Colocalization of HGFR/c-MET and Phosphorylated Forms of This Tyrosine-Kinase Receptor in Glioblastoma

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Abstract

Hepatocyte Growth Factor Receptor/Tyrosine-Protein Kinase Met (HGFR/c-MET) is a glycosylated receptor tyrosine kinase that plays an important role in the development of cancer in different organs including liver, kidney and brain. Mature HGFR/c-MET is a disulfide-linked dimer composed of a 50 kDa extracellular α-chain and a 145 kDa transmembrane β-chain. Interaction of HGF with HGFR/c-MET triggers its internalization and subsequent proteasome-dependent degradation. It also stimulates phosphorylation of tyrosine residues in the cytoplasmic region, which leads to activation of the kinase domain and exposure of docking sites for multiple SH_2-containing molecules. HGFR/c-MET has been implicated in the progression of glioblastoma, which is one of the most aggressive brain tumors. High expression levels of HGFR/c-MET has been shown to be indicative of shorter survival periods. To investigate the spatial distribution of HGFR/c-MET and its phosphorylated forms, we generated antibodies against its extracellular domain, as well as against specific phosphorylation sites in its intracellular kinase domain (Y1234/Y1235) and intracellular multisubstrate docking site (Y1349). Multiplex immunocytochemistry and immunohistochemistry were used to analyze colocalization of these domains in untreated and HGF-treated glioblastoma cell lines and in tissue sections of human glioblastoma. Treatment of U-251 MG, U-87 MG, U-118-MG and A-172 cells with Recombinant Human HGF induced profound phosphorylation of HGFR/c-MET. Phospho-HGFR/c-MET was detected in both the cytoplasm and cell nuclei. We also saw that HGFR/c-MET was colocalized with either phosphorylated form. In addition, the immunoreactive profiles for phospho-HGFR/c-MET (Y1349) and phospho-HGFR/c-MET (Y1234/Y1235) overlapped in certain instances, but not all, indicating the action of HGF on the same cells has different effects on tyrosine phosphorylation in the different domains. In glioblastoma tissue sections, phospho-HGFR/c-MET (Y1234/Y1235) was also detected in cell nuclei. Our data indicate that there is distinct activation of HGFR/c-MET at a single-cell level by phosphorylation that may underly the regulation of tumor progression.

Introduction

Hepatocyte Growth Factor Receptor/Tyrosine-Protein Kinase Met (HGFR/c-MET) is a disulfide-linked dimer consisting of an extracellular β-chain (50 kDa) and a transmembrane α-chain (145 kDa). It is activated following HGF binding to an extracellular ligand-binding domain, which subsequently induces receptor dimerization and transphosphorylation of tyrosine residues in a cytosolic kinase domain (Y1234/Y1235) and a substrate docking domain (Y1349/Y1356). Conventionally, the HGFR/c-MET signaling cascade induces cellular proliferation and differentiation.[1] However, dysregulated HGFR/c-MET signaling, which results from various mechanisms including increased ligand binding and receptor overexpression, is implicated in several cancer types and correlates with malignancy and poor prognosis.[2] In glioblastoma, the most aggressive brain cancer, dysregulated HGFR/c-MET signaling primarily occurs in tumors with a mesenchymal phenotype where it sustains stemness and promotes invasiveness.[3-5]

The role of specific phosphorylated forms of HGFR/c-MET in tumor progression is unknown. Kinase-independent functions of other receptor tyrosine kinases (e.g., EGFR) raise questions about the function of non-phosphorylated HGFR/c-MET in tumorigenesis. Additionally, the influence of the subcellular location of HGFR/c-MET (i.e., cytosol or nucleus) on its function and tumor progression has not been well defined.[6-8] In this study, we investigated the subcellular distribution of HGFR/c-MET and its phosphorylated forms (Y1234/Y1235 and Y1349) in glioblastoma cell lines and human glioblastoma tissue.

We developed antibodies specific to the extracellular domain of HGFR/c-MET, and to the phosphorylation sites in its intracellular kinase domain (Y1234/Y1235) or the multisubstrate docking site (Y1349). Multiplex immunocytochemistry (ICC) or immunohistochemistry (IHC) of HGF-treated glioblastoma cell lines and glioblastoma tissue sections, respectively, with these antibodies indicated partial colocalization of the phosphorylated forms within subcellular compartments (i.e., cytosol and nucleus). Additionally, unphosphorylated HGFR/c-MET only partially colocalized with its phosphorylated forms. Thus, our data indicate that at a single-cell level, HGFR/c-MET activation leads to diverse phosphorylation profiles and triggers differential subcellular distribution of phosphorylated and non-phosphorylated forms of HGFR/c-MET.

Summary

- HGFR/c-MET (total protein), phospho-HGFR/c-MET (Y1349) and phospho-HGFR/c-MET (Y1234/Y1235) are localized to the cell membrane and the perinuclear region.

- Upon stimulation with the HGF protein, the localization and colocalization patterns of non-phosphorylated and phosphorylated proteins change.

- The extent of such changes varies based on the type of glioblastoma cell line.

- Phospho-HGFR/c-MET (Y1234/Y1235) is localized in the perinuclear region in human glioblastoma tissue.

- HGFR/c-MET can be considered a biomarker for human glioblastoma.

Materials

Cells

- A-172 human glioblastoma cell line (ATCC CRL-1573™)

- U-87 MG human glioblastoma/astrocytoma cell line (ATCC HTB-14™)

- U-118-MG human glioblastoma/astrocytoma cell line (ATCC HTB-15™)

- U-251 MG human glioblastoma/astrocytoma cell line (ECACC 09063001)

Tissue

- Human glioblastoma (sample provided by the NCI Cooperative Human Tissue Network (CHTN))

Primary Antibodies

- Mouse Anti-Human HGFR/c-MET Monoclonal Antibody (R&D Systems, Catalog # MAB3581)

- Rabbit Anti-Human/Mouse Phospho-HGFR/c-MET (Y1234/Y1235) Antigen Affinity-Purified Polyclonal Antibody (R&D Systems, Catalog # AF2480)

- Rabbit Anti-Human/Mouse Phospho-HGFR/c-MET (Y1349) Antigen Affinity-Purified Polyclonal Antibody (R&D Systems, Catalog # AF3950)

ICC Secondary Antibodies

- NorthernLights (NL)™ 557-Conjugated Donkey Anti-Mouse IgG Secondary Antibody (R&D Systems, Catalog # NL007)

- Alexa Fluor® 488-conjugated goat anti-rabbit IgG (H+L) Superclonal™ secondary antibody (Invitrogen)

IHC Secondary Antibody

- Goat Anti-Rabbit IgG VisUCyte™ HRP Polymer Antibody (R&D Systems, Catalog # VC003)

IHC Substrate/Chromogen

- Rabbit VisUCyte HRP Polymer-DAB Cell & Tissue Staining Kit (R&D Systems, Catalog # VCTS003)

- DAB Substrate Buffer (BioLegend)

Antibody Diluent

- PBS with 0.3% Triton™ X-100 (MilliporeSigma), 1% NDS, 1% BSA, 0.01% NaN3

Methods

Cell Culture

Cells were cultured with growth media in 75 cm^2 flasks at 37 °C and 5% CO_2. They were disassociated from the flasks using a Trypsin EDTA solution, centrifuged, resuspended with growth media and then plated onto 8-well Culture Slides (Falcon). All cell types were stimulated with Recombinant Human HGF (25 ng/mL; R&D Systems, Catalog # 294-HG) for 30 minutes at 37 °C and 5% CO_2. Stimulated and unstimulated cells were fixed by adding a 10% formalin solution at a ratio of 1:1 with growth media for 20 minutes at room temperature and then rinsed 3 times with PBS.
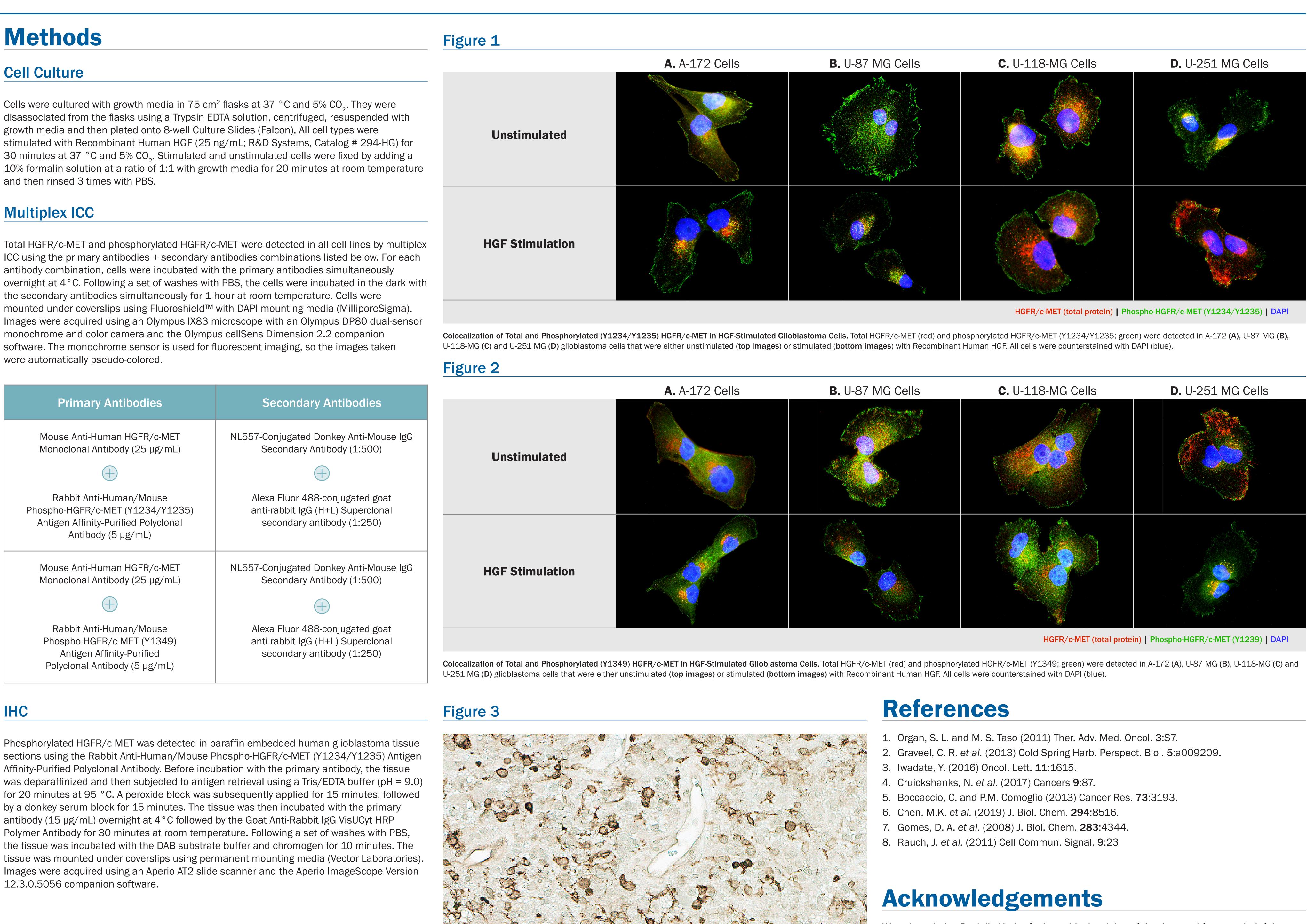
Multiplex ICC

Total HGFR/c-MET and phosphorylated HGFR/c-MET were detected in all cell lines by multiplex ICC using the primary antibodies + secondary antibodies combinations listed below. For each antibody combination, cells were incubated with the primary antibodies simultaneously overnight at 4 °C. Following a set of washes with PBS, the cells were incubated in the dark with the secondary antibodies simultaneously for 1 hour at room temperature. Cells were mounted under coverslips using Fluoroshield™ with DAPI mounting media (MilliporeSigma). Images were acquired using an Olympus IX83 microscope with an Olympus DP80 dual-sensor monochrome and color camera and the Olympus cellSens Dimension 2.2 companion software. The monochrome sensor is used for fluorescent imaging, so the images taken were automatically pseudo-colored.

Primary Antibodies	Secondary Antibodies
Mouse Anti-Human HGFR/c-MET Monoclonal Antibody (25 µg/mL) ⊕ Rabbit Anti-Human/Mouse Phospho-HGFR/c-MET (Y1234/Y1235) Antigen Affinity-Purified Polyclonal Antibody (5 µg/mL)	NL557-Conjugated Donkey Anti-Mouse IgG Secondary Antibody (1:500) ⊕ Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) Superclonal secondary antibody (1:250)
Mouse Anti-Human HGFR/c-MET Monoclonal Antibody (25 µg/mL) ⊕ Rabbit Anti-Human/Mouse Phospho-HGFR/c-MET (Y1349) Antigen Affinity-Purified Polyclonal Antibody (5 µg/mL)	NL557-Conjugated Donkey Anti-Mouse IgG Secondary Antibody (1:500) ⊕ Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) Superclonal secondary antibody (1:250)

IHC

Phosphorylated HGFR/c-MET was detected in paraffin-embedded human glioblastoma tissue sections using the Rabbit Anti-Human/Mouse Phospho-HGFR/c-MET (Y1234/Y1235) Antigen Affinity-Purified Polyclonal Antibody. Before incubation with the primary antibody, the tissue was deparaffinized and then subjected to antigen retrieval using a Tris/EDTA buffer (pH = 9.0) for 20 minutes at 95 °C. A peroxide block was subsequently applied for 15 minutes, followed by a donkey serum block for 15 minutes. The tissue was then incubated with the primary antibody (15 µg/mL) overnight at 4 °C followed by the Goat Anti-Rabbit IgG VisUCyt HRP Polymer Antibody for 30 minutes at room temperature. Following a set of washes with PBS, the tissue was incubated with the DAB substrate buffer and chromogen for 10 minutes. The tissue was mounted under coverslips using permanent mounting media (Vector Laboratories). Images were acquired using an Aperio AT2 slide scanner and the Aperio ImageScope Version 12.3.0.5056 companion software.

Figure 1

Colocalization of Total and Phosphorylated (Y1234/Y1235) HGFR/c-MET in HGF-Stimulated Glioblastoma Cells. Total HGFR/c-MET (red) and phosphorylated HGFR/c-MET (Y1234/Y1235; green) were detected in A-172 (A), U-87 MG (B), U-118-MG (C) and U-251 MG (D) glioblastoma cells that were either unstimulated (top images) or stimulated (bottom images) with Recombinant Human HGF. All cells were counterstained with DAPI (blue).

Figure 2

Colocalization of Total and Phosphorylated (Y1349) HGFR/c-MET in HGF-Stimulated Glioblastoma Cells. Total HGFR/c-MET (red) and phosphorylated HGFR/c-MET (Y1349; green) were detected in A-172 (A), U-87 MG (B), U-118-MG (C) and U-251 MG (D) glioblastoma cells that were either unstimulated (top images) or stimulated (bottom images) with Recombinant Human HGF. All cells were counterstained with DAPI (blue).

Figure 3

Phosphorylated HGFR/c-MET (Y1234/Y1235) in Human Glioblastoma Tissue. Phospho-HGFR/c-MET (Y1234/Y1235; brown) was detected in paraffin-embedded sections of human glioblastoma tissue.

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