Original Article
Apatinib suppresses breast cancer cells proliferation and invasion via angiomotin inhibition

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Abstract: Breast cancer is a leading cause of cancer-related death in the women worldwide. Apatinib is a novel tyrosine kinase inhibitor that selectively binds and inhibits vascular endothelial growth factor receptor 2 (VEGFR-2). The clinical trials have demonstrated the objective efficacy of Apatinib against metastatic breast cancer. However, the underlying mechanism is not well established. In the present study, the breast cell lines, BT-474 and MCF-7, were investigated. The effect of Apatinib on the cell viability was determined using CCK-8 assay. The migration, invasion, cell cycle distribution and the downstream signaling of VEGFR-2 in the cells were determined after 48 h treatment with this drug. Subsequently, Vector of angiomotin (AMOT) cDNA was transfected into MCF-7 cells. The cells were either exposed to Apatinib or vehicle and then examined for cell viabilities, migration, invasion, cell cycle distribution and the downstream signaling of VEGFR-2. Apatinib demonstrated a dose-dependent, significant inhibition of cell viabilities, migration and invasion of BT-474 and MCF-7 cells, with an increase in the percentage of cells in G1 phase and a decrease in S phase. In addition, in MCF-7 cells, Apatinib decreased AMOT expression, accompanied with the decreased expression of LATS1/2, YAP, ERK1/2 phosphorylation and cyclin D1. The inhibitory effect of Apatinib on the cell activities and protein expressions were significantly suppressed by AMOT overexpression. The results of this study indicated that Apatinib inhibited MCF-7 cell proliferation and invasion through AMOT/VEGFR-2 pathway.

Keywords: Breast cancer, Apatinib, Yes-associated protein, invasion, proliferation

Introduction

Breast cancer is the most common cancer in the women worldwide, accounting for approximately 29% of the total new cases in 2016 [1]. Despite the development of various therapies, recurrence and metastasis remain major obstacles for breast cancer treatments [2]. About 40% of breast cancer patients experience recurrence, and about 60-70% of patients display distant metastasis, resulting in the high mortality of about 14% of the total deaths [1, 3].

It is well known that angiogenesis critically contributes to tumor development and metastasis. The physiological and pathological angiogenesis is majorly induced by the vascular endothelial growth factor (VEGF) signaling through its receptor, which is composed of VEGF receptor

1 (VEGFR-1), VEGFR-2 and VEGFR-3. In addition to the principle regulatory role in VEGF signaling, VEGFR-2 plays an important role in the angiogenesis through most pro-angiogenesis factors, leading to functions as the molecular hub to integrate pro-angiogenic signals in tumor microenvironment [4, 5]. Therefore, blocking VEGFR-2 pathway is considered as a potential therapy for tumors. Apatinib, an orally administered small-molecular tyrosine kinase inhibitor (TKI), selectively binds and inhibits VEGFR-2. With encouraging clinical data and manageable toxicities, it was approved and launched in China for treatment of advanced gastric cancer. It has also shown potential antitumor activities in other tumor such as hepatocellular carcinoma and non-small-cell lung cancer. Currently, clinical trials are in progress to investigate the efficacy of Apatinib in the treatment of meta-

static breast cancer. In these trials, Apatinib showed objective efficacy [6, 7]. It is well established that the activity against angiogenesis and vasculature play an important role in the efficacy of Apatinib against metastatic breast cancer. In addition to endothelial cell, VEGFRs are expressed on cancer cells. A few recent studies have revealed that Apatinib induced the apoptosis of tumor cells, such as human intrahepatic cholangiocarcinoma cells [8]. Herein, we hypothesized that Apatinib can induce breast cancer cell apoptosis. Furthermore, the mechanism underlying of the suggested effect was also investigated in this study.

Angiomotin (AMOT), an 80-kDa protein, is mostly expressed in endothelial cells and in some epithelial cells. It has been reported that the expression of AMOT is upregulated in breast cancer tissues and is one of the important features of breast cancer [9]. Associated with a poor clinical outcome in breast cancer, the protein was recommended as a target for therapy [10]. It is well accepted that AMOT exerts functions majorly through regulating Hippo signaling pathway. LATS1/2, a core component in this pathway, is recruited and activated by AMOT. Activation of LATS1/2 results in phosphorylation and sequestering of Yes-associated protein (YAP) in the cytoplasm.

In this study, we investigated the effect of Apatinib on the physiological activities of breast cancer cells. For the first time for breast cancer cells, we report that Apatinib inhibited cell proliferation and invasion, which was mediated by the down-regulated AMOT.

Materials and methods

Cell culture

Human breast cancer cell lines, BT-474 and MCF-7 were obtained from American Type Culture Collection (ATCC, USA), and were maintained in RPMI-1640 medium (Hyclone, USA) containing 10% fetal bovine serum (Gibco, USA) and 0.1 g/L streptomycin and penicillin G (Sigma Aldrich). The cells were cultured at 37°C in humidified atmosphere with carbon dioxide.

Cell transfection

The cDNA encoding the human AMOT was cloned and inserted into pcDNA 3.0 vector. The expression vectors were transfected into MCF-7 cells using Exgen 500 (Euromedex) to produce stable transfectants. The pcDNA 3.0 empty vectors were transfected to generate negative controls. The transfected cells were grown in the medium containing hygromycin B.

Cell viability

The cell viability was determined with Cell Counting Kit-8 (CCK-8; KeyGen, Nanjing, China). BT-474 and MCF-7 cells were seeded in 96-well plates at a density of 1500 cells/well. After incubation overnight, the cells were exposed to Apatinib (Selleck, Texas, USA) or vehicle for 24, 48 and 72 h in the complete medium. The medium was refreshed and 10 μL CCK-8 was added into each well. The cells were incubated for additional 2 h, and the absorbance values were measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). The experiments were repeated in triplicate.

EdU incorporation assay

The cells were seeded in 96-well plates at a density of 1500 cells/well and incubated overnight. The cells were treated with vehicle or Apatinib for 48 h and then were exposed to 50 μmol/L EdU (RiboBio, Guangzhou, China) in culture medium for 2 h. After fixed with 4% paraformaldehyde for 15 min at room temperature and washed with phosphate-buffed saline, the cells were incubated with 100 μL Apollo reaction cocktail (RiboBio, Guangzhou, China) for 30 min and then stained with DAPI (5 μg/mL; Beytime, Nantong, China). Visualization was conducted under a fluorescence microscope (Olympus, Tokyo, Japan).

Wound healing and invasion assay

For the wound-healing assay, the cells were seeded in 6-well plates coated with 10 μg/ml type I collagen (Sigma). As 70% confluence was reached, the cell monolayers were disrupted with a pipette tip, and washed with PBS. Then the monolayers were exposed to vehicle or Apatinib in complete medium for 48 h. The monolayer was photographed right after injury and after treatment using a phase-contrast microscope (Olympus, Tokyo, Japan). The experiments were performed in triplicate for each treatment group. The invasion assay was performed using transwells (8 μm, Costar, Dallas,

Table 1. Primer sequences of quantitative real-time PCR

Genes	Primers	Sequences (5'-3')
GAPDH	Forward	TGTTCGTCATGGGTGTGAAC
	Reverse	ATGGCATGGACTGTGGTCAT
VEGFR-2	Forward	GTGATCGGAAATGACACTGGAG
	Reverse	CATGTTGGTCACTAACAGAAGCA
AMOT	Forward	AGGCAAGAGTTGGAAGGATGC
	Reverse	AGGATGACTTCACGAGGTTCT
YAP	Forward	TAGCCCTGCGTAGCCAGTTA
	Reverse	TCATGCTTAGTCCACTGTCTGT
LATS1	Forward	TTACCAAGATCCTCGACGAGAG
	Reverse	CACATTCCCTGGTTTCATGCT
LATS2	Forward	ACCCCAAAGTTCGGACCTTAT
	Reverse	CATTTGCCGGTTCACTTCTGC
Cyclin D1	Forward	GCTGCGAAGTGGAAACCATC
	Reverse	CCTCCTTCTGCACACATTTGAA
ERK	Forward	TACACCAACCTCTCGTACATCG
	Reverse	CATGTCTGAAGCGCAGTAAGATT

TX, USA) coated with Matrigel (BD Biosciences, San Jose, CA, USA). The cells treated with vehicle or Apatinib for 48 h were seeded into the upper chamber of the transwell at a density of 2×10^4 cells/insert in serum-free culture medium. The lower chambers were filled with culture medium containing 10% FBS. After incubation at 37°C for 24 h, the cells in the top compartment were removed, while those migrated to the underside of the inserts were fixed in methanol and stained with 2% crystal violet. The effects of Apatinib on cells were observed using light microscope (Olympus, Tokyo, Japan) and the representative fields were taken.

Cell cycle distribution assay

After 48-hour treatment with vehicle or Apatinib, the cells were collected and fixed in cold 70% ethanol. The cells were then centrifuged and washed with cold PBS, incubated with RNase A (Sigma, USA) and propidium iodide (50 µg/ml; Sigma, USA) at 4°C for 30 min in the dark, and then analyzed using a FACSCalibur flow cytometer (Becton Dickinson, CA, USA). The residual gaps between the two sides of the wound were monitored by an inverted optical microscope (Olympus, Tokyo, Japan).

Quantitative RT-PCR analysis

To examine the target mRNA expressions, total RNA was extracted from the cultured cells with

TRizol reagent (TaKaRa, Japan) and then 1 µg of total RNA was reversely transcribed into cDNA with Bestar qPCR RT kits (DBI Bioscience, China) based on the manufacturer's instructions. Using DBI Bestar SybrGreen qPCR Master Mix (DBI Bioscience, China), the real time PCR was performed with Stratagene Mx3000P Real-Time PCR system (Agilent Technologies, USA). Amplification was performed in the following condition: initial denaturing at 95°C for 30 s, and then three-step program composed of denaturing at 95°C for 5 s, annealing at 55°C for 30 s and extending at 72°C for 20 s, 40 cycles. The primers were presented in **Table 1**. All reactions were replicated three times. The expression levels of mRNA were normalized to GAPDH expression and were calculated using the delta delta Ct method.

Western blotting

The cells pre-washed with phosphate-buffered saline (PBS), were lysed in RIPA buffer supplemented with protease/phosphatase inhibitor cocktail (cell signaling, USA). The protein concentrations in the lysates were determined with the BCA Protein Assay Kit (Bio-Rad). The lysates were denatured with SDS buffer and 20 µg protein was loaded per lane. The proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, USA). After blocking with 5% skim milk powder, blots were incubated with primary antibodies against AMOT, YAP, p-YAP, LAST1, LAST2, Cyclin D1, ERK1/2, p-ERK1/2 and GAPDH (all from Abcam, Cambridge, US) overnight at 4°C. Following incubation with the peroxidase-conjugated secondary antibodies, ECL detection reagents were used to visualize the target protein bands.

Statistical analysis

Data were presented as mean ± SD. The results were analyzed using SPSS 16.0 software (IBM, Armonk, NY, USA). Statistical analyses were performed using one-way analysis of variance. A value of *P*<0.05 was considered as statistically significant.

Results

Apatinib deregulated the growth and cell cycle distribution in breast cancer cell lines

In order to investigate the effects of Apatinib on breast cancer cells, we initially treated BT-474
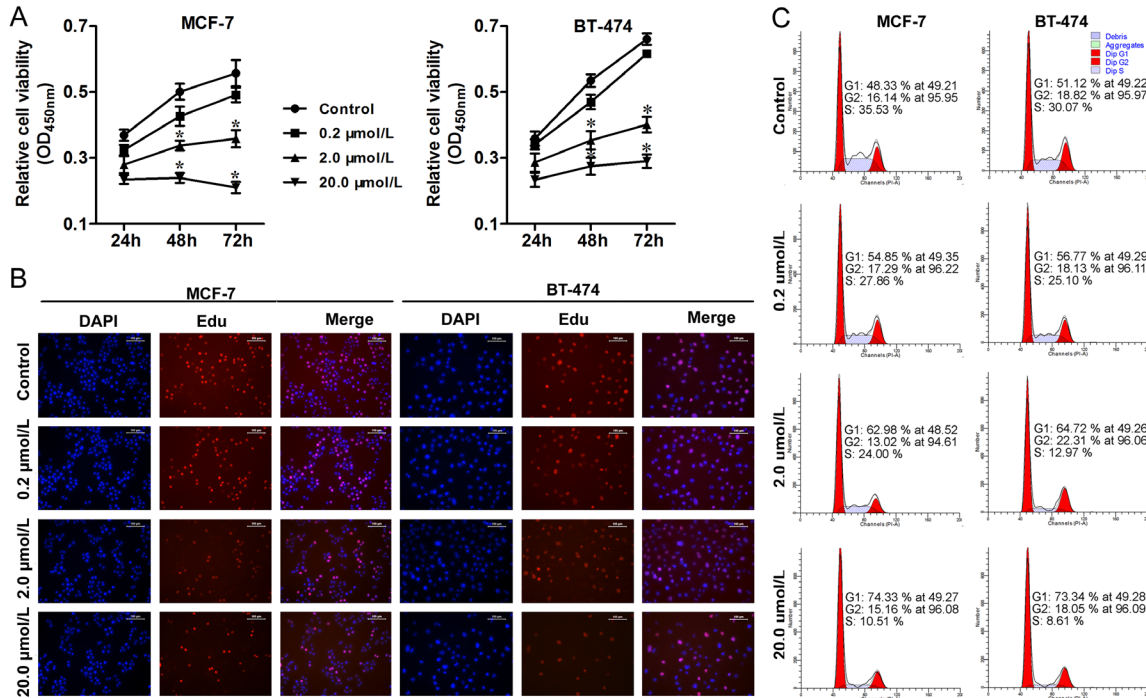
Figure 1. Apatinib inhibited the growth of cells. MCF-7 and BT-474 cells were treated with vehicle or 0.2, 2 or 20 µmol/L of Apatinib. The cell viabilities were determined by CCK-8 assay after 24, 48 or 72 h of treatment (A). After 48 h of treatment, proliferating cells was examined by EdU incorporation assay (Magnification, 100×; red), DAPI (blue) nuclear staining for live cells, and then images were merged (B); The cell cycle distribution was determined with flow cytometry (C). Vertical bars represented SD of the mean values (n = 3). *P<0.05 as compared with the control group.

and MCF-7 cells with various concentrations of Apatinib for 24 h, 48 h and 72 h. Cell viabilities were subsequently examined with CCK-8 assay. As shown in **Figure 1A**, Apatinib at 0.2 µmol/L slightly decreased the growth of BT-474 and MCF-7 cells without statistical significance. At 2.0 and 20.0 µmol/L, the drug exerted marked dose-dependent inhibition of cell growth and with statistically significant inhibition from the start of 48-hour treatment. We then performed an EdU assay on the cells. Apatinib demonstrated a dose-dependent inhibition on the proliferation of the cells (**Figure 1B**). The results indicated that Apatinib inhibited the breast cancer cells viabilities.

To further confirm the hypothesis that inhibited proliferation causes decreased cell viabilities, Apatinib's effects on cell cycle distribution in BT-474 and MCF-7 cells were determined using flow cytometry 48 h after Apatinib treatment. **Figure 1C** shows Apatinib significantly caused cell cycle arrest at G1 in both cells in a concentration-dependent manner. As compared with the control cells (51.12%), the percentage of

BT-474 cells arrested in G1 phase was 56.77%, 64.72% and 73.34%, respectively, when treated with Apatinib at 0.2, 2 and 20 µmol/L. Similarly, Apatinib treatment at 0.2, 2 and 20 µmol/L increased the percentage of MCF-7 cells arrested in G1 phase increased to 54.84%, 62.98% and 74.33%, respectively, from the control of 48.33%. Correspondingly, Apatinib treatment caused a marked decrease in numbers of BT-474 and MCF-7 cells at S phase in a dose-dependent manner. From the control of 30.07 and 35.53% for BT-474 and MCF-7 cells, the percentage of cells in S phase maximally decreased to 8.61% and 10.51% at 20 µmol/L, respectively. All these indicted that Apatinib exerted a cell cycle deregulation effect and resulting in affecting cell growth.

Apatinib inhibited the motility and invasiveness of breast cancer cells

Since cell migration is a critical process in breast cancer progression and metastasis, the effects of Apatinib on the migration and invasiveness of breast cancer cells were observed
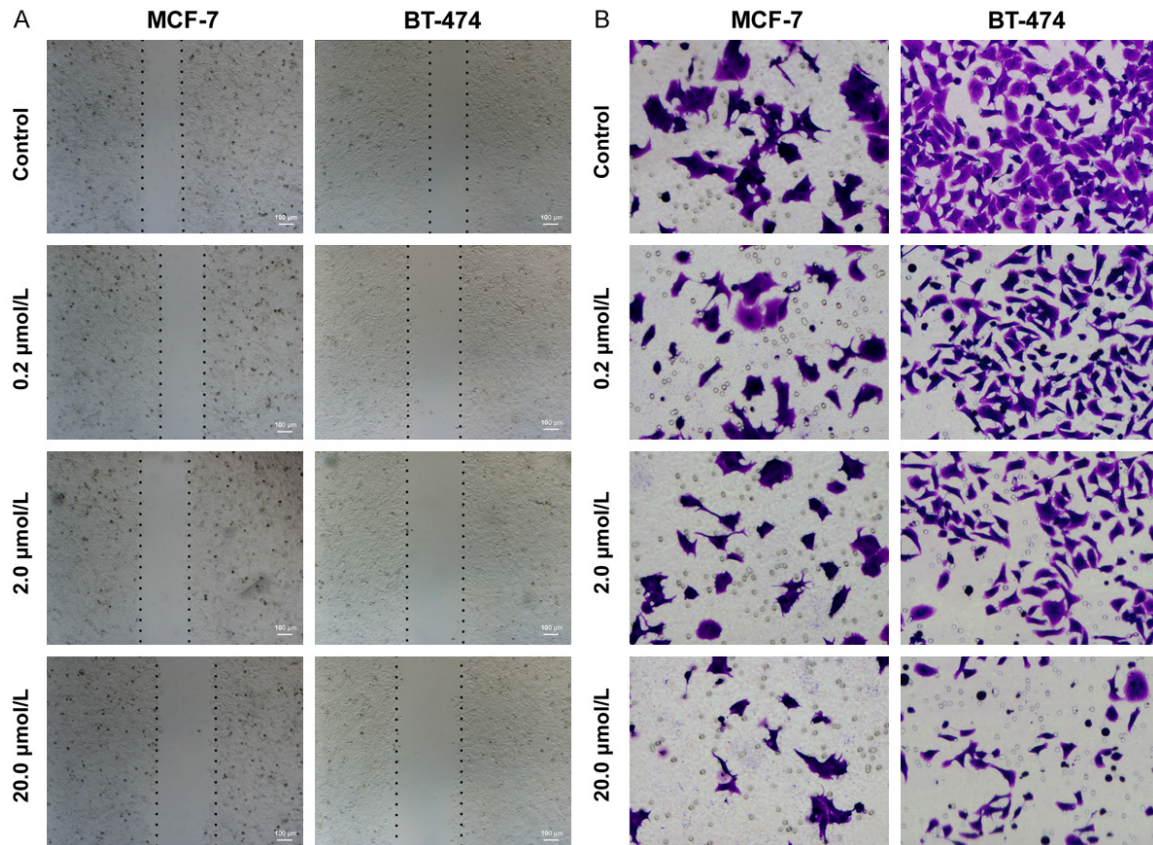
Figure 2. Apatinib inhibited the migration and invasion of breast cancer cells. MCF-7 and T-474 cells were treated with vehicle or 0.2, 2 or 20 µmol/L of Apatinib for 48 h. Then, the cell migration was determined by wound healing assay (Magnification, 40×; A), and the cell invasion was determined by Transwell assay (Magnification, 200×, B).

after 48-hour treatments. As shown in **Figure 2A**, treatment with Apatinib significantly suppressed motility of BT-474 and MCF-7 cells in a wound scratch assay, as compared with the scramble control. Then, the effect of Apatinib on the invasion of BT-474 and MCF-7 cells was examined with Transwell invasion chamber assay. A significant reduction in the number of invading cells was observed in the Apatinib-treated groups, as compared with the controls (**Figure 2B**). These results showed that Apatinib suppressed the motility and invasiveness of both cells.

*Apatinib altered of the downstream signaling cascades of VEGFR-2 in breast cancer cells*

Studies have shown that the up-regulation of AMOT is an important feature in breast cancer, importantly contributing to breast cancer cell proliferation and invasion. Therefore, we analyzed the mRNA expression levels of VEGFR-2 and AMOT, as well as the proteins associated with the pathway, including YAP, p-YAP, LATS1/2, Cyclin D1, ERK1/2 and p-ERK1/2. As expected, Apatinib reduced levels of VEGFR-2 and AMOT in cells at mRNA levels (**Figure 3A**). Increased exposure of the drug decreased the mRNA and protein levels of LATS1/2 (**Figure 3B**). The expression levels of YAP at mRNA were not altered but the level of phosphorylated protein decreased in a concentration-dependent manner after exposure to the drug. In addition, Apatinib treatment did not affect the total levels of ERK1/2, whereas dose-dependently decreased the phosphorylation of the proteins, as well as decreased the levels of Cyclin D1.

Overexpression of AMOT inhibited the effects of Apatinib on breast cancer cells

To investigate whether AMOT inhibition plays a role in the inhibitory effect of Apatinib on the cell growth, MCF-7 cells were transfected with AMOT cDNA. As shown in **Figure 4A** and **4B**, mRNA and protein expression of AMOT were
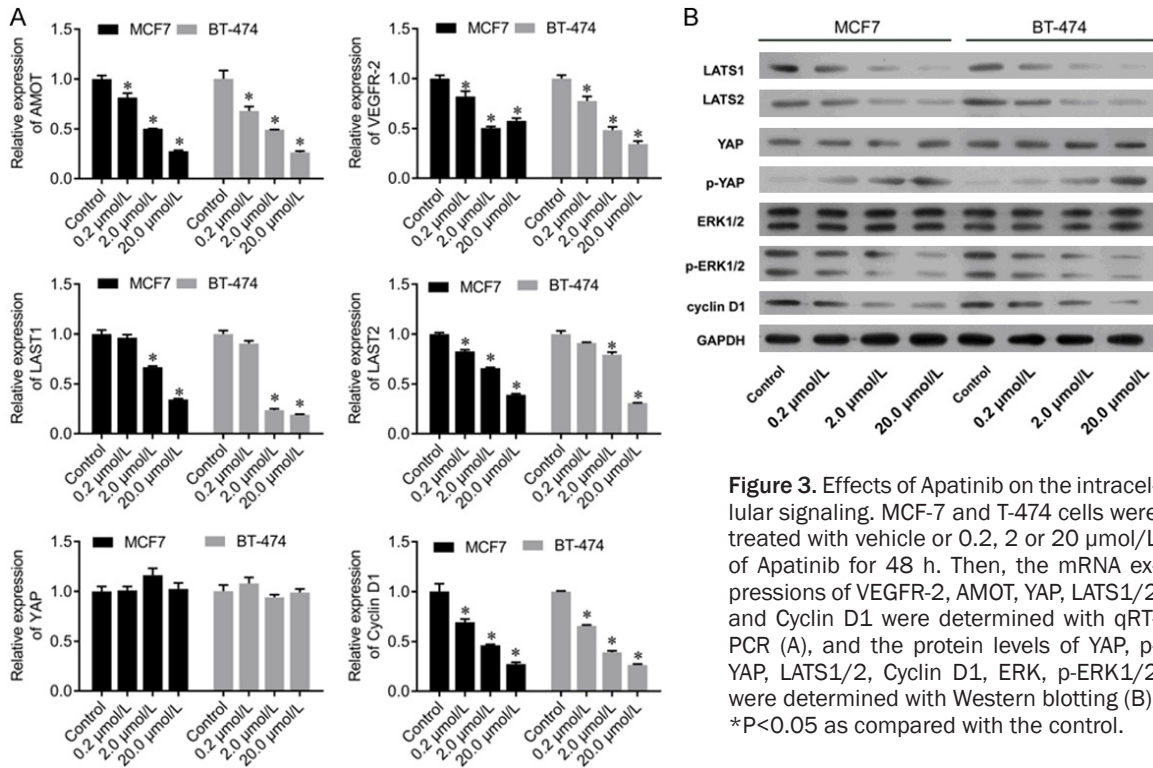
Figure 3. Effects of Apatinib on the intracellular signaling. MCF-7 and T-474 cells were treated with vehicle or 0.2, 2 or 20 μmol/L of Apatinib for 48 h. Then, the mRNA expressions of VEGFR-2, AMOT, YAP, LATS1/2 and Cyclin D1 were determined with qRT-PCR (A), and the protein levels of YAP, p-YAP, LATS1/2, Cyclin D1, ERK, p-ERK1/2 were determined with Western blotting (B). *P<0.05 as compared with the control.

significantly increased in the cDNA transfected cells, as compared with the cells with vector transfection, indicating that AMOT overexpression was successfully established. As 2.0 μmol/L Apatinib induced significant inhibition in cell growth, proliferation and migration, the concentration was used in the further studies. As expected, the overexpression of AMOT significantly increased the viability and proliferation in MCF-7 cells exposed to Apatinib (**Figure 4C** and **4D**). Additionally, the inhibited cell migration and invasion by Apatinib was also significantly suppressed by AMOT overexpression (**Figure 4E** and **4F**). Subsequently, the effect of AMOT overexpression on cell cycle distribution deregulated by Apatinib was examined. As shown in **Figure 4G**, the markedly decreased percentage in S phase and increased in G1 phase by Apatinib treatment were significantly reversed. All these indicated AMOT inhibition play an important role in the anti-proliferation and anti-metastasis of Apatinib.

Overexpression of AMOT inhibited the Apatinib-induced alteration in downstream proteins

With RT-PCR and western blot, the role of AMOT in the altered signaling cascade by Apatinib was investigated. Irrespective whether treated with Apatinib or not, the expressions of LATS1/2 were significantly induced in the cells with AMOT overexpression (**Figure 5A** and **5B**). As shown in **Figure 5B**, AMOT overexpression significantly decreased the phosphorylated levels of YAP protein. As expected, the expressions of ERK1/2 were not affected by AMOT overexpression, whereas the phosphorylated levels were significant increased by the overexpression. Additionally, the decrease in of cyclin D1 expression by Apatinib was significantly inhibited by AMOT overexpression.

Discussion

As a small-molecule TKI selective against VEGFR-2, Apatinib initially became popular as an inhibitor of gastric cancer. Thereafter, with growing evidence, Apatinib was found to inhibit many other types of cancers such as hepatocellular carcinoma and breast cancer. Furthermore, the anticancer effect was attributed to inhibition of angiogenesis via VEGFR-2 in the initial investigations on the biological function of Apatinib in breast cancer cells. The findings of our study which attributes anticancer effect of Apatinib with inhibition of VEGFR-2/AMOT signaling pathway, in addition to inhibition of migration and invasion in breast cancer cells.
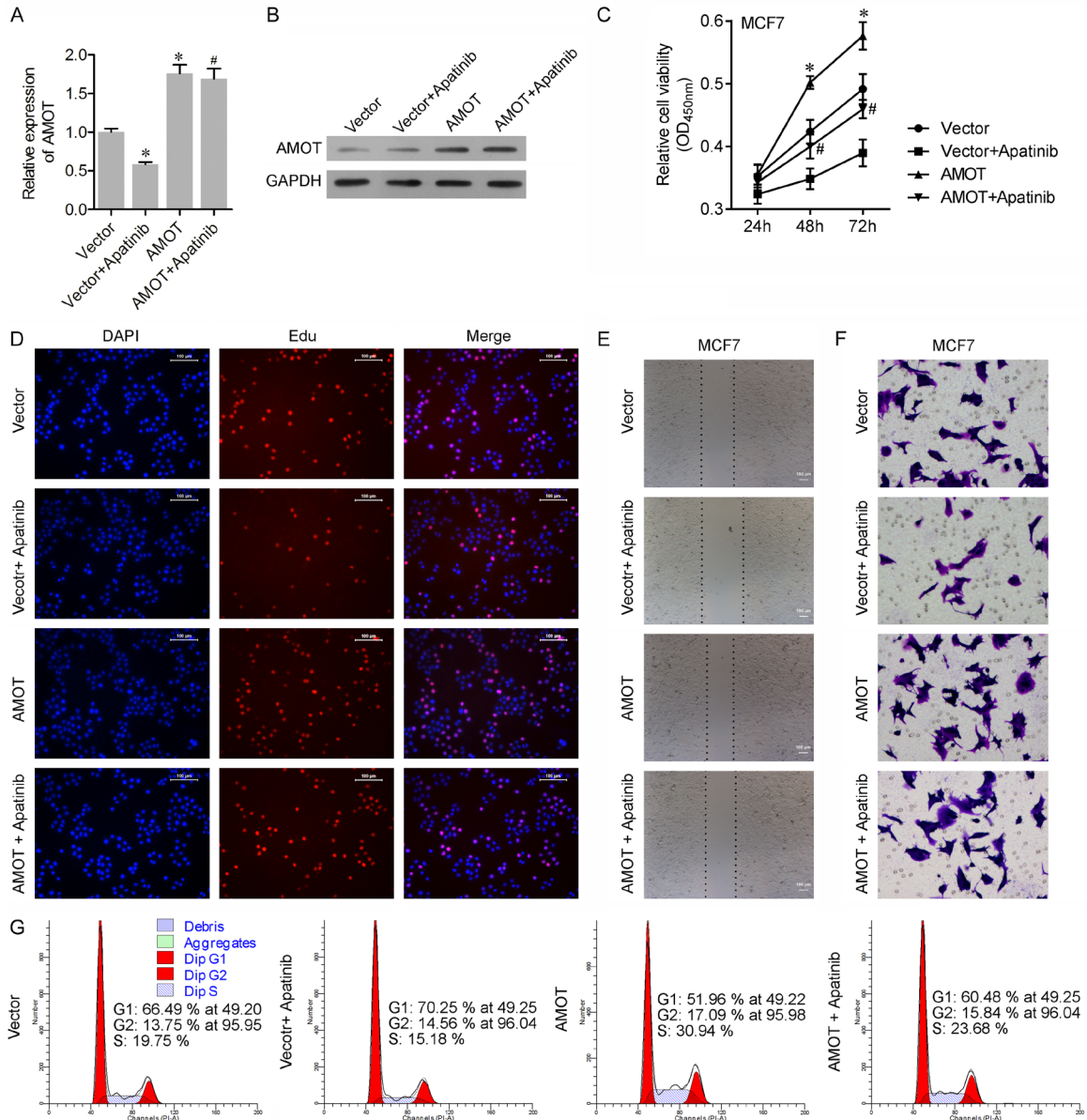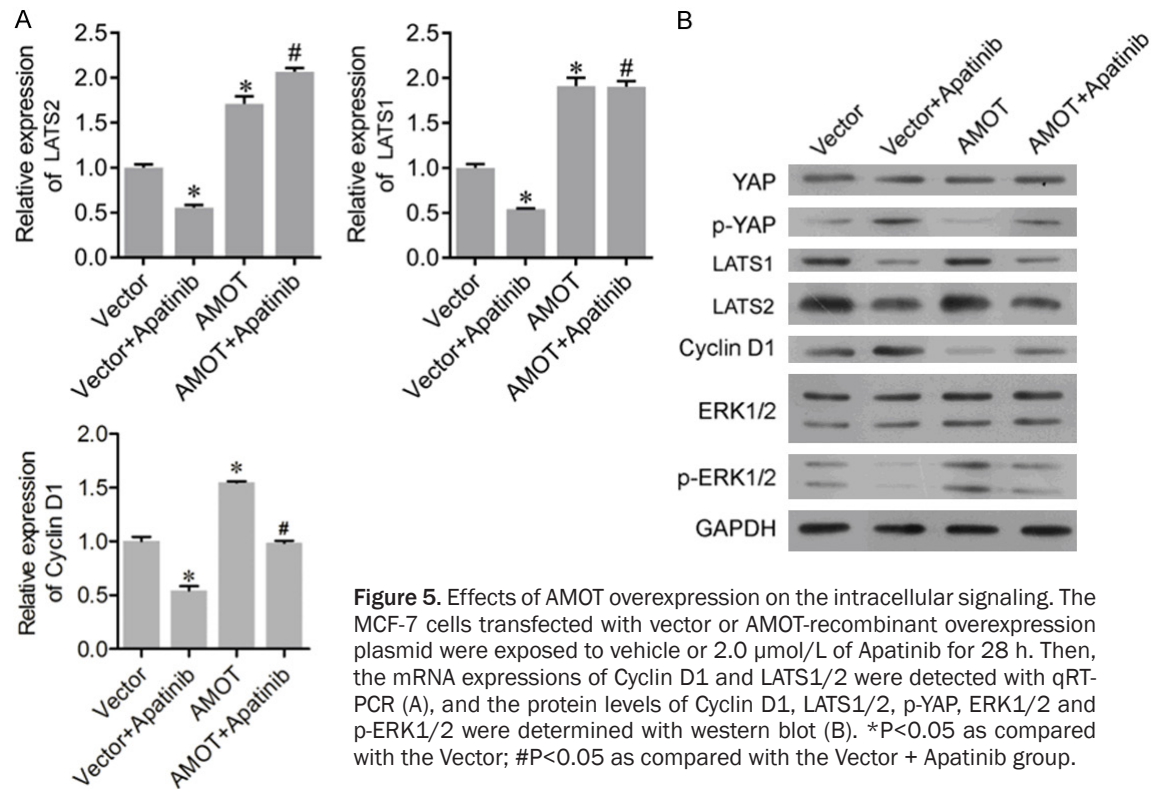
Figure 4. Effects of AMOT overexpression on the inhibited cell viability, migration and invasion by Apatinib. After transfected with vector or AMOT-recombinant overexpression plasmid, the mRNA (A) and protein (B) levels of AMOT in MCF-7 cells were determined by qRT-PCR and Western blotting, respectively. The MCF-7 cells transfected with vector or AMOT-recombinant overexpression plasmid were exposed to vehicle or Apatinib at 2.0 µmol/L for 48 h. Then the viabilities of the cells were determined by CCK-8 assay (C); Proliferating cells were measured by EdU incorporation assay (Magnification, 100×; red), DAPI (blue) nuclear staining for live cells, and then images were merged (D); The cell migration (E) and invasion (F) was determined by wound healing assay (Magnification, 40×) and Transwell assay (Magnification, 200×), respectively; The cell cycle distribution was determined with flow cytometry (G). The values represent the mean ± SD. *P<0.05 as compared with the Vector; #P<0.05 as compared with the Vector + Apatinib group.

VEGFR-2 is mostly expressed in endothelial cells. Due to its important contribution to tumor initiation and development by promoting endothelial precursor cell differentiation and angiogenesis, VEGFR-2 was recognized as an important therapeutic target. Ramucirumab and Apatinib have been developed using this target.

Of late, there has been increasing evidence showing that VEGFR-2 is expressed in various other cancer cells such as osteosarcoma cells, intrahepatic cholangio carcinoma cells, breast, colon and gastric cancer cells [8, 11-13]. However, A few recent studies have suggested that breast cancer cells do not express VEGFR-2

Figure 5. Effects of AMOT overexpression on the intracellular signaling. The MCF-7 cells transfected with vector or AMOT-recombinant overexpression plasmid were exposed to vehicle or 2.0 μmol/L of Apatinib for 28 h. Then, the mRNA expressions of Cyclin D1 and LATS1/2 were detected with qRT-PCR (A), and the protein levels of Cyclin D1, LATS1/2, p-YAP, ERK1/2 and p-ERK1/2 were determined with western blot (B). *$P<0.05$ as compared with the Vector; #$P<0.05$ as compared with the Vector + Apatinib group.

[14]. This discrepancy might may have been due to the different antibodies or assay conditions used in these studies. In the present study, the VEGFR-2 expressions were observed in mRNA and protein. This is consistent with the observations in the previous reports that VE-GFR-2 is expressed in primary breast carcinomas and the cell lines, including T47D, MCF-7 and BT-474 cells [15, 16].

Hippo pathway has been demonstrated to transduce VEGFR-2 signaling, and participate in angiogenesis, tumorigenesis, and metastasis [17, 18]. As a newly identified member in Hippo pathway, AMOT is aberrantly expressed in a variety of cancers. Its expression is significantly increased in the breast cancer and osteosarcoma cells. A research showed that AMOT knockdown significantly reduced cell proliferation and invasion [19]. Consistent with the report, the inhibitory effects were observed after Apatinib inhibited AMOT, and then reversed by AMOT over-expression. Despite this, the role of AMOT in cell proliferation still remains controversy. In lung cancers, decreased expression of AMOT was observed and this down-regulation promoted the proliferation and invasion of lung cancer cells *in vivo* and *in vitro* [20]. The discrepancy may have been due to

the fact that these studies used different cancers and investigated different roles of AMOT on Hippo pathway.

LATS1/2 is the central factor in Hippo signaling pathway. It is well known that AMOT regulates the activation of LATS1/2, instead of the expression. In the present study, significant decrease in LATS1/2 expressions were observed with reduction in AMOT. Consistent with the findings, in MCF-7 cells, AMOT knockdown significantly decreased the expressions of LA-TS1/2. Furthermore, decreased LATS1/2 levels were observed in the breast cancers, which featured high expressions of AMOT. It has been reported that decreased expressions of LA-TS1/2 is attributed to hypermethylation of promoter [21]. Additionally, AMOT was reported to regulate the degradation of LAST1/2 [22].

YAP is the functional output of Hippo signaling. A recent study showed that the expression of YAP levels significantly decreased after AMOT knockdown in breast cancer cells in *in vitro*. Consistently, this study also observed decreased YAP levels after AMOT inhibition by Apatinib, and this reversed after AMOT overexpression. These observations indicated that YAP acts as oncoprotein in MCF-7 cells. On the other hand,

YAP was reported as a tumor suppressor. Furthermore, it was concluded that YAP may function differently in some cells and in the tumors derived from them. This discrepancy might have been due to the varied subcellular localization of YAP between cell types [23]. The expressions of YAP can be regulated at the levels of transcription and degradation. It is generally accepted that activated LATS1/2 leads to phosphorylation and the subsequent degradation of the protein, yet a few studies indicated that AMOT directly interacted with YAP and induced YAP degradation [24]. YAP, depending on the experimental setting, either inhibit or promote its activity [25].

Aberrant activation of ERK are important in cancer cells. Our data showed that phosphorylated ERK1/2 significantly decreased after Apatinib treatment, whereas the inhibitory effect was significantly suppressed, indicating that AMOT is an important factor to mediate the inhibition of ERK by Apatinib. Consistently, a recent study showed that AMOT induced the proliferation of MCF-7 cells *in vitro* via increasing ERK1/2 phosphorylation [24]. Another study suggested that AMOT increase phosphorylated ERK1/2 through Rac1 [26]. Other studies showed that AMOT increased EKR phosphorylation through a YAP-dependent pathway [27, 28]. We also examined the expression of cyclin D1 and found that its expression was significantly decreased after Apatinib treatment, which was significantly reversed by AMOT overexpression. Consistently, AMOT knockdown has been reported to down-regulate cyclin D1 expression. Furthermore, ERK knockdown significantly inhibited ERK [29]. Therefore, cyclin D1 might be regulated by AMOT via ERK.

Conclusion

In conclusion, this study revealed that Apatinib inhibited the proliferation and invasion of MCF-7 cells. Notably, AMOT plays a critical role in the inhibitory activity of Apatinib which was mainly through the activation of the YAP-ERK signaling pathway. In accordance, our study may provide new insight on the role of AMOT as an oncogene in the progression of breast cancers, providing a further information for the therapeutic agent against breast cancer.

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Disclosure of conflict of interest

None.

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