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ORIGINAL ARTICLE

Apatinib strengthens the anti-tumor effect of cisplatin in thyroid carcinoma through downregulating VEGFR2

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Summary

Purpose: *The purpose of this study was to elucidate the influence of Apatinib combined with cisplatin on changing the phenotypes of TPC-1 cells.*

Methods: *VEGFR2 levels in thyroid carcinoma tissues and adjacent normal ones were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Then, the viability of TPC-1 cells induced with different doses of Apatinib and cisplatin was determined by cell counting kit-8 (CCK-8). Migratory and invasive abilities and apoptosis of TPC-1 cells induced with Apatinib combined with cisplatin were assessed. The protein levels of p-VEGFR2, VEGFR2, p-Akt, Akt, p-mTOR and mTOR in TPC-1 cells influenced by the treatment of Apatinib combined with cisplatin were examined by Western blot.*

Results: *VEGFR2 was upregulated in thyroid carcinoma tissues. Cisplatin treatment markedly suppressed viability, migratory and invasive abilities, and stimulated apoptosis of TPC-1 cells, which were further strengthened by combination treatment of Apatinib. Apatinib treatment strengthened the anti-tumor influence of cisplatin on TPC-1 cells through downregulating p-VEGFR2, p-Akt and p-mTOR.*

Conclusions: *Apatinib strengthens the anti-tumor influence of cisplatin in thyroid carcinoma through VEGFR2-Akt-mTOR pathway.*

Key words: *apatinib, VEGFR2, cisplatin, thyroid carcinoma*

Introduction

The incidence of thyroid carcinoma (TC) has gradually increased in recent years [1]. Advanced TC patients suffer a poor life quality because of distant metastasis [2]. Anaplastic thyroid cancer (ATC) is the most fatal TC because of strong invasiveness, high rate of distant metastasis and drug resistance. The incidence of ATC is only 1.5%, while its median survival is 3-9 months [3-5]. Surgical resection of local ATC, and combined postoperative chemotherapy and radiotherapy could effectively prolong the survival of ATC patients. Cisplatin, which kills cancer cells through damaging DNAs, is commonly applied for ATC [6]. However, cisplatin treatment could not achieve a satisfied outcome due to cisplatin resistance. Therefore novel effective strategies for ATC are urgently required.

It is well known that angiogenesis is of significance in tumor development and metastasis. Vascular endothelial growth factor (VEGF) and its homoreceptor VEGFR, are critical mediators in tumor progression [7,8]. VEGFR2 exerts an im-

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portant role in angiogenesis through most pro-angiogenic factors, serving as a key linkage for integrating pro-angiogenic signals in the tumor microenvironment [9]. Therefore, blockage of the VEGFR2 pathway is considered to be a potential tumor treatment.

Apatinib, a novel, small molecular oral drug with anti-angiogenic effects [10], is an inhibitor of VEGFR2 and its downstream pathways [11]. It is reported that Apatinib blocks VEGF pathway, thus inducing tumor cell apoptosis and inhibiting proliferation [12,13]. Besides, Apatinib could suppress the proliferative ability of ATC both *in vivo* and *in vitro* [14]. Nevertheless, the anti-tumor effect and drug-resistance of Apatinib in ATC, and the underlying mechanism, remain to be clarified.

In this paper, we mainly assessed the influences of Apatinib in cisplatin-induced apoptosis stimulation and metastasis inhibition in TPC-1 cells, and potential changes in VEGFR2-Akt-mTOR pathway.

Methods

Clinical samples

ATC tissues and adjacent normal ones were collected from 30 ATC patients and stored at -80°C. Patients and their families in this study have been fully informed and signed the informed consent.

Cell culture

TC cell line TPC-1 (Cell Bank, Shanghai, China) was cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 100 μg/mL penicillin and 100 mg/mL streptomycin. Then the cell line was treated with different doses (0, 5, 10, 20 or 50 μM) of cisplatin and Apatinib.

Cell counting kit-8 (CCK-8)

Cells were inoculated in 96-well plates with 5×10^3 cells/well and induced with cisplatin combined with Apatinib for 72 h. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the

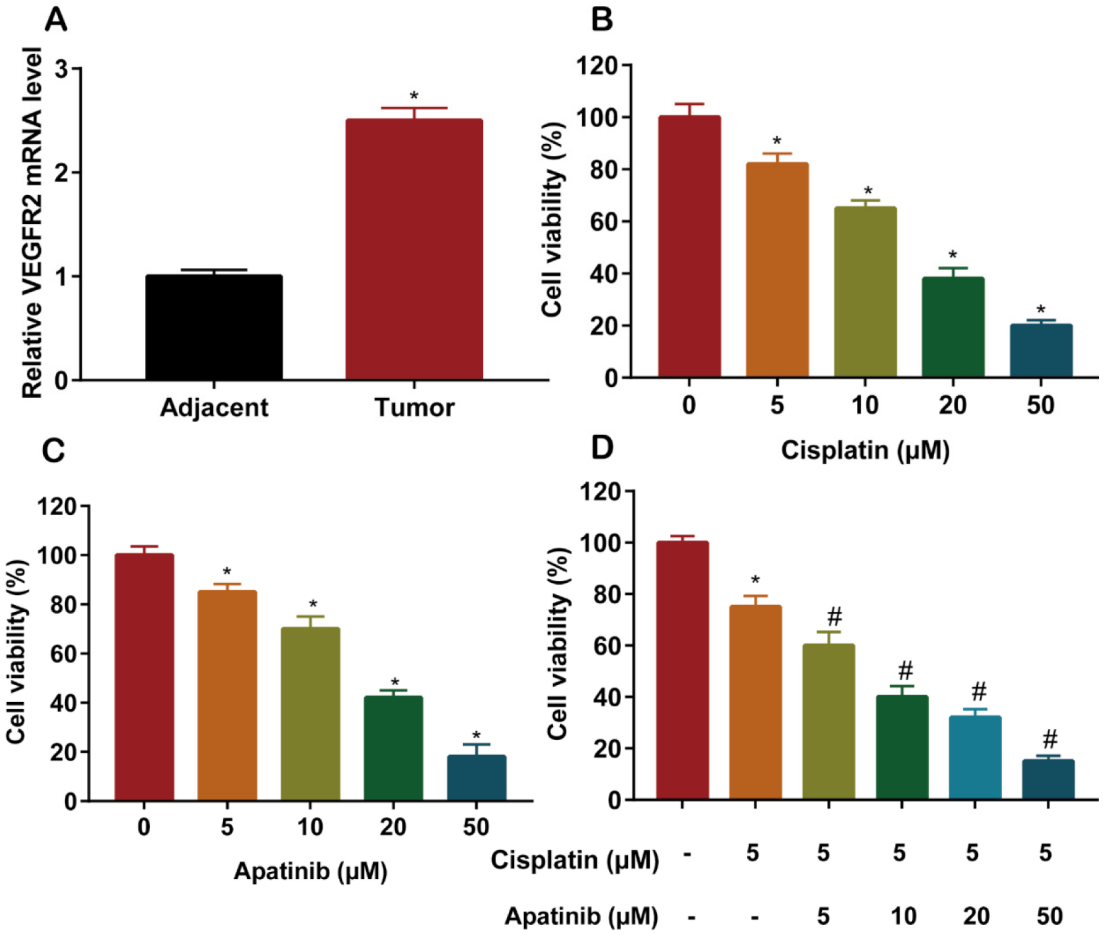

Figure 1. VEGFR2 was upregulated in ATC. **A:** VEGFR2 levels in adjacent normal tissues and ATC tissues (*p<0.05, compared with adjacent). **B:** Viability of TPC-1 cells induced with 0, 5, 10, 20 or 50 μM cisplatin (*p<0.05, compared with 0 μM group). **C:** Viability of TPC-1 cells induced with 0, 5, 10, 20 or 50 μM Apatinib (*p<0.05, compared with 0 μM group). **D:** Viability of TPC-1 cells induced with 5 μM cisplatin combined with 0, 5, 10, 20 or 50 μM Apatinib (*p<0.05, compared with 0 μM group; #p<0.05, compared with 5 μM cisplatin group).

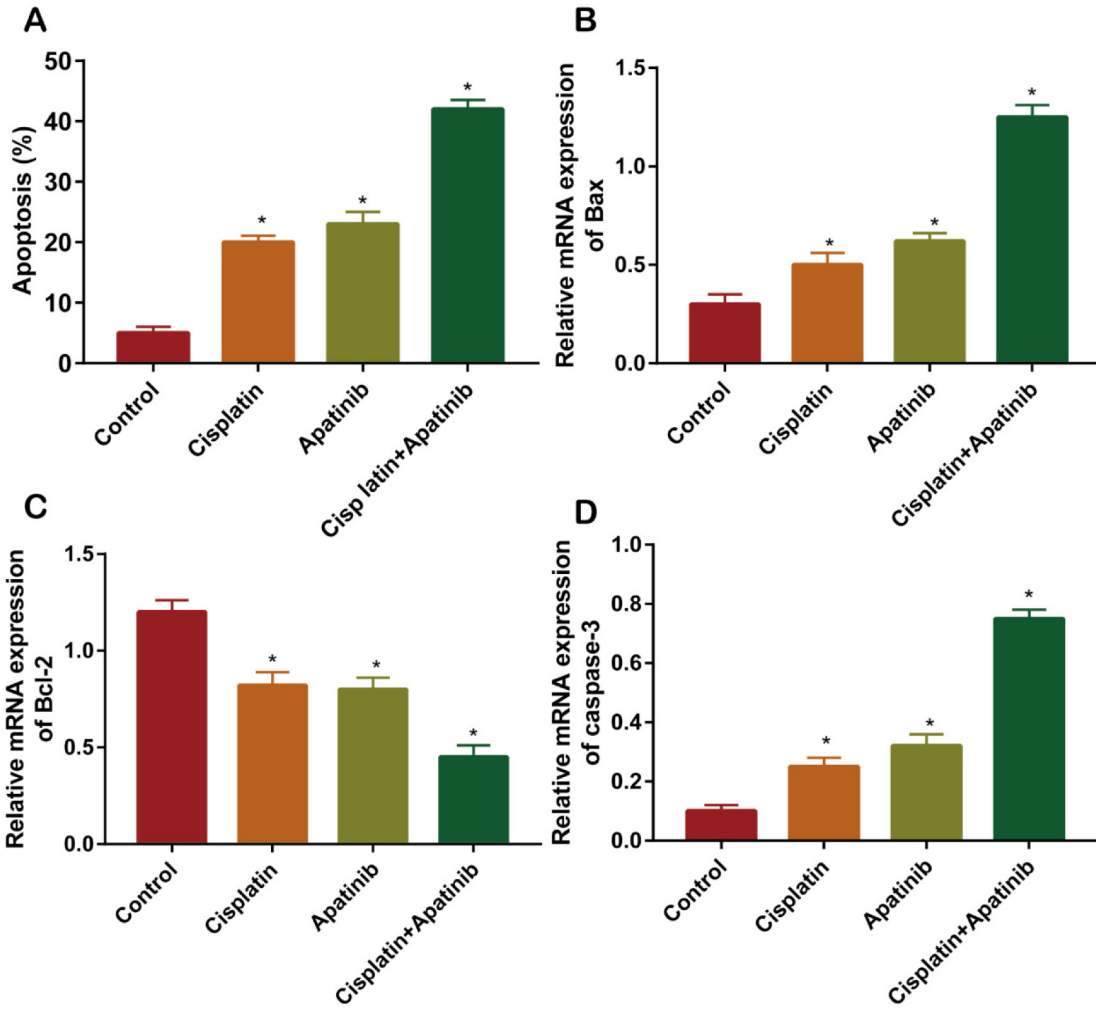

Figure 2. Apatinib strengthened pro-apoptotic influence of cisplatin in TPC-1 cells. **A:** Apoptotic rate of TPC-1 cells treated with blank control, 5 μM cisplatin, 10 μM Apatinib or 5 μM cisplatin combined with 10 μM Apatinib for 72 h. **B-D:** Relative levels of Bax **(B)**, Bcl-2 **(C)** and caspase-3 **(D)** in TPC-1 cells treated with blank control, 5 μM cisplatin, 10 μM Apatinib or 5 μM cisplatin combined with 10 μM Apatinib for 72 h. (*p<0.05, compared with Control group).

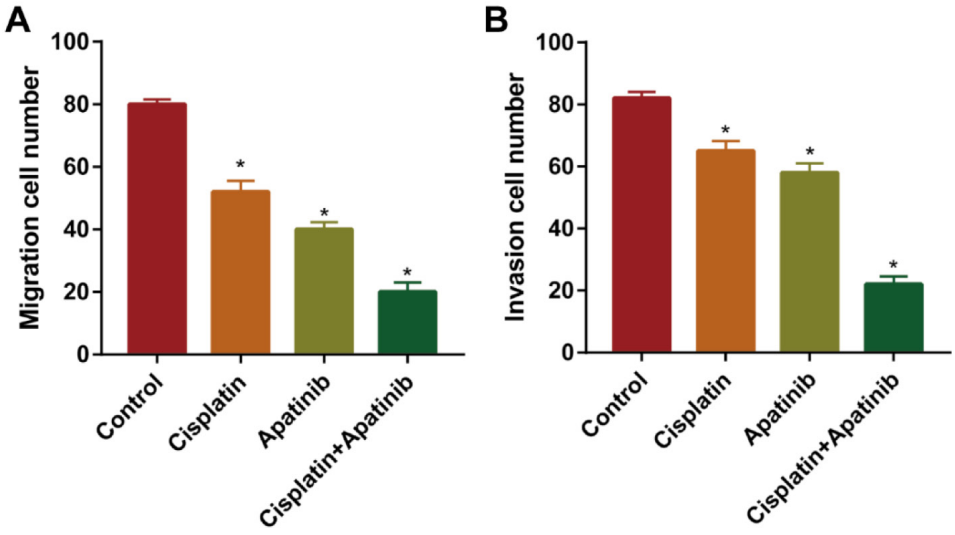

Figure 3. Apatinib strengthened the anti-metastatic influence of cisplatin in TPC-1 cells. Migratory **(A)** and invasive numbers **(B)** of TPC-1 cells treated with blank control, 5 μM cisplatin, 10 μM Apatinib or 5 μM cisplatin combined with 10 μM Apatinib for 72 h. (*p<0.05, compared with Control group).

CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Flow cytometry

Cells were inoculated in 6-well plates with 2×10^6 cells/well. After 48 h treatment of cisplatin combined with Apatinib, cells were collected, washed in pre-cold phosphate buffered saline (PBS) twice, and re-suspended in 500 μL of binding buffer containing 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and 5 μL of propidium iodide (PI) in the dark. Thirty min later, cell apoptosis was determined by flow cytometry at 488 nm excitation and 600 nm emission.

Western blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h, and membranes reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol method (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA. Through reverse tran-

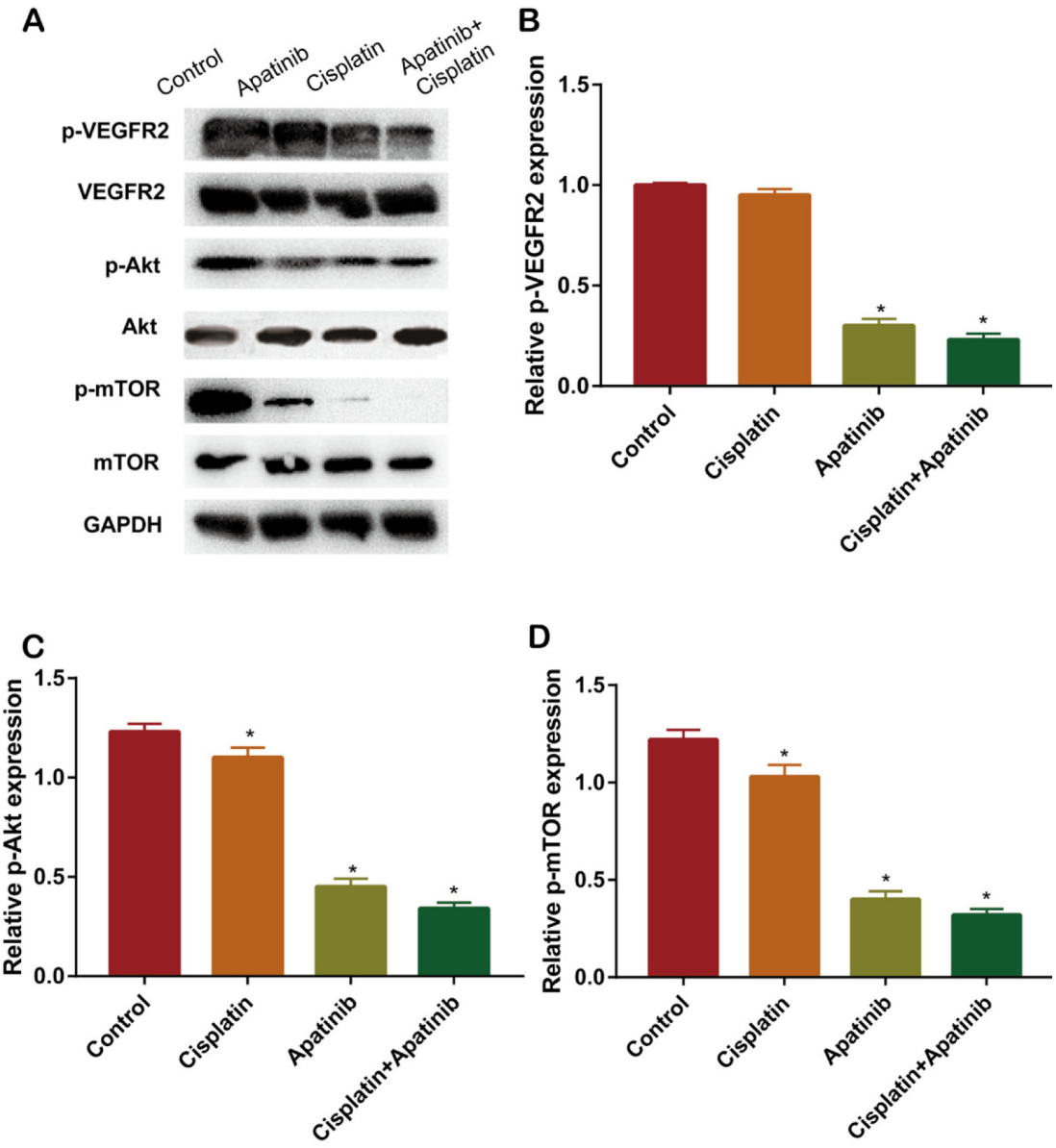

Figure 4. Apatinib strengthened anti-tumor influence of cisplatin on TPC-1 cells through inactivating VEGFR2-Akt-mTOR pathway. **A:** Western blot analyses on p-VEGFR2, VEGFR2, p-Akt, Akt, p-mTOR and mTOR in TPC-1 cells treated with blank control, 5 μM cisplatin, 10 μM Apatinib or 5 μM cisplatin combined with 10 μM Apatinib for 72 h. **B-D:** Relative protein levels of p-VEGFR2 **(B)**, p-Akt **(C)** and p-mTOR **(D)** in TPC-1 cells treated with blank control, 5 μM cisplatin, 10 μM Apatinib or 5 μM cisplatin combined with 10 μM Apatinib for 72 h. (*p<0.05, compared with Control group).

scription of RNA, the extracted complementary deoxyribose nucleic acid (cDNA) was used for PCR detection using the DBI Bestar SybrGreen qPCR Master Mix (DBI Bioscience, Shanghai, China) on Stratagene Mx3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA, USA), with glyceraldheyde 3-phosphate dehydrogenase (GAPDH) as the internal reference.

Transwell assay

Transwell inserts (8 μm, Costar, Dallas, TX, USA) pre-coated with diluted Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were prepared. Cells suspended in serum-free medium were applied on the upper chamber of transwell inserts with 2×10^4 cells per insert. Medium containing 10% FBS was applied in the bottom. After 24 h of cell culture, cells still on the upper side were removed and those penetrating to the bottom were fixed and dyed in 2% crystal violet. Finally, the penetrating cells were captured and calculated.

Statistics

GraphPad Prism 7 (La Jolla, CA, USA) was used for all statistical analyses. Data were expressed as mean ±standard deviation (SD). The t-test was used for analyzing differences between two groups. $P<0.05$ indicated significant difference.

Results

VEGFR2 was upregulated in ATC

Compared with adjacent normal tissues, VEGFR2 was upregulated in ATC tissues (Figure 1A). In TPC-1 cells treated with different doses of cisplatin, cell viability decreased dose-dependently (Figure 1B). Similarly, viability was gradually reduced in TPC-1 cells induced with increased doses of Apatinib (Figure 1C). It is noteworthy that viability was dose-dependently reduced in TPC-1 cells treated with 5 μM cisplatin combined 0, 5, 10, 20 or 50 μM Apatinib (Figure 1D). The above results suggested that Apatinib could strengthen the anti-proliferative effect of cisplatin in TPC-1 cells.

Apatinib strengthened the pro-apoptotic influence of cisplatin on TPC-1 cells

Either cisplatin or Apatinib treatment increased the apoptotic rate in TPC-1 cells. Notably, treatment of Apatinib combined with cisplatin remarkably elevated the apoptotic rate than single treatment (Figure 2A). In addition, the relative levels of Bax and caspase-3 were upregulated by cisplatin or Apatinib treatment, and the uptrend was much more significant after combination treatment (Figure 2B). Moreover, Bcl-2 was much more downregulated following combination treatment compared with those treated with single drug (Figure 2C, 2D). Therefore, Apatinib strengthened the pro-apoptotic influence of cisplatin in TPC-1 cells.

Apatinib strengthened the anti-metastatic influence of cisplatin in TPC-1 cells

After 72 h treatment of 5 μM cisplatin combined with 10 μM Apatinib in TPC-1 cells, migratory and invasive cell numbers were markedly reduced compared with those treated with either cisplatin or Apatinib (Figure 3A,3B). It could be seen that the inhibitory role of cisplatin in migratory and invasive abilities of TPC-1 cells were strengthened by Apatinib.

Apatinib strengthened the anti-tumor influence of cisplatin in TPC-1 cells through inactivating VEGFR2-Akt-mTOR pathway

Western blot analyses uncovered that cisplatin treatment alone could downregulate p-Akt and p-mTOR compared with controls. Notably, Apatinib combined with cisplatin markedly strengthened the downregulation of p-Akt, p-mTOR and p-VEGFR2 (Figure 4A-4D), indicating that Apatinib strengthened the anti-tumor influence of cisplatin in ATC by inactivating VEGFR2-Akt-mTOR pathway.

Discussion

ATC is a rare undifferentiated cancer with an extremely high mortality [15]. The one-year survival of ATC is as low as 20% [16]. Therapeutic efficacy of traditional treatments for ATC, including surgical procedures, chemotherapy and isotope intervention, is far away from satisfaction [17,18]. Tumor angiogenesis is a key event during tumor metastasis [19,20]. Through binding to and activating VEGFR2, VEGF exerts a pro-angiogenesis effect [21]. Previous studies have demonstrated the critical functions of VEGFR2 in multiple types of tumors [22-26]. In this article, we found that VEGFR2 was highly expressed in ATC tissues, suggesting the potential of VEGFR2 to be a promising biomarker.

Apatinib is an anti-angiogenic agent and a tyrosine kinase inhibitor that selectively inhibits VEGFR2 level. It strongly inhibits angiogenesis thus alleviates tumor growth through blocking VEGF and its receptors. Apatinib is reported to be effective in radioactive iodine refractory differentiated thyroid carcinoma and ATC [27,28]. Tumor cell phenotypes are basic features influencing tumor progression [29]. In particular, cell apoptosis is of significance in maintaining cell survival [30]. Here, our experiments suggested that Apatinib remarkably enhanced the role of cisplatin in suppressing viability, migratory and invasive abilities, as well as triggering apoptosis in TPC-1 cells.

Akt pathway is a fundamental signaling pathway in tumor cell survival, which is able to affect various pathological processes [31]. Overactivated Akt/mTOR pathway contributes to an environment favorable to tumor cell survival [32-34]. Feng et al [35] proposed that Apatinib induces cell apoptosis and autophagy in ATC cells through the Akt/mTOR pathway. By blocking VEGFR2-Akt-mTOR pathway, suppression of angiogenesis has become a potential anti-tumor therapy [36,37]. Our findings uncovered that cisplatin treatment could markedly downregulate p-Akt and p-mTOR, while p-VEGFR2 level was not affected. Notably, the treatment of cisplatin combined with Apatinib further reduced the relative levels of p-Akt and p-mTOR, and downregulated p-VEGFR2. Collectively, Apatinib could enhance the anti-tumor effect of cisplatin in ATC through downregulating VEGFR2 to activate the Akt/mTOR pathway.

Conclusions

Apatinib strengthens the anti-tumor role of cisplatin in thyroid carcinoma through VEGFR2-Akt-mTOR pathway.

Conflict of interests

The authors declare no conflict of interests.

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