Targeting FGFR pathway in human hepatocellular carcinoma (HCC) expressing pFGFR and pMET for anti-tumor activity.

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Abbreviation list: FGFR: Fibroblast growth factor, HCC: Human hepatocellular carcinoma, HGF: hepatocyte growth factor, FRS2: fibroblast growth factor receptor substrate 2

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Abstract

The MET receptor tyrosine kinase, the receptor for hepatocyte growth factor (HGF), has been implicated in cancer growth, invasion, migration, angiogenesis, and metastasis in a broad variety of human cancers, including human hepatocellular carcinoma (HCC). Recently, MET was suggested to be a potential target for the personalized treatment of HCC with an active HGF/MET signaling pathway. However, the mechanisms of resistance to MET inhibitors need to be elucidated in order to provide effective treatment. Here, we show that HCC cells exhibit different sensitivities to the MET inhibitor PHA665752, depending on the phosphorylation status of fibroblast growth factor receptor (FGFR). Treatment of cells expressing both phospho-FGFR and phospho-MET, with the inhibitor PHA665752, did not cause growth inhibition and cell death, whereas treatment with AZD4547, a pan-FGFR inhibitor, resulted in decreased colony formation and cleavage of caspase-3. Moreover, silencing of endogenous FGFR1 and FGFR2 by RNA interference of HCC cells expressing phospho-FGFR, phosph-FGFR2, and phospho-MET, overcame the resistance to PHA665752 treatment. Treatment of primary cancer cells from patients with HCC expressing both phospho-FGFR and phospho-MET with PHA665752 did not induce cell death, whereas AZD4547 treatment induced cell death through the cleavage of caspase-3. In addition, treatment of cells resistant to PHA665752 with AZD4547 abrogated the activation of downstream effectors of cell growth, proliferation, and survival. Based on these results, we conclude that the FGFR pathway is critical for HCC survival, and that targeting this pathway with AZD4547 may be beneficial for the treatment of patients with HCC expressing phospho-FGFR and phospho-MET.
Introduction

Human hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide (1). The prognosis of patients with HCC remains poor, with a 1-year survival rate less than 50%, despite recent improvements in overall survival. Treatment with a molecular targeted agent, the multikinase inhibitor sorafenib, has improved the overall survival in patients with HCC, and sorafenib is the only targeted agent shown to prolong the overall survival in randomized controlled trials (2, 3). Nevertheless, sorafenib has demonstrated modest efficacy in patients with HCC in clinical practice. Therefore, a better understanding of the cellular mechanisms in the development and proliferation of HCC is essential.

Hepatocyte growth factor (HGF)-induced activation of the receptor tyrosine kinase, MET, triggers a variety of cellular responses, including proliferation, survival, cytoskeleton rearrangements, cell-cell dissociation, and motility (4). Although many studies have demonstrated that MET overexpression is associated with poor outcomes in HCC (5), definitive evidence that MET inhibition is an effective strategy for the treatment of HCC has not been established. However, You et al. suggested that MET is a potential target for the personalized treatment of HCC with active HGF/MET signaling, by demonstrating that inhibition of MET can significantly inhibit the growth of MET positive HCC tumors (6).

Preclinical data have demonstrated that FGFR-mediated signaling may play a role in the cellular mechanisms underlying the pathogenesis of HCC (7). The main FGFRs expressed in liver tissues are FGFR3 and FGFR4, and several studies have suggested that these receptors may be involved in HCC tumorigenesis (8, 9). Moreover, the levels and activities of several FGFRs are known to be altered in HCC. For example, somatic mutations of FGFR1 have been found in several types of cancers, including gliomas and lung tumors (10, 11). In the case of FGFR2, its mutations have been found in endometrial carcinomas and gastric cancers (12, 13), whereas those of FGFR3 or FGFR4 have been found in bladder carcinomas, multiple myelomas (14-16), and primary rhabdomyosarcomas (17).

Overexpression of the FGFR2 protein in tumors has been associated with advanced clinical
stages (18). Another study reported that FGFR2 overexpression in HCC tumors was associated with a high Ki-67 proliferation index and high levels of serum alpha-fetoprotein, and was linked to the development of multiple sites of metastasis (19). In addition, overexpression of FGFR3 has been associated with a higher grade and poorer differentiation status in HCC tumors, suggesting that increased FGFR3 expression may contribute to advanced HCC tumorigenesis (20). Thus, FGFRs may act as potent regulators in a variety of cancers. A study evaluating clinical HCC samples strongly suggested that FGFR4 was important for the proliferation and survival of HCC tumor cells, as well as for the secretion of alpha-fetoprotein (21).

Although the biological roles of the inter-receptor tyrosine kinase signaling networks are still unclear, FGFR and MET have been recently reported to interact with each other to promote cell growth or acquired resistance to specific inhibitors in gastric cancer cell lines (22). In the present study, we therefore evaluated the response of HCC cell lines and primary HCC cells to a MET inhibitor, to determine whether inhibition of the FGFR pathway can overcome the resistance to MET inhibitors.
Materials and Methods

Cell culture and reagents

All human liver carcinoma cells were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA) who authenticated the identity of the cell lines, except for SNU449 and SNU475, which were obtained from the KCLB (Korean Cell Line Bank, Seoul, Republic of Korea). All human liver carcinoma cell lines were maintained in RPMI-1640 medium or DMEM medium (GIBCO BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; GIBCO BRL), 100 units penicillin, and 100 μL/mL streptomycin in a 5% CO2 incubator at 37°C. PHA665752, a specific MET inhibitor, was purchased from Selleck Chemicals LLC (Houston, TX, USA), and AZD4547, a pan-FGFR inhibitor, was kindly provided by AstraZeneca. Co., Ltd. (Macclesfield, Cheshire, UK).

Human primary hepatocarcinoma cell culture

Specimens of human primary hepatocarcinoma cancer tissues were obtained from patients after written informed consent and approval by the Research Ethics Board at Asan Medical Center. The study protocol followed the tenets of the Declaration of Helsinki. Tumor specimens were minced with scissors, and digested by incubation in Minimum Essential Medium (MEM) (GIBCO BRL) containing 1 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37°C. Cells were washed with medium containing 10% FBS, followed by washing with phosphate-buffered saline (PBS, pH 7.4) to remove FBS. Next, the cells were plated in Human Hepatocyte Basal Medium (HBM) (Lonza, Walkersville, MD, USA) and cultured in a humidified incubator under 5% CO2 at 37 °C.

Cell viability

Cells (3 × 10⁵ cells per plate) were seeded in 60 mm plates and treated with various amounts of PHA665752 or AZD4547 for 48 h. Cell viability was determined using the trypan blue dye exclusion method.
Clonogenic assay

SNU449 and SK-Hep-1 cells, seeded at 3 × 10^2 cells per well in 6-well plates, were treated with AZD4547 or PHA665752 at a concentration of 1 µM for 24 h. The cells were cultured for 14 days. Colonies were fixed with 10% formalin and stained with 0.01% crystal violet dye solution. The numbers of colony-forming cells with a diameter ≥1 mm were counted.

DNA, siRNA, and transfection

MET, FGFR-1, and FGFR-2 cDNAs were purchased from Origene (Rockville, MD, USA). The mutated MET T1191I DNA was generated using a polymerase chain reaction (PCR)-based quick change site-directed mutagenesis kit (Intron Biotechnology, Seoul, Republic of Korea) and wild type MET. All DNA transfections were performed using Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. For siRNA transfection, cells were transfected using Lipofectamine® RNAi MAX reagent (Invitrogen) according to the manufacturer’s protocol. The siRNA sequences for the transfection study were the following: FGFR1-siRNA, 5’-GAA GUG CAU ACA CCG AGA C-3’; FGFR2-siRNA, 5’-AAG UGC UGG CUC UGU UCA AUG-3’; FGFR3-siRNA, 5’-AGA CGA TGC CAC TGA CAA G-3’; and FGFR4-siRNA, 5’-AUA CGG ACA UCA UCC UGU A-3’.

Immunoblot and immunoprecipitation

Total cellular proteins (20 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk in TBS-T buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) and incubated with the following antibodies: anti-Met, anti-FGFR2, and anti-β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phospho-Met (Y1234/1235), anti-phospho-FGFR (pan-pFGFR), anti-phospho-FRS2 (pFRS2), anti-phospho-AKT,
anti-phospho-ERK, anti-phospho-tyrosine, anti-AKT, anti-ERK, and anti-caspase-3 antibodies (Cell Signaling, Beverly, CA, USA); anti-FGFR1 and anti-AFP antibodies (Abcam, Cambridge, MA, USA); and anti-FGFR3 and anti-FGFR4 (Abnova Corporation, Taipei, Taiwan) antibodies. Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Cell Signaling), as appropriate, and immunoreactive proteins were visualized using an enhanced ECL detection reagent (GE Healthcare Bio-Sciences, Uppsala, Sweden).

For immunoprecipitation analyses, cell lysates were immunoprecipitated with anti-FGFR-1, anti-FGFR2, anti-FGFR3, or anti-FGFR4 antibodies for 12 h at 4°C. Protein A/G Plus-Sepharose beads (20 μL) (Santa Cruz Biotechnology) were then added, and the mixtures were incubated for an additional 2 h at 4°C. The immunoprecipitates were washed with Nonidet P-40 lysis buffer, boiled in 2× SDS sample buffer, and analyzed by western blot analysis using anti-phospho-tyrosine antibody.

**Immunofluorescence analysis**

Human primary hepatocarcinoma cells were seeded on glass coverslips. The cells were fixed in 4% paraformaldehyde, and permeabilized-blocked, then incubated with primary antibodies. Following washing, the cells were incubated with Alexa488- or Alexa594-labeled secondary antibodies, and the cells were stained briefly with DAPI. After washing, the coverslips were mounted on glass slides using mounting solution. The slides were analyzed by confocal microscopy (Carl Zeiss, Jena, Germany).

**Immunohistochemistry**

The pMET and pFGFR in tumor tissues and tissue microarrays (TMAs) were detected using immunohistochemistry (IHC). The HCC TMAs were purchased from US Biomax, Inc. (Rockville, MD, USA). Immunohistochemistry was performed according to the manufacturer’s protocol. Antigen retrieval was performed by heating the samples in citrate buffer (pH 6.0) in a microwave. The reaction products were developed with diaminobenzidine (Dako, Hamburg, Germany), followed by
counterstaining with hematoxylin.

Statistical analysis

All data were analyzed using two-tailed Student’s *t*-tests. The level of statistical significance was set at *P*<0.05.
Results

Human hepatoma cancer cells express resistance to a MET inhibitor dependent on the phosphorylation status of FGFR.

It was recently reported that FGFR-expressing gastric cancer cells are sensitive to a MET inhibitor (22), implying that some association exists between MET and FGFR against the sensitivity to the MET inhibitor in a variety of cancer cells. We therefore investigated the dependence of the inhibitory effects of a MET inhibitor on the status of FGFR in human hepatoma cell lines. First, we examined the phosphorylation status of MET and FGFR, including the expression levels of both proteins in multiple human hepatoma cell lines. Two of them, SK-Hep-1 and SNU449 cells, expressed both phospho-MET and phospho-FGFR (Fig. 1A). We examined the inhibitory effects of treatment with a MET inhibitor (PHA665752) or a pan-FGFR inhibitor (AZD4547) in various human hepatoma cell lines. The results demonstrated that SNU449 and SK-Hep-1 cells were sensitive to AZD4547, exhibiting IC\textsubscript{50} values of 84 nM and 92 nM, respectively. By contrast, these cell lines were resistant to PHA665752. Other cell lines were resistant to the two compounds (Fig. 1B). Based on the IC\textsubscript{50} data, we determined the concentration of AZD4547, and in parallel, determined the concentration of PHA665752. These human hepatoma cell lines were selected to determine whether the differences in sensitivities to MET inhibitor were dependent on the presence of phospho-FGFR, and were therefore treated with various doses of each inhibitor that targets MET or FGFR. The results showed that cell growth was inhibited by AZD4547 at lower concentrations, and cell death was induced at higher concentrations (>1 µM) (Fig. 1C and Supplementary Fig 1). Notably, there was almost no change in the population of dead cells in response to PHA665752, a selective MET inhibitor, whereas the population of dead cells after the treatment with AZD4547, a pan-FGFR inhibitor, dramatically increased (Fig. 1C, i). AZD4547 also induced the cleavage of pro-caspase-3 to yield active caspase-3, but not in cells treated with PHA665752 (Fig. 1C, ii). To analyze the effects of AZD4547 or PHA665752 on caspase-3 cleavage, we repeated the western blot analysis. AZD4547 treatment at 1 or 2 µM led to caspase-3 cleavage (Fig. 1C, lower panel). To further characterize the inhibitory effects of
each inhibitor, AZD4547 or PHA665752, on cells that express both phospho-MET and phospho-FGFR, we performed colony-forming assays. Colony formation in SNU449 and SK-Hep1 cells was significantly reduced by AZD4547, whereas a decrease in colony formation was not observed with PHA665752 in both cell lines (Fig. 1D). Taken together, the results indicate that differences in sensitivity to MET inhibitor are dependent on the phosphorylation status of FGFR in human hepatocarcinoma cells.

**MET inhibitor does not inhibit downstream signaling in hepatocarcinoma cells expressing pMET and pFGFR.**

As shown in Fig. 1, SNU449 and SK-Hep1 cells were insensitive to PHA665752, a selective MET inhibitor, whereas both cell lines were sensitive to AZD4547, a pan-FGFR inhibitor. Based upon these results, we investigated the effects of each inhibitor on their downstream signaling pathways. Treatment with AZD4547 caused a decrease of FRS2 (fibroblast growth factor receptor substrate 2), AKT, and ERK phosphorylation in SNU449 cells. However, phosphorylation of these proteins did not decrease after exposure to PHA665752 (Fig. 2A). AZD4547 also blocked the phosphorylation of FRS2, AKT, and ERK in SK-Hep1 cells, whereas PHA665752 did not (Fig. 2B). These results indicated that inhibition of FGFR inhibits the phosphorylation of the downstream signaling molecules FRS2, AKT, and ERK in hepatoma cells expressing pMET and pFGFR.

**FGFR inhibitor, but not MET inhibitor, causes inhibition of FGFR1 or FGFR2 phosphorylation in human hepatocarcinoma cells**

We examined the phosphorylation status of FGFR1, FGFR2, FGFR3, and FGFR4 in SNU449 and SK-Hep1 cells that express both pMET and pFGFR. Notably, phosphorylation of FGFR1 and FGFR2 was observed in both cell types, whereas FGFR3 and FGFR4 phosphorylation was not detected (Fig. 3A), implying that phospho-FGFR1 and FGFR2 may affect sensitivity to the MET inhibitor. We next examined the inhibition of FGFR1 and FGFR2 phosphorylation after treatment of
these cells with AZD4547 or PHA665752 using an immunoprecipitation assay. AZD4547 treatment led to decreases in both p-FGFR1 and FGFR2 in the two selected cell lines, but PHA665752 treatment did not induce a decrease (Fig. 3B).

Silencing of FGFR1 or FGFR2 overcomes the resistance to the MET inhibitor.

We next investigated the effects of members of the FGFR family on the sensitivity to MET inhibitor. We analyzed the dependence of FGFR1 or FGFR2 on the sensitivity to MET inhibitor using constructs expressing FGFR1 or FGFR2 small interfering RNA (siRNA). Initially, SNU449 cells were transfected with FGFR1-siRNA or scrambled siRNA, followed by PHA665752 treatment. The dead cell population was significantly increased in FGFR1-siRNA-treated cells following PHA665752 treatment, but not in scrambled siRNA-treated cells (Fig. 4A, i). Additionally, the cleavage of caspase-3 was significantly increased in FGFR1-siRNA-treated cells following PHA665752 treatment, and the phosphorylation level of FRS2 was decreased after exposure to PHA665752 (Fig. 4A, ii). The results showed that silencing of FGFR1 in SK-Hep1 cells increases cell death (Fig. 4B), indicating that inhibition of FGFR1 can overcome the resistance to MET inhibitor.

As shown in Fig. 4A and B, SNU449 and SK-Hep1 cells expressed phospho-FGFR2 as well as phospho-FGFR1. Thus, we investigated the effect of depletion of FGFR2 by siRNA on the sensitivity to the MET inhibitor. Knockdown of FGFR2 led to increased cell death in SNU449 (Fig. 4C, i) and SK-Hep1 cells (Fig. 4D, i) in response to PHA665752, but not in scrambled siRNA-treated cells, as seen with FGFR1-siRNA. The cleavage of caspase-3 also increased in FGFR2-siRNA-treated cells (Fig. 4C, ii and D, ii), indicating that inhibition of FGFR2 can overcome the resistance to MET inhibitor. To further confirm these conclusions, we co-transfected cells that coexpressed pMET and pFGFR with FGFR1- and 2-siRNA. Cell death was significantly higher in cells co-transfected with FGFR1- and 2-siRNA than in cells transfected with FGFR1- or 2-siRNA (Figs. 4E, i and F, i). Double knockdown also significantly increased the cleaved form of caspase-3 compared with single knockdown (Figs. 4E, ii and F, ii). We next examined the effects of FGFR3 and FGFR4 knockdown...
on the sensitivity to a MET inhibitor. Silencing FGFR3 and FGFR4 did not increase cell death in the absence of PHA665752 (Supplementary Fig. 2), indicating that FGFR3 and FGFR4 do not affect sensitivity to a MET inhibitor. In addition, MET inhibition induced cell death in the absence of AZD4547 (Supplementary Fig. 3). These results indicate that FGFR1 and 2 silencing enhances MET inhibitor sensitivity in human hepatocarcinoma cells.

**FGFR1 or FGFR 2 confers resistance to the MET inhibitor.**

To further characterize the dependence of FGFR1 or FGFR2 on the sensitivity to the MET inhibitor, we co-transfected PLC/PRF5 cells that did not express pMET and pFGFR, with a construct expressing FGFR1 cDNA and a MET CA (constitutively active) mutant or with FGFR2 cDNA and a MET CA mutant, followed by treatment with PHA665752 or AZD4547. There was almost no induction of cell death induced by PHA665752 in cells expressing FGFR1 cDNA and a MET CA mutant, whereas treatment of AZD4547 led to increased cell death (Fig. 5A, i). Similarly, the dead cell population in cells expressing FGFR2 cDNA and a MET CA mutant was significantly increased by AZD4547, but not by PHA665752 (Fig. 5B, ii). Additionally, caspase-3 cleavage was significantly increased in cells expressing FGFR1 or FGFR2 cDNA and a MET CA mutant following AZD4547, but not PHA665752, treatment (Fig. 5A, i and B, ii). Moreover, c-MET altered the growth of these cells (Supplementary Fig. 4). Together, these results indicate that FGFR1 and FGFR2 activities are associated with resistance to the MET inhibitor in human heparocellular carcinoma cells expressing pMET and pFGFR.

**AZD4547 induces cell death in human primary hepatocarcinoma cells expressing pMET and pFGFR.**

Based on the above results, we examined the effect of each inhibitor, PHA665752 or AZD4547, on primary cancer cells from patients with hepatocarcinomas. We first analyzed the phosphorylation status of MET or FGFR. Two out of 14 primary HCC cells, 78019A and 3226B (patient numbers),
coexpressed pMET and pFGFR (Fig. 6A, i). We then performed immunofluorescence analysis on primary HCC cells. The 78019A and 3226B cells expressed pMET, pFGFR, and AFP (Fig. 6A, ii). Human primary HCC cells, which include stromal cells and tumor cells, were obtained from HCC patient tissues. We analyzed the drug sensitivity of cells in the absence or presence of Hep-Par-1 as a HCC marker. Approximately 18% of human primary HCC cells were found to be Hep-Par-1 positive. AZD4547 treatment led to a dose-dependent caspase-3 cleavage in Hep-Par-1 positive cells, but not in Hep-Par-1 negative cells. The Hep-Par-1 negative cells were almost always stromal cells, with no induction of cell death by treatment with AZD4547. These results were confirmed by immunofluorescence analysis, showing that Hep-Par-1 positive cells displayed sensitivity to AZD4547 at 1, 2, and 5 μM (Fig. 6B and C). In addition, AZD4547 induced a decrease of pFRS2, but not pMET, in 3226B and 78019A cells (Fig. 6D and E), indicating that AZD4547 can induce cell death in human primary hepatocarcinoma cells expressing pMET and pFGFR.

Next, we examined the inhibitory effect of each inhibitor on human primary hepatocarcinoma cells expressing pMET alone. PHA665752 induced a decrease of pMET, indicating that the MET inhibitor can act on human primary hepatocarcinoma cells expressing pMET alone (Fig. 6F and G). However, the concentration at which significant induction of caspase-3 cleavage was observed may not be clinically achievable with AZD4547.
Discussion

The receptor tyrosine kinase, MET, that controls cell growth, proliferation, survival, and motility, is constitutively activated and amplified in several cancer types, including non-small cell lung cancers, hepatocarcinomas, and gastric cancers (23). Thus, MET is considered a clinically important target for therapies against these types of cancers. Agents targeting MET have been evaluated and have recently entered clinical trials. Major problems of therapies targeting tyrosine kinases involve the lack of response to treatment of many tumors, and/or the drug resistance that eventually develops. In the present study, we show that AZD4547, a pan-FGFR inhibitor, can overcome the resistance to MET inhibitor in human hepatocarcinoma cells.

It was recently reported that activation of RTK (receptor tyrosine kinase) in gastric cancer cells mediates resistance to MET inhibition, implicating that some association exists between RTK and MET during the induction of drug resistance. Specifically, Kataoka et al. reported that TKI appears to be more effective against human gastric cancer, by blocking inter-RTK signaling networks that depend on MET or FGFR2 (22). Foretinib (GSK1363089), a multi-kinase inhibitor of MET and VEGFRs, shows different sensitivities that are dependent on FGFR expression (22). The FGFR inhibitor that has been most extensively studied in patients with HCC is brivanib, an ATP-competitive dual inhibitor of VEGFR and FGFR1-3 (24). Despite strong preclinical and phase II data of HCC (25, 26), brivanib failed in large randomized phase III trials in both the first- and second-line settings for patients with advanced HCC (27, 28). Nonetheless, FGFR inhibition remains an attractive therapeutic target for HCC, and requires further investigation of its possible clinical applications. Efforts continue to explore the use of multikinase inhibitors that also target FGFR in patients with advanced HCC, and ongoing first-line trials include a randomized phase III study of lenvatinib compared with sorafenib, a randomized phase II trial of dovitinib compared with sorafenib in the Asia-Pacific region, and a phase I/II trial of nintedanib in combination with sorafenib. Conversely, our findings have shown that a pan-FGFR inhibitor, AZD4547, appears to be more effective than a selective MET inhibitor, PHA665752, against HCC expressing phospho-MET and phospho-FGFR. Thus, the inhibition of MET in
hepatocarcinoma cells expressing both pMET and pFGFR has not been effective, unlike that in human gastric cancer cells. Although the most robust effects on inducing cell death in HCC cell lines were seen at concentrations at or above 1 µM, which might be above clinically achievable concentrations of AZD4547, significant effects were also seen at lower concentrations. Similarly, the expression of downstream signaling molecules decreased after exposure to AZD4547 at concentrations above 50 nM.

Based upon these results, we confirmed that an association exists between pMET and pFGFR, using primary cancer cells from patients with hepatocarcinoma. Interestingly, two out of 14 primary cancer cells, 78019A and 3226B, expressed both pMET and pFGFR. According to some reports, MET activation is relevant to hepatitis B virus infection in hepatocarcinoma. Hepatitis B virus X (HBx) protein increases invasiveness via the promoter activity of MET (29). Additionally, hepatitis B and C virus-infected mouse models highly express HGF (30). However, the infectious status of these viruses might not be related to FGFR and MET signaling activation in the 14 primary HCCs (Supplementary Table. 1). Consistent with the results from cancer cell lines, treatment of primary cancer cells that express pMET and pFGFR with PHA665752 as a MET inhibitor was not effective, whereas AZD4547 was more effective against both primary cancer cells, implying that FGFR kinase is more important than MET kinase for hepatocarcinoma cell survival (Fig. 6). Thus, FGFR kinase may serve as a potent target of several therapeutic strategies for human hepatocellular carcinoma. We therefore investigated the expressions of pFGFR and pMET in other primary HCC cell lines.

One limitation of the current study is that it was based on a small sample size of primary HCC samples. The subset of HCC samples with both FGFR and Met signaling activation likely comprises a small proportion of the total HCC population. We analyzed the expression of pFGFR and pMET in a tissue microarray (TMA) by immunohistochemistry. TMA slides were purchased from US Biomax (the donors were from China). Nine of 53 TMA cores (16%) were double positive for the expression of pFGFR and pMET (Supplementary Fig. 5). In addition, other studies reported that MET...
amplification is 27% (31) and FGFR amplification is 31% (32) in HCC. Given the ethnic background of the donors, we expect similar results in Korean patients, but it would be helpful to confirm this possibility using a larger cohort. Nonetheless, our results provide the first evidence regarding the relationship of pMET and pFGFR in HCC.

In conclusion, we have evaluated the effects of AZD4547, a pan-FGFR inhibitor dependent on MET activity/phosphorylation, in human hepatocarcinoma cells. Given the increased effects on inducing HCC cell death at higher concentrations, it is also possible that other FGFR inhibitors, which can be used at higher tolerable doses, even for a shorter time than used with AZD4547, might be more efficacious in such patients. The success of personalized or precision medicine using targeted therapies, based on the particular genotype of cancer, depends on the ability to identify patients who will benefit from specific drugs. Thus, our findings may provide the rationale for clinical development of FGFR inhibitors, such as AZD4547, for the treatment of selected patient populations with hepatocarcinoma that express phospho-MET and phospho-FGFR.
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References


**Figure Legends**

**Figure 1. Effects of MET or pan-FGFR inhibitor on hepatocellular carcinoma (HCC) cell lines.**

A. Expression levels of phospho-MET and phospho-FGFR were evaluated by western blotting of extracts of HCC cell lines with anti-pMET or pFGFR antibodies. β-actin was used as a loading control. B. Various HCC cell lines were seeded in 96-well plates and treated with a MET inhibitor (PHA665752) or a pan-FGFR inhibitor (AZD4547). After incubation for 72 h, MTS assays were performed. C. Differential effects of the MET inhibitor (PHA665752) or pan-FGFR inhibitor (AZD4547) on SNU449 and SK-HEP-1 cells. SNU449 (i) and SK-HEP-1 (ii) cells were treated with different concentrations of PHA665752 or AZD4547 for 48 h. Cell death was determined using the trypan blue dye exclusion assay. Cells were harvested and analyzed by western blotting using antibody against cleaved caspase-3. β-actin was used as a loading control. The graphs present the mean ± s.d. of three separate experiments performed in triplicate. *P<0.05, ***P<0.001. D. Colony formation assays were performed using SNU449 (i) and SK-HEP-1 (ii) cell lines treated with the indicated concentrations of PHA665752 or AZD4547 (upper panel). Data are presented as the mean ± s.d. Representative images are shown (bottom panel). **P<0.01.

**Figure 2. Differential inhibition of downstream signaling molecules of HCC cell lines treated with MET or the pan-FGFR inhibitor.** (A and B). SNU449 (A) and SK-HEP-1 (B) cells were treated with MET or the pan-FGFR inhibitor at the indicated concentrations for 48 h. Cell lysates were analyzed by western blotting using antibodies against phospho-MET, MET, phospho-FRS2, FRS2, phospho-AKT, AKT, phospho-ERK, and ERK. β-actin was used as a loading control.

**Figure 3. Phosphorylation of FGFR1 and FGFR2 mediates the resistance of the MET inhibitor in human HCC cells.** A. SNU449 and SK-HEP-1 cells were harvested and immunoprecipitated with antibodies against FGFR1, FGFR2, FGFR3, and FGFR4. The immunoprecipitates were subjected to western blot analysis using a phospho-tyrosine antibody. The MKN45, KatoIII, HT29, and Huh7 cell
lines were used as positive controls for each antibody. B. SNU449 (i) and SK-HEP-1 (ii) cells were treated with MET or pan-FGFR inhibitor and immunoprecipitated with antibodies directed against FGFR1 or FGFR2. The immunoprecipitates were subjected to western blot analysis using a phospho-tyrosine antibody.

**Figure 4. Silencing of FGFR1 or FGFR2 overcomes the resistance to the MET inhibitor.** (A and B). SNU449 (A) and SK-HEP-1 (B) cells were transfected with siRNA directed against FGFR1 or control siRNA (sc) for 48 h. The transfected cells were treated with the indicated concentrations of PHA665752. Cell death was evaluated using the trypan blue dye exclusion assay. Cells were harvested and analyzed by western blotting using antibodies directed against phospho-MET, phospho-FRS2, and cleaved caspase-3; β-actin was used as loading control. SNU449 (C) and SK-HEP-1 (D) cells were transfected with scrambled siRNA or FGFR2-specific siRNA for 24 h, and then treated with PHA665752 at the indicated concentrations for an additional 48 h. Cell death was determined using the trypan blue dye exclusion assay. Cells were harvested, and immunoblot analyses were performed to determine the expression of phospho-MET, phospho-FRS2, and cleaved caspase-3; β-actin was used as loading control. Error bars indicate the s.d. of three individual experiments. *P<0.05, **P<0.01. SNU449 (E) and SK-Hep1 (F) cells were transfected with FGFR1 siRNA and/or FGFR2 siRNA for 24 h and treated with the specific MET inhibitor PHA665752 (2 μM) for 48 h. Cell death was determined using the trypan blue dye exclusion method. Cell lysates were prepared for western blot analysis using antibodies directed against FGFR1 and FGFR2, phospho-FRS2, and cleaved caspase-3; β-actin was used as loading control. The graph presents the mean ± s.d. **P<0.01.

**Figure 5. Effect of the expression of MET and FGFR1 or FGFR2 on the sensitivity of the MET inhibitor in p-FGFR and p-MET double-negative human HCC cells.** (A). PLC/PRF5 cells were transfected with FGFR1 and MET T1191I-expressing plasmids for 24 h and treated with bFGF (basic fibroblast growth factor) at 20 ng/mL and AZD4547, a pan-FGFR inhibitor, or PHA665752, a specific
MET inhibitor, at the indicated concentrations for another 48 h. Cell death was determined using the trypan blue dye exclusion method. The column and error bars represent the mean ± s.d. **P<0.001.

Cell lysates were prepared for western blot analyses using antibodies directed against FGFR1, FGFR2, phospho-FRS2, phospho-MET, MET, and cleaved caspase-3; β-actin was used as loading control. (B).

Cells were co-transfected with FGFR2 and MET T1191I constructs for 48 h, treated with bFGF at 20 ng/mL, and AZD4547 or PHA665752 at the indicated concentrations for an additional 48 h. The population of dead cells was evaluated by the trypan blue exclusion assay. The column and error bars represent the mean ± s.d. **P<0.01, ***P<0.001. Cell lysates were prepared for western blot analyses using antibodies directed against FGFR1, FGFR2, phospho-FRS2, phospho-MET, MET, and cleaved caspase-3; β-actin was used as loading control.

Figure 6. Effects of MET or pan-FGFR inhibitor on primary cancer cells from patients with hepatocarcinoma. A. (i). Levels of expression of phospho-MET and phospho-FGFR were evaluated by western blotting of primary HCC cells; β-actin was used as loading control. (ii). Immunofluorescence analysis of primary HCC cells. The 78019A and 3226B cells expressed pMET, pFGFR, and AFP (B and C). Differential effects of the MET inhibitor (PHA665752) or pan-FGFR inhibitor (AZD4547) on 3226B (B) and 78019A (C) cells were determined by immunofluorescence analysis. The 3226B and 78019A cells expressed Hep-par-1 and cleaved caspase-3 after AZD4547 treatment at the indicated concentrations as shown by immunofluorescence (D and E). Primary HCC cells 78019A and 3226B were treated with various concentrations of PHA665752 or AZD4547. These cells expressed both phospho-FGFR and phospho-MET. Cells were harvested and analyzed by western blotting using antibodies directed against phospho-MET and phospho-FRS2; β-actin was used as loading control. (F and G). The 34582 (F) and 39214 (G) cells expressed only phospho-MET and not phospho-FRS2. The cells were treated with PHA665752 or AZD4547 at the indicated concentrations. Cells were harvested and analyzed by western blotting using antibodies directed against phospho-MET and cleaved caspase-3; β-actin was used as loading control.
Figure 5

A

i. PLC/PRF5: pFGFR1 (-) / pMET (-)

PLC/PRF5: pFGFR1 (-) / pMET (-)

[Bar charts showing cell death (%) for FGFR1 wt + cMET mt (w FGFR2) and FGFR2 wt + cMET mt (w FGFR2).]

ii. FGFR1 wt + cMET mt

[Western blots showing pMet, pFRS2, FGFR-1, pAKT, cleaved caspase-3, and β-actin levels for AZD4547, PHA665752, and their concentrations (0, 1, 2 μM).]

B

PLC/PRF5: pFGFR2 (-) / pMET (-)

[Bar charts showing cell death (%) for FGFR2 wt + cMET mt (w FGFR2).]

[Western blots showing pMet, pFRS2, FGFR-2, pAKT, cleaved caspase-3, and β-actin levels for AZD4547, PHA665752, and their concentrations (0, 1, 2 μM).]
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