

Anlotinib inhibits the proliferation, migration and invasion, and induces apoptosis of breast cancer cells by downregulating TFAP2C

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Abstract. The vascular endothelial growth factor receptor (VEGFR) network contributes to breast cancer pathogenesis and progression. Anlotinib is a highly potent multi-target tyrosine kinase inhibitor that has been previously shown to exert antitumor effects in various types of cancer. The aim of the present study is to investigate the effect of Anlotinib against breast cancer cells *in vitro* and uncover the possible underlying mechanisms. The human breast cancer cell line MCF-7 was treated with different concentrations of Anlotinib, before cell proliferation, migration, invasion and apoptosis were assessed using colony formation, wound healing, Transwell and TUNEL staining assays. In addition, the expression of transcription factor AP-2 γ (TFAP2C) following Anlotinib stimulation was measured using reverse transcription-quantitative PCR and western blot analysis. TFAP2C was overexpressed in MCF-7 using transfection with a pcDNA3.1 vector, before the aforementioned experiments were repeated. The results revealed that Anlotinib impaired cell viability and colony formation, reduced proliferating cell nuclear antigen, Ki-67, MMP2, MMP9 and Bcl-2 expression levels, and inhibited cell migration and invasion. By contrast, the expression levels of tissue inhibitor of metalloproteinase 1, the frequency of apoptotic cells, the expression of Bax and the cleaved caspase-3/caspase-3 ratio increased in a concentration-dependent manner. Additionally, the expression of TFAP2C decreased after Anlotinib treatment. However, TFAP2C overexpression partially blocked the effects of Anlotinib on the proliferation, migration, invasion and

apoptosis of MCF-7 cells. To conclude, Anlotinib suppressed proliferation, migration and invasion, whilst inducing apoptosis of MCF-7 cells, which may be partially dependent on the inhibition of TFAP2C expression.

Introduction

Malignant tumors of the breast generally occur in the ductal or acinar epithelium. Because of its high morbidity and high mortality, breast cancer is a major disease that seriously impacts on the lives and health of men and women worldwide (1). Surgery is one of the most important treatment options for breast cancer since it is frequently used for breast cancer therapy (2). Although this method can cause deformity or even loss of the breasts of the patient, which can have a great impact on their physical and mental health. Chemotherapy is a reliable method for the treatment of breast malignant tumors. However, multi-drug resistance and metastasis of tumor cells has narrowed the scope of clinical application of chemotherapy drugs to a certain extent (3). Therefore, finding drugs with good curative and anti-metastatic effects in breast cancer has become the focus of breast cancer research.

With the rapid development of life science research, the basic processes of tumor cell signal transduction, cell cycle regulation, apoptosis induction and angiogenesis are gradually being elucidated. Vascular endothelial growth factor receptor (VEGFR) signaling has been identified to play a pivotal role in the regulation of neo-angiogenesis, the process that leads to the formation of new blood vessels from the existing vasculature and that is crucial for tumor growth, invasion and metastasis (4). Therefore, VEGFR has become an important candidate in anti-tumor drug research and a potential target in drug research and development. Drugs directed against the VEGF/VEGFR system can be mainly categorized into two classes, monoclonal antibodies and small-molecule tyrosine kinase inhibitors (TKIs). Since 2018, >20 TKIs have been approved by the Food and Drug Administration (5). These drugs are characterized by high selectivity, high efficacy and low side effects. They are superior to traditional cytotoxicity anti-tumor drugs in the treatment of various cancer types, and some have become first-line drugs for cancer treatment (6).

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Anlotinib is a novel multi-kinase inhibitor that has exhibited efficacy against various types of malignancies in preclinical models, including medullary thyroid cancer, renal cell cancer, gastric cancer and esophageal squamous cell carcinoma (7). It is designed to primarily inhibit VEGFR, platelet-derived growth factor receptors α and β , fibroblast growth factor receptors, c-Kit and RET (8). Anlotinib is currently undergoing phase-II and/or -III clinical development in China, the USA and Italy for soft tissue sarcomas and various cancer types, such as lung cancer, gastric cancer and thyroid cancer (9,10). Another small-molecule TKI that selectively blocks VEGFR-2, Vandetanib, has been approved for use in 2012 for advanced thyroid cancer and was reported to inhibit cell growth and angiogenesis in breast cancer cells (11). Moreover, the effect of Vandetanib on the proliferation and tumor growth response of breast cancer cells was dependent on transcription factor AP-2 γ (TFAP2C) expression (12). A recent study has demonstrated that Anlotinib has good efficacy and low toxicity in a phase-II study evaluating Anlotinib in pre-treated HER-2 negative metastatic breast cancer (13). Additionally, Zhang *et al* (14) suggested that Anlotinib combined with neoadjuvant chemotherapy had good efficacy and safety in the treatment of occult breast cancer and may represent a potential therapeutic option. TFAP2C is a member of the AP-2 transcription factor family, which has been shown to play a critical role in multiple subtypes of breast cancer (15). In breast cancer, data mining of microarray expression studies has shown an association between high levels of TFAP2C mRNA and higher clinical grade and shorter survival times (16,17). However, the effects of Anlotinib on TFAP2C on the progression of breast cancer remain to be elucidated. The aim of the present study was to explore the antitumor effect and mechanism of action of Anlotinib in the human breast cancer cell line MCF-7. Whether Anlotinib could inhibit MCF-7 cell proliferation and migration by regulating TFAP2C expression was also explored.

Materials and methods

Cell culture and treatment. The human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection and cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin and streptomycin at 37°C with 5% CO₂ (all from Gibco; Thermo Fisher Scientific, Inc.).

Cell transfection. The full length of the TFAP2C sequence was cloned into the pcDNA3.1 vector by Gene Script Biotech Co., Ltd. to construct 2 μ g TFAP2C overexpression vector (Ov-TFAP2C). The empty vector pcDNA3.1 (2 μ g) was used as a negative control (Ov-NC). Cells were cultured overnight to reach 60-70% confluence before transfection. The plasmid transfection was performed using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cells were incubated with 5% CO₂ at 37°C and were used in subsequent experiments 48 h post-transfection.

Cell viability measurement. Cell viability was measured using Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology) assays. Control or transfected MCF-7 cells were seeded in 96-well plates at 5x10³ cells/well and incubated

at 37°C for 24 h. The cells were treated with different concentrations of Anlotinib (0, 2, 4 and 6 μ M; >99%; Selleck Chemicals) at 37°C for 24 h. A total of 10 μ l CCK-8 solution was added to each well for 2 h of culture at 37°C. Cell viability was detected using a microplate reader (Bio-Rad Laboratories, Inc.) at the absorbance of 450 nm.

Colony formation assay. For colony formation assays, control or transfected MCF-7 cells (1x10³ cells) were resuspended in 1 ml medium, then cultured in 24-well plates overnight. The attached cells were treated with Anlotinib (0, 2, 4 and 6 μ M) at 37°C for 24 h, then cultured in complete RPMI-1640 medium for an additional 2 weeks. After fixation with 20% methanol for 30 min at room temperature, 0.5% crystal violet was used to stain cells for 5 min at room temperature and the number of colonies with >50 cells was counted manually using a light microscope (magnification, x100).

Wound healing assay. Control or transfected MCF-7 cells were seeded onto 6-well plates (4x10⁵ cells/well) and cultured in normal conditions to 70-80% confluence. The cell surface was scratched with a 100- μ l pipette tip to create an artificial wound, and the medium was replaced with serum-free RPMI-1640 containing Anlotinib (0, 2, 4 and 6 μ M) and culturing for 24 h at 37°C. The cell migration was observed with images captured in each group at 0 and 24 h using a light microscope (magnification, x100; Olympus Corporation). The cell migration rate was calculated using the following equation: (Initial width at 0 h - final width at 24 h)/Initial width at 0 h. The relative migration rate was obtained by normalizing to the control group.

Transwell assay. For Transwell analysis, an 8- μ m Transwell chamber (Corning, Inc.) was coated with 250 mg/ml Matrigel[™] (BD Biosciences) at room temperature for 24 h (18-20), then placed into a 24-well plate. The upper chambers were seeded with control or transfected MCF-7 cells suspended in 200 μ l serum-free DMEM at a density of 1x10⁵ following Anlotinib treatment. RPMI-1640 medium containing 10% FBS was added to the lower chamber for 24 h of incubation at 37°C. Finally, the invading cells into the bottom of chamber were fixed with 4% methanol at 37°C for 10 min, then stained with 0.1% crystal violet solution at 37°C for 15 min. The number of invasive cells in five random fields were counted using a light microscope (magnification, x100).

TUNEL staining. Cell apoptosis was detected using the TUNEL assay kit (Abcam) following the manufacturer's instructions. The control or transfected MCF-7 cells were fixed with 1% paraformaldehyde at room temperature for 15 min after Anlotinib treatment, and then treated with 70% ethanol on ice. After being exposed to the TUNEL detection reagent for 1 h at 37°C, cells were treated with 10 μ g/ml DAPI (Shanghai Haoyang Bio Technology Co., Ltd.) to stain the nucleus at 37°C for 2-3 min and mounted in an anti-fade reagent (Beijing Solarbio Science & Technology Co., Ltd.). In total, three fields of view were selected at random, each with ~300-500 cells and the images were captured using a fluorescence microscope (magnification, x100; Olympus Corporation) and analyzed using the ImageJ software (version 1.48v; National Institutes of Health).

Western blot analysis. Cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology) containing protease inhibitors (Roche Diagnostics), and protein concentration was quantified using a bicinchoninic-acid assay kit (Thermo Fisher Scientific, Inc.). Equal amount of protein samples (20 μg) were separated 12% SDS-PAGE on 12% gels, then transferred onto PVDF membranes (EMD Millipore). After being blocked with 5% non-fat milk for 2 h at room temperature (diluted with PBS-0.1% Tween-20), membranes were incubated overnight at 4°C with the primary antibodies against proliferating cell nuclear antigen (PCNA; 1:5,000, cat. no. ab29; Abcam), Ki-67 (1:1,000, cat. no. ab92742; Abcam), matrix metalloproteinase (MMP)2 (1:1,000, cat. no. ab92536; Abcam), MMP9 (1:1,000, cat. no. ab76003; Abcam), tissue inhibitor of metalloproteinase-1 (TIMP-1; 1:1,000, cat. no. ab211926; Abcam), Bcl-2 (1:2,000, cat. no. ab32124; Abcam), Bax (1:5,000, cat. no. ab32503; Abcam), cleaved caspase-3 (1:500 cat. no. ab32042; Abcam), caspase-3 (1:5,000, cat. no. ab32351; Abcam), TFAP2C (1:1,000, cat. no. ab218107; Abcam), GAPDH (1:5,000, cat. no. ab8245; Abcam) and the corresponding secondary HRP-conjugated goat anti-rabbit IgG (1:5,000, cat. no. 7074; Cell signaling technology) at room temperature for 2 h, followed by visualization with the Pierce™ enhanced chemiluminescence western blotting substrate (Thermo Fisher Scientific, Inc.) and quantitative analysis with the ImageJ software (version 1.48v; National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturers protocol. A total of 5 μg RNA was then reverse transcribed into cDNA using PrimerScript™ RT Master Mix (cat. no. RR036A; Takara Bio, Inc.). The temperature protocol was 37°C for 15 min and reaction at 85°C for 5 sec. The qPCR reactions were performed using TB Green® Premix Ex Taq™ (cat. no. RR420A; Takara Bio, Inc.) on an ABI Prism 7500 sequence detector (Applied Biosystems, Inc.; Thermo Fisher Scientific, Inc.). The thermocycling conditions: Pre-denaturation at 95°C for 30 sec, followed by denaturation at 95°C for 10 sec and annealing at 55°C for 30 sec for 40 cycles. The specific primers used were as follows: TFAP2C forward, 5'-ATCGAAAAATGGAGGCC GGT-3' and reverse, 5'-CGGCTTCACAGACATAGGCA-3'; PCNA forward, 5'-GTTACTGAGGGCGAGAAGCG-3' and reverse, 5'-AAGTCTAGCTGGTTTCGGCT-3'; Ki-67 forward, 5'-CTGACCCTGATGAGAGTGAGGGA-3' and reverse, 5'-GGACAGGTGGAGTGTGCATTA-3' and GAPDH forward, 5'-GCAACCGGGAAGGAAATGAATG-3' and reverse, 5'-CCCAATACGACCAAATCAGAGA-3'. Results were normalized to GAPDH expression, and the comparative Cq method ($2^{-\Delta\Delta Cq}$) was used to calculate the relative gene expression levels (21).

Statistical analysis. GraphPad Prism 8 (GraphPad Software, Inc.) was used for data analysis. Data were generated from three independent experimental repeats. The results are presented as the mean \pm standard deviation. Data analysis was carried out using one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Anlotinib inhibits the proliferation, migration and invasion of breast cancer cell line MCF-7 in a concentration-dependent manner. Different concentrations of Anlotinib (0, 2, 4 and 6 μM) were used to treat MCF-7 cells, and cell viability, colony formation and PCNA and Ki-67 protein expression were assessed. As shown in Fig. 1A, cell viability was significantly reduced following 2, 4 and 6 μM Anlotinib treatment. Moreover, the protein expression of PCNA and Ki-67 was significantly reduced by Anlotinib (Fig. 1B). Anlotinib also significantly reduced the number of colonies in MCF-7 cells (Fig. 1C). Furthermore, wound healing and Transwell assays were used to evaluate the effect of Anlotinib on cell migration and invasion, respectively. As demonstrated in Fig. 1D and E, both the cell migration and invasion rate were significantly inhibited by Anlotinib. Moreover, Anlotinib resulted in a significant decrease in MMP2 and MMP9 expression levels and a significant increase in TIMP-1 expression, further indicating an inhibitory effect on cell migration (Fig. 1F). Thus, Anlotinib exerted a concentration-dependent inhibitory effect on MCF-7 cell proliferation, migration and invasion.

Anlotinib promotes the apoptosis of MCF-7 breast cancer cells. Subsequently, the effect of Anlotinib on MCF-7 cells apoptosis was evaluated using TUNEL staining and western blot assays. As shown in Fig. 2A, cells exposed to 2, 4 and 6 μM Anlotinib exhibited a higher frequency of TUNEL-positive (apoptotic) cells compared with control (0 μM) cells, indicating that Anlotinib could promote apoptosis. This was further evidenced by the decrease in Bcl-2 expression, as well as the increase in Bax expression and cleaved-caspase-3/caspase-3 ratio following treatment with 2, 4 and 6 μM Anlotinib (Fig. 2B).

TFAP2C overexpression partially blocks the effect of Anlotinib on MCF-7 cell proliferation, migration and apoptosis. Next, the effect of Anlotinib on TFAP2C expression was determined. As shown in Fig. 3A and B, both the mRNA and protein expression levels of TFAP2C were reduced in response to Anlotinib treatment, and the effect was concentration-dependent.

TFAP2C was overexpressed in MCF-7 cells (Fig. 3C and D) to explore the changes in the effect of Anlotinib on MCF-7 cells proliferation, migration and apoptosis. Cell viability was higher in Anlotinib + Ov-TFAP2C group compared with that in the Anlotinib + Ov-NC group (Fig. 3E). In addition, Anlotinib inhibited the expression of PCNA and Ki-67, whereas TFAP2C overexpression partially rescued PCNA and Ki-67 expression (Fig. 3F and G). Similarly, the decreased number of colony-forming units caused by Anlotinib was also significantly increased by TFAP2C overexpression (Fig. 3H). These data revealed the protective effects of TFAP2C overexpression against Anlotinib-induced reduction in MCF-7 cell viability. Finally, TFAP2C-overexpressing MCF-7 cells were exposed to 4 μM Anlotinib, then cell migration, invasion and apoptosis were assessed. As shown in Fig. 4A and B, the reduced ability of migration and invasion caused by Anlotinib was reversed following TFAP2C overexpression. Consistently, TFAP2C overexpression also blocked the effect of Anlotinib on MMP2, MMP9 and TIMP-1 expression (Fig. 4C). Furthermore, TFAP2C overexpression significantly reversed

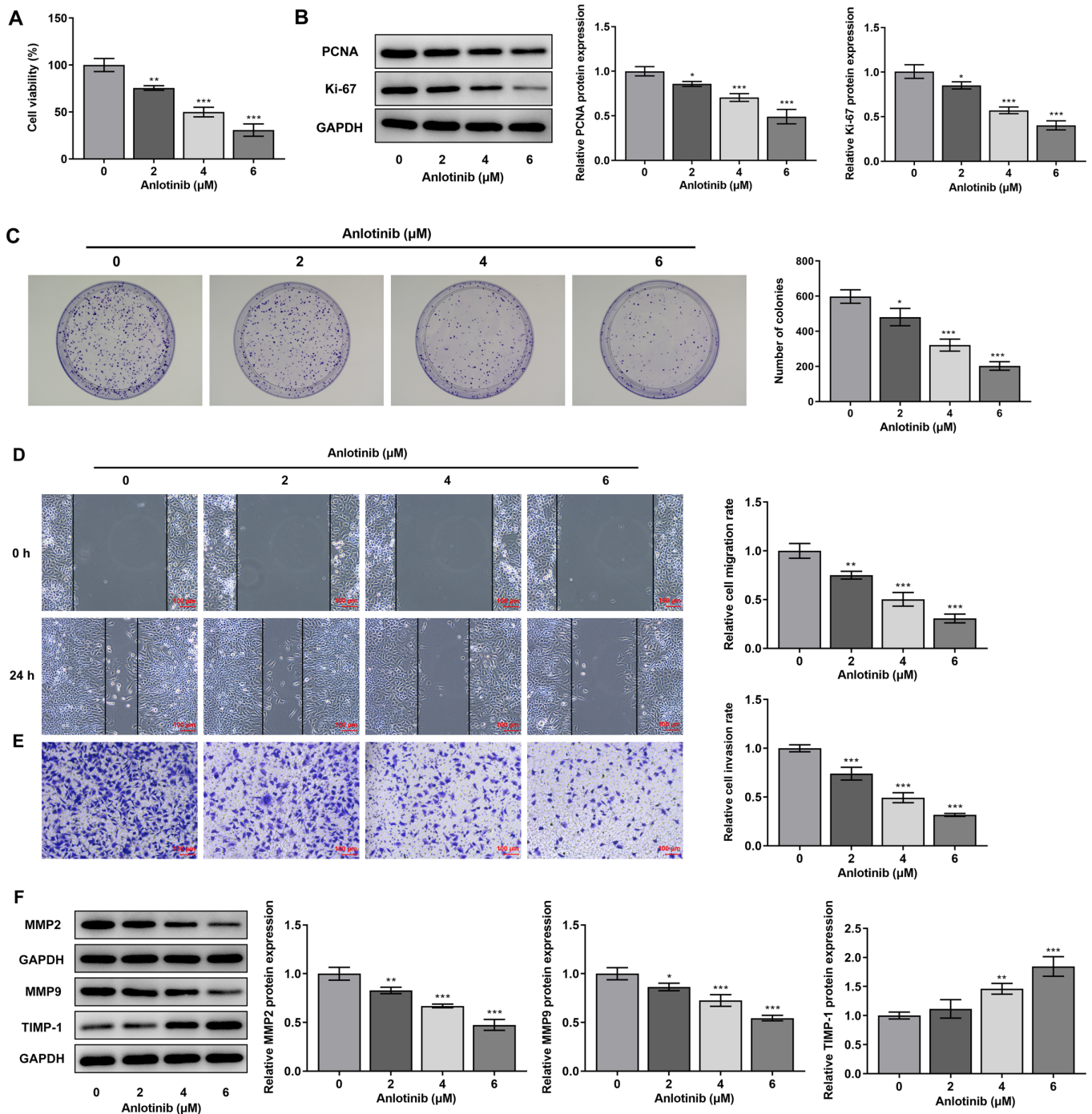


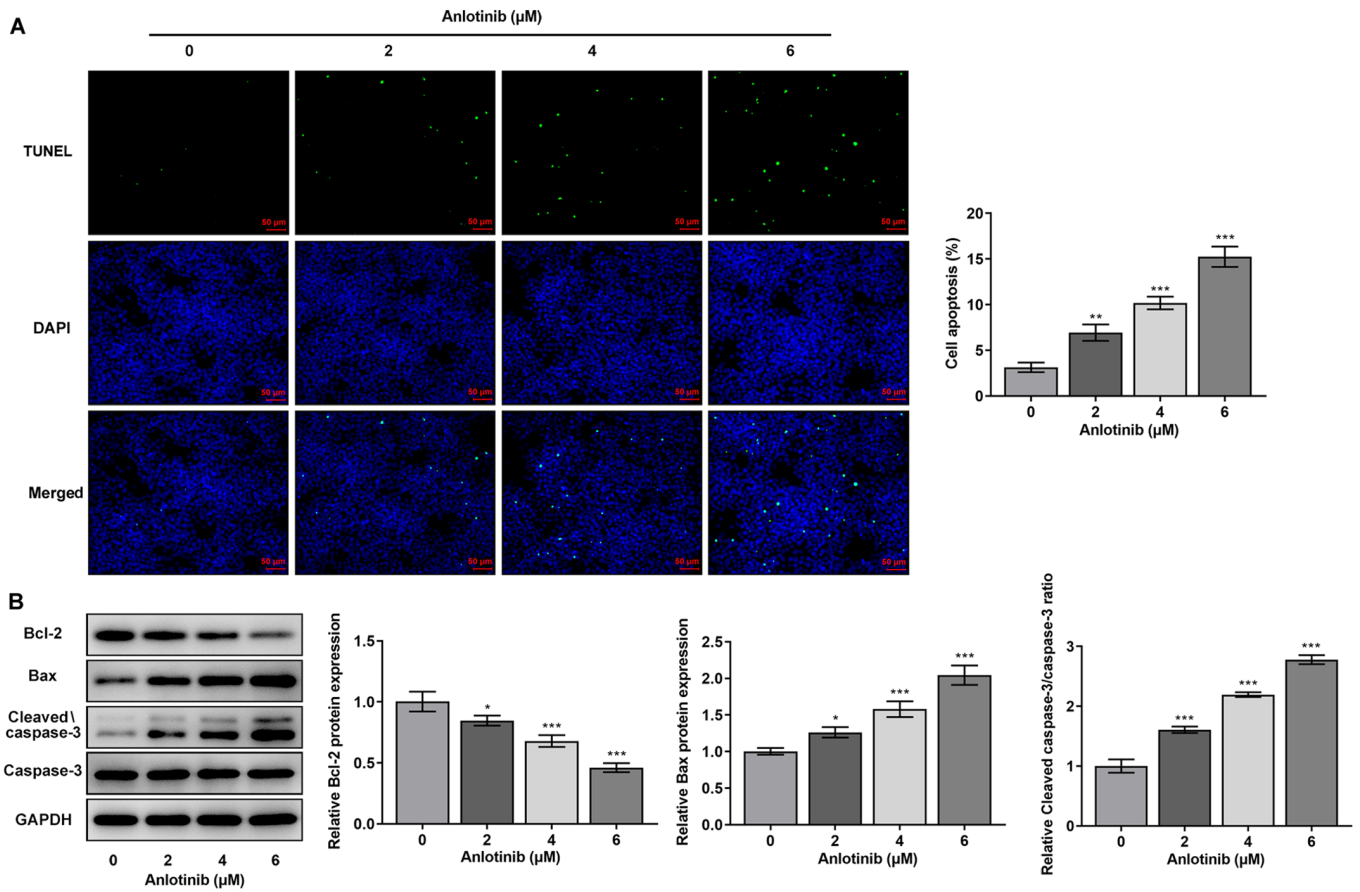
Figure 1. Anlotinib inhibits the proliferation and migration of MCF-7 cells. MCF-7 cells were cultured in medium containing 0, 2, 4 or 6 μM Anlotinib for 24 h. (A) Cell viability was tested using a Cell Counting Kit-8 assay. (B) PCNA and Ki-67 protein expression was measured by western blotting. (C) Cell proliferation was evaluated using colony formation assays. (D) Cell migration was determined using wound healing and Transwell assays. Scale bars, 100 μm . (E) which were quantified. (F) MMP2, MMP9 and TIMP-1 protein expression was detected by western blotting. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. 0 μM Anlotinib. PCNA, proliferating cell nuclear antigen; TIMP-1, tissue inhibitor of metalloproteinase 1.

the effect of Anlotinib on the frequency of apoptotic cells, as well as on the protein expression of Bcl-2, Bax and the cleaved-caspase-3/caspase-3 ratio (Fig. 5A and B).

Discussion

In 2018, the latest data released by the International Agency for Research on Cancer showed that among all cancer types, the incidence and mortality of breast cancer ranked second (22).

Breast cancer is also one of the most important causes of death in women over 45 years of age with malignant tumors (23). In most breast cancer cases, patients have excellent prognosis, whereas ~40% of breast cancer patients undergoing treatment experience recurrence and metastasis (24). The 5-year survival rate of patients with metastatic breast cancer is not higher than 60%, and most patients die due to locoregional recurrence and distant metastasis (25). Therefore, the development of novel effective therapeutic agents for breast cancer remains an



urgent medical requirement. In the present study, the potential therapeutic effect of the novel multi-kinase inhibitor Anlotinib was evaluated in breast cancer cells. The results suggested that Anlotinib had an antitumor effect on breast cancer cells by inhibiting their proliferation, migration, invasion and inducing their apoptosis *in vitro*.

TFAP2C regulates the expression of estrogen receptor α (ER α), which is transcriptionally regulated in breast cancer (26). Thus, the involvement of TFAP2C in breast cancer has been extensively studied (12,26,27). For example, higher TFAP2C expression has been shown to correlate with poor overall survival after 10 years of diagnosis in ER α ⁺ and endocrine therapy-treated subgroups of breast cancer (28). TFAP2C also predicts the outcome in HER2⁺ breast cancer (29). In the present study, Anlotinib inhibited the expression of TFAP2C in a concentration-dependent manner. In luminal breast cancer, the response of cancer cells to Vandetanib is mediated by the TFAP2C target genes epidermal growth factor receptor (EGFR) and RET (12). Similarly, Anlotinib can inhibit the expression of RET, which is a downstream target of TFAP2C in breast cancer (30). Thus, it was hypothesized that Anlotinib may exert its antitumor effect on breast cancer cells by downregulating TFAP2C expression. TFAP2C was overexpressed in MCF-7 cells, which were then subjected cells to Anlotinib treatment. In accordance with our hypothesis, the data revealed that TFAP2C overexpression markedly blocked the antitumor effect of Anlotinib on MCF-7 breast cancer

cells. However, the specific signaling pathways downstream of Anlotinib/TFAP2C have not been investigated in this study. TFAP2C is the key regulator of hormone responsiveness in breast cancer cells through the control of multiple pathways of estrogen signaling (25,30,31). Whether these pathways are involved in the effect of Anlotinib on breast cancer via TFAP2C remains to be elucidated. In addition, the conclusion of this study only came from *in vitro* experiments, lacking the validation of *in vivo* animal models. The effects of knock-down by using small interfering RNA targeting TFAP2C or overexpression of TFAP2C in MCF-7 cells (without Anlotinib treatment) on cell growth, cell migration, invasion, and cell apoptosis will be investigated in future experiments. Moreover, the use of only a TUNEL assay to detect apoptotic cells is another limitation of this study. Future research should aim to strengthen the conclusions of this study using comprehensive analysis and uncover the underlying mechanisms. For instance, whether signaling pathways is associated with Anlotinib in breast cancer should be analyzed.

In conclusion the present study demonstrated that Anlotinib exerts antitumor effects on breast cancer cells through inhibition of cell growth, induction of apoptosis and suppression of cell migration and invasion. Importantly, the effect Anlotinib against breast cancer might be partially dependent on inhibiting TFAP2C expression. Thus, the finding that Anlotinib may be used as an effective antitumor agent for breast cancer treatment was further confirmed.

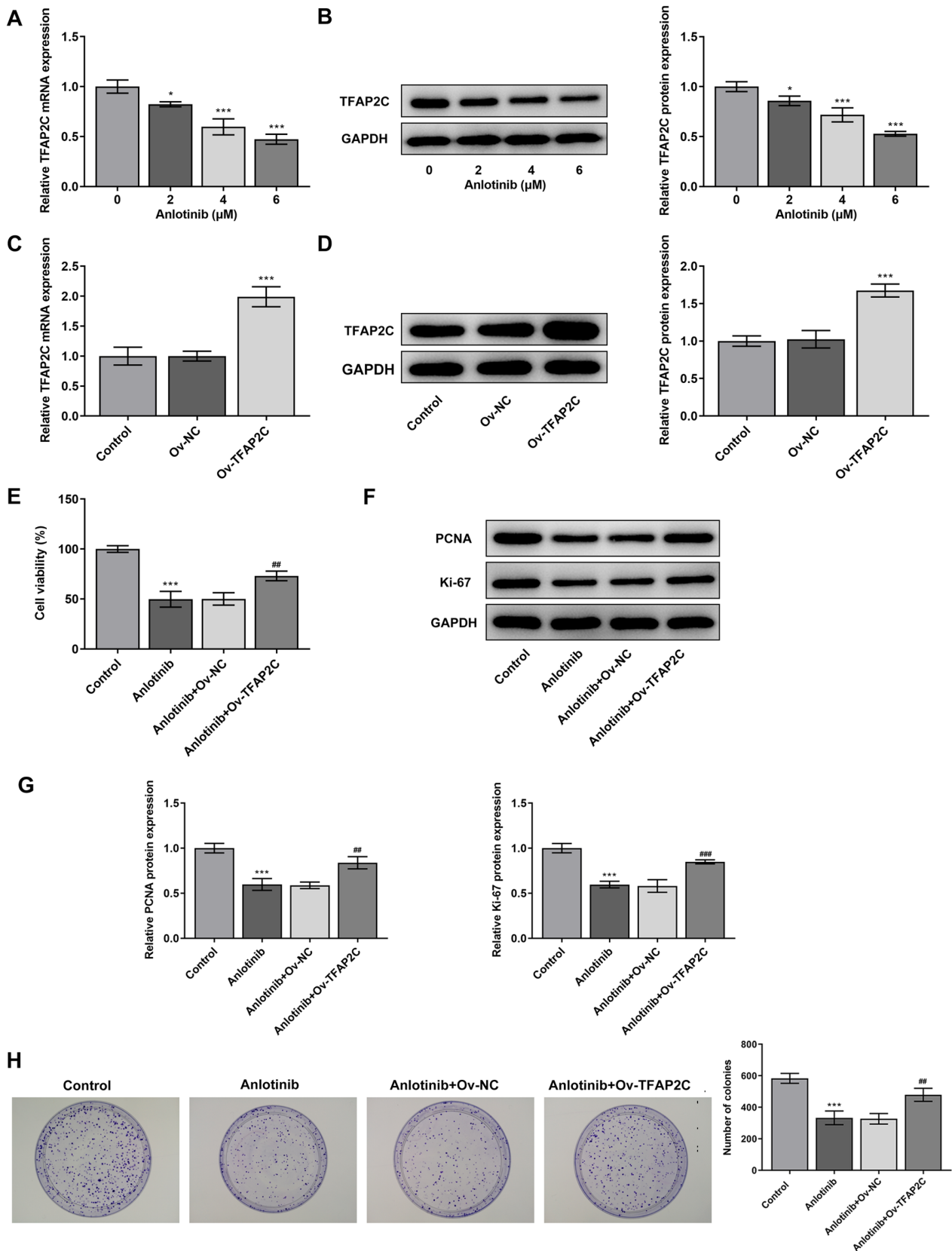


Figure 3. TFAP2C overexpression blocks the inhibitory effect of Anlotinib on MCF-7 cell proliferation. MCF-7 cells were cultured in medium containing 0, 2, 4 or 6 μM Anlotinib for 24 h, then (A) TFAP2C mRNA and (B) protein expression levels were measured by RT-qPCR and western blotting, respectively. Data were obtained from three independent repeated experiments. * $P < 0.05$ and *** $P < 0.001$ vs. 0 μM Anlotinib. MCF-7 cells were transfected with Ov-TFAP2C or Ov-NC, then (C) TFAP2C mRNA and (D) protein expression levels were measured by RT-qPCR and western blotting, respectively. All experiments were independently performed for three times. *** $P < 0.001$ vs. Ov-NC. (E-H) MCF-7 cells transfected with Ov-TFAP2C or Ov-NC were exposed to 4 μM Anlotinib for 24 h, then (E) cell viability was tested using Cell Counting Kit-8 assays, (F and G) the expression of PCNA and Ki-67 was measured by western blotting and (H) cell proliferation was evaluated using colony formation assays. Data were obtained from three independent experiments. *** $P < 0.001$ vs. control; ## $P < 0.01$ and ### $P < 0.001$ vs. Anlotinib + Ov-NC. RT-qPCR, reverse transcription-quantitative PCR; TFAP2C, transcription factor AP-2 γ ; Ov, overexpression; NC, negative control; PCNA, proliferating cell nuclear antigen.

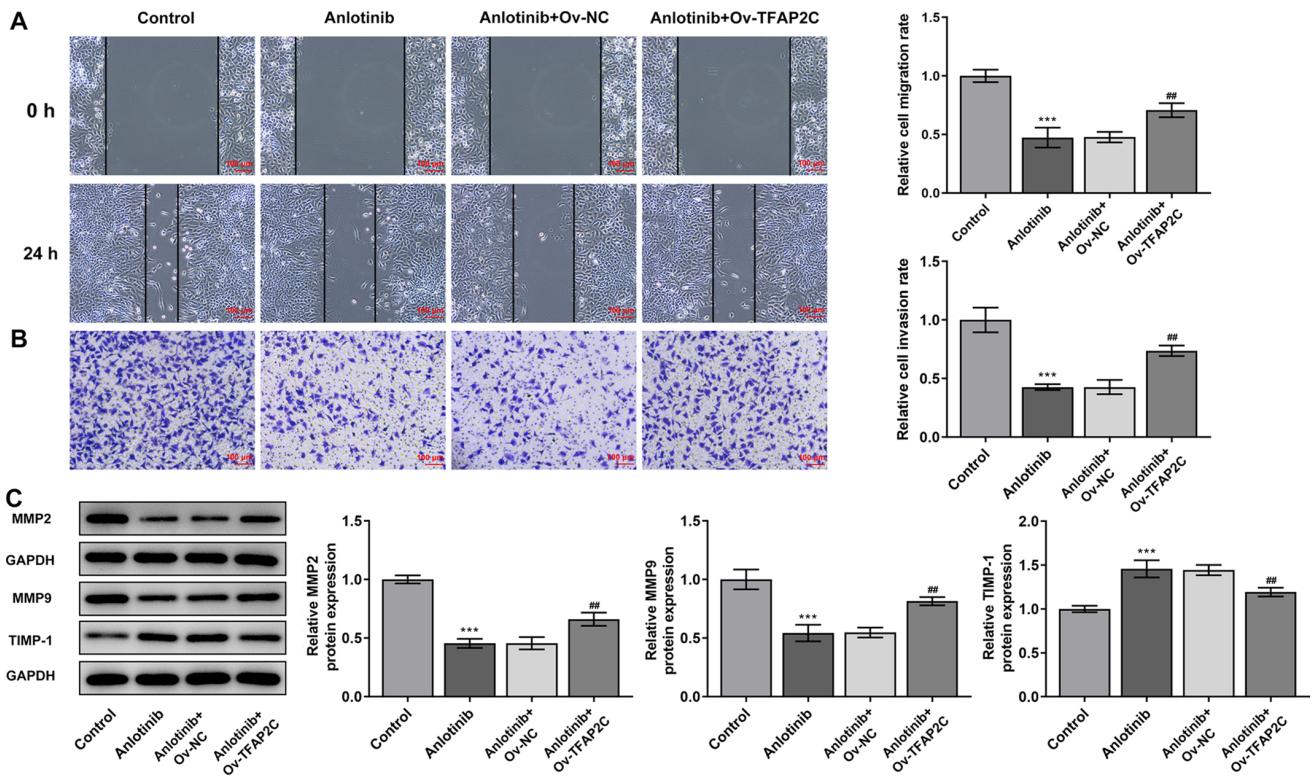


Figure 4. TFAP2C overexpression blocks the effect of Anlotinib on MCF-7 cell migration. MCF-7 cells transfected with Ov-TFAP2C or Ov-NC were exposed to 4 μ M Anlotinib for 24 h. (A) Cell migration was determined using wound healing and Transwell assays. (B) which were quantified. Scale bars, 100 μ m. (C) MMP2, MMP9 and TIMP-1 protein expression was detected by western blotting. Data were obtained from three independent experiments. ***P<0.001 vs. control; ##P<0.01 vs. Anlotinib + Ov-NC. TFAP2C, transcription factor AP-2 γ ; Ov, overexpression; NC, negative control; PCNA, proliferating cell nuclear antigen; TIMP-1, tissue inhibitor of metalloproteinase 1.

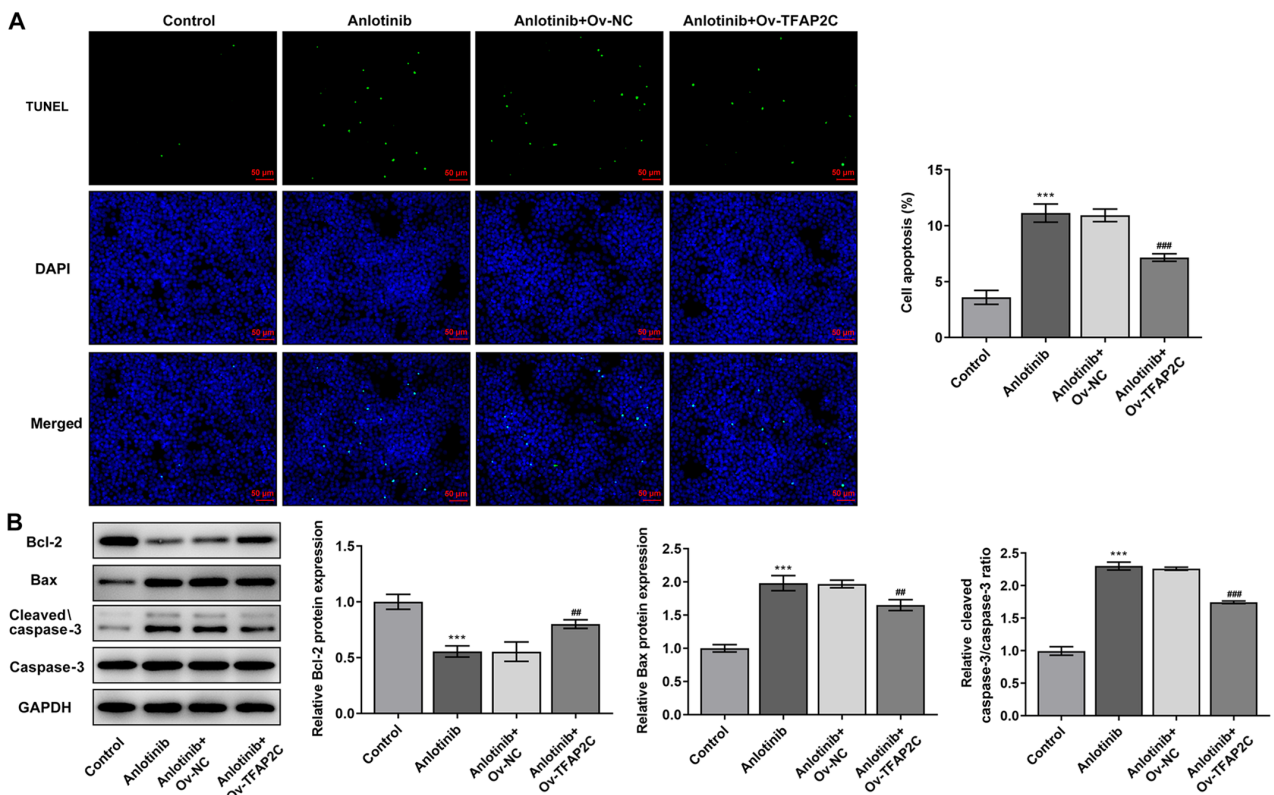


Figure 5. TFAP2C overexpression restores the effect of Anlotinib on MCF-7 cells apoptosis. MCF-7 cells that transfected with Ov-TFAP2C or Ov-NC were exposed to 4 μ M Anlotinib for 24 h. (A) Apoptosis was observed using TUNEL staining. Scale bars, 50 μ m. (B) Bcl-2, Bax, cleaved caspase-3 and caspase-3 protein expression was measured by western blotting. Data were obtained from three independent experiments. ***P<0.001 vs. Control; **P<0.01 and ###P<0.001 vs. Anlotinib + Ov-NC. TFAP2C, transcription factor AP-2 γ ; Ov, overexpression; NC, negative control.

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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

FF and QY designed the experimental study and analyzed the experiment data. FF performed the experiments. FF and QY confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript for submission.

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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