Functional Analysis of c-Met/Hepatocyte Growth Factor Pathway in Malignant Pleural Mesothelioma


Abstract

c-Met receptor tyrosine kinase (RTK) has not been extensively studied in malignant pleural mesothelioma (MPM). In this study, c-Met was overexpressed and activated in most of the mesothelioma cell lines tested. Expression in MPM tissues by immunohistochemistry was increased (82%) in MPM in general compared with normal. c-Met was internalized with its ligand hepatocyte growth factor (HGF) in H28 MPM cells, with robust expression of c-Met. Serum circulating HGF was twice as high in mesothelioma patients as in healthy controls. There was a differential growth response and activation of AKT and extracellular signal–regulated kinase 1/2 in response to HGF for the various cell lines. Dose-dependent inhibition (IC50 < 2.5 μmol/L) of cell growth in mesothelioma cell lines, but not in H2052, H2452, and nonmalignant MeT-5A (IC50 > 10 μmol/L), was observed with the small-molecule c-Met inhibitor SU11274. Furthermore, migration of H28 cells was blocked with both SU11274 and c-Met small interfering RNA. Abrogation of HGF-induced c-Met and downstream signaling was seen in mesothelioma cells. Of the 43 MPM tissues and 7 cell lines, we have identified mutations within the semaphorin domain (N375S, M431V, and N454I), the juxtamembrane domain (T1010I and G1085X), and an alternative spliced product with deletion of the exon 10 of c-Met in some of the samples. Interestingly, we observed that the cell lines H513 and H2596 harboring the T1010I mutation exhibited the most dramatic reduction of cell growth with SU11274 when compared with wild-type H28 and nonmalignant MeT-5A cells. Ultimately, c-Met would be an important target for therapy against MPM.

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive malignancy which can be associated with asbestos exposure. Incidence of this disease is expected to increase dramatically over the next few decades. It has been estimated that 250,000 people will die of MPM in Europe in the next three decades (1) and ~2,500 to 3,000 new cases are diagnosed each year in the United States with an even higher number worldwide. Recently, the multitargeted antifolate pemetrexed has been approved as the front-line agent in combination with cisplatin for the treatment of mesothelioma (2). Nonetheless, the overall outcome of this disease remains very poor despite current available therapies.

New approaches for the treatment of malignant pleural mesothelioma (MPM) are urgently needed. It was reported that the c-Met receptor tyrosine kinase (RTK) is an important molecule in a variety of malignancies (3–6) and overexpression of the receptor confers a poor prognosis (7, 8). Targeting the HGF/c-Met pathway could become a rational therapeutic approach for the malignant mesothelioma. c-Met is a RTK located on chromosome 7q31, and the gene contains 21 exons, and the protein consists of α/β chains, with the β-chain MW of 145 kDa. The ligand for c-Met has been identified as hepatocyte growth factor (HGF). Previously, c-Met has been shown to be overexpressed in MPM tumor tissues as compared with normal pleura (9). The c-Met/HGF axis can be involved in cell growth, cell survival, angiogenesis, cell motility/migration, and invasion and metastasis.

There is now a large body of data supporting the inhibition of RTKs as a new paradigm of therapy for various malignancies. For instance, the small-molecule inhibitor imatinib has been used to target BCR/ABL in chronic myelogenous leukemia and c-Kit in gastrointestinal stromal tumors (10). Epidermal growth factor receptor (EGFR) has been targeted in non–small-cell lung cancer with small-molecule inhibitors gefitinib and erlotinib (11). However, novel targeted therapy against c-Met has not come to clinical fruition. Preclinical data suggest that c-Met can be inhibited with antisense/small interfering RNA (siRNA), peptides/antagonists of HGF, small-molecule tyrosine kinase inhibitors, and antibodies directed against c-Met or HGF. We have previously shown that the small-molecule tyrosine kinase inhibitor SU11274 is specific against c-Met and has an IC50 of 1 to 5 μmol/L against a number of lung cancer cell lines (12).

Because the expression and function of c-Met in MPM is not well known, we evaluated the role of c-Met/HGF in MPM. The potential for therapeutic inhibition of c-Met in MPM is attractive, and we determined that SU11274 and c-Met siRNA against c-Met are effective inhibitors of c-Met in MPM cell lines. We have also determined the mutations of c-Met in MPM. Finally, we
show that serum HGF levels can be higher in MPM patients as compared with control subjects, thus potentially serving as a biomarker in MPM.

Materials and Methods

Reagents and antibodies. SU1274 [(3Z)-N-(3-chlorophenyl)-3-[(3,5-dimethyl-4-[(4-methylpiperazin-1-yl) carbonyl]-1H-pyrrol-2-yl] methylene]-N-methyl-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide] was provided by Pfizer, Inc. (San Diego, CA). Human recombinant HGF was purchased from Calbiochem (Cambridge, MA) and fetal bovine serum (FBS) was from Gemini Bioproducts (Woodland, CA). Cell culture media, penicillin, and streptomycin were obtained from Cellgro (Boehringer Ingelheim, Heidelberg, Germany). Polyclonal phosphorylation site–specific c-Met antibodies and phospho-extracellular signal–regulated kinase (ERK)-1/2 (T185/Y187) antibodies were obtained from Biosource International (Camarillo, CA). Antibody against p-AKT (S473) was obtained from Cell Signaling Technology (Beverly, MA), c-Met (c12) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and v-actin monoclonal antibody and all other chemicals were purchased from Sigma (St. Louis, MO).

Cell lines and cell culture. Malignant pleural mesothelioma (MPM) cell lines H513 (epithelioid) and H2596 (sarcomatoid) were cultured as previously described (13). H28 (epithelioid), H2052 (sarcomatoid), H2452 (biphasic), MSTO-211H (biphasic), and the nonmalignant mesothelial cell line (MeT-5A) were obtained from the American Type Culture Collection (Rockville, MD). MPM cells were cultured routinely in RPMI supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. MeT-5A was cultured in Medium 199 with Earle’s balanced salt solution supplemented with 10% FBS, 0.75 mmol/L l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C.

Immunoblot analysis. To examine c-Met expression in mesothelioma and nonmalignant mesothelial cells under basal conditions, subconfluent cells were cultured in medium supplemented with 10% FBS. The cells were then washed with ice-cold PBS and lysed with lysis buffer. To detect the activation of c-Met, AKT, and ERK1/2, cells grown on six-well plates were washed twice with PBS, then replaced with serum-free RPMI for 24 hours. Then added with HGF (40 ng/ml), and then incubated at 37°C for the specified time durations indicated (0-5 hours). Next, the cells were washed in cold PBS and lysed, followed by SDS-PAGE on a 7.5% gel, and transferred to a nitrocellulose membrane. Immunoblots were then done using standard method as previously described (3, 14). The same membranes were subsequently stripped and reprobed in a similar fashion with different primary antibodies.

Immunohistochemical analysis of c-Met expression by tissue microarray. Expression of c-Met was detected by immunohistochemistry using tissue microarray in tissue cores that were obtained from 21 normal and 43 mesothelioma tumor tissue samples as previously described (15). c-Met Targeted Therapy for Mesothelioma

Table 1. Serum EGF and HGF levels in patients with MPM and in control group

<table>
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<tr>
<th>Characteristics of study subjects</th>
<th>EGF (ng/mL)</th>
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<td></td>
<td>Mean ± SD</td>
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*P < 0.0001, relative to control group.
†P < 0.0001, relative to control group.
IX81 or Axiovert inverted microscopes equipped with a cooled CCD cameras. Control, control siRNA, c-Met siRNA (100 or 200 pmol/mL) transfected, and 2.5 μmol/L SU11274 pretreated (4 hours) H28 cell images were recorded for 2 to 4 hours. Digital video images were saved every minute and cell movement or morphologic changes were analyzed. For movement analysis, the position of cell nuclei was measured every minute with the NIH ImageJ or MetaMorph (Universal Imaging Corp., Downingtown, PA) programs and plotted to show the trace of cell movement. The distance that the cell nucleus transversed for each minute was calculated and the velocity of each cell was determined and compared.

Serum levels of circulating HGF and EGF. The serum HGF and EGF were measured in 23 MPM pretreated patients and 19 healthy controls. The characteristics of all subjects are reported in Table 1. Healthy controls were recruited from a group of blood donors and from nonneoplastic patients with eye diseases, treated in the same hospitals as MPM patients. The diagnosis of MPM was achieved through cytologic or histologic examination of pleural biopsies obtained through thoracoscopy or thoracotomy. A written informed consent was obtained from all patients before enrollment and study protocol was approved by IRB. A detailed questionnaire was administered to all subjects. Information was collected on demographic data, smoking history, occupation, and familial cancer history. Blood samples from all individuals were collected by routine venipuncture and the serum separated by centrifugation was stored at -80°C until assayed. Serum HGF and EGF concentrations were measured with sandwich enzyme immunoassays (ELISA) purchased from R&D Systems (Minneapolis, MN). The reported values represent the mean of duplicate determinations.

Statistical analysis. All reported HGF and EGF values were the mean of duplicate determinations. Means, medians, and SDs were calculated for cancer and control. A cutoff value was calculated on the basis of the mean ± 2 SD of markers frequency in healthy controls. Differences in the values between the groups under study were analyzed using the Kruskal-Wallis test, and the Mann Whitney U test was used for comparison of two groups. A χ² test was used to evaluate the relationship between categorical variables. The Spearman correlation method was used to correlate the HGF level with other variables. The experiments were usually done at least thrice. Statistical significance was tested by two-tailed Student’s t test. A two-tailed P value of <0.05 was considered statistically significant.

Results

Expression of c-Met in cell lines and tumor tissues. The expression of c-Met RTK in mesothelioma cell lines (H28, H2052, H2452, MSTO-211H, H513, and H2596) and a nonmalignant mesothelial cell line MeT-5A using standard immunoblotting with anti-total c-Met polyclonal antibody (C-12). The upper 170-kDa protein band represents the precursor form of the glycosylated c-Met and the lower 145-kDa band is the biologically active transmembrane β-subunit of c-Met (top). Equal loading of lysate is shown by the anti-β-actin immunoblot (bottom). B, the relative signal intensity for c-Met in each lane was determined by densitometry and expressed as arbitrary unit (fold) after the normalization. C, expression of c-Met in the mesothelioma tissue microarray. Immunohistochemical analysis c-Met RTK expression patterns in normal (n = 21) and mesothelioma (n = 66) tissues using anti-c-Met antibody on tissue microarrays. D, weak immunonegative c-Met staining (0) and immunoreactive strong c-Met staining (1+, 2+, and 3+).
HGF induced cell growth, signal transduction, and internalization of c-Met in mesothelioma cell lines. A growth effect of HGF on the various mesothelioma cell lines was also evaluated using MTT assay. The cells were cultured in the presence of serum over 48 hours with or without HGF (40 ng/mL). There was an increased growth effect of HGF in H28, MSTO-211H, and H2596 cells \((P < 0.0001)\) whereas minimal or no growth effect of HGF was observed in H2052, H2452, and H513 cells as well as MeT-5A cells (Fig. 2A).

We used MPM and MeT-5A cell lines to study the downstream signal transduction of the c-Met/HGF axis. Specific phosphorylation of c-Met, ERK1/2, and AKT was determined. MPM cells were responsive to HGF (40 ng/mL) stimulation of c-Met signaling up to 5 hours in a time-dependent fashion (Fig. 2B). Significant induction of tyrosine phosphorylation in c-Met of the autophosphorylation site \((pY1230/1234/1235)\) as well as the regulatory juxtamembrane c-Cbl binding site \((pY1003)\) was evident starting at 15 minutes of HGF stimulation and the phospho-signal declined between 2 and 5 hours. H28 cells that express high c-Met levels, however, had substantial induction of these phosphorylated proteins in response to HGF for up to 5 hours. Moreover, phosphorylation of ERK1/2 \((pT185/pY187)\) and AKT \((S473)\) was significantly induced in a differential manner with different cell lines after HGF stimulation (Fig. 2B). In the MeT-5A cells, there was only phosphorylation of \(pY1003\) of c-Met and \(pT185/pY187\) of ERK1/2 in response to HGF.

Internalization of c-Met was also evaluated with immunofluorescence in H28 cells which express high c-Met levels. Treatment...
of the cells with HGF (40 ng/mL, 15 minutes) led to internalization of c-Met in H28 cells (Fig. 2C). In non-HGF-treated H28 cells, c-Met was primarily distributed in the plasma membrane, whereas in HGF-stimulated cells, the c-Met receptor was readily identifiable in two distinct subcellular locations. An immunofluorescence signal was observed mostly in cytosolic and perinuclear distribution and less in the plasma membrane against c-Met in H28 cells (Fig. 2C).

Effects of SU11274 on mesothelioma cell growth. SU11274 inhibited the cell growth of H28, MSTO-211H, H513, and H2596 cells in a dose-dependent manner with an IC₅₀ between 2 and 3 μmol/L within 48 hours. On the other hand, SU11274 had no inhibitory effect in the H2052 and H2452 mesothelioma cells or in mesothelial cells (MeT-5A) at concentrations even up to 10 μmol/L at 48 hours (Fig. 3). Interestingly, the nonresponding cells did not have a significant alteration in growth with HGF (Fig. 2C).

Effects of SU11274 on migration and HGF-induced signal transduction in H28 cells. In an effort to study the efficacy of SU11274 to block cell migration, we did in vitro wound healing assay and time-lapse video microscopy. When a wound was introduced into subconfluent H28 (with robust c-Met expression) over the course of 24 to 48 hours, motility and migration of untreated was evident with the “wound-closure” by the migrated cells. However, this migration was virtually completely inhibited in H28 cells pretreated with SU11274. Activated migration in c-Met overexpressing mesothelioma cell line (H28) was effectively blocked even with the smaller dose (2.5 μmol/L) of SU11274 (Fig. 4A).

We also examined the effects of SU11274 treatment on H28 mesothelioma cell motility. Migrational velocity of the H28 cells was reduced dramatically with SU11274 (2.5 μmol/L) treatment when compared with untreated cells as observed under time-lapse video microscopy (Fig. 4B).

We examined the tyrosine phosphorylation of c-Met by immunoblotting in H28 mesothelioma cells in the absence or presence of HGF or SU11274. HGF induced tyrosine phosphorylation of c-Met at both the autophosphorylation site (pY1230/1234/12350) and the regulatory juxtamembrane c-Cbl binding site (pY1003). However, both of the tyrosine phospho-epitopes of c-Met were inhibited by pretreatment of SU11274 in a dose-dependent manner up to near basal level without affecting total steady-state c-Met protein level (Fig. 4C). To examine HGF-mediated signaling events downstream of c-Met activation, ERK1/2 and AKT phosphorylation in the presence of SU11274 was analyzed. As shown in Fig. 4C, SU11274 also inhibited HGF-induced phosphorylation of AKT and ERK1/2 in a dose-dependent manner.

Effects of c-Met siRNA on cell growth and migration in mesothelioma cells. To further investigate the role of c-Met in cell growth and migration of mesothelioma, c-Met expression was down-regulated by transfection of cells with siRNAs. H28 and H2596 cells transfected with c-Met siRNA had substantially reduced (>95%) c-Met expression after 72 hours (Fig. 5A). SU11274-mediated down-regulation of c-Met protein expression in H28 and H2596 cells resulted in inhibition of cell growth and viability as determined by MTT assay. Migration in c-Met siRNA–transfected cells was markedly reduced compared with control siRNA–transfected cells (Fig. 5B).

c-Met mutation in mesothelioma. The c-Met gene was analyzed for mutation in malignant mesothelioma (43 primary tumors and 7 cell lines). We identified alterations within the semaphorin domain (N375S, M431V, and N454I), the juxtamembrane domain (T1010I and G1085X), and alternative splice product skipping of entire exon 10 (Fig. 6A-C). All of these mutations were in separate tumor tissue samples. Interestingly, we observed that the cell lines H513 and H2596 harboring the T1010I mutation exhibited the most dramatic reduction of cell growth with SU11274 when compared with the nonmalignant MeT-5A cells and wild-type overexpressing H28 cells (Fig. 6D). Of note, we have previously identified the N375S mutation in non–small-cell lung cancer and the T1010I mutation in both small-cell lung cancer and non–small-cell lung cancer (3, 17).

Serum levels of circulating HGF and EGF in mesothelioma subjects and healthy controls. We analyzed serum samples from 23 subjects with MPM and 19 healthy controls. Median age was 67 years (range, 53-81 years) and 67 years (range, 50-82 years), respectively. Serum levels of HGF and EGF were twice higher in MPM than in healthy controls with statistically significant differences at P < 0.0001 and P < 0.001, respectively (Table 1). No difference was evident in serum concentration of HGF and EGF according to gender, age, or asbestos exposure, neither in cases nor in controls or tumor histology. After setting up a diagnostic cutoff value for the healthy controls mean ± 2 SD, the percentage of positive MPM was 60% (12 of 20) for HGF and 27.8% (5 of 18) for EGF.
Malignant pleural mesothelioma is a difficult illness with current standard therapy involving surgery, chemotherapy, or radiation therapy depending on the extent of the tumor. The link to asbestos in MPM has been established. However, the molecular mechanisms in MPM pathogenesis have not been explored to the fullest extent. Here we analyzed the role of the c-Met RTK in MPM. Table 2 summarizes a majority of our findings within this study.

A number of cell lines had robust expression of c-Met whereas the normal mesothelial cells had a much lower amount of c-Met. The overexpression of c-Met in mesothelioma was also confirmed in MPM tumor tissues. Ligand (HGF)-induced c-Met receptor internalization was observed by immunofluorescence microscopy. There were a number of signal transduction cascade mechanisms that were activated with HGF stimulation such as phosphorylation of c-Met and phosphorylation of ERK1/2 and AKT. Because c-Met is expressed and functional in mesothelioma tumor cell lines, we also determined its specific inhibition with SU11274, a small-molecule c-Met inhibitor. In the mesothelioma cell lines, there was a dose-dependent inhibition of cell growth whereas the nonmalignant MeT-5A was not responsive to SU11274. We observed that the cell lines harboring the T1010I mutation exhibited a dramatic reduction of cell growth with SU11274 when compared with the nonmalignant MeT-5A cells and wild-type overexpressing H28 cells. Finally, the serum levels of HGF were elevated in mesothelioma subjects, implying that this might be an important biomarker in this disease.

Receptor internalization and signal transduction have been studied innumerable for RTKs. However, ours is one of the first studies to show c-Met receptor internalization in response to HGF in mesothelioma cells. Receptor internalization is a tightly controlled process involving endosomes and lysosomes. On HGF stimulation, the receptor is rapidly internalized to early endosomes. At later time points, the localization pattern is more perinuclear, typical of late endosomes/lysosomes. c-Met is itself ubiquitinated after internalization and, interestingly, an oncogenic molecule c-Met inhibitor. In the mesothelioma cell lines, there was a dose-dependent inhibition of cell growth whereas the nonmalignant MeT-5A was not responsive to SU11274. We observed that the cell lines H513 and H2596 harboring the T1010I mutation exhibited a dramatic reduction of cell growth with SU11274 when compared with the nonmalignant MeT-5A cells and wild-type overexpressing H28 cells. Finally, the serum levels of HGF were elevated in mesothelioma subjects, implying that this might be an important biomarker in this disease.

Key Figure 4. Inhibition of cell migration and HGF-induced signal transduction of mesothelioma cells by SU11274. A, SU11274 inhibits migration of H28 cells. H28 cells migrate into an in vitro wound created onto a subconfluent plate. Cells were seen to be migrating into the wound over the subsequent 48 hours, and migration was significantly inhibited in the presence of 2.5 μmol/L SU11274. B, SU11274 reduces velocity of the H28 cells. SU11274 (2.5 μmol/L)-treated H28 cells were observed under time-lapse video microscopy and pictures were taken at 0 to 4 hours. The position of cell nucleus was measured and tracked every minute with the NIH ImageJ or MetaMorph (Universal Imaging) programs and plotted to show the trace of nucleus movement. The distance that the cell nucleus transversed and velocity for each minute were calculated to determine the speed of the movement in control and SU11274-treated H28 cells. C, dose-dependent inhibition of HGF-induced c-Met phosphorylation and downstream AKT and ERK1/2 phosphorylation in H28 cells by SU11274. Serum-starved mesothelioma H28 cells were preincubated with vehicle or the indicated concentrations of SU11274 for 12 hours. Cells were treated with HGF (40 ng/mL) for 15 minutes before lysis. Cell lysates were resolved by 7.5% SDS-PAGE and probed with either antibody. Immunoblots were shown for p-c-Met [Y1230/1234/1235] (top), p-c-Met [Y1003] (second), total c-Met (third), the cell survival proteins p-AKT [S473] (fourth), and p-ERK1/2[pT185/pY187] (fifth). Equal loading of lysate was shown by the anti-α-actin immunoblot (sixth).

Discussion
Malignant pleural mesothelioma is a difficult illness with current standard therapy involving surgery, chemotherapy, or radiation therapy depending on the extent of the tumor. The link to asbestos in MPM has been established. However, the molecular mechanisms in MPM pathogenesis have not been explored to the fullest extent. Here we analyzed the role of the c-Met RTK in MPM. Table 2 summarizes a majority of our findings within this study. A number of cell lines had robust expression of c-Met whereas the normal mesothelial cells had a much lower amount of c-Met. The overexpression of c-Met in mesothelioma was also confirmed in MPM tumor tissues. Ligand (HGF)-induced c-Met receptor internalization was observed by immunofluorescence microscopy. There were a number of signal transduction cascade mechanisms that were activated with HGF stimulation such as phosphorylation of c-Met and phosphorylation of ERK1/2 and AKT. Because c-Met is expressed and functional in mesothelioma tumor cell lines, we also determined its specific inhibition with SU11274, a small-molecule c-Met inhibitor. In the mesothelioma cell lines, there was a dose-dependent inhibition of cell growth whereas the nonmalignant MeT-5A was not responsive to SU11274. We observed that the cell lines H513 and H2596 harboring the T1010I mutation exhibited a dramatic reduction of cell growth with SU11274 when compared with the nonmalignant MeT-5A cells and wild-type overexpressing H28 cells. Finally, the serum levels of HGF were elevated in mesothelioma subjects, implying that this might be an important biomarker in this disease.

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Inhibition of various RTKs offers an exciting opportunity for novel therapeutics, especially in MPM. Unfortunately, clinical trials of imatinib (inhibiting the platelet-derived growth factor receptor and c-Kit pathways) and gefitinib (inhibiting the EGFR pathway) in MPM have not shown any benefit (22). The strategy to target other RTKs, such as c-Met, would be a quite novel approach to inhibit MPM. Previously, c-Met has been shown to be inactivated with c-Met siRNA (23, 24), peptides (25), natural killer cell transcript 4 (26, 27), and antibodies against c-Met (28) or HGF (29). We and others have recently shown that the c-Met tyrosine kinase domain can be inhibited by compounds such as SU11274, PHA665752, and K252a (12, 30, 31). SU11274 treatment in small-cell lung cancer and non–small-cell lung cancer leads to decreased viability, decreased formation of reactive oxygen species, decreased cell motility/migration, and decreased cell signaling to the cytoskeleton (3, 15). SU11274 led to decreased cell growth, motility, and migration and to inhibition of signal transduction by HGF in MPM cell lines. Cell growth of mesothelial cells with HGF was an important determinant to responsiveness to SU11274 (Table 2). As c-Met knockdown with siRNA inhibited cell growth and migration, it indicates that c-Met has an essential role in the cell growth, migration, and progression of mesothelioma. It would now be interesting to pursue the use of inhibitors such as these in vivo. It is also not known if compounds such as SU11274 can synergize with other chemotherapeutic agents such as pemetrexed in MPM.

The results obtained showed that there is a difference in enhancement of growth effects of HGF and the signal transduction effects of HGF in MPM cell lines as shown in Fig. 2 and summarized in Table 2. Interestingly, although the response to HGF in the various cell lines leads to activation of signal transduction, this did not necessarily translate to inhibition of cell growth by the small-molecule inhibitor SU11274. Only cells that were responsive to cell growth by HGF were also responsive to inhibition by SU11274, with the exception of H513 cells. The H513 cell line did not have a growth effect by HGF stimulation. However, by mutational analysis, H513 cells harbored the T1010I c-Met mutation. Despite activation of signaling events by HGF (such as phosphorylation of AKT or ERK1/2), we did not observe increased cell growth in H2052 and H2452 cells. This is of interest because it indicates that in these cells, chronic activation of signaling events involved in a proliferative response eliminates the threshold for ligand-induced proliferation. Nevertheless, it still needs to be determined if there are other biological activities regulated through the c-Met receptor that are regulated by HGF binding.

In two of the cell lines, we also identified a mutation as well as a deletion in the juxtamembrane domain (T1010I). Juxtamembrane domains of RTKs are thought to be key regulators of catalytic functions (32). More recently, the structural basis of the regulatory role (autoinhibition) of the RTK Eph-B2 by the unphosphorylated juxtamembrane domain has been elucidated (32). A germ line mutation of c-Met, P1009S (exon 14), was detected in a patient with gastric carcinoma and is the first such missense mutation to be described affecting the juxtamembrane domain (as opposed to tyrosine kinase domain). The P1009S mutation does not induce ligand-independent activation of c-Met but shows increased persistent response to HGF stimulation when expressed in NIH 3T3 cells (33). Peschand et al. have shown that c-Cbl acts as a negative regulatory protein for c-Met, as well as several other RTKs, by promoting the polyubiquitination of c-Met. The Y1003
juxtamembrane tyrosine, when replaced by phenylalanine, results in the loss of ubiquitination of the Met receptor and has transforming activity in fibroblast and epithelial cells (34). We have also shown that in small-cell lung cancer, R988C and T1010I mutations lead to altered cytoskeletal functions (3). It is unclear at this point what the role of G1085X would be in the pathogenesis of MPM, and this is under evaluation. Besides missense mutations, c-Met-mediated tumorigenesis can be a result of gene amplification, as seen in human gastric carcinoma via the break-fusion-bridge mechanism (35). At this point, it is not known of in some of MPM the gene can be amplified, and this is under evaluation as well.

There were several mutations of the semaphorin domain identified within the MPM tumor tissues. The semaphorin domain is conserved among all semaphorins and is also found present in the plexins and c-Met (36–38). In c-Met, the semaphorin domain is encoded by exon 2. More recently, the three-dimensional conformations of the HGF and heparin-binding sites of c-Met have been established by deletion mutagenesis of the RTK (28). The extracellular ligand–binding domain in the c-Met ectodomain was identified as adopting a seven-blade β-propeller fold for the semaphorin domain of c-Met, homologous to the β-propeller fold template seen in the NH2-terminal domain of α-integrin. These three-dimensional models and functional map of the c-Met ectodomain, along with the mutations identified, would undoubtedly facilitate further development of targeted therapeutics against c-Met.

HGF has recently been shown to be an important serum biomarker in small-cell lung cancer (39). In this study, we also show that HGF can be elevated in patients with MPM. Biomarkers are an important tool in clinical diagnosis, prognosis, and therapeutics. Recently, mesothelin has been shown to be important in MPM (40). It is a highly sensitive positive marker for epithelioid mesotheliomas. There was also a strong statistical correlation between histologic type and HGF expression (41). Similar to mesothelin, sarcomatous differentiated tumors stained much weaker with HGF.

**Figure 6.** Sequence analysis reveals c-Met mutations in human MPM tumors. DNA sequence analysis was done using genomic DNA extracted from mesothelial, MPM cell lines, and MPM tumor samples. Mutations and sequence variants of c-Met were identified in mesothelioma. A, mutations in the MPM mutational analysis are illustrated schematically in the context of the functional domains of c-Met. B and C, samples from patients (T8, T11, T13, and T42; B) and mesothelioma cell lines (HS13 and H2596; C) showed alteration of the c-Met gene. Mutations are indicated in the sequencing chromatogram. No mutations were detected in the normal mesothelial cell line. D, cell lines harboring T1010I mutation exhibited a dramatic reduction of cell growth with SU11274 when compared with wild-type H28 cells and nonmalignant MeT-5A cells.
than their epithelial differentiated counterparts did. It would be important in the future to compare mesothelin levels with HGF levels in MPM.

In conclusion, we show that c-Met is expressed and functional in MPM. c-Met can also be inhibited with siRNA and the small-molecule tyrosine kinase inhibitor SU11274. With further preclinical testing to verify c-Met as an important target in mesothelioma, it would be useful to target this receptor in future clinical trials.

### References


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<td>+</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Cell growth with HGF</td>
<td>+/−</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>p-c-Met (Y1230)/p1835/1837</td>
<td>−</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>p-c-Met (Y1003)</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>p-AKT (S473)</td>
<td>−</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>p-ERK1/2 (pT185/pY187)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
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</table>

### Acknowledgments

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