



Cite this: *RSC Adv.*, 2018, 8, 21451

Apatinib inhibits macrophage-mediated epithelial–mesenchymal transition in lung cancer

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Chemotherapy is one of the main treatment approaches for lung cancer. However, few drugs can be used in the post-first-line treatment of lung cancer. Apatinib is a small molecule inhibitor of vascular endothelial growth factor receptor-2 (VEGFR-2) and is widely used in advanced gastric cancer. This study aimed to investigate the therapeutic effect of apatinib in the second-line treatment of lung cancer and explore its underlying mechanism from the aspect of macrophage-mediated epithelial–mesenchymal transition (EMT). The results showed that apatinib attenuated macrophage-induced EMT and migration of lung cancer cells but not normal lung cells, as demonstrated by the loss of epithelial properties and gain of mesenchymal characteristics. Moreover, apatinib selectively decreased hepatocyte growth factor (HGF) secretion in polarized macrophages. Furthermore, apatinib down-regulated the expression of the HGF-Met signaling pathway in polarized macrophage-stimulated lung cancer cells. Taken together, our study has identified a novel pathway through which apatinib exerts its anti-cancer functions, and provided a molecular basis for apatinib potential applications in the post-first line treatment of lung cancer.

Received 8th February 2018

Accepted 3rd May 2018

DOI: 10.1039/c8ra01231h

rsc.li/rsc-advances

Introduction

Lung cancer is the most frequently diagnosed malignant tumor and the leading cause of cancer death worldwide.^{1,2} Histologically, lung cancer can be divided into small-cell lung cancer and non-small-cell lung cancer (NSCLC), and the latter accounts for more than 70% of lung cancer.^{3,4} In NSCLC, tumor-associated macrophages located in lung carcinomas increase tumor recurrence after surgery and reduce patient survival.^{5,6} Thus, interference with macrophage polarization may shift macrophage function from a tumor facilitator to a tumor suppressor, suggesting a potential mechanism for lung cancer therapy.

To date, EGFR tyrosine kinase inhibitors (EGFR-TKIs) and ALK inhibitors such as gefitinib and afatinib have been suggested as the first-line treatment for NSCLC patients with epidermal growth factor receptor (EGFR) mutation and anaplastic lymphoma kinase (ALK) rearrangements.^{7–9} In the NSCLC patients without genetic driver mutation, platinum-based chemotherapy is the best option for first-line treatment.

Tumor angiogenesis is an essential step for tumor proliferation and metastasis. Vascular endothelial growth factor/vascular endothelial growth factor receptor (VEGF/VEGFR) signaling pathway has been proved to play crucial role in tumor angiogenesis.^{10,11} Thus, VEGF/VEGFR was considered as a target in

cancer therapy. In fact, multiple drugs which target VEGF/VEGFR have received the Food and Drug Administration approval and have shown encouraging efficacy in several solid tumors. For example, it was reported that bevacizumab or ramucirumab combined with chemotherapy significantly improved the progression-free survival (PFS) and overall survival (OS) in NSCLC.^{12,13} However, these anti-angiogenesis drugs were suggested adding to platinum-based doublet chemotherapy as first-line treatment for advanced NSCLC without driver mutation, and there was little evidence for their application in post-first-line monotherapy of advanced lung cancer.

Apatinib is an oral small molecule inhibitor of VEGFR-2, and displays efficiency and safety in breast and gastric cancer therapy.^{14–16} However, its application on other malignant tumors, such as lung carcinoma is just getting started. Recent studies have reported that high dose (500–825 mg d⁻¹) and low dose (250–500 mg d⁻¹) of apatinib showed an efficiency and safety in lung cancer patients.^{17–19} In the present study, we used apatinib as a post-first-line therapy to investigate the underlying mechanism in the aspect of macrophage-mediated EMT. We found that apatinib inhibits macrophage infiltration *in vivo* and suppresses macrophage-induced EMT *in vivo* and *in vitro*. Further studies suggested that apatinib inhibits macrophage-mediated EMT through HGF-Met signaling pathway.

Materials and methods

Reagents

Following reagents (suppliers) were used in this study: apatinib (Selleck, Texas, USA); hepatocyte growth factor (HGF)

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(PeproTech, Suzhou, China); antibodies to CD68, ZO-1, N-cadherin, vimentin, Slug and β -actin (Santa Cruz Biotechnology, USA); antibodies to fibronectin, E-cadherin, Snail, p-Met, Met (Abcam, Cambridge, MA). HRP-conjugated secondary antibodies (Vazyme, Nanjing, China). ELISA kit for HGF, EGF, TGF- β 1 and IL-6 (R&D Systems, Minneapolis, MN).

Cell culture

A549, H1975 and normal human lung cell line BEAS-2B (ATCC, Manassas, USA) were grown in DMEM complemented with 10% FBS (Life Technologies, Grand Island, USA), 100 IU ml⁻¹ penicillin (Sigma, USA), and 100 μ g ml⁻¹ streptomycin (Sigma, USA). All cells were cultured at 37 °C in a 5% CO₂ incubator. Human monocyte cell line THP1 was stimulated with phorbol myristate acetate (PMA, 0.1 μ M, Thermo Scientific, USA) for 3 hours to generate polarized macrophages. Apatinib or DMSO was added to polarized macrophages for 24 hours, followed by a medium exchange and stimulate with LPS for 24 hours. The supernatant was collected and centrifuged for further studies.

Immunohistochemistry

Paraffin-embedded tissue sections were cut to a thickness of 4 μ m. After dewaxing, rehydration and blocking, the sections were incubated with CD68 and fibronectin antibody overnight at 4 °C, followed by incubation with fluorophore-conjugated secondary antibody for 1 h. Sections were visualized with a fluorescent microscope and quantified by ImageJ software as before.⁴²

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Equal amounts of RNA were reversely-transcribed to cDNA with M-MLV Transcriptase (Invitrogen). Then PCR was performed by Premix Ex Taq (Takara, Japan) in a Fast Real-time PCR 7300 System (Applied Biosystems). The PCR specific primers were: 5'-CGAATGGATGAAAGACCCATCC-3' (forward) and 5'-GGAGCCACTGCCCTTCATAGTC-3' (reverse) for N-cadherin; 5'-TTAAACTCCTGGCCTCAAGCAATC-3' (forward) and 5'-TATCTTGGGCAAAGCAACTG-3' (reverse) for E-cadherin; 5'-TGAGTACCGGAGACAGGTGCAGTAGCAG-3' (forward) and 5'-CTTCAACGGCAAAGTTC-3' (reverse) for vimentin; 5'-GCACTGTGATGCCAGTCTA-3' (forward) and 5'-CAGTGAGGGCAAGAGAAAGG-3' (reverse) for slug; 5'-GTT CAATGTGGGACAAGAACATGG-3' (forward) and 5'-GGATTCGGCAGTAATTCTCATTCA-3' (reverse) for HGF; 5'-CCTCACCATAGCTAATCTTGGGACA-3' (forward) and 5'-CACAATCACTTCTGGAGACTGGA-3' (reverse) for MET; 5'-TCGACATGGAGCTGGTGGAAA-3' (forward) and 5'-GGGACTGGC-GAGCCTTAGTT-3' (reverse) for TGF- β 1; 5'-GCTTCACTCTGGAA-GATGCC-3' (forward) and 5'-AAGGAGTGTGGTCACTGTGC-3 (reverse) for TGF- β 2; 5'-TGTGCAATGGCAATTCTGAT-3' (forward) and 5'-GGTACTCCAGAAGACCAGAGGA-3' (reverse) for IL-6; 5'-AAGGACCTCCAGCATCACTGTGTCA-3' (forward) and 5'-CCTTCAAGAGCCGAGCTTCCACGT-3' (reverse) for IL-6R; 5'-CCTGGCACCCAGCACAAAT-3' (forward) and 5'-GCTGATCCA-CATCTGCTGGAA-3' (reverse) for β -actin. The original Ct values were adjusted to GAPDH.

Western blot

Total protein samples from cells were prepared by using RIPA buffer. Equal amounts of protein sample were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, USA). Membranes were blocked with 5% non-fat milk, and then incubated with primary antibodies and corresponding secondary antibodies. The targeted bands in the membranes

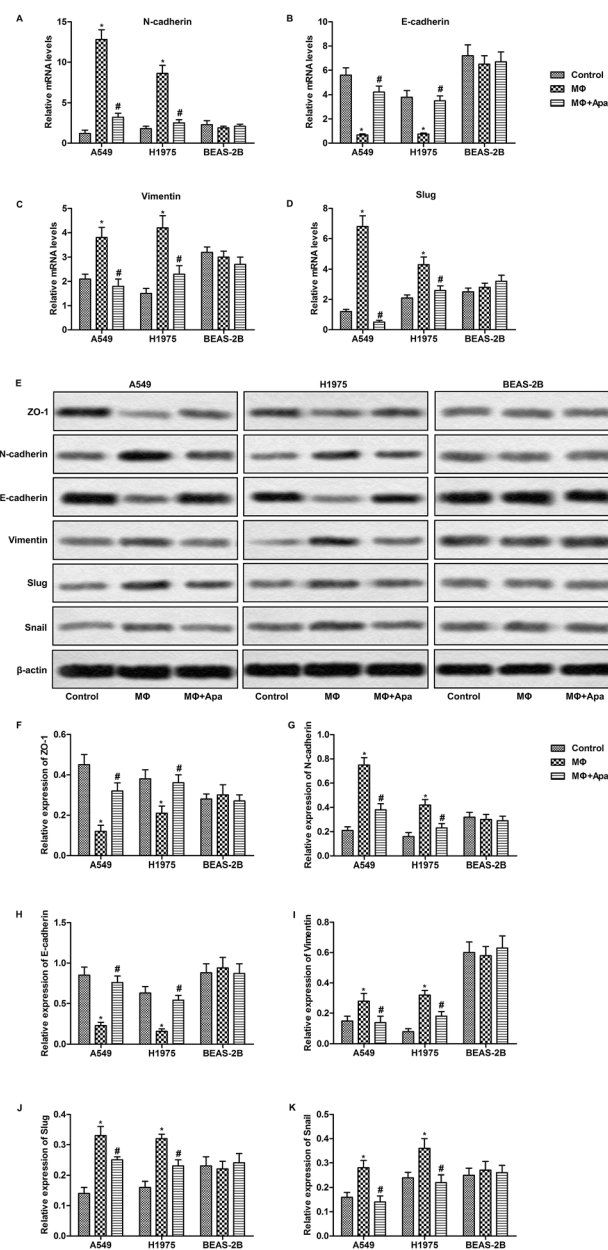
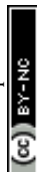


Fig. 1 Apatinib suppresses polarized macrophage-induced EMT in lung cancer cells. A549, H1975 and BEAS-2B cells were treated with the supernatant of polarized macrophages ($M\Phi$) which had been stimulated with LPS (1 ng ml⁻¹) and/or apatinib (10 μ M) for 24 h. (A–D) The mRNA levels of N-cadherin (A), E-cadherin (B), vimentin (C) and Slug (D) were assayed by qPCR. (E) The expressions of ZO-1, N-cadherin, E-cadherin, vimentin, Slug and Snail were detected by western blot. (F–K) Statistical analysis of Fig. 2E. * P < 0.05 versus control. # P < 0.05 versus $M\Phi$.



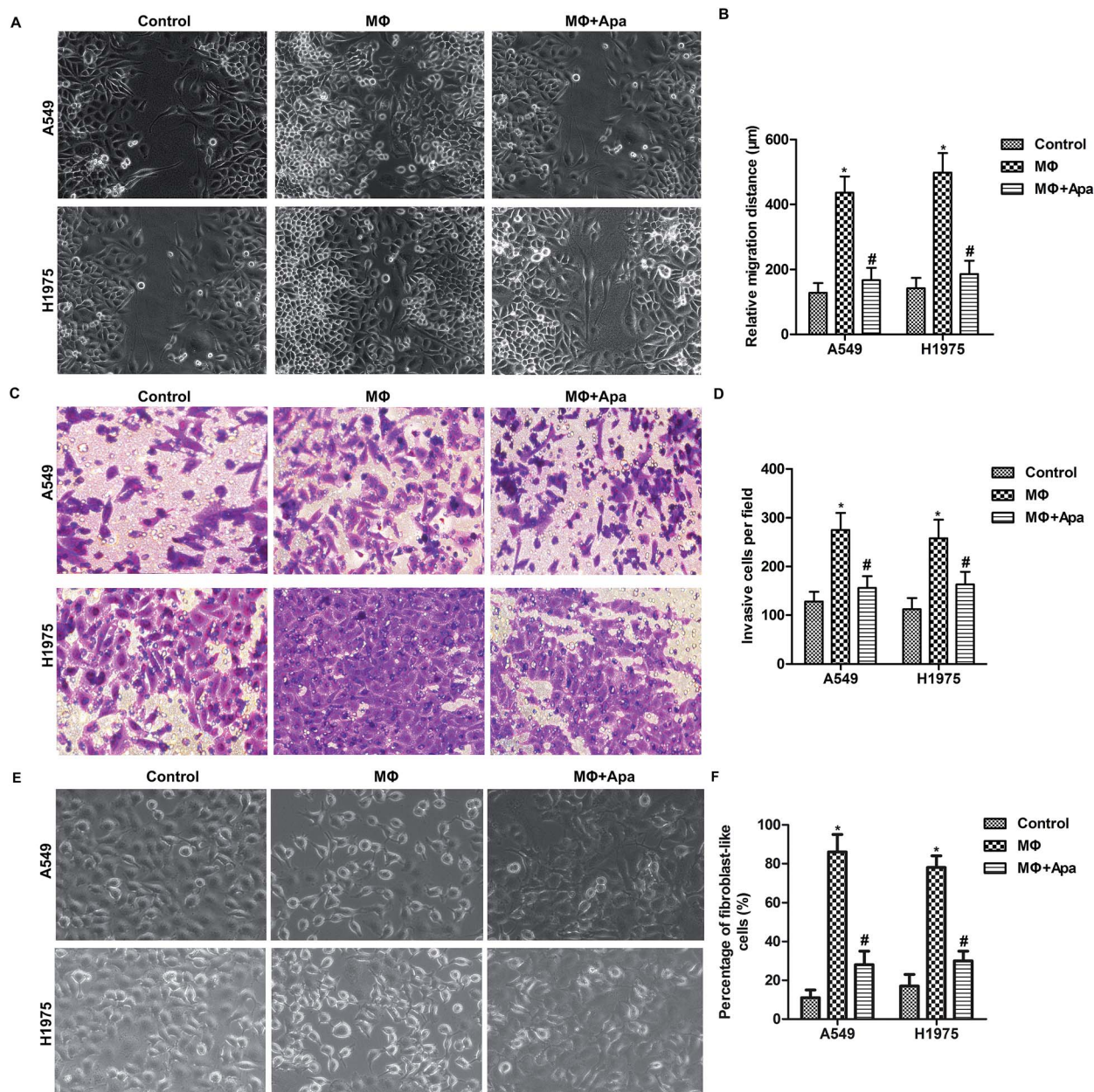


Fig. 2 Apatinib inhibits polarized macrophage-induced migration and invasion of lung cancer cells. A549 and H1975 cells in 12-well plate or transwell chamber were treated with the supernatant of polarized macrophages ($M\Phi$) which had been stimulated with LPS (1 ng ml^{-1}) and/or apatinib ($10 \text{ }\mu\text{M}$) for 24 h. (A) Wound healing assay. (B) Quantification of migration distance. (C) Transwell invasion assay. (D) Quantification of invaded cells. (E) The morphologic characteristics of EMT were detected under microscope. (F) Quantification of EMT cells. * $P < 0.05$ versus control. # $P < 0.05$ versus $M\Phi$.

were visualized using enhanced chemical luminescence (ECL) and quantificated by ImageJ software.

Wound healing assay

Confluent cell monolayer were cultured in a 24-well chamber and stroked with a plastic pipette tip. Cells were washed to remove detached and damaged cells. After treated with the supernatant of polarized macrophages (which have treated with apatinib or DMSO) for 24 h, the cell migrations were monitored microscopically and the migration distance was measured from 5 points per 1 wound area by the ImageJ software.

Transwell invasion assay

Cell invasive capacity was detected using a 24-well plate with transwell chamber ($0.4 \text{ }\mu\text{m}$ pore size, Corning) inserts precoated with Matrigel. Briefly, cells (1×10^5) were seeded into the upper chamber in serum-free medium. Then, complete medium (containing 10% FBS) was added into the lower chamber. After 24 h of indicated treatment, cells in the upper surface of the membranes were removed with cotton swab. The cells that had migrated to the outer side of the membranes were stained with hematoxylin. The stained cells were photographed and counted in 10 random fields.



Enzyme linked immunosorbent assay (ELISA)

The expression of HGF, epidermal growth factor (EGF), Transforming growth factor (TGF)- β and interleukin (IL)-6 were quantified by ELISA according to the manufacturer's instructions (Sigma, St. Louis, USA). Polarized macrophages induced by different concentrations of apatinib were precoated onto ELISA plates and served as the antigen. *o*-Dianisidine was used as substrate and the absorbance of the colored horseradish peroxidase (HRP) product was measured spectrophotometrically at 490 nm by an automated microplate reader (Thermo, Waltham, USA).

Statistical analysis

Data were analyzed by SPSS 19.0 software and the results were expressed as mean \pm standard deviation (SD). The statistical significance of the studies was analyzed using one way ANOVA. The difference was considered significant at $P < 0.05$.

Results

Apatinib suppresses polarized macrophage-induced EMT in lung cancer cells

To investigate the effect of apatinib on macrophages, human monocyte cell line THP1 was treated with PMA to generate polarized macrophage. Then, human lung cancer cell line A549 and H1975 and normal human lung cell line BEAS-2B were cultured with the supernatant from polarized macrophages for 24 h. The expressions of EMT-related genes were detected by qPCR and western blot. As shown in Fig. 1A–D, the supernatant from polarized macrophages significantly increased the mRNA expression of N-cadherin, vimentin and Slug and decreased the level of E-cadherin in A549 and H1975 cells. However, apatinib treated macrophage supernatant effectively corrected these abnormalities. Consistent with the mRNA changes, the supernatant from polarized macrophages significantly decreased the protein expression of epithelial markers (ZO-1 and E-cadherin)

and increased the protein levels of N-cadherin, vimentin, Slug and Snail in A549 and H1975 cells. These changes were reversed when polarized macrophages were pretreated with apatinib (Fig. 1E–K). Similarly, no obvious changes were observed in BEAS-2B cells. These results indicate that apatinib suppresses polarized macrophage-induced EMT only in lung cancer cells.

Apatinib inhibits polarized macrophage-induced migration and invasion of lung cancer cells

To determine whether the effect of apatinib on polarized macrophages leads to an inhibition of cellular migration, wound healing assay and transwell invasion assay were carried out. As shown in Fig. 2A and B, polarized macrophages promoted the migration of A549 and H1975 cells. However, apatinib pretreatment significantly decreased the migration distance in polarized macrophage-stimulated A549 and H1975 cells. In addition, transwell assay showed that polarized macrophages stimulation promoted the invasion of A549 and H1975 cells, which was blocked by apatinib treatment (Fig. 2C and D). In addition, the increased percentage of fibroblast-like cells in *M Φ* were strongly decreased under apatinib treatment (Fig. 2E and F). These data suggest that apatinib inhibits macrophage-induced migration in lung cancer cells.

Apatinib inhibits the expression of HGF in polarized macrophages

To investigate whether cytokine secretion of polarized macrophages contributes to the EMT progression, the expression of EMT-related cytokines in polarized macrophages was analyzed by qPCR and ELISA. The results showed that apatinib notably decreased the level of HGF in polarized macrophages. However, other EMT-related cytokines, such as EGF, TGF- β 1 and IL-6 had no obvious changes in polarized macrophages with or without apatinib stimulation (Fig. 3). Thus, apatinib inhibits macrophage-mediated EMT probably through regulating HGF secretion.

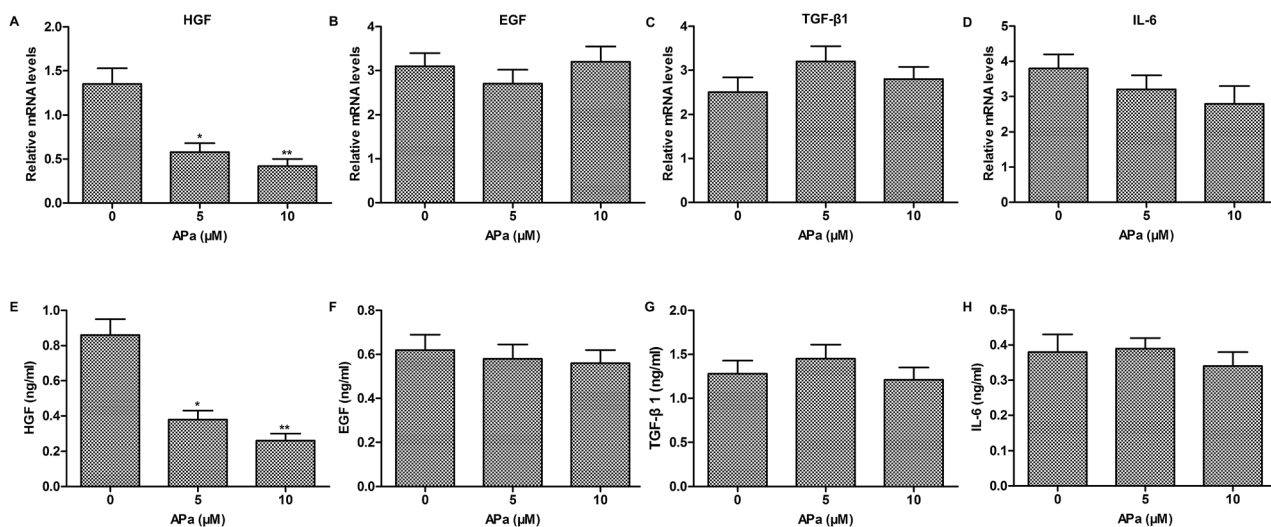


Fig. 3 Cytokine profiles in apatinib-treated macrophages. Polarized macrophages were stimulated with LPS (1 ng ml^{-1}) for 24 h and treated with a series dose of apatinib for 3 h. (A–D) The mRNA levels of HGF, EGF, TGF- β 1 and IL-6 were assayed by qPCR. (E–H) The concentrations of HGF, EGF, TGF- β 1 and IL-6 were detected by ELISA. * $P < 0.05$, ** $P < 0.01$ versus control.



Apatinib inhibits HGF-induced migration and invasion of lung cancer cells

Based on the above observations, the migration and invasion capacities of lung cancer cells were examined after HGF stimulation. As shown in Fig. 4, HGF significantly increased the migration distance and invasion capacity and the percentage of fibroblast-like cells of A549 and H1975 cells. As expected, apatinib treatment effectively corrected these abnormalities. These results further indicate that HGF is involved in the macrophage-mediated EMT.

Apatinib down-regulates HGF-Met signaling in polarized macrophage supernatant-treated lung cancer cells

We next investigated the expression of the downstream receptors of HGF, EGF, TGF- β 1 and IL-6 in polarized macrophage supernatant-treated lung cancer cells. As expected, apatinib dose-dependently inhibited the expression of Met in polarized macrophage-stimulated A549 and H1975 cells but not in BEAS-2B cells (Fig. 5A). However, the expression of EGFR, TGF β R and IL6R was not changed in lung cancer cells or normal lung cells (Fig. 5B–D). Since HGF-Met signaling mainly depends on Met

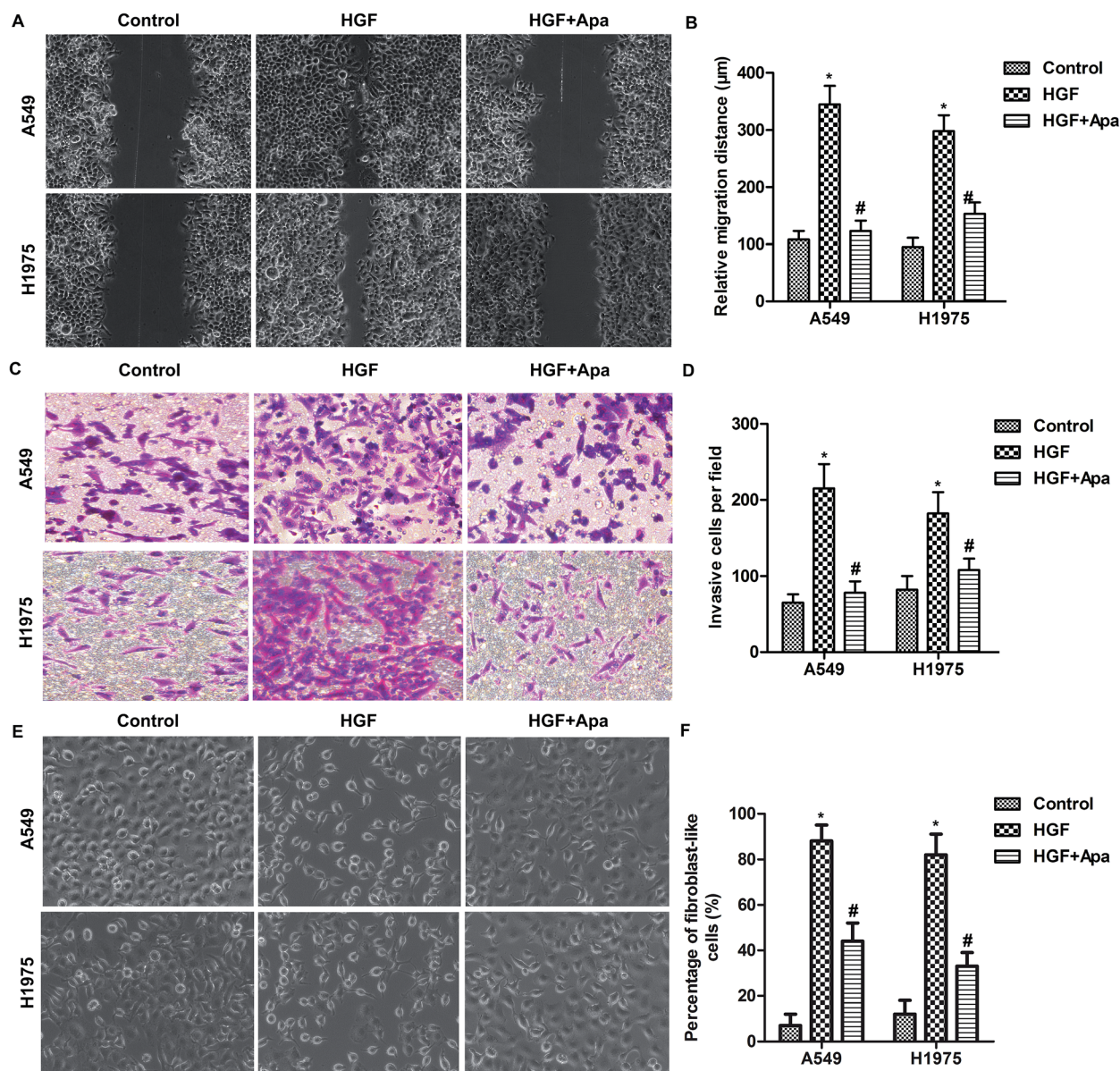


Fig. 4 Apatinib inhibits HGF-induced migration and invasion of lung cancer cells. A549 and H1975 cells in 12-well plate or transwell chamber were treated with HGF (5 ng ml^{-1}) and/or apatinib ($10 \mu\text{M}$) for 24 h. Cells without any treatment were considered as control group. (A) Wound healing assay. (B) Quantification of migration distance. (C) Transwell invasion assay. (D) Quantification of invaded cells. (E) The morphologic characteristics of EMT were detected under microscope. (F) Quantification of EMT cells. * $P < 0.05$ versus control. # $P < 0.05$ versus HGF treated alone.



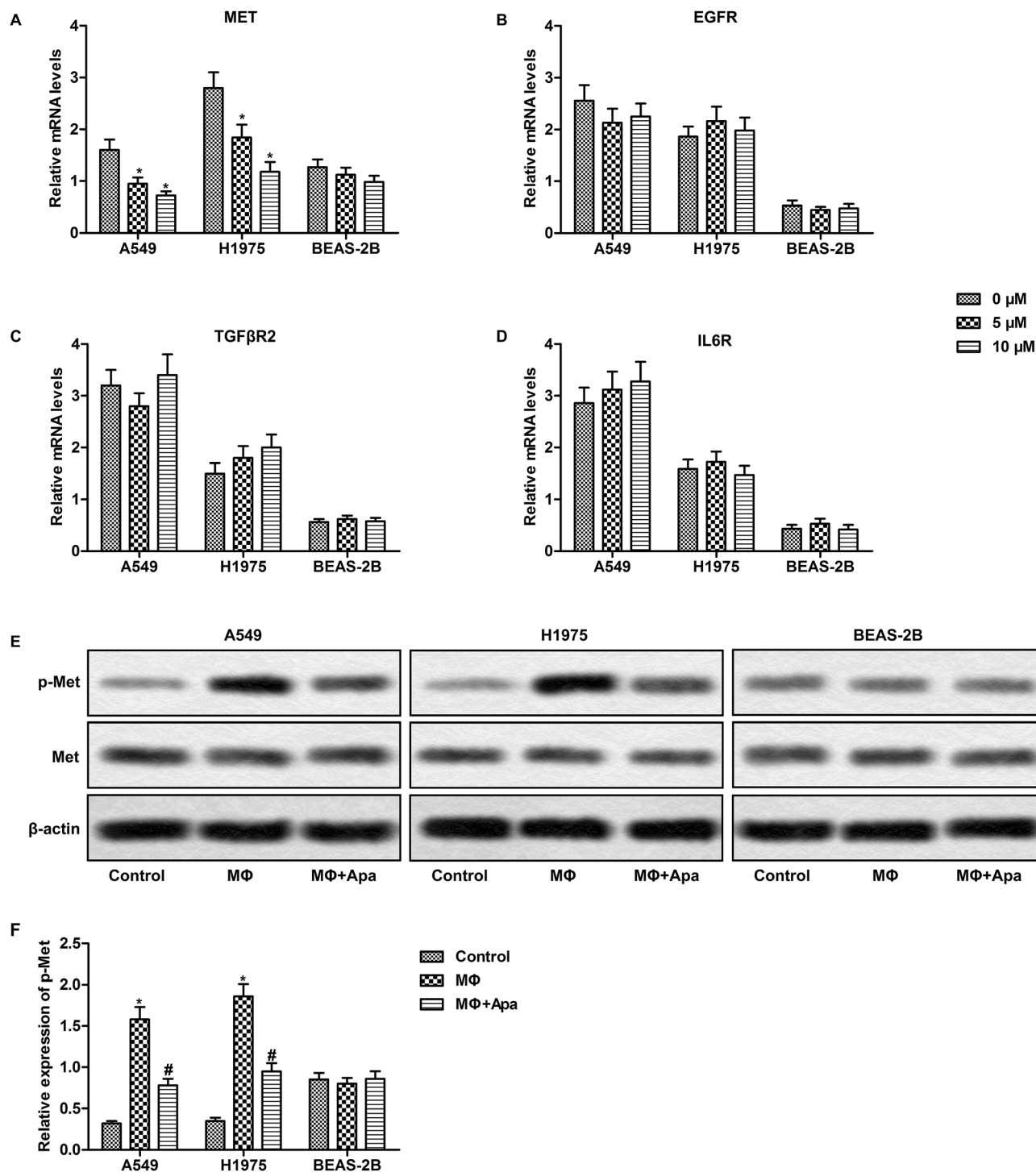


Fig. 5 Apatinib down-regulates HGF-Met signaling in polarized macrophage-treated lung cancer cells. A549, H1975 and BEAS-2B cells were treated with the supernatant of polarized macrophages (*M Φ*) which had been stimulated with LPS (1 ng ml^{-1}) and/or apatinib (0 – $10 \text{ } \mu\text{M}$) for 24 h. (A–D) The mRNA level of MET, EGFR, TGF β R2 and IL6R was detected by qPCR. (E) The phosphorylation of Met was detected by western blot. (F) Statistical analysis of Fig. 2E. * $P < 0.05$ versus control. # $P < 0.05$ versus *M Φ* .

phosphorylation, we next examined the expression of phosphorylated Met. The results showed that apatinib effectively decreased polarized macrophage-induced phosphorylation of Met in A549 and H1975 cells (Fig. 5E and F). Taken together, these results suggest that apatinib blocks polarized macrophage-activated HGF-Met signaling in lung cancer cells.

Discussion

To date, platinum-based chemotherapy is the preferred treatment of advanced lung cancer without genetic driver. However, the efficiency of first-line chemotherapy has reached a threshold and there are fewer options for post-first-line



therapy. Recent studies have reported that combination of anti-angiogenic drugs and chemotherapy could significantly improve PFS and overall OS in advanced NSCLC.^{12,13} It was reported that apatinib inhibits the invasion and migration of lung cancer cells *in vitro* via RET/Src signaling pathway.²⁰ Some clinical cases reports demonstrated that apatinib prolonged the PFS in NSCLC patients.^{16,17} In this study, we found that apatinib treatment significantly attenuated macrophage infiltration and EMT of lung tissue. EMT is a developmental cellular process by which polarized epithelial cells lose epithelial properties and cell–cell adhesions, and gain mesenchymal characteristics,^{21,22} which allows the cancer cell to become more invasive, metastatic and form a secondary metastasis.²³ E-cadherin and ZO-1 are required for the formation of stable adherens junction. During EMT, epithelial cells lose E-cadherin and convert into spindle shaped mesenchymal cells by acquiring N-cadherin.^{24,25} It was reported that E/N-cadherin switch play a crucial role in cancer progression. Slug and Snail are key regulators of E-cadherin and it was demonstrated that all these factors promotes tumor growth, recurrence and metastasis.^{26,27} Vimentin and fibronectin are mesenchymal markers and are overexpressed in cancer cells.^{28–30} In the present study, we found that apatinib significantly reversed macrophage-induced increase of N-cadherin, vimentin, Slug and Snail and decrease of ZO-1 and E-cadherin. Furthermore, apatinib also attenuated EMT-mediated migration and invasion of lung cancer cells.

Previous studies have reported that macrophage derived cytokines stimulate the progression of EMT.³¹ Thus, the expression of EMT-related cytokines was examined in polarized macrophages. Among these cytokines, TGF- β 1 is considered to be the most common activator of EMT.³² It promotes EMT through various pathways, such as smad2/3 and Wnt/ β -catenin.^{33,34} However, the expression of TGF- β 1 is not changed in polarized macrophages after apatinib stimulation. TGF- β 1 interacts with other cytokines to regulate the progression of EMT, such as IL-6 and EGF, and EGF also directly induces EMT.^{35,36} Similarly, the expression of EGF and IL-6 was not significantly altered by apatinib stimulation. Only the production of HGF was consistent with EMT progression. These data indicated that apatinib attenuated macrophage-induced EMT through inhibiting HGF expression.

HGF is a multifunctional growth factor originally isolated from rat platelets.³⁷ HGF-Met activation is considered to promote tumor cell proliferation and survival and EMT.³⁸ HGF and Met are also localized at sites of pathological angiogenesis and are a potent endothelial mitogens.³⁹ Many studies have reported that inhibition of HGF-Met pathway could attenuate the progression of EGFR-TKI-resistant lung cancer.^{40,41} Overexpression of Met activates ERBB3/PI3K/AKT signaling in EGFR mutant lung cancers, which lead to EGFR-TKI-resistance. In the present study, the results showed that apatinib not only inhibited HGF secretion in polarized macrophages, but also suppressed Met activation in polarized macrophage-stimulated lung cancer cells, suggesting that apatinib attenuated macrophage-induced EMT through HGF-Met signaling pathway. This study reported that apatinib may act as a potential VEGFR inhibitor in suppressing cancer progression. We are now applying a commonly used

inhibitor of MET and VEGFRs, Foretinib (GSK1363089), as a positive control of apatinib in lung cancer cells A549 and H1975, in order to convince the effect of apatinib on HGF expressions and EMT in lung cancer. In this study we first reported that apatinib inhibits macrophage-mediated EMT in lung cancers. Our study not only provided a new insight into apatinib's application in post-first-line therapy of lung cancer, but also elaborated a novel mechanism of apatinib's tumor-suppressive effect in treatment of lung cancer.

Conclusions

In this study we first reported that apatinib inhibits macrophage-mediated EMT in lung cancers. Our study not only provided a new insight into apatinib's application in post-first-line therapy of lung cancer, but also elaborated a novel mechanism of apatinib's tumor-suppressive effect in treatment of lung cancer.

Conflicts of interest

There are no conflicts to declare.

Abbreviations

VEGFR	Vascular endothelial growth factor receptor
EMT	Epithelial–mesenchymal transition
HGF	Hepatocyte growth factor
NSCLC	Non-small-cell lung cancer
EGFR	Epidermal growth factor receptor
ALK	Anaplastic lymphoma kinase
PFS	Progression-free survival
OS	Overall survival

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