Apatinib suppresses the migration, invasion and angiogenesis of hepatocellular carcinoma cells by blocking VEGF and PI3K/AKT signaling pathways

JIFU SONG, ZHIBIN GUAN, CHAO SONG, MAOJIANG LI, ZHIWEI GAO and YONGLI ZHAO

Department of Radiotherapy, Qingdao Jiaozhou City Central Hospital, Jiaozhou, Qingdao 266300, P.R. China

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Abstract. Hepatocellular carcinoma (HCC) is a commonly diagnosed malignancy worldwide with poor prognosis and high metastasis and recurrence rates. Although apatinib has been demonstrated to have potential antitumor activity in multiple solid tumors, the underlying mechanism of apatinib in HCC treatment remains to be elucidated. In the present study, apatinib were used to treat HCC cells transfected with or without VEGFR2 overexpression vectors. The proliferation of HCC cells was detected by MTT assay. The migration and invasion of HCC cells were detected by wound healing assay and Transwell assay. The ability of angiogenesis of HCC cells were detected by tube formation assay. The related protein expression levels were detected by western blotting. The present study aims to investigate the effect and potential mechanism of apatinib on the migration, invasion and angiogenesis of HCC cells. It was found that apatinib treatment significantly inhibited the proliferation, migration and invasion of Hep3b cells and suppressed angiogenesis in HUVECs. In addition, apatinib inhibited the epithelial-mesenchymal transition of Hep3b cells by increasing the expression of the epithelial hallmarks E-cadherin and α-catenin and decreased the expression of the mesenchymal hallmarks N-cadherin and vimentin. These effects were associated with the downregulation of VEGF and VEGFR2 and suppression of the PI3K/AKT signaling pathway. Thus, apatinib inhibited cell migration, invasion and angiogenesis by blocking the VEGF and PI3K/AKT pathways, supporting an effective therapeutic strategy in the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the severest types of human cancer and is the third-leading cause of cancer-related mortality worldwide (1). Although the treatment of HCC has improved, the long-term prognosis of patients with HCC is still poor, mainly due to the advanced stages of the disease at the time of diagnosis and its high metastasis and recurrence rates (2). Therefore, potential drugs with fewer side effects that could significantly inhibit the growth of existing tumors and prevent cancer cell metastasis, invasion and angiogenesis are required for treating HCC (3).

The epithelial-mesenchymal transition (EMT) is an important mechanism for epithelial-derived tumor cells to become malignant and acquire an invasive phenotype (4,5). It is accompanied by the downregulation of the epithelial marker E-cadherin and the upregulation of the mesenchymal markers vimentin and N-cadherin (6,7). Previous studies have identified that metastatic cancer cells induce EMT by modulating their cell shape or adhesive properties, thereby affecting cell adhesion and migration (8,9). Therefore, EMT serves an important role in tumorigenesis, invasion and metastasis.

VEGF is a signal protein released by epithelial cells and serves an important role in cell proliferation and neovascularization in several cancers, including HCC (10). In addition to the well-known effects of VEGF on angiogenesis, VEGF signaling serves an important role in promoting the proliferation and inhibiting the apoptosis in tumor cells (11). In HCC, multivariate analyses suggests that only a strong VEGF expression in tissue is significantly associated with metastatic recurrence (12). VEGF can stimulate the activation of the VEGFR2-dependent mTOR pathway to promote angiogenesis in lung cancer cells (13). Furthermore, there is evidence that the proliferation, migration, invasion and adhesion of non-small cell lung cancer (NSCLC) cells are significantly inhibited by blocking the VEGF/VEGFR2 pathway (11).

Apatinib is a tyrosine kinase inhibitor that selectively inhibits VEGFR2, resulting in the blocking of the intracellular VEGF signaling pathway (14). As a new oral antiangiogenic agent, apatinib has shown encouraging clinical results in treating various solid tumors (15-17). Apatinib has been identified as the only effective drug for patients with terminal gastric cancer without chemotherapy indications in a phase III clinical trial (18). In a phase II clinical trial, apatinib monotherapy is effective and safe in advanced HCC (19). However, the underlying mechanism of apatinib against HCC remains to be elucidated.

The present study explored the effect and potential mechanism of apatinib in HCC cell migration, invasion and

Correspondence to: Dr Yongli Zhao, Department of Radiotherapy, Qingdao Jiaozhou City Central Hospital, 29 Xuzhou Road, Jiaozhou, Qingdao 266300, P.R. China
E-mail: yonglizhao0807@163.com

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angiogenesis using the Hep3b cell line. It was found that apatinib reduced the proliferation, migration and invasion of Hep3b cells by regulating VEGF and PI3K-AKT signaling pathways.

Materials and methods

Cell culture and reagents. The human HCC cell line Hep3b was purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured in a humidified chamber at 37˚C with 5% CO_2. Apatinib was purchased from Jiangsu Hengrui Medicine Co., Ltd. and was dissolved in dimethyl sulfoxide (DMSO).

Cell transfection. VEGFR2 overexpression vector and empty vector (EV) were purchased from VectorBuilder. VEGFR2 were amplified by using the sense primer 5'-TGTCGTTGTAGGGTATAGGATTTATGAT-3' and the anti-sense primer 5'-ATACTTGTCGTCTGATTCTCCAGGTTTC-3'. Following the manufacturer's instructions for Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), Hep3b cells at 80% confluence were transfected with 1 μg/ml VEGFR2 expression vector or EV. After incubated at 37˚C with 5% CO_2 for 24 h for 24 h, cells successfully transfected with the vectors were used for subsequent experiments; the transfection efficiency was verified by fluorescence microscopy (Fig. S1).

MTT assay. After Hep3b cells were cultured overnight in 96-well plates ($1x10^5$ cells/well), the following three treatment conditions were set: i) Cells treated with 0, 20, 40 and 60 μM apatinib for 24 h; ii) cells treated with 40 μM apatinib for 12, 24, 48 and 72 h; and iii) cells transfected with VEGF overexpression vector or EV for 2 h following treatment with 40 μM apatinib for 24 h. Following culture for another 24 h, 10 mg/ml MTT was added into each well and incubated for another 4 h. Cells were centrifuged at 1,000 x g for 5 min at room temperature. DMSO (100 μl) was added into each well and incubated for 30 min to dissolve the formazan product. The absorbance value of each well was measured at a 490 nm wavelength.

Transwell assay. Hep3b cells were resuspended in RPMI-1640 supplemented with 1% FBS and $1x10^4$ Hep3b cells were seeded into the top chamber and treated under two conditions: i) Cells treated with 40 μM apatinib for 24 h; and ii) cells transfected with VEGF overexpression vector or EV for 2 h following treatment with 40 μM apatinib for 24 h. Cells in the upper chamber were then gently removed and the invaded cells were collected and fixed for 30 min using 4% paraformaldehyde, stained with 0.1% crystal violet for 30 min at room temperature and washed three times with PBS. The number of cells was then counted under an optical microscope (x100 magnification; Olympus Corporation).

Wound healing assay. Hep3b cells were seeded into 6-well plates at $2x10^5$ cells/well and two treatment conditions were set: i) Cells treated with 40 μM apatinib for 24 h; and ii) cells transfected with VEGF overexpression vector or EV for 2 h following treatment with 40 μM apatinib for 24 h. The wound gap on the cell monolayer was created using a 200 μl pipette tip and cultured in serum-free RPMI-1640. An optical microscope at x100 magnification was used for imaging and the migration of cells was observed at 24 h after wound scratching.

Matrigel in vitro HUVEC tube formation assay. The conditioned media (CM) of Hep3b cells were collected and stored at -80˚C. HUVECs were trypsinised and seeded ($5.0x10^4$ cells per well) into Matrigel-coated wells (coated with Matrigel at 4˚C, then incubated for 30 min at 37˚C) with 250 μl of CM from Hep3b cells. Following incubation at 37˚C for 24 h, six different fields were randomly chosen in each well and images were captured.

Western blotting analysis. Hep3b cells were washed in PBS and lysed using the protein extraction reagent RIPA (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration of proteins was measured by BCA kit (cat. no. ab102536; Abcam). Equivalent amounts of proteins (30 μg) from each sample were electrophoresed on SDS-polyacrylamide gel (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane, blocked in 4% skim milk for 2 h at room temperature and incubated with the following specific primary antibodies: E-cadherin antibody (ab219332 1:1,000; Abcam), α-catenin antibody (ab51032 1:2,000; Abcam), N-cadherin (ab76011, 1:5000 dilution, Abcam), vimentin (ab92547 1:1,000; Abcam), p-PI3K (ab182651 1:1,000; Abcam), PI3K (ab227204 1:1,000; Abcam), p-AKT (ab38449, 1:500 dilution, Abcam), AKT (ab18785 1:1,000; Abcam), VEGF (ab214424 1:1,000; Abcam), VEGFR2 (ab221679 1:1,000; Abcam), Snail (ab53519 1:1,000; Abcam), Slug (ab27568 1:1,000; Abcam) and MPP9 (ab38898 1:1,000; Abcam) overnight at 4˚C. β-actin (ab8277 1:1,000; Abcam) was used as internal reference. Then, the membranes were incubated in HRP-linked goat anti-rabbit IgG secondary antibody (ab97051; 1:10,000; Abcam) for 2 h at room temperature. Immunoreactivity was visualized by a colorimetric reaction using an ECL substrate buffer (EMD Millipore) and membranes were scanned with Gel Doz EZ imager (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are shown as the mean ± standard deviation. Statistical analysis was performed using Tukey's post hoc test for one-way analysis of variance using the SPSS 16.0 software package (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Apatinib inhibits the proliferation of Hep3b cells. Compared with the control group, 10, 20, 40 and 60 μM apatinib treatment significantly reduced the proliferation of Hep3b cells (Fig. 1A). Treatment with 40 μM apatinib for 24 h inhibited the proliferation of Hep3b cells and a greater decrease in

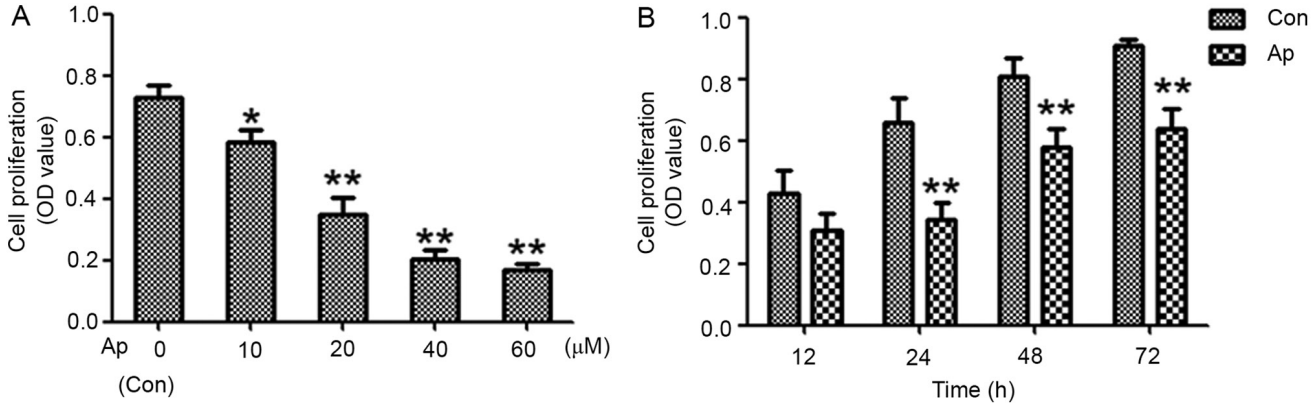

Figure 1. Apatinib inhibited the proliferation of Hep3b cells. (A) Hep3b cells were incubated for 24 h in the presence of various concentrations of apatinib. (B) Hep3b cells were incubated in the absence or presence of 40 μM apatinib for the indicated time interval. Values are shown as mean ± standard deviation (n=3); *P<0.05, **P<0.01 vs. Con group. Con, control; OD, optical density; Ap, apatinib.

cell proliferation was observed upon treatment with apatinib for 48 and 72 h (Fig. 1B). These results indicated that apatinib decreases the proliferation of Hep3b cells in a dose- and time-dependent manner.

Apatinib inhibits the migration and invasion of Hep3b cells. Previous studies have revealed that apatinib reduces tumor cell metastasis by inhibiting EMT (20,21). The present study investigated the metastatic ability of Hep3b cells following treatment with 40 μM apatinib using the Transwell and wound healing assays. It was found that apatinib treatment was sufficient to reduce invasion of Hep3b cells (Fig. 2A) and inhibit migration (Fig. 2B). EMT-related protein levels in Hep3b cells treated with or without 40 μM apatinib were detected by western blotting. The results showed that apatinib significantly induced protein expression of E-cadherin and α-catenin and reduced protein expression of N-cadherin, vimentin, Snail, Slug and MMP9 in Hep3b cells (Fig. 2C). The PI3K/Akt pathway activation is known to be involved in tumor cell invasion and metastasis in response to various growth factors, including EMT (22,23). In the present study, the treatment of 40 μM apatinib significantly inhibited the activation of the PI3K/AKT pathway (Fig. 2D). These data demonstrated that apatinib inhibited migration and invasion by suppressing the PI3K/AKT pathway-dependent EMT in Hep3b cells.

Apatinib inhibits the angiogenesis of HUVEC cells. Next, it was determined whether the conditioned medium from Hep3b cells could regulate tube formation in HUVECs. After 24 h of incubation, CM from Hep3b cells treated with apatinib has decreased the extent of tube formation by HUVECs compared with the control group (Fig. 3A). To further elucidate the underlying molecular mechanism of apatinib on angiogenesis, protein expression levels of VEGF and VEGFR2 were determined upon cell treatment. It was found that treatment of Hep3b cells with 40 μM apatinib led to decreases in VEGF and VEGFR2 protein levels (Fig. 3B).

VEGFR2 overexpression abolishes the inhibitory effect of apatinib on Hep3b cell migration and invasion. Knowing that apatinib inhibited expression of VEGF and VEGFR2 and signaling activity in Hep3b cells, the role of VEGF signaling in apatinib-treated Hep3b cells was next determined. VEGFR2 overexpression significantly counteracted the inhibitory effects of apatinib on the proliferation, migration and invasion of Hep3b cells (Fig. 4A-C). VEGFR2 overexpression significantly induced EMT in apatinib-treated Hep3b cells via the downregulation of E-cadherin and α-catenin and upregulation of N-cadherin, vimentin, Snail, Slug and MMP9 (Fig. 4D). In addition, VEGFR2 overexpression significantly induced the activation of the PI3K/AKT pathway by increasing the levels of phosphorylated PI3K and AKT in Hep3b cells (Fig. 4E).

VEGFR2 overexpression abolishes the inhibitory effect of apatinib on the angiogenesis of HUVEC cells. After 24 h of incubation, CM from Hep3b cells transfected with VEGFR2 overexpression vector combined with apatinib treatment increased the extent of tube formation by HUVECs compared the Ap group (Fig. 5A). The protein expression levels of VEGF and VEGFR2 in Hep3b cells transfected with the VEGF overexpression vector combined with apatinib treatment were higher than the Ap group (Fig. 5B).

Discussion

Liver cancer is one of the three most lethal cancers. Although patients with HCC exhibit increased survival rates following curative resection, the prognosis of patients with HCC remains poor due to tumor metastasis and invasiveness (24). Apatinib is a highly selective inhibitor of multiple tyrosine kinases and one of the latest agents with encouraging preclinical and clinical data in treating solid tumors (25). Previous studies have shown that apatinib treatment leads to apoptosis and autophagy and inhibits EMT and metastasis in osteosarcoma cells (26,27). The present study found that apatinib significantly inhibited the proliferation, invasion and metastasis of Hep3b cells. This was consistent with a previous study on cholangiocarcinoma, which found that apatinib inhibits cellular migration and invasion via the PI3K/AKT pathway (28). The PI3K/AKT signaling pathway serves an important role in regulating tumor growth, angiogenesis, apoptosis, invasion and metastasis (29). Abnormal activation of the PI3K/Akt/mTOR signaling pathway occurs in ~45% of HCC cases and is associated with the poor prognosis in patients with HCC through related

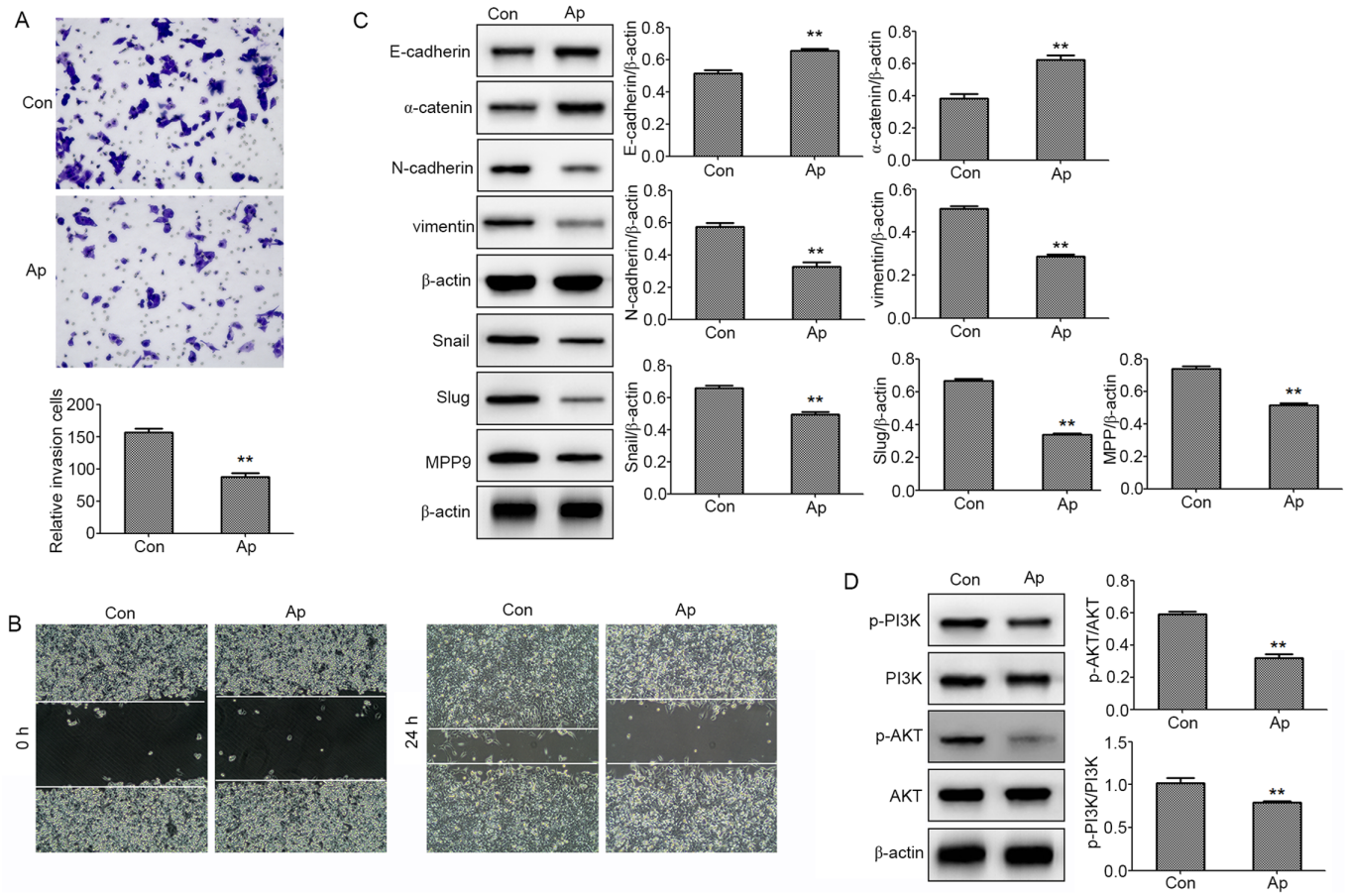

Figure 2. Apatinib inhibited the migration and invasion of Hep3b cells. (A) Invasion of Hep3b cells incubation with apatinib for 24 was determined by Transwell assay (magnification, x100). (B) Migration of Hep3b cells in response to apatinib was determined by wound scratch assay at 0 and 24 h under microscope (magnification, x100). (C) Western blotting was used to measure the expression level of EMT-related proteins E-cadherin, α-catenin, N-cadherin, Vimentin, Snail, Slug and MMP9 (D) Western blotting was used to measure the expression level of p-PI3K, PI3K, p-AKT and AKT. Values are shown as mean ± standard deviation (n=3); **P<0.01 vs. Con group. EMT, epithelial-mesenchymal transition; p-, phosphorylated; Con, control; Ap, apatinib.

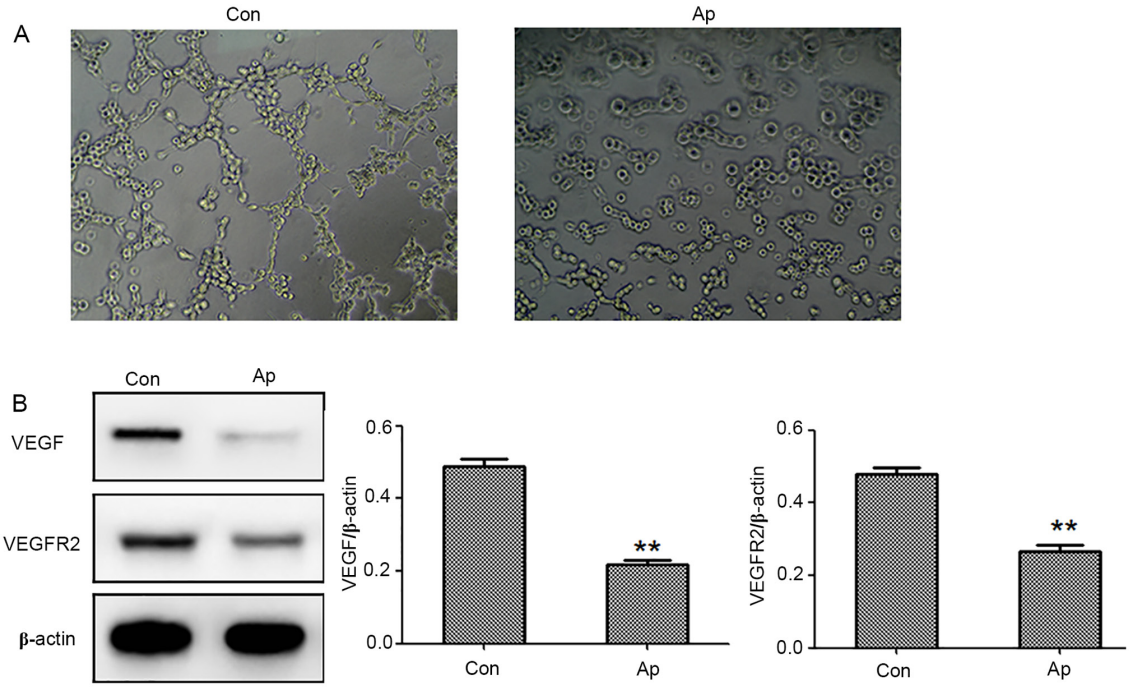

Figure 3. Apatinib inhibited the tubule formation of HUVECs. (A) Effects Hep3b cells-CM on HUVEC tube formation on Matrigel after 24 h were recorded with a bright field microscope (magnification, x100). (B) Western blotting was used to measure the protein expression level of VEGF and VEGFR2 in Hep3b cells. Values are shown as mean ± standard deviation (n=3); **P<0.01 vs. Con group. HUVECs, human umbilical vein endothelial cells; Con, control; Ap, apatinib.

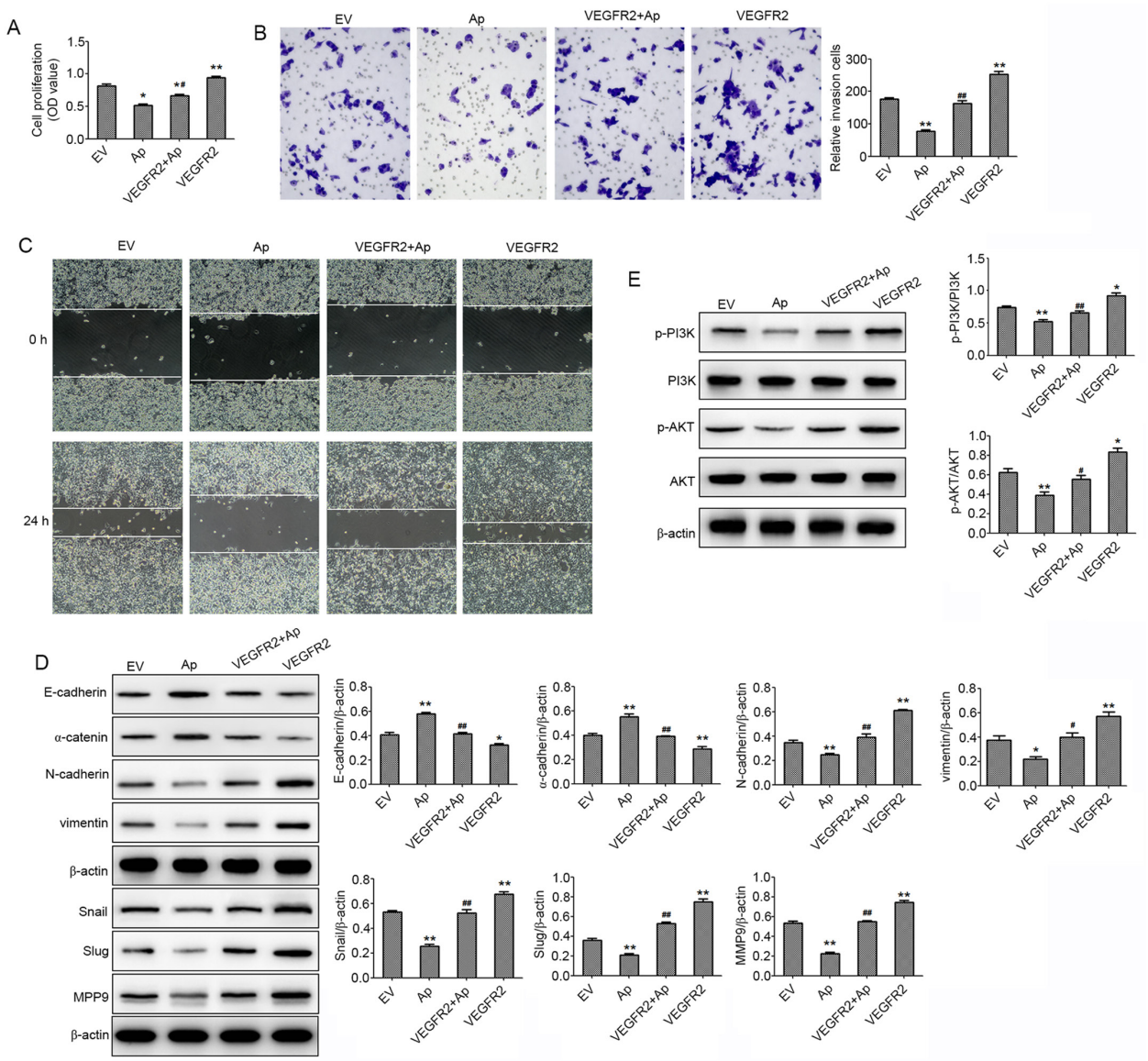

Figure 4. VEGFR2 overexpression suppresses the inhibitory effect of apatinib on Hep3b cell proliferation, migration and invasion. (A) The proliferation of Hep3b cells were detected by MTT assay. (B) Invasion of Hep3b cells was determined by Transwell assay (magnification, x100). (C) Migration of Hep3b cells was determined by wound scratch assay at 0 and 24 h under microscope (magnification, x100). (D) Western blotting was used to measure the expression level of EMT-related proteins E-cadherin, α-catenin, N-cadherin, Vimentin, Snail, Slug and MMP9. (E) Western blotting was used to measure the expression level of p-PI3K, PI3K, p-AKT and AKT. Values are shown as mean ± standard deviation (n=3); *P<0.05 or **P<0.01 vs. EV group; $^{\#}$P<0.05 or $^{\#\#}$P<0.01 vs. Ap group. EMT, epithelial-mesenchymal transition; p-, phosphorylated; EV, empty vector; Ap, apatinib.

independent prognostic factors, such as vascular invasion, metastasis stage and tumor differentiation (30,31). The activation of the PI3K/AKT pathway is reported to enhance the invasion and metastasis of HCC cells (32). The present study found that apatinib treatment reduced the phosphorylation of PI3K and AKT in Hep3b cells, indicating that a strong inhibitory effect of apatinib on Hep3b cell migration and invasion is associated with the inhibition of the PI3K/AKT signaling pathway. Similar results have found in NSCLC cells, in which the apatinib treatment synergistically reduced proliferation and inhibited the migration and invasion of NSCLC cells (33).

A previous study reported that apatinib treatment significantly attenuated macrophage infiltration and EMT of lung tissue (20). EMT is a fundamental process during which tumor cells acquire the capacity of migration and invasion (34). It is mainly characterized by the downregulation of cell adhesion molecules [including E-cadherin, α-catenin and zonula occludens-1 (ZO-1)], transformation of the cytokeratin cytoskeleton into vimentin and morphological characteristics of mesenchymal cells (35). E-cadherin, ZO-1 and α-catenin are necessary to form stable adherens junctions (36). During the EMT process, epithelial cells lose E-cadherin and transform into spindle shaped mesenchymal cells by acquiring N-cadherin. Vimentin and fibronectin are mesenchymal markers that are overexpressed in cancer cells, demonstrating that these factors promote tumor growth, metastasis and recurrence (37). Zheng *et al* (27) suggested that apatinib inhibits the migration and invasion of osteosarcoma by targeting STAT3 pathway to inhibit EMT. The present study found that the apatinib treatment increased the expressions of the epithelial hallmarks E-cadherin and α-catenin and decreased the expressions of the mesenchymal hallmarks N-cadherin, Vimentinin, Snail, Slug and MMP9 in Hep3b cells. These results indicated

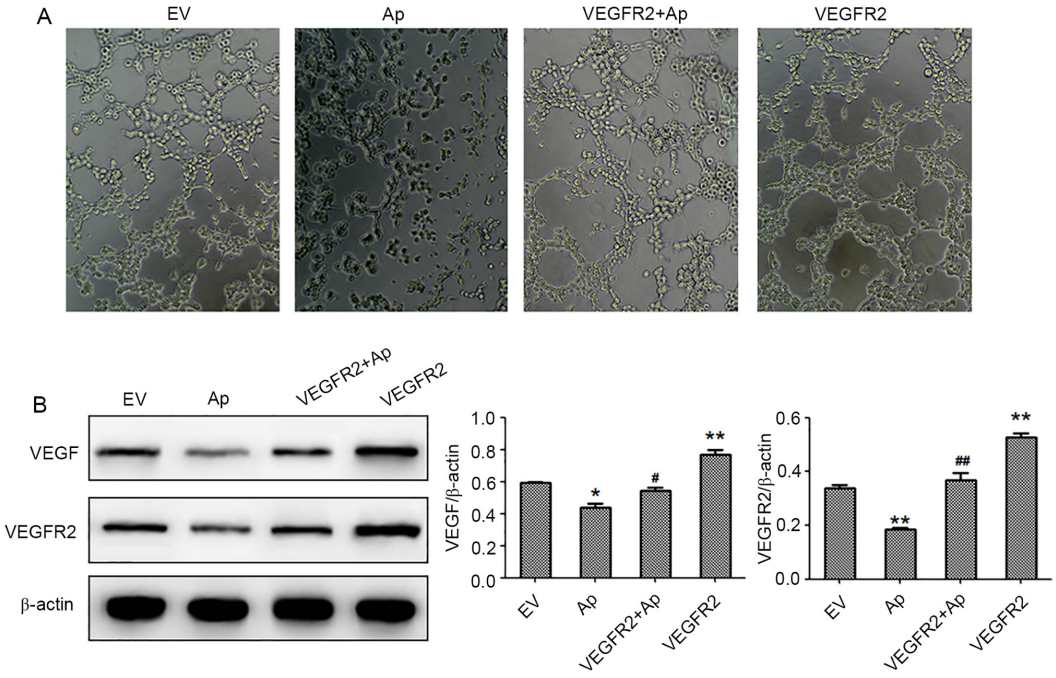

Figure 5. VEGFR2 overexpression suppresses the inhibitory effect of apatinib on the angiogenesis of HUVEC cells. (A) Effects Hep3b cells-CM on HUVEC tube formation on Matrigel after 24 h were recorded with a bright field microscope (magnification, x100). (B) Western blotting was used to measure the protein expression level of VEGF and VEGFR2 in Hep3b cells. Values are shown as mean ± standard deviation (n=3); *P<0.05 or **P<0.01 vs. EV group; $^{\#}$P<0.05 or $^{\#\#}$P<0.01 vs. Ap group. HUVECs, human umbilical vein endothelial cells; CM, conditioned media; EV, empty vector; Ap, apatinib.

that apatinib attenuates the migration and invasion of Hep3b cells by regulating EMT.

HCC is a typical hypervascular tumor with a high VEGFR expression (38). VEGF promotes angiogenesis by inducing proliferation and migration of endothelial cells (39). Tumor angiogenesis provides essential growth that requires nutrients and oxygen for tumor occurrence, development and metastasis and which are closely associated with tumor stage and prognosis (40-42). Therefore, studies have been conducted to explore agents that target the VEGF axis in advanced HCC (43-45). It has been reported that apatinib effectively inhibits the proliferation, migration and tube formation of HUVEC by blocking the VEGF axis (14). Chen *et al* (21) reported that the inhibitory effects of apatinib on EMT and tumorigenesis may be associated with the downregulation of the expression levels of MMP2/9, VEGF and VEGFR2 in HCC. Similarly, apatinib had an inhibitory effect on the VEGF/VEGFR pathway, Hep3b cell migration, invasion and tube formation in the present study (46). A previous study reported that the PI3K/Akt/NF-κB pathway can regulate the invasion of carcinoma cells via upregulation of VEGF, indicating that VEGF is the downstream target of the PI3K/AKT pathway in regulating cancer invasion and metastasis (47). In addition, apatinib treatment inhibits tumor growth and angiogenesis in anaplastic thyroid carcinoma via suppression of the AKT pathway way (48). The present study found that apatinib inhibited the expressions of VEGF and VEGFR2 and reduced the activation of PI3K/AKT. Conversely, the VEGFR2 overexpression markedly increased the activation of the PI3K/AKT pathway. These results indicated that the anti-angiogenic effect of apatinib in Hep3b cells may be mediated, at least in part, by preventing the VEGF expression and subsequent decrease in PI3K/AKT pathway activation.

In conclusion, the data presented in the current study revealed that apatinib suppressed the proliferation, migration and invasion of Hep3b cells by inhibiting the PI3K-AKT pathway-mediated EMT. In addition, it suppressed the expression and activity of pro-angiogenic factors VEGF and VEGFR-2, which may also involve the activation of the downstream PI3K-AKT signaling pathway, resulting in the inhibition of angiogenesis. These findings indicated that apatinib has a great potential for use as an antitumor agent in patients with HCC by inhibiting cell migration, invasion and angiogenesis by blocking the VEGF and PI3K/AKT pathways. A further study on the effect apatinib effect on other liver cancer cell lines and *in vivo* animal experiment will be of great interest to fully elucidate the underlying signaling pathways involved in its anti-cancer effect.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JS, ZG and YZ designed the experiments. JS, CS and ML performed most of the experiments. JS drafted the manuscript.

ZG contributed to the analysis and interpretation of the data. YZ modified the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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