Original Paper

Apatinib Inhibits Angiogenesis Via Suppressing Akt/GSK3 β /ANG Signaling Pathway in Anaplastic Thyroid Cancer

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Key Words

Apatinib • Angiogenin • Angiogenesis • Akt • GSK3 β • Anaplastic thyroid carcinoma

Abstract

Background/Aims: Anaplastic thyroid carcinoma (ATC) is one of the most lethal human malignancies, and there is no efficient method to slow its process. Apatinib, a novel tyrosine kinase inhibitor (TKI), has been confirmed for its efficacy and safety in the treatment of advanced gastric carcinoma patients. However, the effects of Apatinib in ATC are still unknown.

Methods: In this study, we explored the effects and mechanisms of Apatinib on tumor growth and angiogenesis *in vitro* and *in vivo* in ATC cells. Angiogenesis antibodies array was utilized to detect the expression of angiogenesis-related genes after Apatinib treatment in ATC cells. In addition, we used Akt activator, Akt inhibitor and GSK3 β inhibitor to further study the mechanism for how Apatinib suppressed angiogenesis. **Results:** Apatinib treatment could suppress the growth of ATC cells in a dose- and time-dependent manner via inducing apoptosis and blocking cell cycle progression at G0/G1 phase. Moreover, Apatinib treatment decreased the expression of angiogenin (ANG) and inhibited angiogenesis of ATC cells *in vitro* and *in vivo*. We further confirmed that recombinant human ANG (rhANG) significantly abrogated Apatinib-mediated anti-angiogenic ability in ATC cells. Additionally, Apatinib treatment decreased the level of p-Akt and p-GSK3 β . Moreover, the Apatinib-mediated decrease of ANG and anti-angiogenic ability were partly reversed when an Akt activator, SC79, was administered. Furthermore, the anti-angiogenic ability of Apatinib can be enhanced in the presence of Akt inhibitor, and the inhibition of GSK3 β attenuated the anti-angiogenic ability of Apatinib. **Conclusion:** Our results demonstrated that Apatinib treatment inhibited tumor growth, and Apatinib-induced suppression of Akt/GSK3 β /ANG signaling pathway may play an important role in the inhibition of angiogenesis in ATC, supporting a potential therapeutic approach for using Apatinib in the treatment of ATC.

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Introduction

Anaplastic thyroid carcinoma (ATC) is one of the most lethal human malignant tumors, characterized as high levels of rapid proliferation, extrathyroidal invasion, and distant metastasis. More importantly, unlike differentiated thyroid carcinoma, ATC has been proved to be resistant to conventional treatment, including surgical, chemical, isotopic interventions [1, 2]. Therefore, ATC is the major cause of all thyroid carcinoma-related deaths, with a median survival of 3-9 months and a 20% 1-year survival rate [3, 4]. 90% of patients with ATC have extraglandular spread at the time of diagnosis, and 75% of the patients will develop distant metastasis during their disease course [5]. Neovascularization is necessary for ATC growth and dissemination [3, 6].

Angiogenin (ANG) is a 14.2-kDa secreted single chain basic protein composed of 123 amino acids [7]. ANG has been confirmed to be involved in several stages of angiogenesis in malignant carcinoma via its multi-functional effects in signaling pathway networks [8], which also could apparently enhance angiogenesis of ATC in our primary study.

Apatinib, a novel tyrosine kinase inhibitor (TKI), can inhibit multiple tumor related kinases, such as vascular endothelial growth factor receptor-2 (VEGFR-2), rearranged during transfection (RET), platelet-derived growth factor- β (PDGFR- β), stem cell factor receptor (c-Kit), and v-Src sarcoma viral oncogene homolog (c-Src) [9]. Apatinib has highly selective competition in the ATP binding site of VEGFR-2, then blocking downstream signaling pathways and inhibiting tumor angiogenesis [10]. Considering apparent neovascularization in ATC, in this study, we explored the role of Apatinib in cytotoxicity and angiogenesis of ATC. Our results showed that Apatinib could efficiently inhibit ATC cells growth via inducing both apoptosis and cell cycle arrest. Furthermore, Apatinib-induced angiogenesis inhibition in ATC cells was mediated by regulation of Akt/GSK3 β /ANG signaling pathway. These findings launched a new insight that Apatinib can be further studied as a potential agent for ATC treatments.

Materials and Methods

Cell Culture and Reagents

Human ATC cell lines CAL-62, BHT-101, HTh-7, KHM-5M, BHT-101 and 8305C were obtained from China Center for Type Culture Collection (CCTCC, Wuhan, China). CAL-62, HTh-7, BHT-101 and 8305C cells were cultured in DMEM medium with 10% fetal bovine serum (Gibco, USA); C643 and KHM-5M cells were maintained in RPMI-1640 medium with the same components [11-13]. All cells incubated at 37°C and 5% CO₂ (Shanghai Medical Instruments, China). Apatinib was obtained from Hengrui Medicine Co. Ltd. (Jiangsu, China), then dissolved in DMSO and diluted with DMEM or 1640 medium to achieve a desired concentration for vitro experiments. Apatinib was dissolved in CMC for vivo experiments. Prior to each treatment, cells were plated overnight and were in similar subconfluent density at the time of drug exposure. SC79, API-2 and LiCl were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

CCK-8 Assays of Cell Viability and Colony Formation Assay

The cytotoxicity of Apatinib was estimated using the CCK-8 assay (Cell Counting Kit-8, Dojindo, Kumamoto, Japan). Cells were plated in 96-well plates at 3,000 cells per well 24 h before treatment. The cells were then treated with Apatinib at 0.5, 1, 5, 10, 20, 40 and 80 μ M for 24, 48, or 72 h, respectively. DMSO was added to cultures as a solvent control. For the colony formation assay, 1,000 cells were plated in 6-well plate and cultured at 37°C for 14 days. The colonies were observed by staining with 0.1% crystal violet in methanol for 30 min. For the colony formation in soft agar, 1000 cells suspended in 0.7% soft agar were plated on the surface of 1.2% soft agar in 6-well plate and cultured at 37°C for 2 weeks.

Apoptosis Detection and Cell Cycle Analyses

Flow cytometric assays of apoptosis and cell cycle were performed as previously described [14]. Briefly, cells were treated with 5 or 10 μ M Apatinib for 24 h. Then both attached and the floating cells were

harvested, washed twice with ice-cold PBS and suspended in 100 μ l binding buffer. Cells were incubated with 3 μ l FITC-Annexin V and 5 μ l PI at room temperature for 15 minutes in the dark. Next we added an additional 300 μ l 1 $\times$ Binding Buffer to each sample. The cells were harvested and suspended in ice-cold PBS after treatment for cell cycle distribution analysis. Single-cell suspensions were fixed with 70% cold ethanol at 4°C overnight. Afterwards samples were washed twice with cold PBS and incubated with PI (50 μ g/ml) for 30 min in the dark, then analyzed by flow cytometer.

Endothelial Tube Formation Assay

HUVEC cells were cultured in tumor supernatant and plated in 96-well plate coated with 50 μ l matrigel (BD Bioscience, CA, USA), approximately 1×10^4 per well. Tumor supernatant was collected from both control groups and Apatinib treatment groups cultured after 24 h. After 6 h of incubation at 37°C with 5% CO₂, tubules were photographed by microscopy and evaluated by Image Pro Plus software.

Western blot analysis

After treatment, cells were harvested and digested in RIPA buffer with the presence of Protease Inhibitor Cocktail (Pierce, USA), and protein concentration was quantified using the BCA Assays (Thermo Scientific). After electrophoresis in SDS-PAGE and transferring to PVDF membranes (Bio-Rad, Hercules, CA), proteins were then incubated with primary and secondary antibodies. The primary antibodies were listed as follows: ANG, AKT, p-AKT, GSK3 β , p-GSK3 β Ser9, GAPDH.

Angiogenesis antibody array

The samples from CAL-62/control and CAL-62/Apatinib (10 μ M) groups were collected for human angiogenesis array analysis. The levels of 43 angiogenesis-associated proteins were analyzed by using the Human Angiogenesis Antibody Array C1000 (RayBiotech, Norcross, Ga) according to the manufacturer's protocol. Fold-change was cut-off at 1.5x; significance testing was performed by t-test.

Quantitative real-time RT-PCR

ANG mRNA expression was determined by real-time PCR using SYBR Premix Ex Taq (TaKaRa, Japan) as previously described [15]. Primers pairs utilized were following: ANG: 5'-CTGGGCGTTTGTGTTGGTC-3' and 5'-GGTTTGGCATCATAGTGCTGG-3'; GAPDH: 5'-CATGAGAAGTATGACAACAGCCT-3' and 5'-AGTCCTTCCACGATACCAAAGT-3'.

Chick Embryo Chorioallantoic Membrane (CAM) Assay

Fertilized chicken eggs (Xing-Huo farm, Shanghai, China) were incubated at 37°C in a 70% humidified environment. On the 6th day, an opening was punctured on the shell and was sealed with transparent tape. Next day, a piece of sterilized filter paper disk (0.5 cm diameter) was placed on the surface of the CAM. 30- μ l supernatant from CAL-62 and CAL-62/Apatinib (10 μ M) was dropped onto the filter paper disk and sealed with transparent tape for the next consecutive 3 days. On the 10th day, the eggs were photographed with MacroPATH dissecting microscope (Milestone, Italy) and the number of blood vessels around the filter paper disc was assessed.

Xenograft Tumor Model, Immunohistochemistry and TUNEL Assay

Four-week-old male BALB/c nude mice were purchased from the Institute of Zoology, Chinese Academy of Sciences of Shanghai. Approximately 5×10^6 CAL-62 cells were injected into the right flank of nude mice. Two weeks after inoculation, the mice were randomly divided into either the Apatinib treatment or control group (N= 5 per group). Mice were administered a daily oral gavage with 50 mg/kg Apatinib or vehicle-only solution.

Tumor dimensions were measured using a Vernier caliper, and the tumor volume was calculated using the following formula: $V = \pi/6$ (length) $\times$ (Width) $\times$ (Width)/2. Tumor size were measured every 3 days. Three days after the last treatment, the animals were euthanized by cervical decapitation and tumors were removed and weighed. Tumor dimensions were measured using a Vernier caliper, and the tumor volume was calculated using the following formula: $V =$ (length) $\times$ (Width) $\times$ (Width)/2. The immunohistochemistry of xenograft tumor in nude mice was conducted as previously described [16]. Slides were incubated by antibodies including Ki-67 (1:100, Abcam) CD31 (1:200, Santa Cruz) and ANG (1:200, Santa Cruz). The

apoptotic cells were detected by the TUNEL assay using In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, USA) according to the manufacturer's protocol and nuclei were detected by DAPI staining.

Statistical Analysis

Data is expressed as the means $\pm$ SD. An analysis of variance (ANOVA) and Student's t test were used for comparison among groups. The Mann-Whitney U test was used for comparison of tumor volume. Categorical data is evaluated with the chi-square test or Fisher exact test. A P-value less than 0.05 was considered to be significant.

Results

Apatinib inhibits ATC cells proliferation

To analyze the effects of Apatinib on the proliferation of ATC cell lines, ATC cells were treated with a series of concentrations of Apatinib for 24 h, 48 h and 72 h, respectively. Apatinib performed a dose- and time-dependent manner in inhibiting the cell viability (Fig. 1A). The IC_{50} values for six ATC cell lines with Apatinib for 24 h, 48 h and 72 h were shown in Table 1. Contact-dependent growth assay by cloning formation was performed to further confirm the effect of Apatinib on inhibiting ATC cells proliferation. We observed the fewer clone number in CAL-62 and BHT-101 cells after Apatinib treatment (Fig. 1B and 1C). 3D growth model in soft agar colony formation also confirmed the effect of Apatinib on proliferation of ATC cells (Fig. 1D and 1E).

Apatinib induces apoptosis in ATC cell lines

As assessed by flow cytometry, treatment of Apatinib significantly induced apoptosis as compared with the control group in CAL-62 cells and especially in BHT-101 cells (Fig. 2A and 2B). The apoptotic rates were $5.86 \pm 0.29\%$ and $13.31 \pm 0.46\%$ in CAL-62 and BHT-101 cells respectively after 10 μ M of Apatinib treatment, which were remarkably higher than those in the control group (CAL-62: $5.86 \pm 0.29\%$ vs $2.23 \pm 0.24\%$, $p < 0.05$; and BHT-101: $13.31 \pm 0.46\%$ vs $3.93 \pm 0.28\%$, $p < 0.05$). In addition, the apoptotic rate increased with respect to the concentrations of Apatinib. Thus, our data demonstrated that Apatinib induced apoptosis in ATC cell lines in a dose-dependent manner.

Apatinib blocks cell cycle in ATC cell lines

We next detected cell cycle progression in CAL-62 and BHT-101 cells after Apatinib treatment by flow cytometry. The percentage of the S phase proportion decreased conspicuously and G0/G1 phase increased after Apatinib treatment both in CAL-62 and BHT-101 cells (CAL-62: $16.43 \pm 1.10\%$ (10 μ M Apatinib) vs $32.57 \pm 2.09\%$ (control), $p < 0.05$; and BHT-101: $9.74 \pm 1.16\%$ (10 μ M Apatinib) vs $30.91 \pm 2.08\%$ (control), $p < 0.05$, Fig. 2C and 2D). This data indicated that Apatinib could markedly inhibit G1-S transition in ATC cell lines. Thus, our results herein illustrated that Apatinib may function as an antitumor drug by inhibiting proliferation of cancer cells.

Apatinib inhibits HUVEC cell migration and tubular formation of HUVEC cells

CAL-62 and BHT-101 cells treated with 5 or 10 μ M of Apatinib for 24 h. To culture HUVEC cells, tumor supernatant was collected from CAL-62/Apatinib (5 μ M), CAL-62/Apatinib (10 μ M), BHT-101/Apatinib (5 μ M), BHT-101/Apatinib (10 μ M) and their corresponding control groups. 1×10^5 HUVEC cells were plated into the upper chamber of transwell coated with or without matrigel; incubated for 24 h at 37°C with 5% CO₂. With Apatinib concentra-

Table 1. IC_{50} values of Apatinib in various ATC cell lines. IC_{50} , 50% inhibitory concentration; ATC, anaplastic thyroid carcinoma

Cell lines	IC_{50} (μ M)		
	24 h	48 h	72 h
CAL-62	68.44	38.64	16.47
C643	62.68	25.32	11.02
KHM-5M	70.83	37.26	17.36
HTh-7	>80	41.26	22.11
BHT-101	59.45	15.11	10.93
8305C	64.65	24.23	17.28

Fig. 1. The effects of Apatinib on cell proliferation. (A) Cells were cultured in control conditions (DMSO) or in the present of a series of concentration of Apatinib for 24 h, 48 h and 72 h. Cell viability was assessed by CCK-8 assay. The data are averages of triplicate results from representative experiments. (B) Representative images of contact-dependent clone formation. (C) The columns represent the mean $\pm$ SD of the three independent experiments. (D) Representative images of clone formation in soft agar. (E) Data represent the mean $\pm$ SD and are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with control group.

tion increasing, the migration ability of HUVEC cells was gradually reduced both in CAL-62 and BHT-101 cells (Fig. 3A and 3B). A similar tendency was observed in the invasive ability of HUVEC cells (Fig. 3C and 3D). meanwhile, 1×10^4 HUVEC cells were plated into 96-well plate coated with matrigel and incubated for 4 h at 37°C with 5% CO_2 . We observed less tubular formation in Apatinib treatment groups compared with the control groups (Fig. 3E and 3F). Thus, these results suggested that Apatinib inhibited tumor angiogenesis in ATC by inhibiting HUVEC migration, invasion, and tubular formation.

Apatinib represses the expression of angiogenesis-related genes in ATC cells.

To investigate the mechanisms involved in Apatinib inhibition of angiogenesis, we used Angiogenesis antibody array to detect the expression of angiogenesis-related genes after Apatinib treatment (10 μM) (Fig. 4A and 4B). Apatinib significantly downregulated the expression of ANG, VEGFR2 and TNF- α in ATC cells (Fig. 4C). These results indicated that the anti-angiogenic effects of Apatinib were executed through a mechanism involving multiple genes. ANG has been recognized as an important angiogenic factor in the development of human malignant tumors. In our study, quantification data showed that ANG protein expression was reduced in a dose-dependent manner after Apatinib treatment (Fig. 4D). Furthermore, Apatinib-mediated anti-angiogenic effect was significantly attenuated in the

Fig. 2. Apatinib promotes apoptosis and blocks cell cycle in ATC cells. (A) The ratio of apoptotic cells was measured in CAL-62 and BHT-101 cells after treatment with the indicate concentrations of Apatinib for 24 h. The results were representative of three independent experiments. (B) The percentage of apoptotic cells was investigated using Annexin V-FITC and PI. (AnV+) PI- cells were considered early apoptotic and (AnV+) PI+ cells were considered late apoptotic. The columns represent the mean $\pm$ SD of the three independent experiments. ** $p < 0.01$, compared with control. (C) CAL-62 and BHT-101 cells were treated with Apatinib in indicate concentration for 24 h. Then cell cycle phase distribution was detected by flow cytometry. The results were representative of three independent experiments. (D) The percentages of cells in G1, S, and G2-M were shown as histograms.

Fig. 3. Effects of Apatinib on migration, invasion and tubular formation of HUVECs. (A) Apatinib treatment inhibited the migration of HUVECs. (B) Histograms showed the numbers of migration cells. Data are represented as mean $\pm$ SD of three independent experiments. (C) Treatment of Apatinib inhibited the invasion of HUVECs. (D) Histograms showed the numbers of invasion cells. (E) Less tubules were observed in the Apatinib treatment groups compared with the control groups. (F) Bar charts showed mean tubular lengths relative to control. Data are represented as mean $\pm$ SD of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

presence of recombinant human ANG (rhANG) treatment (Fig. 4E). All these results showed that ANG could promote angiogenesis and the expression of ANG could be inhibited by Apatinib treatment.

Akt/GSK3 β /ANG signaling is involved in the anti-angiogenic effects of Apatinib

Recent research has shown activated GSK3 β can decrease HIF-1 α to inhibit angiogenesis, and GSK3 β may be inhibited by AKT via phosphorylation of its Ser9 and Ser21 [17, 18]. The activity of AKT performs an important role in angiogenesis [19]. Therefore, the level of AKT and GSK3 β was examined; and the results showed that the phosphorylation of these proteins decreased in a concentration-dependent manner in response to Apatinib in CAL-62 and BHT-101 cells (Fig. 5A).

To determine whether Akt/GSK3 β signaling participates in Apatinib-induced reduction of ANG and angiogenesis, CAL-62 cells were subsequently treated together with Apatinib and SC79 (the activator of Akt). The Apatinib-induced decrease of p-GSK3 β and ANG were reversed through the activation of Akt, resulting in a significantly improved angiogenesis (Fig. 5B-D). To further examine the role of Akt in Apatinib-mediated inhibition of angiogenesis, API-2, an inhibitor of Akt, was utilized to deactivate Akt. Deactivation of Akt decreased the level of p-GSK3 β , ANG and angiogenesis (Fig. 5E-G). Additionally, LiCl, an inhibitor of GSK3 β , was used to verify that GSK3 β was involved in the anti-angiogenic ability of Apatinib. The reduction of Apatinib-mediated ANG and angiogenesis was reversed by LiCl (Fig. 5H-J).

Fig. 4. Apatinib decreases the level of multiple angiogenesis-related protein. (A) Antibody arrays of protein expression of angiogenesis-related genes after Apatinib treatment. (B) The heat map of antibody arrays. Up and down arrows, protein expression significantly upregulated or downregulated, respectively, by 1.5-fold. (C) Analysis of antibody array grey levels. (D) The level of ANG after treatment of Apatinib in ATC cells was assayed by western blotting. (E) HUVEC cells were treated with supernatant from different group for tube formation assay. RhANG significantly enhanced tube formation. * $p < 0.05$, ** $p < 0.01$.

These results showed that Apatinib may repress ANG production and inhibit angiogenesis by suppressing AKT/GSK3β pathway in ATC cells.

Fig. 5. Apatinib decreases ANG expression and angiogenesis via the AKT/GSK3β signaling pathway. (A) The expression of total and phosphorylated AKT and GSK3β in CAL-62 and BHT-101 cells with or without Apatinib treatment for 24 h. CAL-62 and BHT-101 cells were treated with Apatinib (10 μM) or together with SC79 (4 μg/ml) or API-2 (2 μM) or LiCl (5 mM) up to 24h. (B, E and H) Cells were lysed and subjected to immunoblotting with the indicated antibodies. (C, F and I) The level of ANG was measured by qPCR. (D, G and J) Tube formation activity was detected and the relative tube length was shown as a bar chart. Data are represented as mean ± SD of three independent experiments. *p<0.05; **p<0.01.

Fig. 6. Apatinib inhibits tumor growth and angiogenesis in vivo. (A) A chick embryo chorioallantoic membrane assay was used to observe the effects of Apatinib on angiogenesis. (B) The number of blood vessels surrounding the filter paper disc was assessed. Each bar represents the mean $\pm$ SD from three independent experiments. (C) A representative tumor of xenograft mice injected with 5×10^6 CAL-62 cells. The mice were given by gavage once three days for 4 weeks with Apatinib or CMC. (D) Tumor volumes were measured every 3 days. (E) The weights of tumors in nude mice. (F) Representative images of TUNEL assay and IHC staining of Ki-67, CD31 and ANG were performed on serial sections of tumors from control groups and Apatinib group. * $p < 0.05$, ** $p < 0.01$.

Apatinib inhibits tumor growth and angiogenesis in vivo

Based on the results above, a traditional chick embryo chorioallantoic membrane (CAM) assay was used for observing the effects of Apatinib on angiogenesis. The vessel numbers treated with CAL-62/Apatinib ($10 \mu\text{M}$) supernatant were significantly reduced, compared

with control groups (16.33 ± 2.52 vs 9.33 ± 1.53 , $P < 0.05$; Fig. 6A and 6B). Then, tumor xenograft mouse model was utilized to further confirm the effects of Apatinib. CAL-62 cells were subcutaneously injected into the right flank of athymic nude mice (Fig. 6C). As expected, the xenografts grew significantly slower in Apatinib group than those in the control group ($189.03 \pm 105.36 \text{ mm}^3$ vs. $989.88 \pm 191.92 \text{ mm}^3$, $p < 0.01$; Fig. 6D and 6E). The average tumor weight of xenografts was also lighter ($0.154 \pm 0.077 \text{ g}$ vs. $0.884 \pm 0.134 \text{ g}$, $p < 0.01$; Fig. 6D and 6E). The expressions of Ki-67, CD31 and ANG in xenografts were detected by IHC, and cell apoptosis were assayed by TUNEL (Fig. 6F). The expressions of Ki-67, CD31 and ANG were significantly reduced in Apatinib group compared to control group. Moreover, Apatinib treatment led to an increase in the number of apoptotic cells suggested by TUNEL assay. Thus, Apatinib could reduce tumor growth, repress ANG expression and inhibit angiogenesis in a xenograft model of ATC.

Discussion

Although ATC is a rather rare malignant tumor, it is notorious for its high levels of metastasis and poor prognosis [3]. Traditional treatment methods are mostly palliative, and adjuvant chemotherapy may prolong survival somewhat and improve quality of life. Several clinical trials have proven that Apatinib, a selective VEGFR2 inhibitor, is an effective drug in terminal gastric cancer, non-small-cell lung cancer (NSCLC) and breast cancer [20-22]. In this study, Apatinib was shown to exert a cytostatic effect on ATC cell lines via inducing cell apoptosis *in vitro* and *in vivo*. Liu et al [23]. reported that Apatinib could promote autophagy and apoptosis through VEGFR2/STAT3/BCL-2 signaling in osteosarcoma, and Peng et al [24]. indicated that Apatinib could inhibit VEGF signaling and promote apoptosis in intrahepatic cholangiocarcinoma.

Angiogenesis is a key step in cancer metastasis, promoting cancer progression via synthesis of new blood vessels [25-27]. ATC is prone to hematogenous metastasis to the lung and bone, and anti-angiogenesis is one of the leading mechanisms for Apatinib in tumor therapy [9, 28-30]. In our study, evidenced by HUVEC migration and tube formation assay, Apatinib could effectively inhibit angiogenesis in ATC *in vitro*. CAM assay and detection of CD31 expression in xenografts demonstrated similar effects *in vivo*. Our results suggested Apatinib could efficiently reduce angiogenesis in ATC and could be a potential therapeutic approach in ATC. Apatinib could inhibit angiogenesis by interfering with VEGF signaling [31]. However, in addition to the inhibition of VEGF signaling network, multiple regulation pathways may be involved in the anti-angiogenic effects of Apatinib.

Based on our pioneering research in ATC, antibody array and Western blot analyses showed that ANG was closely related to ATC and could be inhibited by Apatinib treatment. High serum levels of ANG were also observed in patients with divergent cancers, including breast cancer [32], bladder carcinoma [33], melanoma [34], B non-Hodgkin lymphoma [35], and prostate cancer [36]. As an essential factor in several stages of angiogenesis in malignant carcinoma, ANG can translocate rapidly to the nucleus and cleave tRNA, which promotes endothelial cell activities including migration, proliferation, and tube formation [8, 37, 38]. In our study, ANG could attenuate the anti-angiogenic effects of Apatinib, further confirming the anti-angiogenesis effects of Apatinib through reducing ANG expression. What's more, the comprehensive regulatory mechanisms of how Apatinib inhibited ANG level should be elaborated.

The Akt signaling pathway is one of the major survival gateways of tumor cells, and its expression rate is frequently high in many cases of carcinoma [39]. Increasing reports suggest that the dysregulation of this pathway has been involved in several pathological processes, including tumorigenesis, proliferation, invasion, cell cycle, apoptosis, angiogenesis, metastasis and drug resistance [40]. Our results showed that the level of p-Akt was decreased after Apatinib treatment. Through utilizing the activator SC79 of Akt and the inhibitor API-2 of Akt, we confirmed that Akt signaling was involved in the anti-angiogenic

effects of Apatinib. The Akt signaling pathway may closely interact with mTOR, VEGF and PDGF to influence angiogenesis [19].

GSK3 β , a serine/threonine kinase, is another downstream effector of Akt. It can be phosphorylated and deactivated by Akt [40]. GSK3 β is also a nodal point of convergent signaling pathways in regulating angiogenesis [41, 42]. In this study, in addition to Akt, p-GSK3 β were reduced by Apatinib administration as well. The inhibitor LiCl of GSK3 β could restore the anti-angiogenic effects of Apatinib. Previous studies have shown that the Akt/GSK3 β signaling pathway could regulate cyclin D1, AM or other downstream targets to promote angiogenesis [43, 44]. Our study found that ANG may be a downstream target of Akt/GSK3 β signaling pathway. Activation of the Akt/GSK3 β signaling pathway increased ANG expression, and this effect could be inhibited by Apatinib treatment.

In summary, our study demonstrates that Apatinib could inhibit ATC cells proliferation in a dose- and time- dependent manner through inducing apoptosis and blocking cell cycle. Suppression of AKT/GSK3 β /ANG signaling pathway is involved in the anti-angiogenic effects of Apatinib. Our research may establish a novel strategy in ATC treatment and Apatinib is a potential therapeutic agent.

Abbreviations

ATC (anaplastic thyroid cancer); ANG (angiogenin); TKI (tyrosine kinase inhibitor); VEGFR-2 (vascular endothelial growth factor receptor-2); RET (rearranged during transfection); PDGFR- β (platelet-derived growth factor- β); c-Kit (stem cell factor receptor); c-Src (v-Src sarcoma viral oncogene homolog); CAM (chick embryo chorioallantoic membrane).

Disclosure Statement

The authors declare no conflicts of interest.

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