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Role of c-Met in Cancer: Emphasis on Lung Cancer

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Lung cancer remains the leading cause of cancer death. It is often diagnosed at late stages and is treated systemically with cytotoxic chemotherapy, which is generally ineffective. Research efforts have focused on developing therapies targeted to growth factor receptor pathways, such as epidermal growth factor receptor (EGFR), but the results from clinical trials overall show very small improvements in survival. Research on signaling pathways dysregulated in lung cancer is ongoing, including investigation of the hepatocyte growth factor receptor (HGFR) or c-Met. Protein tyrosine kinases, such as EGFR and c-Met, are a family of oncogenes that regulate important cellular processes, such as differentiation, proliferation, cell cycle, motility, and apoptosis. Hepatocyte growth factor (HGF), a ligand for c-Met, is secreted by mesodermal cells during development. It produces multiple effects upon binding to its receptor (HGFR/c-Met) and regulates proliferation, motility, mitogenesis, and morphogenesis. Studies in cell lines isolated from various tumors show that several intracellular pathways participate in c-Met signaling, including growth factor receptor-bound protein 2 (Grb2), mitogen-activated protein (MAP) kinase, phosphoinositol 3-kinase (PI3K), and phospholipase C- γ (PLC- γ). c-Met is overexpressed in many tumors. However, overexpression may not be sufficient to cause increased activity; the receptor needs to be activated. In some cases, the kinases are constitutively active due to mutations in the gene. The cytoskeletal protein paxillin also appears to be activated along with c-Met. Correlative studies from patient tissue samples and cell lines have rendered the same information, indicating that the signaling pathways dysregulated are complex and interdependent. Mutations in human c-Met have been exogenously expressed in *Caenorhabditis elegans*, which can serve as a model for determining the role of gene mutations in a whole organism. Several inhibitors of c-Met/HGF binding are in development, including some in phase I trials. Their effectiveness in improving cancer outcomes will be determined in the near future.

The c-Met receptor is a tyrosine kinase receptor for hepatocyte growth factor (HGF), also known as scatter factor (SF). HGF is a heparin-binding protein that shares structural domains with enzymes of the blood clotting cascade. HGF, secreted by cells of mesodermal origin, has powerful mitogenic, motogenic, and morphogenic activity on epithelial and endothelial cells. Upon binding c-Met, HGF, which is a natural ligand for c-Met, produces multiple biological responses, including proliferation, survival, motility, and morphogenesis.¹⁻⁵ HGF binds c-Met receptor at the N-terminal domain, which is also

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STATEMENT OF CONFLICT OF INTEREST:

known as the semaphorin domain. The C-terminal domain of the receptor encompasses the tyrosine kinase region with a number of tyrosine phosphorylation sites. When the c-Met receptor tyrosine kinase is activated, either through ligand binding or through other means, it leads to a plethora of changes within the cell, making this receptor a desirable therapeutic target.

Many investigators have shown that the c-Met receptor can be overexpressed, potentially mutated, and/or amplified in cancer cells and plays an important role in epithelial mesenchymal transition. Changes in c-Met expression levels, and/or mutation/amplification of the receptor, and/or changes in kinase activity can occur in solid tumors, such as lung cancer, mesothelioma, colon cancer, head and neck cancer, esophageal cancer, gastric cancer, pancreatic cancer, sarcomas, thyroid cancer, ovarian cancer, breast cancer, cervical cancer, brain tumors, and especially hereditary papillary renal cell carcinomas.^{1,4,6,7} Activating mutations are found within the tyrosine kinase domain.⁸ The c-Met receptor tyrosine kinase was found to be overexpressed in non-small cell lung cancer (NSCLC), in which the p140-beta form showed robust overexpression. In small cell lung cancer (SCLC), there is heterogeneity of expression of c-Met, with overexpression in some of the tumor tissues.⁹⁻¹¹

c-Met BINDING ACTIVITY

The c-Met receptor tyrosine kinase, upon binding HGF, homodimerizes and cross-transactivates the tyrosine kinase domain as well as the juxtamembrane domain. Upon ligand binding, autophosphorylation of the c-Met receptor occurs on tyrosine residues. These residues are present in the activation loop of the tyrosine kinase domain. This step results in an activated docking site that recruits intracellular adaptor molecules by using, for example, the Src homology-2 domains and other recognition motifs. These are signal transduction molecules, such as growth factor receptor-bound protein 2 (Grb2), Gab1, phosphoinositol 3-kinase (PI3K) (stimulating the phosphorylation of PIP2 to PIP3), phospholipase C- γ , Shc, Src, Shp2, and SHIP2.^{2,4} Phosphorylation activates downstream signaling tyrosine kinases, as well as serine threonine kinases, PDK, and AKT, and also recruits Ras, Raf, and the Gab1/Grb2/SOS pathway proteins, which can then ultimately activate the MEK/ERK pathway. This chain of events results in many biological changes, such as regulation of transcription and gene expression, as well as growth, differentiation, survival, reduced apoptosis, and regulation of cytoskeletal function (Figure 1).

Studying HGF/c-Met biology and dissecting the pathways activated by ligand binding are relevant to cancer therapeutics. The strategy is to use the knowledge gained from biology to find targeted therapies in oncology. A particularly useful agent is a small molecule inhibitor that can cross the cell membrane and, in this case, abrogate the HGF/c-Met axis and its activation, thereby leading to apoptosis or even cellular senescence. Below are described some of the studies that have led to more information on the dysregulated pathways associated with c-Met in a variety of cancer cells.

c-Met RECEPTOR BINDING AND FUNCTION

At the N-terminus of the c-Met oncogene is the semaphorin (SEMA) domain that can bind to the ligand HGF. There are two important domains at the C-terminus, the tyrosine kinase and the juxtamembrane regions. In c-Met, many of the tyrosine phosphorylation sites can be activated upon ligand HGF binding. In lung cancer cells derived from either NSCLC or SCLC treated with either HGF or control, the tyrosine pY1003 from the juxtamembrane domain was more phosphorylated in the presence of HGF over time compared to control.¹² This phosphorylation event was relevant for binding to the E3 ubiquitin ligase Cbl. Several such phosphorylation events are revealed upon ligand binding. The phosphorylation of the

pY1313, which is the YXXM motif, is important in binding to the p85SH2 domain of the PI3K.¹³ Autoactivation of the tyrosine kinase domain can occur at the catalytic site, which is the pY1230/1234/1235 site. Other biological functions are also important with c-Met receptor activation. These functions are activated by the Grb2 binding to the YXN motif that is available on pY1349. Cell morphogenesis occurs with the PLC- γ binding site at YXXL motif, which is important for binding to pY1365.

DISSECTING c-Met IN TUMOR TISSUES

Studying these processes using cell lines is worthwhile because findings in the cell lines potentially reflect the mechanisms in the tumor tissues. This requires a reverse translation approach. That is, findings from patient samples must be brought back to the laboratory to study the processes that are altered. Tumor tissue samples can be examined, and the results can be linked to clinical information so as to determine the genetics, type and stage of tumor, and success of therapy.¹⁴

Tumor tissue analysis accomplished over the past 5 years in our laboratory has led to the observation that the relative expression of c-Met increases as cells progress from normal to cancer cells. The c-Met receptor is overexpressed as the cells proceed from being normal cells and move into metaplasia, dysplasia, and frank carcinoma.^{9,15} Lung cancer tumors show overexpression of c-Met when compared to normal adjacent lung tissue. Overexpression was observed in colon cancer, renal cancer, and breast cancer, among others.¹⁶⁻²⁵ In addition to measuring c-Met overexpression, it is also relevant to measure the degree of phosphorylation and activity resulting from the phosphorylated sites. For example, in lung cancer, activated phospho c-Met has been observed and identified in the tumor tissues.^{26,27}

In SCLC, the increased activity of c-Met and phosphorylated c-Met is identified by the robust overexpression in tumor tissues as compared to the normal adjacent lung tissue.¹⁰ In normal cells, there are at least two different tyrosine phosphorylation sites responsible for c-Met activity. This activity can be controlled by the juxtamembrane domain, which contains a negative regulator of the tyrosine kinase activity. The juxtamembrane domain can be mutated or deleted in lung cancer, which leads to increased tyrosine kinase activity.¹⁰

Lung cancer incidence has been strongly correlated to smoking; thus a diagnosis of chronic obstructive pulmonary disease can be an important indication for those who develop lung cancers from smoking. It has been shown in pancreatic and lung cancer that c-Met may play a critical role in the function of solid tumor stem cells.²⁸ Kim et al initially showed that stem cells in lung cancers can reside in the bronchial-alveolar junction.²⁹ We have shown that the early progenitor cells at the bronchial-alveolar junction have a high expression of c-Met.³⁰

Our laboratory has been investigating the early events of metastasis through the study of cell motility, migration, and invasion. We have shown that upon stimulation of c-Met with HGF in lung cancer (and other tumors), the cells have enhanced cell motility/migration/invasion. This is effected, in part, by the actin cytoskeleton and the actin binding proteins/focal adhesion proteins.^{11,31}

Figure 2 shows interactions of the extracellular matrix in which the integrin is tightly linked to the actin cytoskeleton and to the focal adhesion proteins paxillin, p125FAK, talin, and tensin. It is a representative example of interactions that can lead to increased adhesion, migration, motility, and transformation, and decreased apoptosis. Paxillin, which we initially cloned, is a 68-kd focal adhesion protein that was initially identified in a v-src-transformed chicken embryo fibroblast.³² It is important in growth factor receptors, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR),

interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and c-Met signaling. It interacts with various oncogenes, such as *v-src*, *v-crk*, *E6*, and *BCR/ABL*. Paxillin plays a role in cervical cancer developing from the human papilloma virus, E6 transformation in head and neck cancers, and the non-p53 mechanism-mediated transformation through E6.³³⁻³⁵ BCR/ABL plays a very important role in the development of chronic myeloid leukemia, but in this event, BCR/ABL activates paxillin downstream.³⁶

Paxillin has five leucine-rich (LD) motifs at the N-terminus, important in binding to adaptor proteins and oncogenes. Towards the C-terminus, paxillin has LIM domains, which are zinc finger domains important for binding to the focal adhesion proteins and to the cytoskeletal structure. In experiments examining phosphorylated c-Met and its downstream biomarkers, paxillin was found to be heavily phosphorylated. Interestingly, there can be domain mutations of the paxillin gene in lung cancer. Also, the amplification of c-Met in lung cancer appears to be concordant with amplification of paxillin in lung cancer tumor tissues.

Based on extensive biochemical analyses and global proteomics analyses, we have shown that HGF activation of c-Met results in a plethora of effects, such as activation of signal transduction molecules, leading to various biological effects controlling cell proliferation and differentiation, cell survival and apoptosis, and cytoskeleton-related functions. Beyond examining the expression levels of the protein, we also determined the activation of c-Met and the functions of cell motility, invasion, and metastatic properties. It was shown that c-Met receptor tyrosine kinase was sometimes mutated in the tyrosine kinase domain, such as germline mutations in hereditary papillary renal cells³⁷ and later as somatic mutations in sporadic renal cell carcinomas.³⁸ A multiplex polymerase chain reaction technique was designed to study the mutations in c-Met in the whole gene.

Based on the results from our laboratory over the past decade, we were able to show that in thoracic malignancies, such as SCLC, NSCLC, and malignant mesothelioma, clusters of mutations and single-nucleotide polymorphisms (SNPs) were present, some in the semaphorin domain and others in the juxtamembrane domain.¹² However, no mutations were found in the tyrosine kinase domain, and activating mutations could be found only within the semaphorin domain and the juxtamembrane domain. This is unlike head and neck cancers, glioblastomas, and the hereditary papillary renal cells, in which activating tyrosine kinase domain mutations can be present.

In the experiments to search for mutations in c-Met, we used paxillin as a control and found that paxillin itself was mutated in lung cancer cells. Paxillin, a downstream effector of c-Met receptor tyrosine kinase, had a somatic mutation.³⁹ Using a large cohort containing samples from Caucasians, African Americans, and Taiwanese patients, we were able to show activating mutations within the LIM domains and between the LD1 and LD2 motifs for paxillin. The structure and function of the mutated protein is being analyzed currently. c-Met and paxillin cooperate to produce downstream effects, but mutated paxillin itself can lead to an angiogenic phenotype. The H522 cell lines lack c-Met and paxillin, and when this cell line was transduced with the wild-type paxillin, some angiogenesis and invasion was observed. The introduction of the A127T paxillin-activating mutation in mouse xenograft model resulted in an invasive and an angiogenic phenotype.³⁹

SCLC mutations in the juxtamembrane domain are not infrequent and are related to enhanced tumorigenicity, altered morphology in vitro with less adhesion, and disorganized architecture, leading to increased cell survival and increased motility and migration. The pY31 phosphorylation site on paxillin is HGF-ligand independent and constitutively active with increased tyrosine phosphorylation.¹⁰

MODELING MUTATIONS VIA *CAENORHABDITIS ELEGANS*

In addition to using an in vitro system with cell lines and tailoring mouse models to mimic disease, we have also employed a strategy using *Caenorhabditis elegans* to analyze mutations and functionality of genes mutated in human cancers. Transgenic *C. elegans* worms with mutations of the c-Met were generated to evaluate the role of human c-Met and mutants in a multicellular organism in a high-throughput fashion. A multivulval phenotype can represent the “cancer phenotype” within *C. elegans*, and vulval morphology can be used to define alterations in c-Met. For example, wild-type N2 adult hermaphrodite *C. elegans* has a normal vulva; however, transgenic nematodes expressing wild-type human c-Met genes have ectopic hypodermal growth in the posterior region. In the transgenic worm with R988C mutant c-Met construct, there was a tumor-like growth of the vulva-forming cells, whereas in animals expressing human T1010I mutant c-Met gene, the adult hermaphrodites are vulva-less. Over a course of time, this multivulva does not extrude out the miniature worms, giving rise to a “bag of worms.” Ultimately, these worms “explode” after a period of time, which can be followed by microscopy.⁴⁰

Based on these studies, we can also determine how the c-Met receptor impacts lung cancer. Many previous studies have revealed that EGFR mutations can occur a number of times in nonsmokers, but we have shown that c-Met receptor mutations occur mostly in smokers. This suggests a synergism between c-Met and nicotine or c-Met and smoke (and related toxins). The synergism between the mutated c-Met and wild-type c-Met in the presence of nicotine exposure resulted in an altered phenotype similar to the multivulva phenotype.⁴⁰ This is quite important, as the addictive smoking gene has been recently identified.^{41,42} It is a SNP on 15q24 that a majority of smokers may have: 50% will have a variant allele, 10% will have two variant alleles, and the remaining 40% will have the wild-type allele. If one variant allele is present, the relative risk of lung cancer is greater than 30%, whereas, if two variant alleles are present, the risk of developing lung cancer is greater than 80%.⁴²

The *C. elegans* system can also be used to study other toxins, such as environmental toxins. These worms can be exposed to asbestos and a mesothelioma-type of phenotype can be observed.⁴⁰ Similarly, drugs can be tested in the *C. elegans* system, because the drugs are absorbed through the gut. Small molecule inhibitors are being tested in this manner. In the *C. elegans* modeling system, there is synergism with nicotine with the wild-type allele and, more specifically, with the mutations on c-Met. This may reflect the phenotype and the genotype that is seen in the patient population.

DETERMINING c-Met AS A VALID THERAPEUTIC TARGET

In addition to overexpression of c-Met and mutations in c-Met in lung cancer, there can also be amplification of c-Met. Although this mutation may not necessarily be concordant with changes in the EGFR, they are concordant with paxillin. In our sample set, we observed that c-Met and paxillin activity amplification was a concordant event in lung cancer. Preclinical studies point to therapeutic inhibition of the c-Met receptor tyrosine kinase and its ligand HGF. There are a number of small molecule inhibitors against c-Met,^{43,44} the HGF antibody, RNAi, and micro-RNA (Table 1), and some of them are in phase I and phase II clinical trials.^{45,46} There are also some monoclonal antibodies against c-Met receptor and HGF. These agents show activity against the receptor or the tyrosine kinase or can have multiple targets.

The proof of principle that c-Met is an important therapeutic target in terms of invasion and metastasis was shown in an experiment with siRNA. On an immunoblot, the siRNA inhibition of c-Met transcription showed dramatic downregulation of the c-Met receptor. This was reflected in the percent of cell viability, where a considerable decrease was

observed. SU11274 (an inhibitor of c-Met) showed a dramatic decrease in cell viability in vitro for SCLC cell lines H69 and H345, with an IC_{50} of 2.5–5 $\mu\text{mol/L}$ compared to control. The H69 cell line has a juxtamembrane domain mutation, so it appears to be more sensitive to small tyrosine kinase inhibition. The c-Met inhibitor PHA-665752 reduced the size of H69 tumors induced in nude mice compared to control.⁴⁷ Tumor growth and shrinkage were observed using magnetic resonance imaging.

Similarly, tumor sections from these mice can be stained and examined initially and at discrete time periods to examine changes. For example, in A549 xenografts, a K-ras–mutated cell line, initially there was angiogenesis inhibition and thereafter tumor regression.⁴⁷

Finally, there is cooperation between the c-Met and EGFR tyrosine kinases. Crosstalk between these two receptors can be measured by cell viability as well as biological functions, such as cell motility/migration.^{48,49} HGF and EGF stimulation cause synergistic phosphorylation of AKT and downstream p70-S6 kinase or the mammalian target of rapamycin (mTOR) pathway in NSCLC cells. Gefitinib and SU11274 could inhibit the effect of c-Met and EGF receptors.⁵⁰

CONCLUSION

In summary, the c-Met receptor is highly expressed and activated in cancers. Its activity is augmented by synergism with other proteins, such as paxillin, or with EGFR in lung cancer. Several inhibitors appear promising in early clinical trials. The outcome of these trials will determine their utility in treating these cancers.

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Figure 1. c-Met receptor and its domains and phosphorylation sites. A schematic of the signaling cascade of c-Met upon ligand binding of HGF.⁵¹ The domains of c-Met (HGFR) are represented in this figure. The SEMA region is the ligand binding domain. The phosphorylation and tyrosine kinase (TK) sites are in the C-terminus, in the juxtamembrane region, and in the TK domain. Signaling requires dimerization of the receptor, which occurs upon ligand (HGF) binding. Many pathways are activated, as shown downstream of the dimerized receptor (blue) leading to motility, migration, proliferation, and morphogenesis. Reprinted with permission from the American Society of Clinical Oncology. Salgia R: American Society of Clinical Oncology Educational Book 2008;113-8.

Figure 2.

Cell motility regulators: focal adhesion proteins.⁵² Paxillin interacts with the integrin $\alpha_4\beta_1$ and is a key mediator in cytoskeletal interactions. A complex formed by paxillin and other cytoskeletal members talin, vinculin, FAK, and CRKL can lead to membrane reorganization and separation of focal adhesions leading to migration and motility. Constitutively active paxillin can mediate these functions more aggressively. The many functions of the cytoskeletal protein complex are shown above. Reprinted with permission from Begell House Journals. Sattler M, Pisick E, Morrison PT, Salgia R. Crit Rev Oncol 2000;11:63-76.

Table 1**Met inhibitors in development**

Monoclonal antibodies		
OA-505	Genentech	MET receptor antibody
AMG-102	Amgen	HGF ligand antibody
Small molecules		
PF-02341066	Pfizer	ALK, Tie2, RON, Axl
ARQ-197	Arqule	Selective, only non-ATP inhibitor
XL-184 XL-880 (aka GSK1363089)	Exelixis Exelixis, GSK	VEGFR, PDGFR, c-Kit, Flt3, Tie2, RET
MGCD265	Methygene	VEGFR 1,2,3, Tie2, RON
MK2461	Merck	Tie2, RON
MP-470	SuperGen	c-Kit, PDGFR
SGX-523	SGX	Selective
JNJ-38877605	Johnson & Johnson	Selective