for all patients who underwent peripheral blood sampling. All continuous variables (hemoglobin, albumin, lactate dehydrogenase, and tumor markers) are summarized as median (range).

<i>Disease site (Total N)</i>	<i>Ethnicity</i>	<i>Age mean (range)</i>	<i>Sex</i>	<i>Prior therapies (N)</i>	<i>Sites of metastases (N)</i>	<i>Hemoglobin (g/dL) Median (range)</i>	<i>Albumin (g/dL) Median (range)</i>	<i>LDH (IU/L) Median (range)</i>	<i>Tumor marker* Median (range)</i>
Prostate (10)	7 W, 3 AA	72 (53-83)	10M	GnRH agonist (9), bicalutamide (9), surgical castration (1), abiraterone or enzalutamide (9), sipuleucel-T (5), docetaxel (9), cabazitaxel (4)	LNs (5), Liver (5), Lung (2), Bone (10)	11.4 (8.3, 13.8)	3.8 (2.7, 4.7)	215 (158, 2812)	172 (9, 466)
Renal cell (10)	10 W	61 (35-69)	8M	VEGF-targeted therapy (9), IL-2 (1), Gemcitabine/cispl atin (1)	LNs (6), Liver (4), Lung (8), CNS (2), Bone (6)	11.8 (8.8, 14)	3.4 (2.9, 4.3)	173 (103, 1131)	
Bladder (8)	7 W, 1 AA	66 (56-77)	6M	Gemcitabine/cispl atin (6), carboplatin (2), radiation (3)	LNs (7), Liver (1), Lung (5), Bone (5)	11.5 (9.7, 12.3)	3.6 (3.3, 4.4)	129 (102, 738)	
Gastric (7)	5 W, 2 AA	70 (50-82)	4M	5-FU/oxaliplatin (6), Trastuzumab (2), carboplatin- paclitaxel (1)	LNs (4), Liver (4), Lung (2), Bone (1), Ovaries (1)	10 (8.7, 13.6)	3.1 (1.3, 4.1)	n/a	
Colon (10)	10 W	55 (46-68)	5M	EGFR-therapy (6), 5-FU (10), oxaliplatin (10), bevacizumab (9), irinotecan (9), regorafenib (5), afibercept (3), mitomycin C (2), radiation (2)	LNs (9), Liver (7), Lung (9), CNS (1), Bone (1), Peritoneum (4)	12.1 (8.9, 13.4)	3.5 (2.8, 4.4)	221 (169, 668)	23 (1.5, 1433)
Pancreas (7)	5 W, 2 AA	66 (54-73)	3M	5-FU (6), oxaliplatin (4), gemcitabine (5), nab-paclitaxel (2), irinotecan (1), ruxolitinib (1)	LNs (3), Liver (3), Lung (6), Pleura (2), Peritoneum (1)	12.7 (8.1, 15.7)	3.2 (2.6, 4.3)	n/a	63 (6, 608663)

* Tumor markers: PSA in prostate cancer, CEA in colon cancer, and CA 19-9 in pancreatic cancer.

Units are ng/mL for PSA and CEA; units/mL for CA 19-9.

Abbreviations: VEGF: vascular endothelial growth factor; IL-2: interleukin-2; EGFR: epidermal growth factor receptor; 5-FU: 5-fluorouracil; LDH: lactate dehydrogenase; W: White; AA: African American; M: male; CEA: carcinoembryonic antigen; CA 19-9: cancer antigen 19-9

Table 2. Cell types captured per disease site. N: number; CTCs: circulating tumor cells; EpCAM: epithelial cell adhesion molecule; CK: cytokeratin.

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

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

Figure legends

Figure 1. Depiction of c-MET circulating tumor cells (1A), 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 (1B). Spiking studies with percentage of cells captured for each cell line in c-MET and EpCAM assays (1C). *MET* amplified cell line: SNU5; c-MET high-expressing, EpCAM negative cell lines: BT549, HeLa; c-MET high-expressing, EpCAM positive cell lines: PC3, AT549; c-MET low-expressing cell line: SiHA; c-MET negative: LnCAP.

Figure 2. Enumeration of circulating tumor cells captured either by c-MET (2A) or EpCAM (2B), separated by disease site.

Figure 3. c-MET CTCs isolated from patient A with renal cell carcinoma, with 3 and 1 c-MET CTCs in duplicate samples (3A) and patient B with urothelial carcinoma, with 0 and 4 c-MET CTCs in duplicate samples (3B). Representative c-MET CTCs isolated from patient C with colorectal cancer, 7 and 2 c-MET CTCs in duplicate samples (3C) and from patient D with gastroesophageal adenocarcinoma, with 52 and 90 c-MET CTCs in duplicate samples (3D). First column is the combined fluorescent image of c-MET PE and DAPI, second column is c-MET PE staining, third column is DAPI, fourth column is CD45 linked to APC, and last column depicts cytokeratins linked to FITC.

Figure 4. DNA FISH for centromere of chromosome 7, *MET* gene, and DAPI on single circulating tumor cells from patient A with renal cell carcinoma (4A), patient D with gastric cancer (4B), patient C with colorectal cancer (4C), as well as white blood cell control (4D). Trisomy 7 found in patient A, and polysomy 7 and *MET* amplification found in patients B and C. WBC control with diploid chromosome 7 and 2 copies of *MET* gene.

Figure 1

1C

Cell line	c-MET/EpCAM status	% cells captured c-MET assay mean (std dev)	% cells captured EpCAM assay mean (std dev)
SNU5	<i>MET</i> amplified	65% (15)	70% (21)
A549	c-MET high-expressing, EpCAM positive	32% (17)	51% (N/A)
SiHA	c-MET low expressing, EpCAM positive	18% (8)	23% (N/A)
HeLa	c-MET high-expressing, EpCAM negative	66% (15)	0% (0)
BT549	c-MET high-expressing, EpCAM negative	35% (N/A)	0% (0)
PC3	c-MET high-expressing, EpCAM positive	40% (2)	88% (N/A)
LnCAP	c-MET negative	0% (0)	N/A

Figure 2

Figure 3

Figure 4

Molecular Cancer Research

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