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Expression and Mutational Analysis of *MET* in Human Solid Cancers

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Abstract

MET receptor tyrosine kinase and its ligand hepatocyte growth factor (HGF) regulate a variety of cellular functions, many of which can be dysregulated in human cancers. Activated MET signaling can lead to cell motility and scattering, angiogenesis, proliferation, branching morphogenesis, invasion, and eventual metastasis. We performed systematic analysis of the expression of the MET receptor and its ligand HGF in tumor tissue microarrays (TMA) from human solid cancers. Standard immunohistochemistry and a computerized automated scoring system were used. DNA sequencing for *MET* mutations in both non-kinase and kinase domains was also performed. *MET* was differentially overexpressed in human solid cancers. The ligand HGF was widely expressed in both tumor, primarily intra-tumoral, and non-malignant tissues. The MET/HGF likely is functional and may be activated in autocrine fashion in vivo. MET and SCF were found to be positively stained in the bronchioalveolar junctions of lung tumors. A number of novel mutations of *MET* were identified, particularly in the extracellular semaphorin domain and the juxtamembrane domain. MET-HGF pathway can be assayed in TMAs and is often overexpressed in a wide variety of human solid cancers. *MET* can be activated through overexpression, mutation, or autocrine signaling in malignant cells. Mutations in the non-kinase regions of *MET* might play important role in tumorigenesis and tumor progression. *MET* would be an important therapeutic anti-tumor target to be inhibited, and in lung cancer, MET may represent a cancer early progenitor cell marker.

INTRODUCTION

The *MET* protooncogene encodes the transmembrane tyrosine kinase receptor for the hepatocyte growth factor, HGF (also known as scatter factor, SF). The MET/HGF signaling pathway regulates a wide variety of cellular functions including cell proliferation, survival,

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apoptosis, scattering and motility, invasion, angiogenesis, and branching morphogenesis (Ma et al., 2003b). Dysregulated MET/HGF signaling leads to an abnormally activated cellular invasive program that plays a role in cellular transformation, epithelial-mesenchymal transition (EMT), tumor invasion, progression and metastasis. The ligand HGF binds to MET with high affinity and induces a sequence of cytoplasmic phosphorylation on multiple serine and tyrosine residues crucial in the eventual signal transduction. The role of *MET* in human cancer was first established with the identification of germline *MET* kinase domain mutations in papillary renal cell carcinoma (Schmidt et al., 1997; Schmidt et al., 1999). Dysregulated MET kinase activity is correlated with aggressive tumor traits such as abnormal proliferation and survival, increased cell motility and migration, tumor invasion and metastasis (Ma et al., 2003b). A number of missense mutations of *MET* kinase domain have been reported in a variety of human cancers. *MET* kinase domain mutations have been found to occur more frequently in metastatic tumor tissues when compared to the primary tumors (Di Renzo et al., 2000). Recent analysis of the *MET* gene in human cancers including lung cancer, mesothelioma and melanoma identified novel extracellular semaphorin (sema) domain and also juxtamembrane domain missense mutations (Ma et al., 2003a, 2005a; Jagadeeswaran et al., 2006; Puri et al., 2007). The functional implications of these mutations proximal of the tyrosine kinase domain of MET are not fully understood.

MET receptor is widely expressed among epithelial tissues (Ma et al., 2003b). Besides activating mutations, MET signaling pathway can be activated by overexpression of the kinase itself or its ligand HGF. MET/HGF signaling can be driven by autocrine, paracrine or endocrine loop regulation. The source of HGF production is primarily located in the stromal tissue sites such as the liver and bone marrow. Our previous studies have shown overexpression of MET in both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (Ma et al., 2003a, 2005a). In addition, overexpression of MET in malignant pleural mesothelioma cell lines and also tumor tissues have also been identified (Jagadeeswaran et al., 2006). There has been a recent report suggesting *MET* gene amplification can drive the dependency of cell survival and proliferation upon the MET signaling in lung cancer cell lines (Lutterbach et al., 2007). Moreover, *MET* amplification found in some gastric cancer cell lines has been correlated to hypersensitivity to the MET inhibitor PHA665752 (Smolen et al., 2006). Here we undertook a systematic comprehensive analysis of the relative receptor protein expression of MET and its ligand HGF in a variety of human solid cancers using tumor tissue microarray (TMA). We found that MET was overexpressed primarily in tumor tissues compared to the non-neoplastic controls. Co-expression of MET and stem cell factor (SCF) was identified within the lung bronchioalveolar duct junction as well as that in the lung TMA with potential implication as lung cancer early progenitor cells. On the other hand, HGF was more widely expressed both in normal and malignant tumor tissues and was predominantly intra-tumoral rather than in the adjacent stromal tissues of the tumor. We also performed tumor tissue mutational analysis of the *MET* gene on several human cancer types and show that the non-kinase sema domain and juxtamembrane domain were sometimes mutated.

MATERIALS AND METHODS

Tumor Tissues and TMA

Tumor and non-neoplastic tissue microarrays were generated as previously described (Ma et al., 2007). The following human tumor tissue microarrays were included in this study: breast (adenocarcinoma), lung (non-small cell) cancer, colon (adenocarcinoma), renal cell cancer, ovarian (36 adenocarcinomas, 1 small cell and 3 endometroid carcinomas), and non-neoplastic tissues from a wide variety of 25 organs. The total number of tumor tissue cores on each array was forty, and ten normal tissue controls were present in each array. The

University of Chicago (UC) Tissue Bank at the Department of Pathology has implemented standardization of all aspects of patient tissue collection, processing, pathology verification and quality control. Every case was collected within 1 hour after tissue excision and fixed for a standard time-period depending on the tissue size, but not exceeding 24 hours. The only fixative routinely used in the UC tissue banking is 10% neutral buffered formalin. All tissue microarrays used in this study were generated from Tissue Bank research samples, and validated to highly stringent quality specifications.

Antibodies and Immunohistochemistry (IHC)

IHC staining was performed using standard techniques as previously described (Ma et al., 2005a). For this study, we tested 7 commercially available antibodies: MET antibodies from Santa Cruz (Santa Cruz, CA) (1) and Zymed-Invitrogen (Carlsbad, CA) (2), and HGF antibodies from R&D Systems (Minneapolis, MN) (2), Santa Cruz (Santa Cruz, CA) (1) and Sigma-Aldrich (St. Louis, MO) (1). We determined the best antibodies according to our criteria which were then subsequently used in our study as described below. The antibodies used here against MET receptor was purchased from Zymed-Invitrogen (Carlsbad, CA), and HGF from Santa Cruz (Santa Cruz, CA). The phosphospecific antibodies against p-MET (Y1003: this commercial amino acid sequence nomenclature based on the common exon 10 alternative spliced variant is equivalent to the Y1021 epitope in the full-length MET) was purchased from Upstate/Millipore (Charlottesville, VA). Cytoplasmic and membranous expression of MET and phospho-MET was quantified manually in each core at x400 magnification by two pathologists (AM, AH) and with Automated Cellular Imaging System (ACIS, Clariant, CA). The ACIS system measures the intensity of the staining based on three related color parameters: the color defined by hue, the "darkness" defined as luminosity, and the density of the color defined as saturation. ACIS software for TMA was programmed by an experienced user-pathologist by setting the color-specific thresholds to determine and calculate staining intensity and the ratio of positively stained cells to the entire area of selection (Fig. 1C). MET and phospho-MET intensity measurements were translated into the 4-tier system as negative (0), weak (1+), moderate (2+), or strong (3+) staining and compared with manual scoring. The comparison between manual and automated imager scoring showed strong positive correlation ranging from 0.7 to 0.94 in different tumor types. Spearman correlation coefficient for all studied tumors was 0.897 ($P<0.01$). High concordance (77–91%) was observed between individual scores performed manually by two pathologists (MT and AM). Any discrepancies in manual scores were resolved by reviewing the slides with a third pathologist (AH) under the multihead microscope.

Non-neoplastic and neoplastic TMAs were included to be screened for expression of MET, phospho-MET and HGF. Each TMA was immunostained using primary MET (Zymed), phospho-MET (Y1003) (Upstate/Millipore), and HGF (Santa Cruz) antibodies. After incubation for 1 hour, the slides were incubated for 30 min with secondary IgG conjugated to a horseradish peroxidase-labeled polymer (Envision+ System). Reactions were developed with 3,3'-DAB chromogen and counterstained with hematoxylin. Negative controls were prepared by substituting the primary antibody with nonimmune mouse or rabbit serum.

DNA Sequencing

Tumor samples were collected according to Institutional Review Board (IRB) approved protocol. For *MET* mutational analysis, RNA was extracted from tumor tissues of lung adenocarcinoma (n=3), renal carcinoma (n=14) and ovarian carcinoma (n=3) using standard techniques. cDNA was generated from the tumor RNA for subsequent direct DNA sequencing. cDNA was also generated from RNA extracted from melanoma cell line (K008; n=1) for the mutational analysis. Direct DNA sequencing of the tumor tissue DNA was

performed as previously described (Ma et al., 2003a, 2005a; Jagadeeswaran et al., 2006). Mutational analysis was performed using the Mutation Surveyor software as previously described (Ma et al., 2005a).

RESULTS

Tumor Microarray Expression Analysis of MET in Human Solid Cancers

In order to profile the protein expression pattern of MET receptor in human cancers, a panel of tumor microarrays from different human solid cancer types (breast, colon, lung, ovarian and renal cancers) were used for the immunohistochemistry study (Fig. 1A). Non-malignant tissues microarray was included as control (Fig. 1B). Cytoplasmic and membranous expression of MET and phospho-MET was quantified manually in each core at 400X magnification by two pathologists (AM, AH) and with Automated Cellular Imaging System (ACIS) (see Methods) (Fig. 1C). Expression of MET receptor in the tumor microarrays was quantitated in 4-tier score system as negative (0), weak (1+), moderate (2+), or strong (3+) staining (Fig. 2A). Expression of the MET receptor in the breast cancer TMA was found to be: 0, 65% (24/37); 1+, 19% (7/37); 2+, 14% (5/37); 3+, 3% (1/37). Hence, 16% (6/37) of breast cancer tissues overexpressed MET. For colon cancer, 3% (1/40) of tumor tissues had no expression (0), whereas 20% (8/40) had 1+, 45% (18/40) had 2+, and 33% (13/40) had 3+ MET expression. Seventy-eight percent (31/40) of colon cancer tissues overexpressed MET. For lung cancer, 28% (1/40) of tumor tissues had no expression (0), whereas 33% (13/40) had 1+, 35% (14/40) had 2+, and 5% (2/40) had 3+ MET expression. Forty percent (16/40) of lung cancer tissues overexpressed MET. For ovarian cancer, 33% (13/40) of tumor tissue samples had no MET expression (0), whereas 38% (15/40) had 1+, 18% (7/40) had 2+, and 13% (5/40) had 3+ expression of the receptor MET. Thirty percent (12/40) of ovarian cancer samples overexpressed MET. In renal cancer, 15% (6/40) of tumor tissues had no MET expression (0), whereas 15% (6/40) had 1+, 45% (18/40) had 2+, and 25% (10/40) had 3+ expression. A total of 70% (28/40) renal cancer samples had overexpression of MET. Lastly, in the non-neoplastic TMA, 70% (32/46) of samples was found to have no MET expression (0), whereas 11% (5/46) had 1+, 17% (8/46) had 2+, and 2% (1/46) had 3+ expression.

A number of “fresh frozen” tissue samples were also used for IHC staining. IHC staining in fresh frozen samples from two adenocarcinomas and one squamous cell lung carcinoma was performed. Representative images of MET, HGF and phospho-MET (P-MET) expression are shown in Fig. 1D. The tyrosine phosphorylation of P-MET in the formalin-fixed lung tumor tissues in our study was relatively well-preserved as compared to that in the fresh-frozen tissues. We have also tested several different antibodies for IHC against MET, HGF and phospho-MET on the same tumor tissue for comparison and controls (Fig. 1E).

Interestingly, we also identified a tissue specific localization pattern of MET receptor expression in human cancers (Fig. 2B). Different proportions of the tumor specimen cores expressed MET in either cytoplasmic/membranous combined versus cytoplasmic localization alone. Highest cytoplasmic alone expression of MET receptor was observed in breast cancer, up to 90%, followed by colon cancer with approximately 50% cytoplasmic expression. The cytoplasmic alone expression of MET in lung, ovarian and renal cancers were 7%, 11% and 6% respectively.

Tumor Microarray Expression Analysis Expression of Phospho-MET in Human Solid Cancers

Ligand-induced activation of the MET receptor can be assayed through the activated phosphorylation of the juxtamembrane tyrosine phosphosite Y1003 of the receptor, which is

inducible by the ligand HGF binding. Expression of phospho-MET in human solid cancers identified in the tumor microarray immunohistochemical staining study is summarized as follows (Fig. 2B). In breast cancer, 23% expressed phospho-MET (1+, 6/39; 2+, 1/39; 3+, 2/39) while 77% did not (30/39). In colon cancer, 8% expressed phospho-MET (1+, 1/39; 2+, 2/39; 3+ 0/39) while 92% did not (36/39). In lung cancer, 73% expressed phospho-MET (1+, 14/40; 2+, 13/40; 3+, 2/40) while 27% (11/40) did not. In ovarian cancer, 33% expressed phospho-MET (1+, 6/40; 2+, 6/40; 3+, 1/40) while 67% (27/40) did not. Lastly, in renal cancer, 18% expressed phospho-MET (1+, 6/40; 2+, 0/40; 3+, 1/40) while 82% (33/40) did not. Expression pattern of the p-MET [Y1230/1234/1235] in these human cancer tumor microarrays followed a similar pattern (data not shown). Hence, in the descending order of expression of phospho-MET, lung cancer had the highest percentage, followed by ovarian, breast, renal and colon cancers.

Tumor Microarray Expression Analysis Expression of HGF in Human Solid Cancers

In the five human cancer TMAs, HGF expression was found to be widely present in all tumor tissues (Fig. 3). Sixty-seven percent (67%, 25/37) of breast cancer, 67% (26/39) of colon cancer, 5% (2/40) of lung cancer, 37% (15/40) of ovarian cancer, 45% (18/40) of renal cancer expressed 3+ HGF in the TMA. In addition, 2+ expression of HGF was observed in 24% (9/37) of breast cancer, 28% (11/39) of colon cancer, 45% (18/40) of lung cancer, 57% (23/40) of ovarian cancer, and 65% (26/40) of renal cancer. Finally, 1+ HGF expression was seen in 8% (3/37) of breast cancer, 5% (2/39) of colon cancer, 47% (19/40) of lung cancer, 5% (2/40) of ovarian cancer and 15% (6/40) of renal cancer TMA. Interestingly, in the non-neoplastic TMA, 4% (2/46) of samples was found to have no HGF expression (0), whereas 15% (7/46) had 1+, 46% (21/46) had 2+, and 35% (16/46) had 3+ HGF expression.

Mutations of *MET* in Human Cancers

A number of novel mutations of *MET* in small cell lung cancer, non-small cell lung cancer (adenocarcinoma), mesothelioma and also melanoma have been identified recently (Ma et al., 2003a, 2005a; Jagadeeswaran et al., 2006; Puri et al., 2007). The mutations were found to be predominantly located in the non-kinase domain, namely the extracellular sema domain and the short cytoplasmic juxtamembrane domain. Here, we performed cDNA direct sequencing screening for *MET* mutations in a number of melanoma, lung, renal and ovarian carcinoma samples. We further identified several *MET* missense mutations within the extracellular sema domain: D370V (heterozygous; INP313), and N375S (heterozygous; INP313) from lung adenocarcinoma, and V136I (heterozygous; R3) from a renal carcinoma. In addition, novel missense mutations in the melanoma cell line (K008) in the sema domain: N375S (heterozygous), and in the juxtamembrane domain: G983V (heterozygous) and R1000S (heterozygous) were identified. A mutation within exon 13 was also found in 2 renal carcinomas: Q978L (heterozygous; R3 and R4). No mutation was found from the ovarian carcinoma tissues. Figure 4 summarizes the previously identified *MET* mutations in human solid cancers and the newly identified human cancer mutations in this study. Notably, kinase domain mutations have been found in cancers such as renal cell carcinoma, glioma, hepatocellular carcinoma, and squamous cell carcinoma of the head and neck. Our recent mutational analysis of *MET* identified mutations in thoracic malignancies and melanoma clustered predominantly in the sema domain and juxtamembrane domain. Here, we show that sema and juxtamembrane mutations can also occur in solid tumors such as renal cell carcinoma and melanoma other than thoracic malignancies.

MET Expression in Lung Cancer Early Progenitor Cells

A growing body of evidence supports the concept of cancer stem cells which may play a crucial role in both tumorigenesis and therapeutic resistance to novel targeted inhibition. MET-HGF signaling pathway functions to stimulate cell motility, migration, scattering, and

cell invasion. Both HGF and MET have been found to be expressed in adult mesenchymal stem and progenitor cells. These cells can be mobilized and induced to undergo chemotactic migration by HGF. More recently, Kim et al. (2005) reported the identification of lung bronchioalveolar stem cells at the bronchioalveolar duct junction. These cells are thought to be anatomically and functionally distinct lung epithelial cell subpopulations with “stemness” properties of self-renewal and multipotent in clonality. MET-HGF signaling axis may potentially serve to drive the mobilization and dissemination of both normal and lung cancer stem cells. We examined to see if MET expression can be identified in the progenitor cell population in lung tissues. Using immunohistochemical staining with the anti-total MET antibody, we identified the MET expressing potential lung progenitor cells located in the bronchioalveolar duct junction of the lung tissue (Fig. 5A). In addition, immunohistochemical analysis was also performed using the antibody against SCF in conjunction with c-MET. A distinct cell population consistently overexpressing MET and SCF (clone EP665Y, 1:250, Abcam, MA) in areas with bronchiolar and alveolar junction damage in inflammatory areas in the human lung TMA was identified. Even though the precise role of SCF in lung tumorigenesis is largely unknown, SCF was identified in this study as one of the most sensitive markers of cells highly suggestive for the progenitor role. Immunohistochemical staining with KIT, CD34, POU5F1 and CD10 antibodies was also determined to detect the potential early progenitor cells in lung cancer. However, the value of these markers in lung cancers was not relevant due to their staining of primarily hematopoietic or embryonic stem cells. Representative images of immunohistochemistry with SCF in lung TMA are shown in Figure 5B.

DISCUSSION

The MET/HGF signaling pathway can be activated by overexpression of the ligand HGF or the MET receptor kinase, *MET* genomic amplification, or its activating mutations. We have previously shown that MET/HGF pathway is functionally expressed in lung cancer, mesothelioma and melanoma (Ma et al., 2003a, 2005a, 2005b; Jagadeeswaran et al., 2006; Puri et al., 2007). In addition, various novel mutations of the MET receptor have been found to cluster within the “hotspots” of the extracellular sema domain and also juxtamembrane domain. To investigate further the role of MET/HGF pathway in human solid tumors, we utilized tumor tissue microarrays to systematically examine for the expression of the ligand HGF and receptor MET in breast, lung, colon, kidney and ovarian carcinomas. While MET receptor is more preferentially expressed and overexpressed in malignant tumors compared to normal non-malignant tissues, the ligand HGF is relatively more ubiquitously expressed. Moreover, the immunostaining pattern of HGF indicated that its expression is primarily intra-tumoral rather than stromal expression. Results of this study suggest that HGF and MET may function in an autocrine fashions in solid tumors. The immunostaining pattern for HGF was primarily cytoplasmic, consistent with the fact that the growth factor is a secreted protein most abundant within the cytoplasm. On the other hand, there are cytoplasmic and membranous localization of MET, which appears to have tissue specificity as well.

Olivero et al. (1996) reported in a previous study that MET was found 2 to 10-fold increased in 25% of primary NSCLC samples comparing to adjacent normal tissues. The ligand HGF was found to be 10 to 100-fold overexpressed in the carcinoma samples ($P<0.0001$) (Olivero et al., 1996). Many studies have also been conducted in the past years to examine expression/overexpression of MET and its ligand HGF in a number of tumor tissues. Nonetheless, the methodologies and scoring criteria were not necessarily uniform. The use of tumor microarray as a research platform for more comprehensive and high throughput immunohistochemical analyses of tumor biomarkers has only been made available in more recent years. MET was found be expressed at various levels in 87% of various renal cell carcinomas but not in transitional cell carcinomas (Natali et al., 1996). There was also report

of the mutant *MET* allele found in hereditary papillary cell renal cell tumors being duplicated and overexpressed in tumors cells (Fischer et al., 1998). Further studies to examine the temporal relationship between *MET* mutation and its genomic amplification/receptor overexpression would be of great interest. Recently, it has been proposed that kinase mutant *EGFR* occurs during the early phase of lung adenocarcinoma tumorigenesis followed eventually by genomic amplification with increased *EGFR* gene copy number of the mutant allele as a late event (Soh et al., 2008). In colon cancer, *MET* gene amplification and expression level have also been correlated with advanced stage disease/liver metastases and as predictor of tumor invasion and lymph node metastases (Takeuchi et al., 2003; Zeng et al., 2008). Similarly, *MET* overexpression has been observed in ovarian cancers (Di Renzo et al., 1994; Wong et al., 2001; Sawada et al., 2007) and breast cancers (Garcia et al., 2007). *MET* expression were found to be a negative prognostic factor in ovarian cancer (Sawada et al., 2007) and correlated with worse outcome in breast cancer patients with negative lymph node (Tolgay Ocal et al., 2003). *MET* was also found to be commonly expressed and sometimes overexpressed in both SCLC and NSCLC in our earlier studies using standard immunohistochemistry and immunoblotting in tumor tissues and cell lines (Maulik et al., 2002; Ma et al., 2003a, 2005a). In our current study using TMA of various human solid cancers, consistent with previous reports, we found that *MET* is frequently overexpressed in the tumor tissues. Interestingly, HGF was found to be quite ubiquitously overexpressed as well across all tumor types examined. With regard to phospho-*MET* expression, lung cancer had the highest percentage of expression, followed by ovarian, breast, renal and colon cancers in a descending order

Oncogenic potential of various activating *MET* tyrosine kinase mutations identified in hereditary papillary renal carcinoma have been confirmed by a number of in vitro, and in vivo xenograft and transgenic experiments. Targeted mutations in the murine *Met* locus (knock-in mice), with the mutant *Met* allele expressed under the endogenous *Met* promoter resulted in various kinase mutant *Met* lines developed uniquely different tumor profiles, including carcinomas, sarcomas, and lymphomas (Graveel et al., 2004). These data strongly support the role of activating *Met* mutations in promoting tumorigenesis in vivo. Most interestingly, different mutations in the *Met* tyrosine kinase domain can also influence the types of cancers that eventually develop (Graveel et al., 2004). Hence, there might be tissue-specificity in *MET* driven tumorigenesis that is more dependent on the specific types of the kinase mutations rather than the mutant-receptor kinase activity itself. We show here that sema domain and juxtamembrane domain mutations can occur in human cancers more frequently than previously recognized. Juxtamembrane domain mutations of *MET*, R988C and T1010I, have been shown to be activating in our previous studies, supporting their role as “driver mutation” rather than merely “passenger mutation” (Ma et al., 2003a). This has now been shown in the *C. elegans* model, with synergism of these *MET* mutations with nicotine (Siddiqui et al., 2008). However, the mechanisms of the signaling alteration and effects on tumorigenesis of these *MET* mutations found outside of the tyrosine kinase domain remain much less understood than the kinase domain mutations. Extracellular domain alternative spliced variant of *EGFR*, *EGFRvIII*, has been known to be involved in glioblastoma and has recently been also shown to exist in a subset of lung adenocarcinoma patients (Ji et al., 2006). Recently, extracellular domain missense mutations of *EGFR* have been found to occur in glioblastomas and are in fact tumorigenic (Lee et al., 2006). Further studies to define the role of non-kinase domain mutations of *MET* in human cancer tumorigenesis, tumor progression, invasion and metastasis would be of high significance. Moreover, further characterization of the role and sensitivity of targeted therapeutics against the non-kinase mutated *MET* receptor would be crucial in moving forward in bringing full fruition to *MET* targeted drugs, including antibodies and small molecule inhibitors.

Evidence supporting the role of aberrant MET-HGF signaling in human oncogenesis and tumor progression suggests that inhibitors of this signaling pathway are attractive strategy for novel therapeutics. Our study here is the first attempt of a comprehensive analysis of MET receptor expression in human solid cancers with the use of multiple TMAs systematically. The results support the role of MET/HGF signaling and their mutations, especially in the non-kinase domains, in human cancers. Specific inhibitors against MET-HGF/SF signaling likely would have important therapeutic potential for the treatment of cancers in which MET activity contributes to the invasive/metastatic phenotype. A number of inhibitory strategies have been under development to inhibit MET/HGF signaling pathway in pre-clinical settings (Christensen et al., 2003; Ma, et al. 2003b, 2005a, 2005b; Kong-Beltran et al., 2004; Janne et al., 2005; Shepherd et al., 2005).

MET is a unique receptor tyrosine kinase important in regulating many cellular functions that might mediate tumor invasion and metastasis when deregulated. It serves a versatile role in regulating numerous biological functions in response to HGF/SF, including mitogenesis, cell scattering, cell motility and migration, branching morphogenesis, invasion, and eventual metastasis. Aberrant MET-HGF/SF signaling plays a significant role in the pathogenesis of many types of human cancers. MET-HGF/SF pathway is an attractive therapeutic target for inhibition in human malignancies harboring activating signaling pathway of MET/HGF. Various parameters of predictor of response to kinase inhibitors, especially the EGFR targeting gefitinib and erlotinib, have been under rigorous testing (Bell et al., 2005; Eberhard et al., 2005; Haber et al., 2005; Kobayashi et al., 2005; Pao et al., 2005). These include receptor overexpression, gene copy number and amplification, as well as kinase domain mutations. Our previous data showed that that MET overexpression in lung cancer cell lines such as H1838, H1993 and SW900 resulted in a more sensitive phenotype to cell viability inhibition by the MET inhibitor SU11274 (Ma et al., 2005a). *MET* gene amplification has also been recently correlated to enhanced sensitivity to PHA665752 inhibition in gastric cancer cell lines (Smolen et al., 2006). Acquired *MET* amplification has also been linked to approximately 22% of non-T790M mediated secondary gefitinib resistance in NSCLC patients (Engelman et al., 2007), although it can also occur concurrently with T790M-EGFR as well (Bean et al., 2007). With improved and more high-throughput comprehensive analysis of signaling protein targets expression in human cancer TMAs, it would be important to study the concordance of *MET* gene amplification and receptor overexpression in various cancer types with the intent to best select patient subpopulation most likely to respond to MET targeting inhibitors. Incorporating such analysis in future clinical trials involving therapeutic MET inhibitors or antibodies would facilitate the understanding into the mechanism of response and resistance to MET therapeutic inhibition.

It has been shown in the injury models that the normal lung contains anatomically and functionally distinct epithelial stem cell population that was termed bronchioalveolar stem cells (BASCs) (Kim et al., 2005). While the existence of cancer stem cell population, which carries such similar stem cell properties as self-renewal, and differentiation, remain somewhat controversial, there are now more emerging evidence in various tumor types supporting of this view (Hope et al., 2004; Burger et al., 2005; Liu et al., 2005; Wang et al., 2006; Ho et al., 2007; Korkaya and Wicha 2007; Yang et al., 2008). A number of cancer stem cell markers have been implicated from these studies, including CD34, CD44, SCF, KIT, POU5F1, and CD10. While the identity of stem cell subpopulations can be illustrated in single-cells clonogenic transplantation in vivo assay, identification of stem cells in paraffin tissues is admittedly quite difficult. Our IHC data in this study showing specific staining of MET and SCF in the bronchioalveolar junctions of normal lung can lung cancer in TMA is interesting. Even though the role of SCF in lung tumorigenesis is largely unknown, we identified SCF as one of the most sensitive markers of cells highly suggestive

for the progenitor role. Taken together, these results serve as further supporting argument of the existence of lung cancer progenitor cells, which presumably contribute ultimately to the tumor recurrence after initial response from chemotherapy. The identification of MET/SCF expression in the bronchioalveolar junction where the putative lung stem cells are located supports the notion that the subset of MET expressing cancer stem/progenitor cells may be responsible for the role to undergo clonal cell expansion that eventually drives the tumor cells invasion and metastasis. Since cancer stem cells are also implicated in tumor cell recurrence and chemoresistance, targeting MET overexpression using novel inhibitors or antibodies might have a potential role in circumventing tumor recurrence or chemoresistance that contribute predominantly to most human solid cancer morbidity and mortality.

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MET

Before

After

Figure 1.

(A), Human solid cancer tumor tissue microarrays (TMA): Breast cancer (breast adenocarcinoma: n=40); Lung cancer (non-small cell lung carcinoma: n=40); Colon cancer (adenocarcinoma, n=30; adenocarcinoma, metastatic, n=10); Kidney cancer (renal cell, n=1; renal cell, metastatic, n=3; renal cell papillary, n=6; renal cell papillary, metastatic, n=1; renal cell chromophobes, n=3; renal cell, clear cell, n=21, renal cell, clear cell, metastatic, n=3; renal cell, clear cell, sarcomatoid, n=2); Ovarian cancer (36 adenocarcinomas, 1 small cell and 3 endometrioid carcinomas); (B), Non-neoplastic tissues (multi-organ array including 2 tissue cores of each of 25 organs). Examples of 12 non-neoplastic tissues cores with MET IHC staining are shown here. (C), Automated Cellular Imaging System (ACIS). The ACIS system measures the intensity of the staining based on three related color parameters: the color defined by hue, the "darkness" defined as luminosity, and the density of the color defined as saturation. ACIS software for TMA was programmed by an experienced user-pathologist by setting the color-specific thresholds to determine and calculate staining intensity and the ratio of positively stained cells to the entire area of selection. (D), Expression of MET, HGF and phospho-MET (Y1003) in frozen sections of lung adenocarcinomas (cases 1&2) and squamous cell carcinoma (case 3). Original magnification x200. (E), Comparative IHC on the same tumor sample using 3 different c-MET antibodies (magnification x100). (a) MET from Zymed (clone 3D4) shows strong signal only within tumor tissue. (b) MET from Santa Cruz (rabbit polyclonal) shows moderately strong signal with non-specific reactivity in lung macrophages and inflammatory

stromal cells. (c) MET staining with another Zymed antibody (Rabbit polyclonal) exhibit weak signal in lung tumor and moderate non-specific positivity of necrotic areas and alveolar macrophages. Simultaneous expression of MET (d), HGF (e) and phospho-MET (f) in non-small cell lung carcinoma (magnification x200).

Figure 2.

Tumor microarray expression analysis of MET in human solid cancers. Tumor microarrays from human solid cancers were used for immunostaining with anti-total-MET and also phosphospecific MET antibodies as described in the Materials and Methods. Cytoplasmic and membranous expression of MET and phospho-MET was quantified manually in each core at x400 magnification by two pathologists (AM, AH) and with Automated Cellular Imaging System (ACIS, Clarent, CA) as described in the Materials and Methods. MET and phospho-MET intensity measurements were translated into the 4-tier system as negative (0), weak (1+), moderate (2+), or strong (3+) staining and compared with manual scoring. Combined quantitative analyses of the expression of total-MET and phospho-MET in different human solid cancers are shown here. (A), Total-MET expression. (B), Tumor specific localization of MET expression. (C), Phospho-MET expression.

Figure 3.

Tumor microarray expression analysis of HGF in human solid cancers. Tumor microarrays from human solid cancers were used for immunostaining with anti-HGF antibody as described in the Materials and Methods. Similar to the analyses shown in Figure 2 above, expression of HGF was quantified manually in each core at x400 magnification by two pathologists (AM, AH) and with Automated Cellular Imaging System (ACIS, Clarent, CA). HGF intensity measurements were translated into the 4-tier system as negative (0), weak (1+), moderate (2+), or strong (3+) staining and compared with manual scoring. Combined quantitative analyses of the expression of HGF in different human solid cancers are shown here. **(A)**, TMA analysis of HGF expression. **(B)**, HGF expression in human solid cancers.

Figure 4.

Mutations of *MET* in human solid tumors. MET receptor is shown in the schematic diagram highlighting different functional domains of the receptor: extracellular semaphorin (Sema) domain, PSI domain, the four IPT-repeats, transmembrane (TM) domain, juxtamembrane (JM) domain and cytoplasmic tyrosine kinase (TK) domain. The *MET* mutations identified in different human solid cancers in this study are represented in the top. Summary of various mutations of *MET* previously reported in human solid cancers, including renal cell carcinomas (both sporadic and hereditary), gastric carcinoma, hepatocellular carcinoma, glioma, squamous cell carcinoma of the head and neck, SCLC, NSCLC, mesothelioma and melanoma, are shown in the bottom for comparison (see references: Ma et al., 2003a, 2003b, 2005a; Jagadeeswaran et al., 2006; Puri et al., 2007).

Figure 5.

MET expression at the bronchioalveolar junction of normal lung and lung tumors with implication of lung progenitor cells. (A), Relative location of MET expression in normal lung tissues was examined using immunostaining with anti-MET antibody on a normal lung tissue showing more proximal airways including the bronchioalveolar junction. The strong positive immuno-expression of MET selectively at the bronchioalveolar junction (see arrows) may imply a role of MET in the biology of lung progenitor cells. (B), Identification of bronchioalveolar progenitor cells in lung TMA using stem cell factor marker (SCF, Rabbit monoclonal, Abcam). SCF positivity of regenerating epithelial cells in area of inflammation and fibrosis (*a*, x100; *d* – same, higher magnification (x400)). Bronchioalveolar lung carcinoma with almost identical staining pattern with SCF and MET markers (*b* – SCF, x100; *c* – MET, x100; *e* – SCF, x400; *f* – MET, x400).

Table 1

Detailed summary of IHC staining conditions.

Antibody	Clone	Species	Dilution	Antigen retrieval	Positive controls
MET	3D4	mouse	1:100	HIER, Citrate buffer, pH=6	Colorectal Cancer and Malignant Melanoma
HGF (H-145)	polyclonal	rabbit	1:25	HIER, EDTA buffer, pH=9	Normal Hepatocytes
Phospho- MET (Y1003)	Polyclonal	rabbit	1:50	HIER, EDTA buffer, pH=9	Colorectal Cancer

For this study, we tested 7 commercially available antibodies: MET antibodies from Santa Cruz (1) and Zymed-Invitrogen (2), and HGF antibodies from R&D Systems (2), Santa Cruz (1) and Sigma-Aldrich (1). We determined the best antibodies according to our criteria which were then used in this study. The conditions used for the immunohistochemical staining using the respective antibodies are outlined here. Examples of comparative IHC on the same tumor sample using 3 different MET antibodies, as well as the high resolution images with simultaneous expression of MET, HGF and phospho-MET in non-small cell lung carcinoma is shown in Figure 1E.