

Quantitative immunofluorescence assessment of MET and epithelial-to-mesenchymal transition (EMT) biomarker modulation by antiangiogenic inhibitors in xenograft tumor tissues

biomarker modulation by antiangiogenic inhibitors in xenograft tumor tissues

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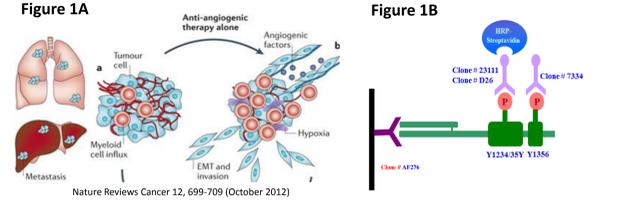


Introduction

Inhibition of VEGF signaling by pazopanib reduces tumor growth in some preclinical models. However, angiogenic inhibition is often accompanied by enhanced hypoxic conditions in tumors that lead to increased c-Met expression and activity as well as to an epithelial-to-mesenchymal transition (EMT)-like phenotype, which leads ultimately to tumor invasion and metastasis (Fig. 1A, Ref 1). Co-inhibition of c-Met can block these effects, providing a potential mechanism to overcome increased invasion in the face of antiangiogenic therapy.

We have previously reported the development of ELISA assays for intact MET, pY1235-MET, and pY1356-MET in tumor lysates (Fig. 1B) (Ref 2) and measured drug-related biomarker changes induced by the anti-VEGF and anti-MET combination therapy in gastric xenograft models (Ref 3).

Here, we report the development of a multiplex quantitative immunofluorescence assay (qIFA) for simultaneous detection of pY1235-MET and total MET in formalin-fixed paraffin-embedded (FFPE) tissues to determine whether pY1235-MET or total MET is induced by anti-VEGF inhibitors due to increased EMT transition and whether this effect could be reversed by the drug combination with a MET inhibitor, tivantinib (ARQ197) in FFPE tissue samples.



Methods

Reagents. Mouse monoclonal antibody against total MET (clone Met4) was from Van Andel Research Institute Technologies, Inc. Two rabbit monoclonal antibodies specific to pY1235-MET (clone 23111 with undetectable reactivity to pY1234-MET) and pY1356-MET were developed at Epitomics Inc., by using peptide antigens corresponding to the MET sequence surrounding these amino acids. E-Cadherin-AF488 mouse monoclonal antibody (clone 32) was from BD Biosciences, and Vimentin-AF647 (clone V9) was from Santa Cruz Biotechnology.

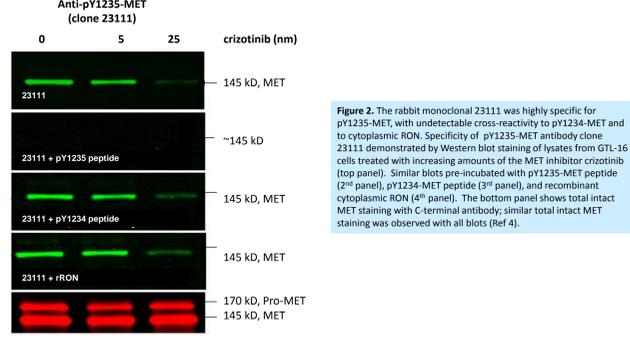
Animal models and drug administration. Athymic nude mice (NCr; Animal Production Program, FNLCR) were implanted with the human cancer cell lines A549 (lung carcinoma), MDA-MB-231 (breast carcinoma), HT-29 (colon carcinoma), HT-29 (colon carcinoma; MET amplified), MNK 45 (gastric carcinoma; MET amplified), and SNU5 (gastric carcinoma; MET amplified) by subcutaneous injection.

MET inhibitors. PF 02341066 (NSC D479769-y, crizotinib), tivantinib (NSC 758242), and VEGFR inhibitor pazopanib (NSC 737754) were synthesized at the Developmental Therapeutics Program (DTP), NCI. PF02341066 and pazopanib were administered by oral gavage in a saline vehicle, and PHA 665752 by intraperitoneal (IP) injections in a vehicle composed of 10% DMSO in saline. Tivantinib was administered orally in a PEG 400:20% vitamin E TPMS solution (60:40) vehicle.

Xenograft biopsy and tumor quarter collection. Whole xenograft tumors were collected on the same schedule as tumor biopsies by standard dissection methods. Specimens were cut into two to four equal pieces with fine-point scissors and placed into Sarstedt tubes that were pre-cooled in liquid nitrogen and was immediately flash frozen in an o-ring sealed, conical bottom screw-capped 1.5-ml Sarstedt cryovial by touching the biopsy to the inside of the tube within one minute of excision. Tubes were pre-cooled on liquid nitrogen.

MET immunofluorescence analysis. HT29, GTL16, and A549 cancer cell lines (FNLCR Repository) were treated with DMSO (0.1% w/v), 100 nM crizotinib, or 100 nM sorafenib for 24 h at 37°C. Frozen tissues or cells were fixed in 10% neutral buffered formalin for 24 h (Sigma Aldrich), and then embedded in paraffin. Paraffin-embedded tissue was cut into 5 µm sections and placed on slides, and staining was performed in a Bond Max Autostainer (Leica Microsystems). Antigen retrieval was performed using Bond Epitope Retrieval Solution 2 (Leica) at 100°C for 10 min. For antigen detection, 10 µg/ml rabbit monoclonal anti-pY1235-MET (clone 23111) was used as the primary antibody, followed by 10 µg/ml goat anti-rabbit AF488 (Life Technologies) as the secondary antibody. Multiple immunofluorescence was performed on formalin-fixed paraffin-embedded tumor tissues: 10 µg/ml rabbit monoclonal anti-pY1235-MET (clone 23111) and 5 µg/ml mouse monoclonal anti-intact MET (clone MET4, Van Andel Research Institute) were used as primary antibodies, followed by 10 µg/ml goat anti-rabbit AF488 and 10 µg/ml anti-mouse AF660 (Life Technologies) secondary antibodies. Image acquisition and analysis were performed on a wide-field fluorescent and confocal microscope (Nikon 90i Andor Camera, NIS Elements Software).

Demonstration of pY1235-MET antibody specificity by Western Analysis



References

1. Semnino and McDonal (Oct 2012) Controlling escape from angiogenesis inhibitors. Nature Reviews Cancer 12, 699-709
 2. Srivastava et al. (ASCO 2011) Development and validation of biomarker assays to assess pharmacodynamic modulation of MET
 3. Srivastava et al. (AACR 2012) Preclinical assessment of MET modulation by a VEGFR/MET inhibitor combination that shows additive antitumor efficacy
 4. Srivastava et al. (2014) Development and Validation of MET Assays to Assess Pharmacodynamic Response in Tumor Tissues (submitted)

Results

Demonstration of pY1235-MET IFA staining specificity using specific MET and non-specific TK inhibitors on FFPE tissues by confocal microscopy:

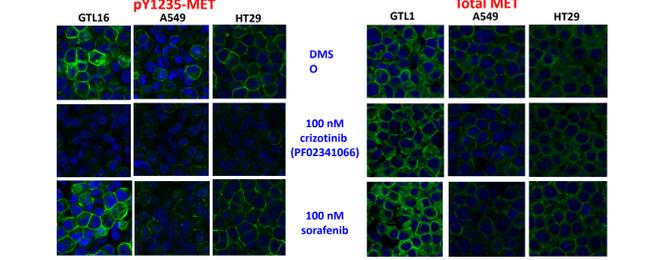


Figure 3. Specificity of the pY1235-MET monoclonal antibody is demonstrated in formalin-fixed, paraffin-embedded GTL16 (MET-amplified), A549, and HT-29 (paracrine or autocrine) cancer cells treated *in vitro* with 100 nM crizotinib (specific MET-kinase inhibitor) or 100 nM sorafenib (nonspecific TK inhibitor) for 4 h. Total MET staining of the same tissue sections using Met4 Mab shows differences in expression levels among the various cell lines, but no changes in expression were observed with drug treatment. Staining was performed in a Bond Max Autostainer (Leica Microsystems), and slides were imaged on a confocal microscope (Nikon 90i Andor Camera, NIS Elements Software).

HGF specifically induces pY1235-MET IFA staining, which was completely inhibited by crizotinib in MET-expressing cell lines *in vitro*

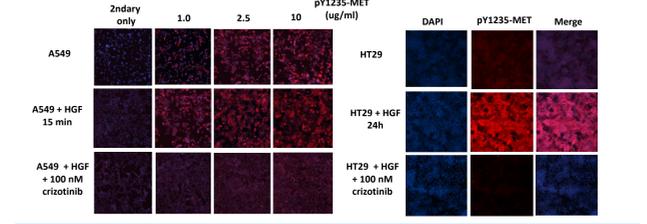
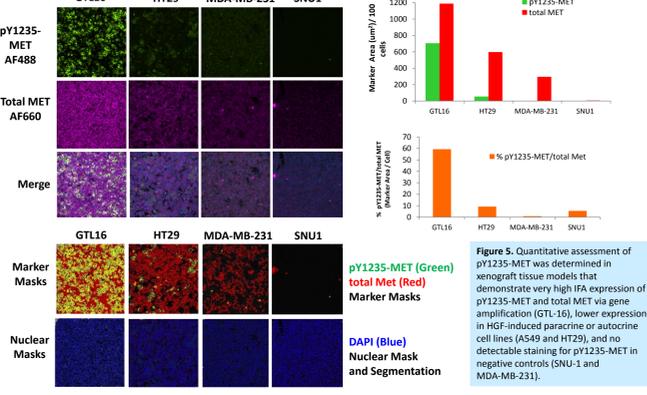


Figure 4. Immunofluorescence staining of pY1235-MET on A549 and HT29 cell lines was specifically induced by HGF stimulation but completely blocked by treatment with crizotinib *in vitro*. A549 or HT29 cells were grown on chamber slides to 80% confluency before being serum starved for 24 h. Cells were incubated with or without 100 nM crizotinib for 4 h prior to stimulation with 20 ng/ml HGF for 15 min (A549) or for 24 h (HT29) in serum-free media. Cells were fixed in 10% NBF for 15 min, permeabilized in 0.3% Triton X-100, and stained with increasing concentrations of pY1235-MET antibody (A549), followed by 10 µg/ml anti-rabbit AF488 secondary. Slides were imaged with a Nikon 90i Andor Camera, and analyzed using NIS Elements Software.

Multiplex IFA staining and Definiens quantitation analysis of pY1235-MET and total MET on FFPE tissues



pY1235-MET and total MET IFA staining of FFPE renal cell carcinoma tissue

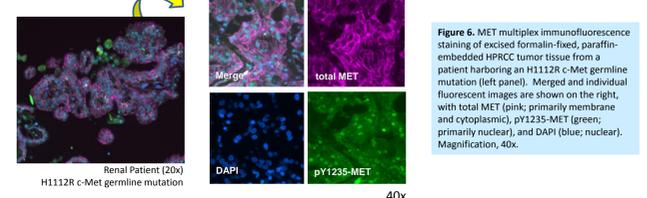
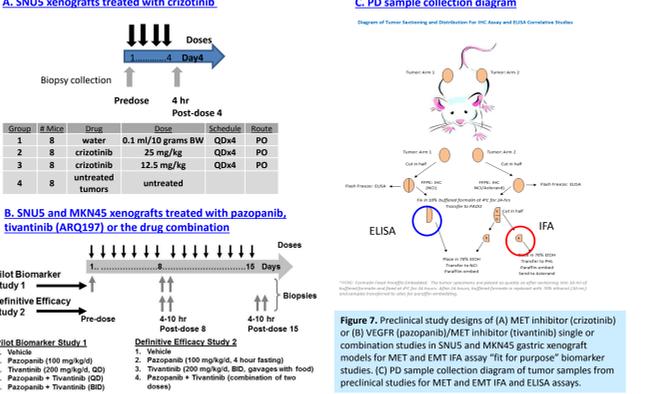
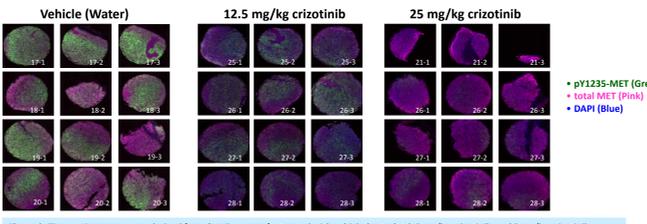


Figure 6. MET multiplex immunofluorescence staining of excised formalin-fixed, paraffin-embedded HPRCC tumor tissue from a patient harboring an H1129R c-Met germline mutation (left panel). Merged and individual fluorescent images are shown on the right, with total MET (pink; primarily membrane and cytoplasmic), pY1235-MET (green; primarily nuclear), and DAPI (blue; nuclear). Magnification, 40x.

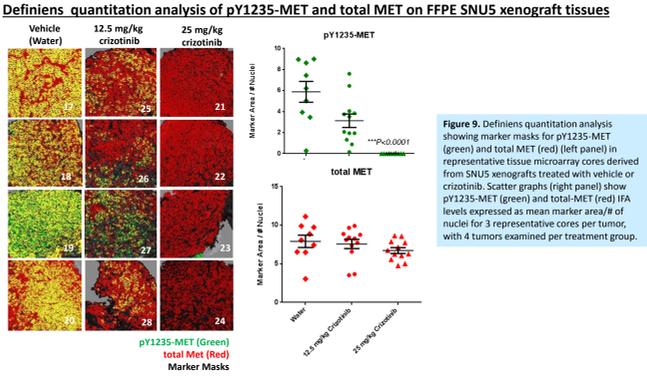
Preclinical study designs of MET inhibitor and VEGFR/MET inhibitor combination studies



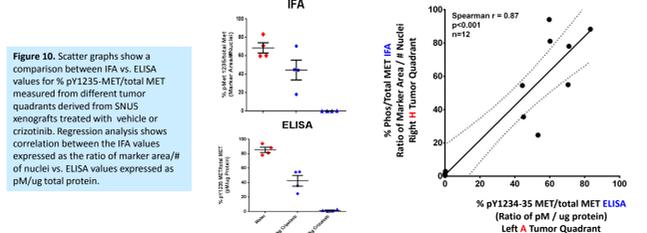
Tissue microarray multiplex staining of pY1235-MET and total MET on FFPE tissues from SNU5 xenografts treated with crizotinib *in vivo*



Definiens quantitation analysis of pY1235-MET and total MET on FFPE SNU5 xenograft tissues

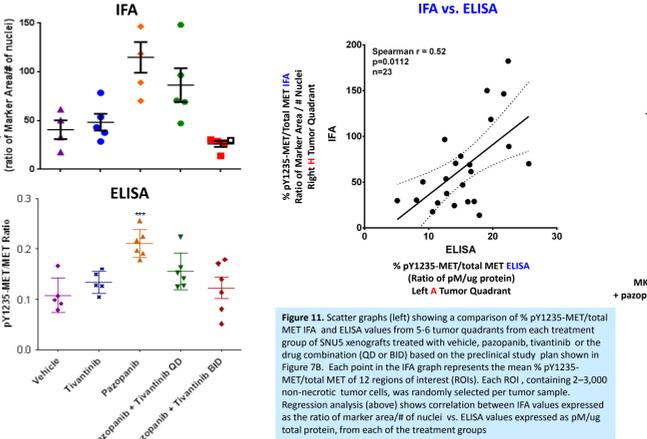


Regression analysis showing concordance between % pY1235-MET/total MET IFA and ELISA data for SNU5 xenograft tissues treated with crizotinib *in vivo*



Results

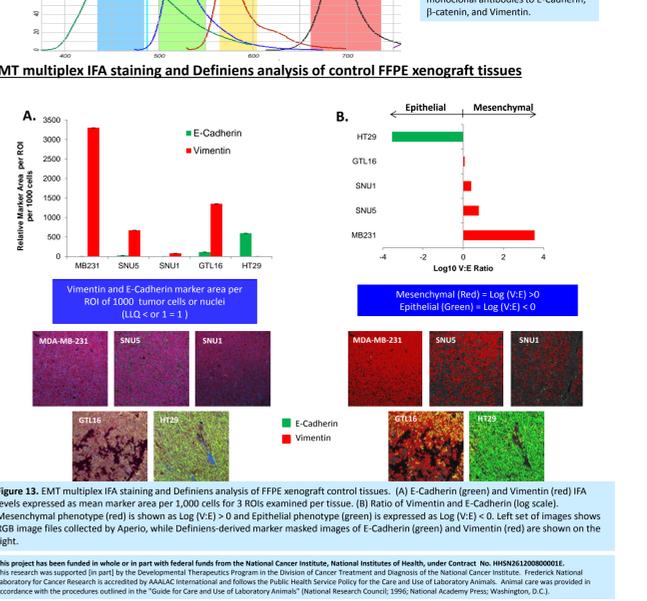
MET multiplex IFA vs ELISA analysis of %pY1235-MET/total MET in FFPE tissues from SNU5 xenografts treated with VEGF inhibitor (pazopanib), MET inhibitor (tivantinib; ARQ197) or the drug combination



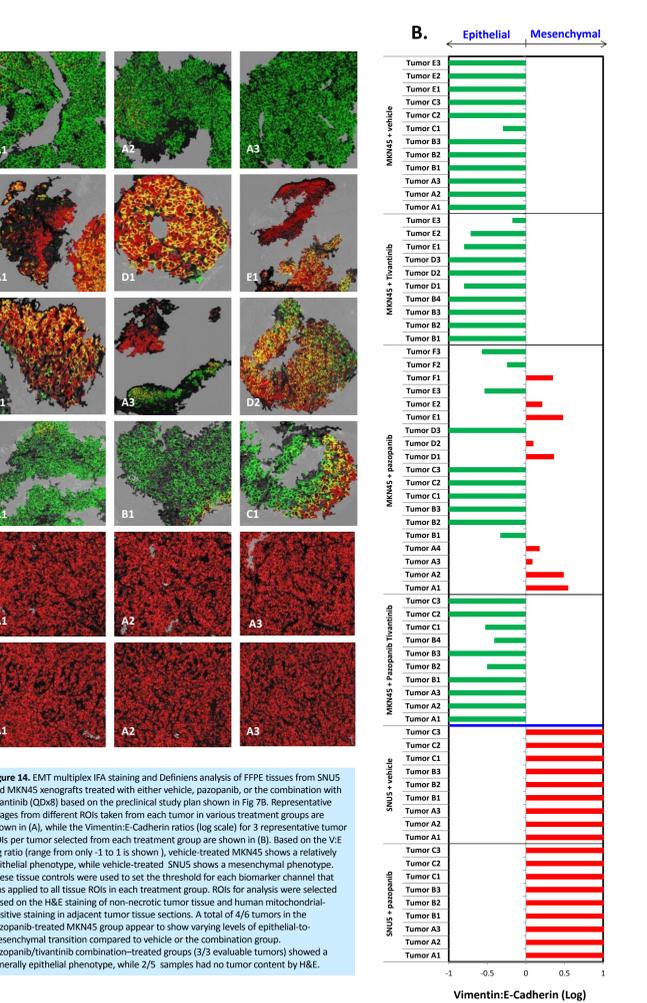
EMT Multiplex IFA Assay Panel

Direct Conjugate Mab	Source/Vendor	Mab Clone	Direct Conjugate Total mg	Total Vol ml	Direct Conjugate Mab Conc (mg/ml)	Mixes Alexa-Fluor per mole protein	Catalog #	Lot #
E-Cadherin-AF488	BD Bioscience	36 Mouse Mab	0.2 mg	4.0 ml	0.05 mg/ml	4.0	560061	39472
Beta-catenin-AF546	Epitomics Custom Conjugation	Rabbit Mab	3.69 mg	3.0 ml	1.23 mg/ml	7.0	C47215	96780A Lot 42
Vimentin-AF647	Santa Cruz Biotechnology	V9 Mouse Mab	5.0 mg	5 ml	1 mg/ml	3.0	sc-6260 AF647	K0813

EMT Multiplex IFA staining and Definiens analysis of control FFPE xenograft tissues



Definiens analysis of EMT IFA multiplex-stained SNU5 and MNK45 FFPE tissues treated with VEGF inhibitor (pazopanib), MET inhibitor (tivantinib) or the drug combination *in vivo*



Summary and Conclusions

MET IFA

- We have developed a specific and sensitive multiplex pY1235-MET and total c-Met IFA on FFPE tissues.
- We have demonstrated assay "fit for purpose" application of the multiplex MET IFA using SNU5 xenograft tissues treated with crizotinib as well as with pazopanib, tivantinib, or the drug combination.
- We have shown by IFA that tivantinib had no activity on its own in inhibiting pY1235-MET in SNU5 xenografts, that pazopanib induced pY1235-MET compared to vehicle, and that the drug combination inhibited pY1235-MET compared to pazopanib.
- We have used Definiens analysis to numerate % pY1235-MET/total MET levels in tissue sections and have shown good concordance with ELISA data taken from correlated tumor samples from the same sets of experiments.

EMT IFA

- EMT IFA analysis of gastric tumors showed that vehicle-treated MNK45 has a relatively epithelial phenotype, while vehicle-treated SNU5 showed a mesenchymal phenotype.
- Pazopanib-treated MNK45 (4/6 tumors) appears to show varying levels of epithelial-to-mesenchymal transition compared to vehicle.
- Pazopanib/tivantinib combination-treated groups (3/3 evaluable tumors) showed a generally more epithelial phenotype, while 2/5 samples had no tumor content by H&E.