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RESEARCH

Targeting MET by Tyrosine Kinase Inhibitor Suppresses Growth and Invasion of Nasopharyngeal Carcinoma Cell Lines

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Abstract Nasopharyngeal carcinoma (NPC) represents a common cancer in endemic areas with high invasive and metastatic potential. It is now known that the HGF-MET signaling pathway plays an important role in mediating the invasive growth of many different types of cancer, including head and neck squamous cell carcinoma. HGF has been shown to stimulate NPC cell growth and invasion in cell line model. The current study aims at demonstrating the effect of MET inhibition by small molecule tyrosine kinase inhibitor PHA665752 on the growth and invasive potential of NPC cell lines. NPC cell lines were used for immunohistochemistry for the MET protein, as well as western blot analysis on MET together with its downstream cascade signaling proteins after treatment with PHA665752. The effect on cell growth, migration and invasion after PHA665752 treatment was also studied. MET inhibition by PHA665752 resulted in highly significant inhibition on NPC cell growth, migration and invasion *in vitro*. Down-regulation of phospho-MET, phospho-Akt, phospho-MAPK, phospho-STAT3, cyclin D1, β-catenin and PCNA was detected in NPC cells after PHA665752 treatment. MET inhibition with tyrosine kinase inhibitor resulted in suppression of NPC cell growth and invasive potential via down-regulation of a variety of signaling onco-proteins. MET is an important therapeutic target for NPC that warrants further studies and clinical trials.

Keywords Nasopharyngeal carcinoma · MET · Growth · Invasion · Tyrosine kinase inhibitor

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Introduction

Nasopharyngeal carcinoma (NPC) is a specific type of head and neck cancer that is endemic in South East Asia, especially in the Cantonese-speaking Chinese population residing in the southern part of China. The etiology of NPC is thought to be multi-factorial including environmental factors such as consumption of salted fish, genetic susceptibility as well as infection by Epstein-barr virus (EBV) [1]. Three types of NPC are recognized by the World Health Organization (WHO): type I, differentiated, keratinizing squamous cell carcinoma; type II, nonkeratinizing carcinoma and type III, undifferentiated carcinoma, with type III accounting for the majority (95%) of NPC cases in Southern China and Hong Kong. The WHO type III NPC is always associated with EBV infection. Expression of EBV-encoded viral onco-proteins, such as Latent Membrane Protein 1 (LMP-1), confers cancer cells growth and survival advantages [2]. Treatment of NPC remains a challenge although major breakthrough in radiation techniques [3, 4] and the use of concurrent chemoradiation [5] have improved the rate of local control and survival in recent years. The hepatocyte growth factor (HGF) cell signaling pathway with its tyrosine kinase receptor MET has been shown to play an important role in mediating cancer growth

and invasion in many different types of malignancies [6, 7]. We have reviewed how the HGF-MET signaling pathway promotes invasive growth of head and neck squamous cell carcinoma, a type of cancer with high metastatic potential [8]. High MET expression was detected in NPC in two major reports [9, 10], and the association of MET expression with cervical lymph node metastases, more advanced clinical stage and poorer survival was established. The HGF-MET signaling pathway has been demonstrated to increase the invasive potential of NPC in cell line model [11], and possibly via down-regulation of E-cadherin resulting in the loss of cell-to-cell adhesion [12]. In the current study, we aim to demonstrate the effect of MET inhibition by small molecule tyrosine kinase inhibitor PHA665752 on the growth and invasion of NPC cells *in vitro*, as well as the changes in the downstream signaling cascade of the MET receptor after MET inhibition by drug molecule. This will serve as a pre-clinical model for the design of phase I clinical trial on MET targeted therapy in NPC patients.

Materials and Methods

Reagents

Antibodies used in this study included: Rabbit-anti-human anti-MET for immunohistochemistry (dilution 1:25, Upstate, USA) and western blot (Santa Cruz Biotechnology Inc., USA). Anti-phospho-MET, anti-phsopho-STAT3 (Tyr), anti-phospho-STAT3 (Ser), anti-Akt, anti-phospho-Akt, anti-MAPK, anti-phospho-MAPK, anti-Src and anti-β-catenin antibodies for western blot were purchased from the same company (Cell Signaling Technology Inc., Danvers). Other antibodies for western blot included: anti-Cyclin D1 (Thermo Scientific, Waltham, MA), anti-PCNA (Abcam Inc., USA), and anti-actin (Calibiochem, Gibbstown, NJ). Secondary antibodies for western blot were purchased from Invitrogen (Carlsbad, CA).

Cell Culture

The nasopharyngeal carcinoma (NPC) cell line HONE-1 was derived from NPC patients with poorly differentiated NPC [13]. HONE-1-EBV cell line was established by introducing the EBV genome into the parental cell line HONE-1 [14]. All NPC cell lines were routinely maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, Thermo Scientific, Waltham, MA), 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate (all from Invitrogen, Carlsbad, CA). Cells were cultured at 37°C and 5% CO_2 in cell culture incubator. HONE-1-EBV cells were maintained in selection media containing 400 μg/ml G418 (Invitrogen, Carlsbad, CA). A HONE-1-LMP1 cell line was established by retroviral transfection of the LMP1 gene (the B95.8 LMP1 variant) into the parental HONE-1 cell line. Stable cell lines were established and maintained in selection medium (400 ng/ml puromycin; Merck, New Jersey, USA). The LMP1-B95.8-expressing cell line was named HONE-1-B95.8.

Immunohistochemistry

Rabbit monoclonal anti-MET antibody (Upstate, USA) for immunohistochemistry on the MET receptor protein was used. All patients have been consented for tissue collection for research at the Tumor Bank, Department of Clinical Oncology, the Chinese University of Hong Kong, according to the approved Ethics Approval of Research Protocol. Serial sections of nasopharyngeal carcinoma tissue samples from two patients were prepared, and antigen retrieval was performed using Bond Epitope Retrieval Solution 2 on the Bond-max automated immunostainer (Vision BioSystems, Mount Waverley, Australia) at 100°C for 25 min. Staining was performed according to a standard protocol in the immunostainer. Anti- MET antibody was used at a dilution of 1:25. A polymer detection system was selected to avoid the problem of nonspecific endogenous biotin staining. Normal nasopharyngeal epithelium adjacent to the cancer tissue was used as a control, and negative controls were performed by replacing the antibody with Tris buffered saline. The stained slides were evaluated (400× magnification) under light microscope.

Treatment with MET Tyrosine Kinase Inhibitor PHA665752

PHA665752 was purchased from Tocris Bioscience., USA. HONE-1, HONE-1-EBV, HONE-1-B95.8 cell lines were plated at a density of 4×10^5 cells/plate in 5 cm^2 culture dishes for 24 h. Culture media containing PHA665752 with concentrations of 2, 5, 10 μM/L were then added directly to the cells and incubated for 24–48 h. Vehicle control for PHA665752 treatment was DMSO. Cells were then collected and lysed for western blot analysis.

Western Blot

Cells from cell lines were lysed with the western lysis buffer (1% Nonidet-P40, 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate buffer (pH 7.2), 0.25 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin) for 5 min at 4°C. The lysate was then centrifuged at 4°C, 1200 rpm for 15 min. Supernatant was collected for protein quantitation. Protein quantitation was performed using the Protein Assay Solution (BioRad Laboratories, Hercules, CA) and the bovine serum albumin of known concentration as the standard. 50 μg of protein was then resolved on a 10% SDS-PAGE gel and transferred onto the Trans-Blot nitrocellulose membrane

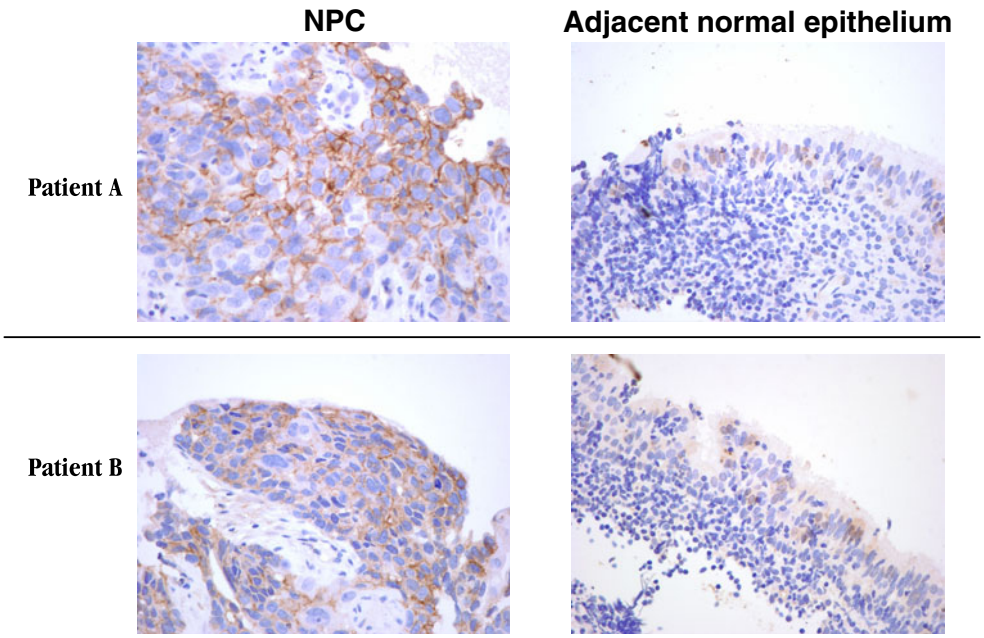

Fig. 1 Stronger immunostaining for MET in NPC cells compared with adjacent normal nasopharyngeal epithelium in two patients

(BioRad Laboratories, Hercules, CA) using the semi-dry transfer machine (BioRad Laboratories, Hercules, CA). After protein transfer, the membrane was then blocked overnight with a blocking solution containing 5% non-fat dry milk, 0.2% Tween 20 in 1×PBS (TBST). The membrane was incubated with the primary antibody at 4°C for 2 h and then washed with TBST three times for 10 min each. The membrane was further incubated with the secondary antibody for 1 h at room temperature and then washed another three times for 10 min each. The blot was developed with SuperSignal Chemiluminesence Kit (Thermal Scientifics, USA) by autoradiography.

MTT Cell Viability Assay

To determine survival of NPC cells in response to PHA665752 treatment, MTT assays were performed in 24-well plates. MTT solution was prepared from MTT powder (Sigma, St. Louis, MO) in phosphate-buffered saline (final concentration, 5 mg/ml). 48 h after the PHA665752 treatment, MTT solution was added to each well and incubated at 37°C for 1 h. MTT solution was then removed, and dimethyl-sulfoxide was added to each well. The optical density of the contents of each well was determined using a microplate reader set at 570 nm. The percentage of cell death was a function of the optical density obtained from the microplate reader.

Transwell Migration Assay

Migration of NPC cells was evaluated *in vitro* using Biocoat 8-μm pore size cell culture inserts. NPC cells were seeded onto the upper chamber at a density of 7×10^4 cells/chamber (HONE-1 and HONE-1-B95.8) and maintained in serum-free medium supplemented with 10 μM/L PHA665752. The cell-containing chamber was immersed in a lower chamber containing serum-free medium (SFM). Cells were incubated for 48 h at 37°C in a 5% CO_2 incubator. Non-migrating cells retaining in the upper chamber were removed with a cotton swab. The migrating cells were stained with 1% Toludine Blue O (Sigma, St. Louis, MO) in 1% Borax (USB, Cleveland, OH) and ten random fields were counted under the light microscope at 200X magnification. Triplicate experiments were performed.

Matrigel Invasion Assay

Invasion of NPC cells was evaluated *in vitro* using Matrigel-coated modified Boyden inserts with a pore size of 8 μm (Becton Dickinson/Biocoat, Bedford, MA), similar to the migration assay. NPC cells were seeded onto the upper chamber at a density of 7×10^4 cells/chamber (HONE-1 and HONE-1-B95.8) and maintained in serum-free medium supplemented with 10 μM/L PHA665752. The cell-containing chamber was immersed in a lower chamber containing serum-free medium (SFM). Cells were incubated for 48 h at 37°C in a 5% CO_2 incubator. Non-invading cells retaining in the upper chamber were removed with a cotton swab. The invading cells were stained with 1% Toludine Blue O (Sigma, St. Louis, MO) in 1% Borax (USB, Cleveland, OH) and ten random fields were counted under the light microscope at 200X magnification. Triplicate experiments were performed.

Statistical Analysis

Statistical analyses were performed using PRISM4 software (GraphPad, La Jolla, CA), and the *p*-values were obtained

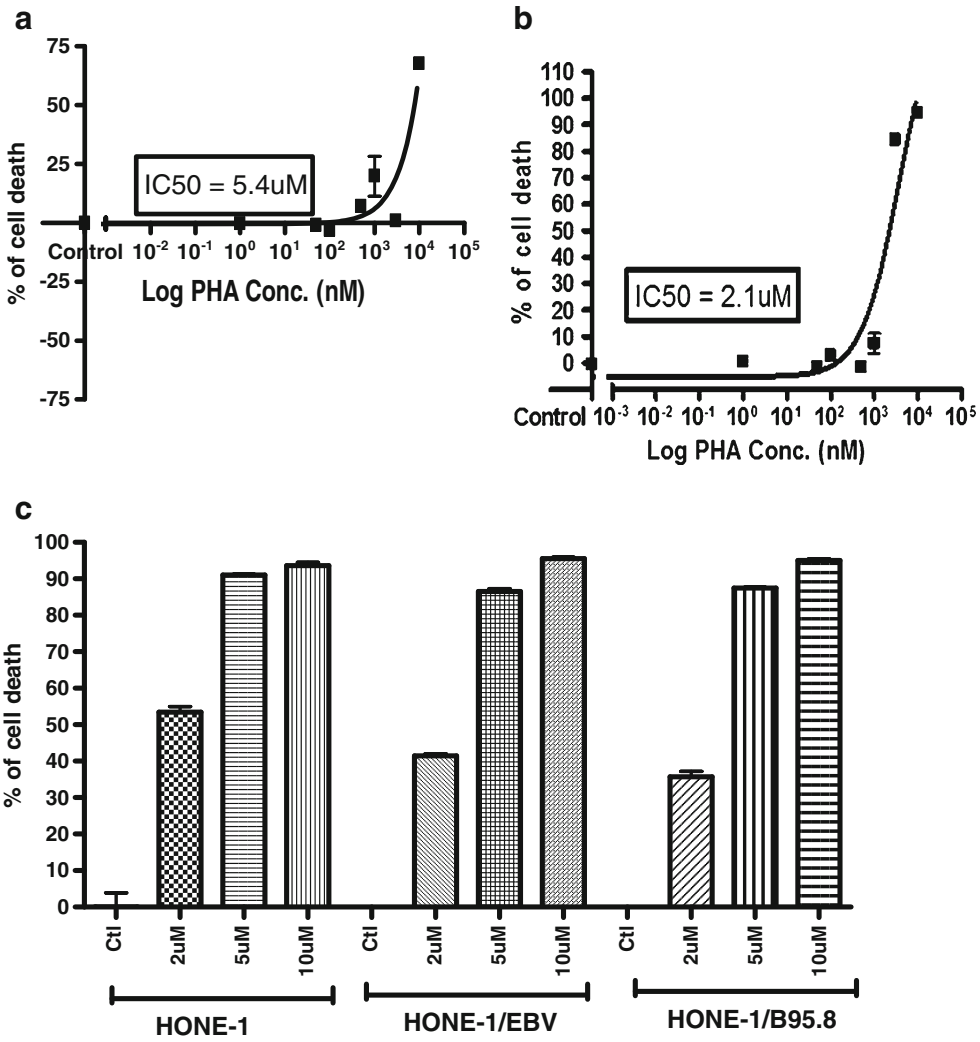

Fig. 2 **a** PHA665752 inhibited NPC cell growth (HONE-1) in a dose -dependent manner. The IC50 was 5.4 uM/L. Log PHA Conc. (nM) = Log scale of PHA665752 concentration in nM/L. **b** PHA665752 inhibited NPC cell growth (HONE-1-EBV) in a dose -dependent manner. The IC50 was 2.1 uM/L. Log PHA Conc. (nM) = Log scale of PHA665752 concentration in nM/L. **c** Treatment with PHA665752 on NPC cell lines. In HONE-1 cell line, cell growth was inhibited by PHA665752 at 2 μM/L (p=0.0002), 5 μM/L (p<0.0001) and 10 μM/L (p<0.0001). In HONE-1-EBV cell line, cell growth was inhibited by PHA665752 at 2 μM/L (p=0.0026), 5 μM/L (p=0.0002) and 10 μM/L (p=0.0001). In HONE-1-B95.8 cell line, cell growth was inhibited by PHA665752 at 2 μM/L (p<0.0001), 5 μM/L (p<0.0001) and 10 μM/L (p<0.0001). (Ctl = control, 2 uM=2 μM/L, 5 uM=5 μM/L, 10 uM=10 μM/L)

by the Wilcox-Mann–Whitney test. Results were considered as statistically significant with a p-value<0.05.

Results

MET is Overexpressed in NPC Tumor Cells Compared with Adjacent Normal Nasopharyngeal Epithelium

MET expression in NPC tumor cells has been reported in a study by Qian et al. [10]. In their study, most of the tumor cells from 66 patients showed MET expression and northern blot analysis demonstrated that MET expression in at least four NPC cell lines (including HONE-1) was stronger than a control cell line. This is in line with our early results when MET expression in multiple NPC cell lines was shown to be stronger than a negative control (normal esophageal squamous epithelial cell line Het-1A). NPC tumor samples from two patients who have given their consent for tissue collection were selected for further immunostaining with anti-MET antibody to further confirm MET overexpression in NPC cells. As shown in Fig. 1, MET expression is observed as strong focal positive membrane staining in the NPC tumor cells but not in the adjacent normal epithelium in the same patient.

MET Inhibition with PHA665752 Decreased Cell Growth in NPC Cell Lines

The importance of the MET signaling pathway in NPC cell growth and survival was examined by testing the inhibitory effect of the small molecule PHA665752 which inhibits the tyrosine kinase activity of MET. Percentage of cell death or degree of growth inhibition was calculated as a function of the optical density generated in the MTT cell viability assay after treatment with PHA665752. As shown in Figs. 2a and b, in two NPC cell lines tested (HONE-1 and HONE-1-EBV), PHA665752 inhibited NPC cell growth in a dose-dependent manner. The degree of growth inhibition was proportional to the percentage of cell death shown in the MTT assay and

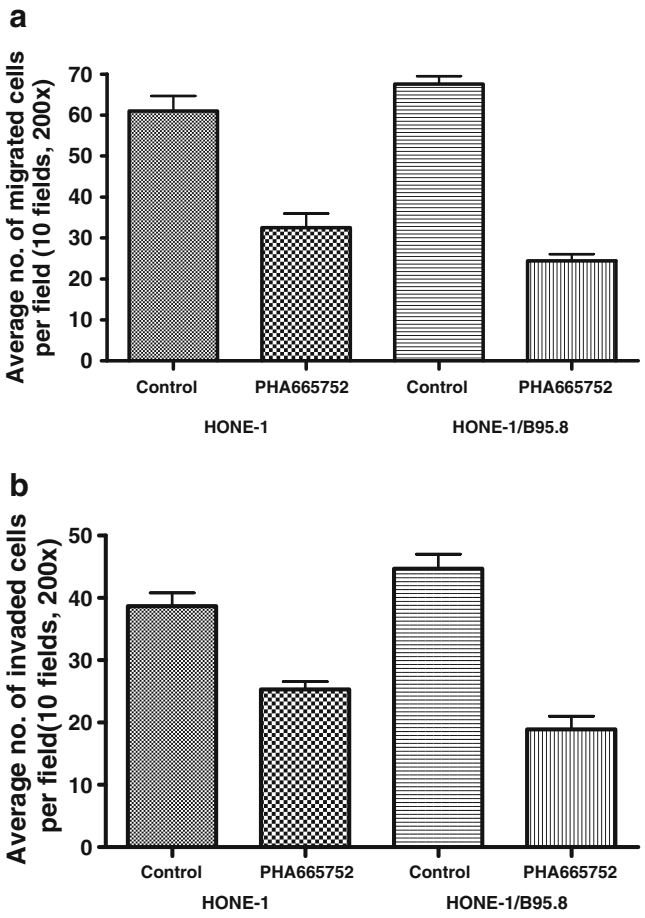

Fig. 3 **a** Treatment with PHA665752 resulted in decreased cell migration in both HONE-1 ($p<0.0001$) and HONE-1-B95.8 ($p<0.0001$) cell lines when compared to control. **b** Treatment with PHA665752 resulted in decreased cell invasion in both HONE-1 ($p<0.0001$) and HONE-1-B95.8 ($p<0.0001$) cell lines when compared to control

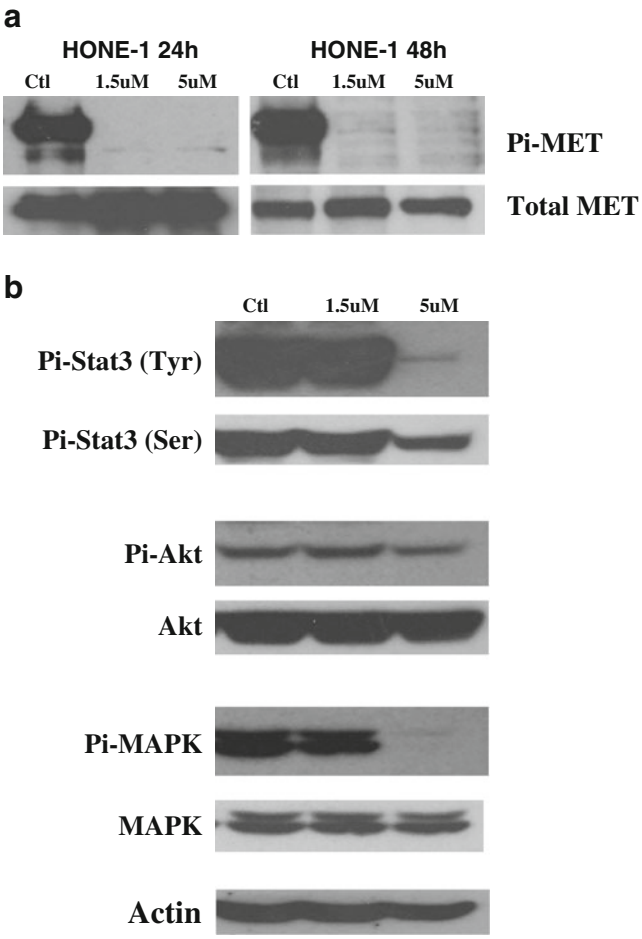

Fig. 4 **a** Western blot analysis on MET and phospho-MET (Pi-MET) protein expression in HONE-1 NPC cell line with two concentrations of PHA665752 treatment. Ctl = control; 1.5 uM=1.5 uM/L; 5 uM= 5 uM/L. **b** Western blot analysis on phospho-STAT3, phospho-Akt and phospho-MAPK protein expression in HONE-1 NPC cell line with two concentrations of PHA665752 treatment. Ctl = control; 1.5 uM= 1.5 uM/L; 5 uM=5 uM/L; Tyr = Tyrosine; Ser = Serine

expressed as such. The IC50 for PHA665752 was about 5 μM/L for HONE-1 and about 2 μM/L for HONE-1-EBV. Both of these concentrations together with 10 μM/L were selected for further PHA665752 treatment on NPC cell lines. As shown in Fig. 2c, treatment with all three concentrations of PHA665752 resulted in highly statistically significant cell death and growth inhibition when compared to control.

MET Inhibition with PHA665752 Decreased Cell Migration and Invasion in NPC Cell Lines

In addition to cell growth, the effect of MET inhibition by PHA665752 on NPC cell migration and invasion was also examined. As 10 μM/L of PHA665752 resulted in the most statistically significant growth inhibition on NPC cells, we adopted this concentration for further testing this molecule on NPC cell migration and invasion by the transwell migration and matrigel invasion assays. As shown in Fig. 3, after treating both HONE-1 and HONE-1-B95.8 NPC cell lines with 10 μM/L of PHA665752 for 48 h, a highly statistically significant reduction in both cancer cell migration and invasion was demonstrated ($p<0.0001$).

MET Inhibition by PHA665752 Resulted in Down-Regulation of the MET Signaling Pathway

Various concentrations of PHA665752 were used to treat the HONE-1 NPC cell line and cell lysate was then obtained for western blot analysis to study the effect of MET receptor tyrosine kinase inhibition on its downstream signaling proteins in NPC cells. As shown in Figs. 4a and b, both 1.5 μM/L and 5 μM/L of PHA665752 after 24 and 48 h of treatment resulted in down-regulation of the expression of the activated, phosphorylated form of MET. 5 μM/L PHA665752 was able to down-regulate the expression of

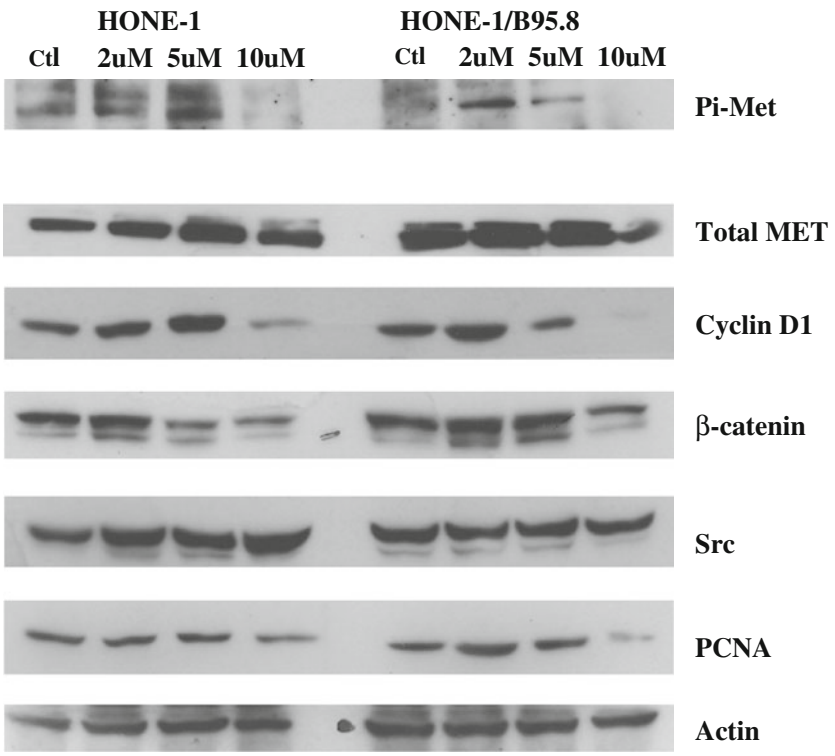

Fig. 5 Western blot analysis on phospho-MET (Pi-MET), cyclin D1, β-catenin, Src and PCNA protein expression in HONE-1 and HONE-1-B95.8 cell lines with various concentrations of PHA665752 treatment. Ctl = control; 2 uM=2 uM/L; 5 uM=1.5 uM/L; 10 uM=10 uM/L

phospho-STAT3, phospho-Akt, and phospho-MAPK. These results suggest that in NPC cells, activated MET protein mediates its carcinogenic effects via the STAT3, Akt and MAPK signaling cascades.

Identification of Other Cell Signaling Proteins in NPC Cell Line that are Regulated by MET Inhibition

To identify additional cell signaling proteins that are regulated by MET inhibition, our laboratory has previously generated a stable clone of HONE-1 NPC cell line that has been transfected with small hairpin RNA (shRNA) which targets and inhibits MET protein translation and expression. The lysate from the stable clone was subjected to proteomic analysis with a highly sensitive, high through-put protein assay, the reverse phase protein array (RPPA) [15]. High performance antibodies against a panel of 70 cell signaling proteins were used in the assay and changes in the expression levels of these candidate proteins after MET down-regulation by the specific shRNA was studied. It was found that MET down-regulation by shRNA resulted in down-regulation of a number of cell signaling proteins that are known to be involved in cancer cell proliferation and invasion, including beta-catenin, PCNA, Src and cyclin D1. Validation of these results was performed in the current study by treating the HONE-1 and HONE-1-B95.8 cell lines with the MET tyrosine kinase inhibitor PHA665752. As shown in Fig. 5, treatment of both cell lines with PHA665752 at 10 μM/L resulted in down-regulation of MET phosphorylation, cyclin D1 expression, beta-catenin expression (more marked in HONE-1 cell line) and PCNA expression (more marked in HONE-1-B95.8 cell line) in the western blot analysis. These results suggest that in line with the preliminary data shown by the RPPA assay, cyclin D1, beta-catenin as well as PCNA could be other important signaling proteins in the downstream cascade of MET.

Discussion

Since the discovery of the MET protein in the 1980s, numerous work done over the past three decades provided strong evidence on the importance of this signaling pathway in cancer development and progression. Of particular interest is that it does not only mediate cancer cell proliferation, but also promotes cell invasion and metastasis in a variety of cancer. Nasopharyngeal carcinoma (NPC) remains one of the major cancers in endemic areas. Because of its invasive phenotype and high metastatic potential, patients with this cancer are still suffering from significant morbidity and mortality. It is therefore essential to search for better treatment for this devastating disease. As the importance of the MET signaling pathway has been well demonstrated in other types of head and neck cancers

arising from the squamous epithelium, it is hypothesized that this pathway is also important in NPC oncogenesis and may be an attractive therapeutic target for NPC treatment. In the current study, overexpression of MET in NPC cells was demonstrated which confirmed similar results reported in previous larger series. The data shown in the current study provided strong evidence that NPC cells over-express MET protein receptor, and inhibition of MET by a small molecule tyrosine kinase inhibitor suppressed NPC cell growth, migration and invasion *in vitro* via down-regulation of its downstream signaling cascades including the MAPK, Akt and STAT3 pathways. Further onco-proteins down-stream of MET were also identified (cyclin D1, beta-catenin and PCNA) which could serve as potential biomarkers for response to MET targeted treatment for NPC. In the current study, NPC cell lines were engineered by re-introducing EBV back to the genome before further analysis. This is because in clinical settings many NPC cases are associated with EBV infection and hence a cell line with more stable EBV expression will mimic NPC *in vivo* to a greater extent. Indeed, the HONE-1-B95.8 cell line which was transfected with the EBV genome containing the carcinogenic LMP-1 sequence behaved more aggressively with higher degree of cell invasion. This is consistent with previous reports that LMP-1 could up-regulate the MET signaling pathway in NPC cells conferring higher invasive potential.

The mechanism of MET signaling pathway activation in NPC remains unclear. Preliminary data from our laboratory showed that HGF mRNA was not detected in NPC cells, suggesting that it is unlikely to act via an autocrine mechanism. Gene sequencing and in-situ hybridization studies to search for MET gene mutation and amplification in NPC is currently underway. Further studies on using xenograft models to test the *in vivo* effects of MET targeted therapy on NPC development and invasion will further confirm the importance of the MET signaling pathway in this cancer and the potential clinical value of MET targeted therapy. In fact, different therapeutic strategies have already been developed to target the MET signaling pathway in many cancer types including head and neck squamous cell carcinoma [8]. These strategies, which include biological antagonist, monoclonal antibody against HGF or MET as well as small molecule tyrosine kinase inhibitor, have entered phase I and II clinical trials some of which have demonstrated early encouraging results.

In conclusion, the current study demonstrated that inhibition of the MET signaling pathway in NPC by small molecule tyrosine kinase inhibitor resulted in suppression of cancer growth and invasion. This provided a pre-clinical model for the design of Phase I clinical study on the use of MET targeted therapy in treating patients with NPC in the near future.

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Conflict of interest No conflict of interest declared.
No financial declaration.

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