Increased HGF Expression Induces Resistance to c-MET Tyrosine Kinase Inhibitors in Gastric Cancer

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Abstract. Background: Increased expression of hepatocyte growth factor (HGF) and MET proto-oncogene (c-MET) is associated with poor prognosis in various cancer types. Recently, it was reported that the expression of HGF induces resistance to tyrosine kinase inhibitors (TKIs) targeting epidermal growth factor receptor, human epidermal receptor receptor 2, and b-raf proto-oncogene. Here, we investigated the effects of HGF overexpression in gastric cancer cells in the absence or presence of c-MET TKIs. Materials and Methods: The effects of c-MET TKIs in gastric cancer cells with and without c-MET overexpression were determined in gastric cancer cell lines with various cell biology methods. Results: Compared to the control, cells with induced expression of HGF showed increase in anchorage-independent colony formation (p<0.001). The c-MET TKIs inhibited HGF/c-MET downstream signaling, cell proliferation, migration and invasion, and triggered cell-cycle arrest in Hs746T cells. However, HGF-transfected cells were less affected. Conclusion: c-MET TKIs had inhibitory effects only on cells overexpressing c-MET. Furthermore, overexpression of HGF resulted in resistance to c-MET TKIs through an autocrine manner in gastric cancer cells.

Cancer is the second leading cause of death worldwide (1). Although gastric cancer incidence and death rates have been decreasing, approximately 952,000 cases and 723,000 deaths occurred in 2012. The incidence of gastric cancer in Korea was the highest in 2013 following that of thyroid cancer (2). Receptor tyrosine kinases (RTKs) play major roles in normal growth, mammalian development, and regeneration processes. Dysregulation of RTKs by gene amplification, chromosomal rearrangement, gene mutation and transcriptional up-regulation can result in various human malignancies (3). Among the RTKs, c-MET protein encoded by the MET proto-oncogene, was identified as the receptor of hepatocyte growth factor (HGF) in the 1980s (4). HGF, also known as scatter factor, was originally discovered as a potent mitogen for hepatocytes (5). c-MET and HGF are generally expressed in numerous tissue types, and they are essential for organ development and regeneration (5, 6).

HGF consists of two heterodimeric molecules, an α-chain and a β-chain. The α-chain contains the high-affinity binding site, NK1. NK1 is composed of the amino-terminal hairpin loop (N domain) and the first kringle domain 1 (K1), and can recognize the immunoglobulin region of c-MET. The NK1 dimer induces c-MET dimerization and the β-chain of HGF binds to the semaphorin domain of the receptor, which triggers c-MET activation (7). Activated c-MET phosphorylates adapter proteins, such as growth factor receptor bound protein 2 (GRB2), GRB2-associated binding protein 1 (GAB1), and src homology 2 domain containing transforming protein (SHP), which subsequently mediate activation of other multiple signal transducers, such as phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K), mitogen-activated protein kinase 1 (ERK), mitogen-activated protein kinase 8 (JNK), signal transducer and activator of transcription 3 (STAT3), AKT serine threonine kinase (AKT), nuclear factor-kappa B, phospholipase Cγ, focal adhesion kinase, and β-catenin (8, 9). HGF and c-MET modulate biological functions via these signaling transductions. These diverse functions include regulatory processes of cell spreading, motility, invasion, survival, proliferation, transformation, and angiogenesis (7, 8). However, dysregulation of HGF and c-MET signaling has causative effects on the progression of human cancer. Oncogenic HGF–c-MET signaling promotes tumor cell scattering, dissociation, invasion, tumor growth, and immune tolerance (4, 6). HGF, normally produced in stromal cells, creates a paracrine loop of HGF–c-MET with c-MET overexpression. Moreover, the production of HGF itself in cancer cells activates c-MET in an autocrine manner (6, 10).
According to The Cancer Genome Atlas data, genetic alterations resulting in c-MET and HGF amplification occur in 3.8% and 2.5% of gastric cancers, respectively (11-13). Aberrant expression of c-MET is frequently observed in gastric cancer and patients with c-MET overexpression have poor overall survival compared to those with c-MET-negative tumors (14, 15). In addition, elevated HGF levels are associated with poor survival in patients with gastric cancer (16-18).

There have been a number of studies of RTKs as therapeutic targets, including gefitinib and erlotinib, which are tyrosine kinase inhibitors (TKIs) of epidermal growth factor receptor (EGFR); human epidermal growth factor receptor 2 (HER2) TKIs, including trastuzumab; and imatinib which is a KIT proto-oncogene receptor tyrosine kinase TKI (19). However, de novo resistance to these TKIs occurs because of c-MET amplification or HGF overexpression (20-22). c-MET overexpression contributes to tumor development via crosstalk between other receptors, such as EGFR, HER2, and HER3 (4, 21). Moreover, overexpression of HGF stimulates c-MET activation which trans-phosphorylates other receptors (4). Accordingly, it is important to consider HGF status when therapeutic strategies targeting the HGF–c-MET axis are applied (23).

Many pre-clinical studies have been directed towards the development of anticancer drugs targeting the HGF–c-MET pathway (24). In these studies, several different strategies were used to inhibit HGF and c-MET, including antagonistic HGF truncated isoforms such as NK2 and NK4, neutralizing monoclonal antibodies against HGF or c-MET such as AMG102 and h224G11, and small-molecule c-MET kinase inhibitors such as ARQ197 (7, 25). Among the small-molecule inhibitors of c-MET, JNJ-38877605 and PHA-665752 are highly selective TKIs as well as being ATP-competitive inhibitors (7, 26).

Details regarding the mechanism of HGF-induced resistance to c-MET TKIs remain unclear, especially in gastric cancer. In the present study, we investigated the effects of endogenous HGF overexpression in gastric cancer cells with and without c-MET inhibitors JNJ-38877605 and PHA-665752.

Materials and Methods

Cell lines and patient tissue samples. Human gastric cancer cell lines (IM95, IM95m, Hs746T, MKN1, SNU620, SNU668, and SNU719) were used in this study. IM95 and IM95m cell lines were obtained from the Japanese Collection of Research Bioreresources (JCRB) Cell Bank, and the other cell lines were obtained from Korea Cell Line Bank (Seoul, Korea) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM; HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) and 100 μg/ml of streptomycin (HyClone), and 100 units/ml of penicillin, and 100 μg/ml of streptomycin (HyClone), and cells were cultured at 37°C with 5% CO₂. Surgically resected consecutive gastric cancer tissues and paired normal tissues (n=37) were obtained during surgery from 2009 to 2013 at Seoul National University Hospital.

HGF gene transfection. HGF human cDNA clones and the pCMV6-Entry vector were purchased from Origene (Rockville, MD, USA). Cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s instructions. Twenty-four hours after transfection, cells were washed and selected in G418 sulfate (Gibco-BRL, Grand Island, NY, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen). To generate cDNA, total RNA (1 μg) was reverse transcribed using GoScript™ reverse-transcription system (Promega, Madison, WI, USA) and oligo d(T) primers (Invitrogen) in an ABI Veriti 96-well thermal cycler (Applied Biosystems, Foster city, CA, USA). TaqMan gene expression assays were used for determination of HGF, MET, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems). qRT-PCR analysis was performed in an ABI 7500 real-time PCR system (Applied Biosystems). Relative mRNA expression was normalized with expression of the endogenous control gene GAPDH.

c-MET inhibitor treatment. c-MET inhibitors, JNJ-38877605 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and PHA-665752 (Sigma–Aldrich) were dissolved in distilled water and dimethyl sulfoxide (Sigma–Aldrich) respectively. Their 50% inhibitory concentration values against the vector control and HGF-transfected cells of Hs746T and MKN1 cell lines were calculated using GraphPad Prism program version 5.01 (GraphPad Software Inc., La Jolla, CA, USA).

Western blot analysis and antibodies. All tissue and cell lysates were obtained in Pro-Prep for cell/tissue protein extraction solution (Intron Biotechnology, Seongnam, Korea). The rabbit anti-c-MET (1:100; Santa Cruz), rabbit anti-p-MET (Y1234) (1:100; Santa Cruz), mouse anti-HGF (1:1,000; GalaxyBiotech, Sunnyvale CA, USA), rabbit anti-AKT (1:300; Cell signaling Technology Inc., Beverly, MA), rabbit anti-p-AKT (1:300; Cell Signaling), rabbit anti-ERK (1:500; Cell Signaling), rabbit anti-p-ERK (1:500; Cell Signaling), rabbit anti-β-actin (1:5,000; Sigma–Aldrich) were used as primary antibodies. After overnight incubation with primary antibodies, blots were subsequently incubated with horser anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies. After washing blots, antigens–antibody complexes were visualized using ECL kits (Pierce, Rockford, IL, USA).

Cell growth assay. The transfected cells were seeded at 3x10³ cells per well into 96-well plates. Each cell line was treated with a different concentration of c-MET inhibitor and incubated for 24 h at 37°C. After adding 10 μl of Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Tokyo, Japan) and incubating for 2 h at 37°C, the absorbance at 450 nm was measured using a spectrophotometer (Thermo Labsystems, Beverly, MA, USA).

Migration and invasion assay. The cell migration assay was performed using BD BioCoat Control Cell Culture Inserts in 24-well plates (BD Biosciences, San Jose, CA, USA). For the invasion assay, a BD BioCoat Matrigel Invasion Chamber was used. Cells (1x10⁵) in serum-free RPMI-1640 medium were placed into the upper chamber. RPMI-1640 medium containing 10% fetal bovine serum
with or without inhibitors JNJ-38877605 or PHA-665752, was placed into the lower chamber. After 24 h of incubation, the number of cells on the lower side of the filters were stained with hematoxylin and eosin and then counted under a microscope. All experiments were performed in triplicate.

Wound-healing assay. Transfected cells were seeded and grown to confluence in 60-mm dishes in RPMI-1640 medium. After incubation for 24 h, wounds were created at three places using a sterile tip and the medium was replaced with fresh containing inhibitors. Cells were photographed by microscopy at 24 h and 48 h after incubation. All experiments were performed in triplicate.

Cell-cycle analysis. Cells were fixed in 100% cold ethanol, resuspended in 250 μl of cold phosphate-buffered saline containing 200 μg/ml of RNase and incubated at 37°C for 2 h. After incubation, 40 μg/ml of propidium iodide (Sigma–Aldrich) was added and stained samples were analyzed by flow cytometry on a FACSCalibur (BD Biosciences). All experiments were performed in triplicate.

Anchorage-independent soft agar colony formation. On the bottom of the 35-mm culture dishes, 1% agarose diluted in 2× RPMI-1640 medium containing each of the c-MET inhibitors or vehicle control was deposited. Cells (5x10^3) were added directly to 0.7% agarose in 2× RPMI-1640 medium and overlaid on the bottom layer. Cells were maintained in complete RPMI-1640 medium for 4 weeks. Surviving colonies were stained with 0.005% crystal violet (Sigma–Aldrich) in 20% methanol. Stained colonies were photographed and counted under a microscope.

Immunohistochemistry (IHC). We immunostained tissue microarray (TMA) slides, which represented 59 gastric cancer cases treated at the Seoul National University College of Medicine. All processing was performed using a BOND-MAX automated immunostainer and a Bond Polymer Refine Detection kit (Leica Microsystems, Wetzlar, Germany). IHC staining for HGF and c-MET was performed using mouse anti-HGF (GalaxyBiotech) and rabbit anti-c-MET (Ventana Medical Systems, Tucson, AZ, USA) monoclonal primary antibodies. The intensity of membranous staining was scored as 0: negative, 1: weak, 2: moderate, or 3: strong. Tumors with a score of 0 were considered to have negative expression and tumors with scores 1, 2 and 3 were considered to have positive expression.

Fluorescent in situ hybridization (FISH). To determine c-MET gene copy number of 7q1.2, FISH was performed using the TMA and Vysis LSI D7S486/CEP 7 probe (Abbott Laboratories, Chicago, IL, USA). The Vysis LSI D7S486 probe covering 7q31.1 – 31.3 was used to detect the target, and CEP 7, mapping to 7p11.1 - 7q11.1, was used as a control probe. The signals were detected by fluorescence microscopy. Gene amplification was strictly defined by a mean D7S486/CEP7 copy number ratio of >2.2 (29).

RNA in situ hybridization (ISH). RNA ISH for HGF and c-MET was performed with RNAscope FFPE assay kit (Advanced Cell Diagnostics, Inc., Hayward, CA, USA) according to the manufacturer’s instructions. TMA sections were pretreated with heat and protease digestion followed by hybridization with a target probe for HGF or c-MET. Thereafter, a horseradish peroxidase-based signal amplification system was hybridized to the HGF or c-MET probes before color development with 3,3'-diaminobenzidine tetrahydrochloride. The presence of brown punctate dots was measured as positive staining in the nucleus or cytoplasm. As a positive and negative controls, the housekeeping gene ubiquitin C (UBC) and the bacterial gene DapB, respectively, were used. HGF and c-MET staining was graded on the basis of the percentage of tumor cells positive for HGF or c-MET as follows: grade 0, 0-5%; grade 1, 5-10%; grade 2, 10-25%; and grade 3, 25-100%. Where the percentage of tumor cells expressing HGF or c-MET was more than 5% (grade 1 or more), the sample was considered as positive for HGF or c-MET.

Statistical analysis. All statistical calculations were performed using SPSS PASW Statistics software version 18.0 (SPSS Inc., Chicago, IL, USA). The significance of differences between two groups was determined by Student’s t-test or Fisher’s exact test (two-sided). Pearson’s Chi-square test was used to assess the significance of correlation between two factors. In all tests, the level of significance was set at p<0.05.

Results

Expression of HGF and c-MET in gastric cancer. In order to examine the mRNA expression of HGF in gastric cancer cell lines, we performed qRT-PCR. High HGF expression was observed in both IM95 and IM95m cells but not in SNU620, SNU668, SNU719, Hs746T, and MKN1 cells (Figure 1A). We also validated the mRNA expression of c-MET in gastric cancer cell lines using qRT-PCR (Figure 1B). Hs746T and SNU620 cells had high expression of c-MET mRNA, but IM95, IM95m, MKN1, SNU668, and SNU719 had low expression.

We then selected Hs746T as c-MET-positive cells and MKN1 as c-MET-negative cells. In this study, we transfected HGF gene and control vectors into Hs746T and MKN1 cells. Overexpression of HGF was confirmed by qRT-PCR and western blot analysis after stable cell lines were established. PCR products were loaded into an agarose gel and electrophoresed because the mRNA level of HGF in control cells was too low to be detected using the qRT-PCR system (Figure 1C). HGF expression was increased in HGF-transfected Hs746T and MKN1 cells (Figure 1D). Expression of c-MET in HGF-transfected cells was evaluated by qRT-PCR and western blot analysis. In HGF-transfected cell lines, MET mRNA and protein expression was not increased compared to controls (Figure 1E and F).

Next, we investigated mRNA and protein expression of HGF in gastric cancer tissues. Using western blot analysis, we quantified HGF protein expression in tumor tissues by normalizing the values with their paired normal tissues after adjusting to β-actin expression (Figure 2A). qRT-PCR was performed to determine the mRNA expression of HGF in gastric cancer tissues, and expression of tumors was also normalized with their paired normal tissues (data not shown). High HGF protein expression was observed in 36.4% (12/33) of samples. Fourteen (41.2%) out of 34 samples had high

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mRNA expression of HGF. There was no correlation between HGF protein and mRNA expression (p=0.076) (Figure 2B).

HGF protein and RNA expression was investigated in 59 gastric cancer tissues specimens using IHC (Figure 2C, i-iii) and RNA ISH (Figure 2C, iv-vi). Of these specimens, 83.1% and 84.7% were positive for HGF protein and RNA staining respectively. However, there was no significant correlation between them (p=0.163) (Table I). Protein and RNA expression of c-MET was also examined by IHC (Figure 2D, i-iii) and RNA ISH (Figure 2D, iv-vi) in gastric cancer tissues. Positive staining for c-MET protein and RNA was observed in 6.8% and 28.8% of samples, respectively, and there was significant correlation between them (p=0.005) (Table II). MET gene amplification was detected using FISH in 59 gastric cancer tissue samples (Figure 2E). Among the specimens, MET amplification was detected in only one case (1.7%).

Cell viability of HGF-transfected cells with c-MET inhibitors. Before we treated cells with c-MET inhibitors, we determined the half-maximal inhibitory concentration (IC_{50}) in Hs746T (Figure 3A, upper panel) and MKN1 cells (Figure 3A, lower panel). We treated Hs746T and MKN1 cells with serial concentrations of the c-MET inhibitors and cell viability was investigated for 72 h. MKN1 cells were not affected by either

Figure 1. Expression of hepatocyte growth factor (HGF) and MET proto-oncogene (MET) in gastric cancer cells. mRNA (RT-PCR) expression of HGF (A) and MET (B) in gastric cancer cell lines was evaluated using real-time reverse transcription polymerase chain reaction. Expression of HGF (C) and MET (E) mRNA in MKN1 and Hs746T cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Protein expression of HGF (D) and c-MET (F) was detected by western blot analysis in HGF-transfected Hs746T and MKN1 cells. Data are the mean, error bars represent the SD.
of the c-MET inhibitors regardless of HGF induction. On the other hand, cell survival of Hs746T cells was influenced by the treatment with c-MET inhibitors. The IC_{50} value of c-MET inhibitors was higher against HGF-transfected cells compared with that of the control cells. The IC_{50} of JNJ-38877605 was 46.7 nM against HGF-transfected cells and 22.8 nM against control cells. PHA-665752 IC_{50} was 117 nM against HGF-transfected cells and 48.2 nM against control cells.

Figure 2. Expression of hepatocyte growth factor (HGF) and the MET proto-oncogene (MET) in gastric cancer tissues. A: HGF protein expression in gastric cancer tissues was evaluated by western blot analysis. IM95 cell line was used as a positive control for HGF overexpression. B: Relationship between HGF mRNA and protein expression in gastric cancer tissues. N indicates adjacent normal tissue and T is tumor tissue. Representative immunohistochemistry (i-iii), and RNA in situ hybridization (iv-vi) for HGF (C) and MET (D) expression in gastric cancer tissues. i: Adjacent normal tissues displaying negative protein expression. Gastric carcinomas displaying negative (ii) and positive (iii) expression of protein. iv: Adjacent normal tissues showing negative expression of RNA. Gastric carcinomas displaying negative (v) and positive (vi) RNA expression. E: Fluorescent in situ hybridization analysis of gastric cancer tissues. Gastric carcinomas showing positive (left) and negative (right) MET amplification. The MET signal is presented in red and the endogenous control signal is shown in green. Magnification, ×100.
We investigated the effect of c-MET inhibitors on the growth of Hs746T cells in a time-dependent manner (Figure 3B). Cells were treated with 50 nM of c-MET inhibitor or control vehicle. A cell viability assay using CCK-8 was then performed every 12 h. It appeared that the growth of HGF-transfected cells at 72 h was higher than that of the control cells in the absence of c-MET inhibitor; however, this finding was not significant. The cell growth differences between control and HGF-transfected cells were significant when they were treated with c-MET inhibitors. Especially, the growth was significantly different when the exposure time of JNJ-38877605 increased from 48 h to 72 h (both \( p<0.001 \)). PHA-665752 had a significant impact on cell growth when cells were treated with it for 60 h (\( p=0.013 \)) or 72 h (\( p<0.001 \)).

These results suggested that c-MET inhibitors had an inhibitory effect on cell survival only in the cells harboring c-MET expression. Therefore, not MKN1 cells, but Hs746T cells were affected by c-MET inhibitors. In addition, HGF-transfected Hs746T cells were less sensitive to the c-MET inhibitors.

Effect of HGF expression on HGF–c-MET signaling with c-MET inhibition. To determine the effects of HGF expression on HGF–c-MET downstream molecules, we treated Hs746T and MKN1 cells with 0, 25, and 50 nM of c-MET inhibitor. Cells were then harvested after 24 h and 48 h following JNJ-38877605 or PHA-665752 treatment and western blot analysis was performed. As shown in Figure 4A, c-MET activation was not increased in HGF-transfected Hs746T cells and the treatment with JNJ-38877605 had no effect on HGF expression. In control cells, 24-h treatment with JNJ-38877605 resulted in a dose-dependent decrease of p-MET expression (Figure 4A, left). In HGF-transfected cells, p-MET was slightly inhibited by JNJ-38877605 following 24-h treatment. The inhibitor triggered inhibition of p-AKT and p-ERK in both control and HGF-transfected cells after 24 h. However, the expression of p-AKT and p-ERK in HGF-transfected cells was higher than that of the control cells. This result suggests that induction of HGF expression mediates c-MET activation and reduces sensitivity to the inhibitor. In addition, JNJ-38877605 treatment for 48 h showed that p-MET expression was more effectively inhibited in control cells, but remained in HGF-transfected cells because of resistance (Figure 4A, right). The results from PHA-665752 treatment were consistent with those of JNJ-38877605 treatment (Figure 4B). Treatment with PHA-665752 reduced expression of p-MET and its downstream molecules, and HGF-transfected cells were less sensitive. The inhibitory impact of PHA-665752 on HGF–c-MET downstream molecules was more effective at 24 h than 48 h.

MKN1 control and HGF-transfected cells were also treated with c-MET inhibitor (Figure 4C). The phosphorylation of ERK declined using 50 nM of PHA-665752 for 24 h in MKN1 control and HGF-transfected cells. Nevertheless, except for that change, there was no remarkable difference induced by transfection. As a result, MKN1 cells did not respond to the c-MET inhibitors. The c-MET inhibitors were unlikely to block HGF–c-MET signaling because of low c-MET expression in MKN1 cells.

Taken together, these data indicate that PHA-665752 and JNJ-38877605 have an inhibitory impact on activation of HGF–c-MET downstream molecules in cells with c-MET expression. HGF-transfected Hs746T cells were less sensitive to c-MET inhibitors compared to control cells.

Migration and invasion of HGF-transfected cells with c-MET inhibitors. In order to validate the effects of altered HGF expression on cell migration and cell invasion, we seeded Hs746T cells on migration and invasion chambers with and without c-MET inhibitors. In the absence of inhibitors, induced HGF expression did not affect cell migration (Figure 5A). On the other hand, c-MET inhibitors effectively reduced the number of migrating control cells compared to HGF-transfected cells (\( p<0.001 \)).

Results from the Matrigel invasion assay showed that HGF expression prevented the inhibitory effect of c-MET inhibitor (Figure 5B). The expression of HGF did not have an impact on cell invasion when there was no inhibitor present. Treatment with inhibitor caused a decrease in invasion by control cells. When treated with c-MET inhibitor, the number of invading cells was significantly higher in HGF-transfected cells than control cells (\( p<0.001 \)).
In concordance with the results from the migration assay, the wound-healing assay revealed that the c-MET inhibitors inhibited migration of control cells but not of HGF-transfected cells (Figure 5C). These data indicate that the expression of HGF has a protective effect on cell migration and cell invasion against the inhibitor.

Protective effect of HGF expression on cell-cycle arrest and colony formation in soft agar. To identify whether c-MET inhibition affects cell-cycle regulation, we performed a cell-cycle analysis of Hs746T cells using flow cytometry. The cell population in each phase was not affected by HGF expression in the absence of inhibitor (Figure 6A and B). In control cells, both inhibitors induced the accumulation of cells in G0/G1 phases and reduced the percentage in the G2/M phase. However, the inhibitors had no impact on the cell cycle in HGF-transfected cells. The differences between control and HGF induced cells were statistically significant ($p<0.001$). These results suggest that Hs746T cells underwent a G0/G1 growth arrest in response to JNJ-38877605 and PHA-665752 treatment. Furthermore, the expression HGF had a protective effect on cell-cycle arrest in the presence of inhibitors.

Next, a soft agar colony formation analysis was conducted in order to examine single-cell proliferation in an anchorage-independent manner. The results from the colony formation analysis showed that the expression of HGF significantly promoted clonogenicity ($p<0.001$) (Figure 6C). The number of colonies of control and HGF-transfected cells was reduced by treatment with JNJ-38877605.

Discussion

Aberrant activation of the HGF–c-MET pathway promotes tumorigenesis and is associated with cancer cell invasion, lymph node metastasis, and poor prognosis (6, 27). In many studies, it has been reported that overexpression of HGF and c-MET contributes to resistance to other therapies, such as TKIs targeting EGFR, BRAF, and HER2, as well as chemotherapy and radiotherapy (21, 28). Therefore, HGF–c-MET-targeting therapies have emerged as attractive strategies, and a large number of clinical trials involving c-MET TKIs are ongoing (10, 25, 28). However, in the case of c-MET TKIs, the inhibitory effect is valid only in cells harboring constitutively activated c-MET (26, 29). Among c-MET-negative gastric cancer cell lines, IM95 cells are sensitive to c-MET TKIs (10). This is because IM95 cells have high expression of HGF, which triggers c-MET activation in an autocrine manner and responses to c-MET TKIs (10, 30). In this study, the effect of altered HGF expression on c-MET inhibition was analyzed in terms of functional significance in gastric cancer cells.
Figure 4. Effect of hepatocyte growth factor (HGF) and MET proto-oncogene (MET) tyrosine kinase inhibitors on molecules downstream of HGF–c-MET. The expression of HGF–c-MET downstream molecules was detected by western blot analysis. Hs746T control and HGF-transfected cells were treated with JNJ-38877605 (A) or PHA-665752 (B) in a dose-dependent manner for 24 h and 48 h. C: MKN1 cells were treated with c-MET inhibitors, JNJ-38877605 and PHA-665752.
HGF expression in gastric cancer cell lines was determined using qRT-PCR and western blot analysis. We confirmed that IM95 and IM95m cells showed high levels of HGF expression as mentioned above and other cell lines were negative for HGF. The expression of c-MET was also screened in gastric cancer cells. We then selected MKN1 and Hs746T cells, which had low expression of HGF, in order to establish stable HGF-transfected cells.

Figure 5. Effect of hepatocyte growth factor (HGF) expression on cell migration and cell invasion. A: Cell migration was analyzed using pore chambers. B: Cell invasion analysis was performed using Matrigel chambers. C: Cell migration was investigated using a wound-healing assay. Forty-eight hours after scratching the cells, wound healing was abrogated in the presence of inhibitor only in control cells. Data are the mean, error bars represent the SD. *Significantly different at p<0.001.
In concordance with previous studies, Hs746T cells overexpressing c-MET were sensitive to c-MET TKIs, JNJ-38877605 and PHA-665752, and MKN1 cells were not responsive. In HGF-transfected cells, the growth of Hs746T cells, but not MKN1 cells was inhibited by c-MET inhibitors. The activation of HGF–c-MET downstream

Figure 6. Effect of hepatocyte growth factor (HGF) expression on cell-cycle regulation and anchorage-independent colony formation. Cells were treated with inhibitors JNJ-38877605 (A) or PHA-665752 (B) or control vehicle. After 24 h, cells were stained with propidium iodide and cell-cycle analysis was performed using flow cytometry. C: Cells were seeded in soft agar with or without inhibitors. After 4 weeks of incubation, cells were stained with 0.005% crystal violet, and the number of colonies was counted. Data are the mean, error bars represent the SD.*Significantly different at p<0.001.
suppresses HGF-stimulated cell mitogenesis and induces phosphorylation of c-MET and its downstream molecules for AKT and p-ERK, and suppresses anchorage-independent differentiation, cell survival, and motility by inhibiting p-AKT and p-ERK, and suppresses anchorage-independent growth of gastric, lung, and glioma cancer cell lines (4, 6).

In cancer cells with MET amplification and overexpression, c-MET is constitutively activated regardless of HGF status (26). Meanwhile, c-MET activation becomes HGF-dependent where the kinase activity of c-MET is inhibited, which suggests that HGF is implicated in resistance to drugs targeting c-MET (28). In the present study, the protein level of phosphorylated c-MET was not augmented by altered HGF expression because c-MET is basically activated in Hs746T cells. We also observed that c-MET phosphorylation was inhibited by c-MET inhibitors in HGF-transfected Hs746T cells. The expression of HGF–c-MET downstream molecules, p-AKT and p-ERK, was inhibited in HGF-transfected cells by treatment with inhibitors. This means that the activation of c-MET became dependent on HGF in the presence of inhibitors. Consistent with these findings, functional assays indicated that the overexpression of HGF did not influence cell migration, invasion, or cell cycle in the absence of inhibitors. On the other hand, all these functions declined in control cells treated with inhibitors. Although treatment with JNJ-38877605 reduced cell invasion in HGF-transfected cells, this inhibition was more effective in control cells and the difference was statistically significant. Additionally, the inhibitors did not have any impact on cell migration and cell-cycle arrest in HGF-transfected cells.

JNJ-38877605 and PHA-665752 are ATP-competitive small-molecule c-MET inhibitors. Such molecules block the ATP-binding site competitively in the kinase domain of the c-MET receptor (31). It was reported that another ATP-competitive c-MET inhibitor, tivantinib (ARQ 197), has the ability to delay the start of the ATP-related reaction, nevertheless autophosphorylation eventually rises after a lag phase (32). In this study, the inhibitory effects of JNJ-38877605 and PHA-665752 on c-MET downstream molecules were more effective after 24 h than after 48 h. This is because the inhibitors effectively blocked phosphorylation of c-MET and its downstream molecules for 24 h but not as long as 48 h.

It was reported that highly selective PHA-665752 suppresses HGF-stimulated cell mitogenesis and induces G1/S arrest in the cell cycle (6). Although the inhibitory mechanisms of PHA-665752 are different for each cell type, PHA-665752 generally regulates morphogenic differentiation, cell survival, and motility by inhibiting p-AKT and p-ERK, and suppresses anchorage-independent growth of gastric, lung, and glioma cancer cell lines (4, 6).

In the present study, the anchorage-independent soft agar colony formation assay showed that the number of colonies was higher in HGF-transfected Hs746T cells compared to that of the control cells, even in the absence of inhibitors. The differences between the control and HGF-transfected cells was still remarkable following exposure to inhibitors. Since HGF expression is required for anchorage-independent colony formation (25), we assumed that it was not sufficient for Hs746T control cells to form colonies in soft agar, as they only had overexpression of c-MET not HGF.

In conclusion, we found that HGF, through an autocrine effect, promoted anchorage-independent colony formation in gastric cancer cells which harbored amplification and overexpression of MET. Moreover, HGF overexpression conferred resistance to TKIs targeting c-MET. Cells with overexpression of HGF were less sensitive to inhibition of c-MET TKIs on cell growth, HGF–c-MET downstream signaling, cell migration, cell invasion, colony formation, and cell-cycle regulation. More studies are necessary to reveal the mechanisms underlying HGF-induced intrinsic and acquired resistance to TKIs, including c-MET TKIs.

References


