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High-MET status in non-small cell lung tumors correlates with receptor phosphorylation but not with the serum level of soluble form

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ABSTRACT

Objectives: The receptor tyrosine kinase MET is essential to embryonic development and organ regeneration. Its deregulation is associated with tumorigenesis. While *MET* gene amplification and mutations leading to MET self-activation concern only a few patients, a high MET level has been found in about half of the non-small cell lung cancers (NSCLCs) tested. How this affects MET activation in tumors is unclear. Also uncertain is the prognostic value, in cancer, of a phenomenon well described in cell models: MET shedding, i.e. its cleavage by membrane proteases leading to release of a soluble fragment into the medium.
Materials and methods: A prospective cohort of 39 NSCLC patients was constituted at diagnosis or soon after. Normal tissues, tumor tissues, and blood samples were obtained. This allowed, for the same patient, synchronous determination of (i) the MET level in the tumor, (ii) receptor phosphorylation, and (iii) the concentration of soluble MET fragment (sMET) in the serum.
Results: After confirming the adequacy of an ELISA for measuring the serum level of sMET, we found no correlation between this level and the concentration of MET in tumors, as evaluated by immunohistochemistry and western blotting. Nevertheless, all but one tumor displaying a high MET level also displayed receptor phosphorylation, restricted to a small number of tumor cells.
Conclusion: Our results thus demonstrate that the serum level of sMET is not indicative of the amount of MET present in the tumor cells and cannot be used as a biomarker for therapeutic purposes. However, MET scoring of tumor biopsies could be a first step prior to determination of MET receptor activation in high-MET tumors.

1. Introduction

MET is a receptor tyrosine kinase (RTK) present predominantly in cells of epithelial origin, activated by its stromal ligand, the hepatocyte growth factor/scatter factor (HGF/SF) [1]. HGF and MET are essential to embryonic development, since knockout of either one affects epithelial organ, placenta, muscle, and neuron formation [2–5]. Conditional knockout of MET in the lung inhibits alveolar development, possibly because of decreased alveolar epithelial cell proliferation and survival [6].

Aberrant MET and HGF signaling is involved in promoting tumorigenesis and metastasis [7]. MET is overproduced in half of all non-small-cell lung cancers (NSCLCs) which is associated with poor prognosis [8,9]. Furthermore, MET mutations have been discovered in a variety of cancers. In renal cancers, most are located in the kinase domain and cause kinase activition [10]. Recently discovered in lung cancers are various mutations, leading to exon skipping and to deletion in the regulatory juxtamembrane domain [11,12]. Furthermore, in about 5–20% of NSCLCs displaying a mutated Epidermal Growth Factor Receptor (EGFR) gene, acquired resistance to EGFR inhibitors involves amplification of the *MET* gene,

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associated with MET self-activation [13]. In recent phase III clinical trials evaluating the anticancer effect of anti-MET antibodies or tyrosine kinase inhibitors (TKIs) in patients stratified according to the amount of MET detected by immunohistochemistry (IHC), these agents have proved ineffective. Yet several studies have revealed encouraging responses in a limited number of patients displaying *MET* gene amplification or mutations [12,14–18].

Upon ligand binding and subsequent dimerization, MET autophosphorylates tyrosine residues located notably in its kinase domains [19,20]. MET phosphorylation has been detected in tumor samples, including NSCLC samples, but whether the MET level correlates with MET phosphorylation remains controversial [21,22].

Downregulation of the MET receptor is an essential desensitization mechanism. In addition to the well-known ligand-dependent degradation of MET, we have demonstrated that MET undergoes several proteolytic cleavages promoting its downregulation [23–25]. For instance, under steady-state condition, MET is processed by Presenilin-Regulated Intramembrane Proteolysis (PS-RIP). This proteolytic process involves a first cleavage by ADAM metalloproteases, leading to generation of an N-terminal fragment (soluble MET; sMET), which is released into the extracellular medium, and a membrane-anchored p55MET fragment, which is degraded by the lysosome [26], or further cleaved by γ-secretase, yielding an intracellular p50MET fragment degraded by the proteasome [27]. These cleavages help reduce the half-life of the receptor. HER2 likewise undergoes proteolytic cleavages by membrane metalloproteases, leading to generation of a soluble HER2. Interestingly, this soluble fragment is detectable in the sera of patients with breast cancer, where its level correlates with the level of full-length HER2. It is thus a potential serum biomarker of HER2 accumulation [28,29]. We thus wondered whether the sMET fragment might provide a relevant biomarker of MET accumulation. To answer this question, we have characterized in the same NSCLC patients the level of MET receptor in paraffin-embedded sections and frozen tumors, its phosphorylation state, and the serum concentration of sMET fragment.

2. Materials and methods

2.1. Cell cultures

The breast cancer cell line MDA-MB-231 and the gastric cancer cell line GTL-16 were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and antibiotics. The lung cancer cell line EBC-1 was cultured in EMEM medium supplemented with 10% FBS and antibiotics. MCF-10A mammary epithelial cells were cultured in DMEM and HAM's F12 vol/vol (Life Technologies) supplemented with 5% horse serum (Life Technologies), 500 ng/ml hydrocortisone (Calbiochem), 20 ng/ml epidermal growth factor (Peprotech), 10 μg/ml insulin (Sigma Aldrich), 100 ng/ml cholera toxin (Calbiochem), and 1% antibiotics. Primary human endothelial cells (HUVECs) were cultured in EGM-2 medium (Lonza). Primary human keratinocytes (NHEKs) were cultured in KGM gold keratinocyte growth medium (00192060, Lonza). The lung cell line p339 (6CFSMEO-) was cultured in MEM, 10% FCS, antibiotics.

2.2. Antibodies

Rabbit polyclonal antibodies directed against phosphorylated (Y1234/1235) MET (#3126) were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibody against the kinase domain of MET (3D4) was purchased from Life Technologies. Mouse monoclonal antibody against GAPDH (6C5-32233) was purchased from Santa Cruz Biotechnology. Rabbit monoclonal antibody directed against the C-terminal tail of MET (SP44) used for immunohistochemistry (IHC) was purchased from Roche (Schlieren, Switzerland). HRP-conjugated monoclonal antibody directed against the histidine tag was purchased from Invitrogen. Mouse monoclonal antibody directed against the N-terminal domain of MET (Met extra) (DL-21) was kindly provided by Dr. Silvia Giordano (University of Torino Medical School, Italy).

2.3. Tumor sample preparation

Tumor samples were divided into two parts; frozen in a Snapfrost fast freezing system (Excilone, Aperio, CA, USA) and stored at −80 °C; formalin-fixed and paraffin-embedded (FFPE). For western blot analysis, frozen samples were sliced into pieces about 1.2 mm in diameter and transferred into Lysing Matrix type D tubes containing ceramic beads (MP Biomedicals, Santa Ana, CA, USA) in the presence of RIPA buffer (50 mM Tris-HCl pH = 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.5% sodium deoxycholate, 0.1% SDS, and 1% NP-40). The samples were lysed with a FastPrep homogenizer (MP Biomedicals) (4 cycles of 40 s at 6 m/s, each followed by a 5 min pause on ice). The samples were then centrifuged and proteins in the supernatant were quantified by the BCA Protein Assay (Pierce, Rockford, IL, USA). Protein were analyzed by western blotting. For IHC, FFPE tissue sections were stained with Hematein/Eosin/Safran and IHC was performed in an automated immunostainer Benchmark XT (Ventana Medical Systems) with an antibody against the intracellular domain of MET (SP44 CONFIRM, Ventana Medical Systems). MET was scored according to the study of Spigel et al. [8] (score 3: high-intensity staining of at least 50% of the tumor; score 2: moderate staining of at least 50% of the tumor and high-intensity staining of less than 50%; score 1: weak staining of at least 50% of the tumor and moderate or strong staining of less than 50%; score 0: no staining or staining at any intensity of less than 50% of the tumor).

2.4. Western blotting

Western blotting was performed as previously described [30].

2.5. RNA interference

A suspension of 400,000 cells was incubated for 20 min with a mix of 4.5 μl/ml Lipofectamine 2000 (Invitrogen) and 3 nM (MCF10A cells) or 120 nM (GTL16 cells) siRNA. The cells were then plated in complete medium in a 6-well plate. The MET-targeting siRNAs were a pool of three stealth siRNAs (Invitrogen) [5′-CCAUUUCAACUGAGUUUGCUGUUAA-3′, 5′-UCCAGAAGAUCAGUUUCCUAAUUCA-3′, 5′CCGAGGGAAUCAUCAUGAAAGAUUU-3′]. A negative control Stealth siRNA was also purchased from Invitrogen.

2.6. Enzyme-linked immunosorbent assay

The concentration of sMET in conditioned medium was measured by an ELISA performed with the c-MET (soluble) ELISA Kit, human (Novex KHO2031). Briefly, patient serum was diluted 1:100 and conditioned serum-free medium from cell lines was diluted 1:100 for EBC-1 and GTL-16 cells or 1:3 for the other cells. One hundred microliters of the dilution was incubated for 2 h in a plate coated with antibodies against human soluble MET. Detection was done with an anti-human soluble MET antibody-biotin/streptavidin-HRP. After incubation for 30 min with chromogenic substrate, the plate was read at 450 nm with a Multiskan RC (Thermo Labsystem) spectrophotometer.