Pathology - Research and Practice 215 (2019) 152422

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Apatinib enhanced anti-tumor activity of cisplatin on triple-negative breast cancer through inhibition of VEGFR-2

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ARTICLE INFO

Keywords:
Triple-Negative breast cancer
Apatinib
Cisplatin
VEGFR2

ABSTRACT

Background: Triple-negative breast cancer (TNBC) was known as a fast-growing and an aggressive tumor. Cisplatin is the effective cytotoxic drug used for the treatment of TNBC. In addition, apatinib, a VEGFR2 inhibitor, exhibits antitumor activity in patients with TNBC. However, the effects of combination of apatinib with cisplatin on TNBC remain unclear. Thus, this study aimed to investigate the effects of apatinib in combination with cisplatin on MDA-MB-231 cells.

Methods: Immunohistochemistry was used to detect the expression of VEGFR2. In addition, CCK-8, flow cytometric, transwell assays were used to measure the cell proliferation, apoptosis, migration and invasion, respectively. Moreover, western blotting was used to detect the expressions of Bax, active caspase 3, p-VEGFR2, p-Akt and p-mTOR.

Results: VEGFR2 was significantly upreguated in patients with TNBC. In addition, the inhibitory effects of cisplatin on the proliferation, migration and invasion of MDA-MB-231 cells were enhanced by apatinib. Moreover, apatinib increased cisplatin-induced apoptosis on MDA-MB-231 cells via increasing the level of Bax and active caspase 3 and decreasing the expression of Bcl-2. Importantly, apatinib enhanced anti-tumor effect of cisplatin on MDA-MB-231 cells via inhibiting the levels of p-VEGFR2, p-Akt and p-mTOR.

Conclusion: Our findings indicated that apatinib enhanced the anti-tumor effects of cisplatin on MDA-MB-231 cells via inhibition of VEGFR2. Thus, the combination of apatinib with cisplatin may serve as a potential approach in the treatment of patients with TNBC.

1. Introduction

Breast cancer is the most leading malignant tumor in women all over the world [1]. According to the estimation, there are 252,710 new cases and 40,610 deaths cases in America [1]. Triple-negative breast cancer (TNBC) accounts for approximately 15% of all breast cancers, which is a fast-growing and an aggressive tumor [2]. Molecular profiling has identified TNBC as a disease which contains several intrinsic subtypes, such as “basal-like” and Her2-enriched [3]. Triple-negative basal-like (TNBL) are characterized by lacking estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth receptor 2 (Her2) proteins expression [4]. The “basal-like” subtype is more commonly negative for all 3 markers (ER, PR and HER2), which belong to the “triple-negative” phenotypic classification [4]. Previous study has suggested that TNBC has a relatively high rate of recurrence and distant metastasis, with poor overall survival (OS) [5]. The common therapeutic option is chemotherapy for the treatment of TNBC [6]. However, TNBC develops resistance to chemotherapy treatment widely [7]. Therefore, investigations of novel effective therapies for TNBC are urgently needed.

Cisplatin (cis-diamminedichloroplatinum II), a most common chemotherapeutic drug, which kills cancer cells by damaging their DNA [8]. Ciaplatin is widely used to combat multiple cancers, including breast, ovary, testes and bladder cancer [8]. Some studies have showed that cisplatin is an important cytotoxic drug for treatment of TNBC [9,10]. However, its clinical usefulness is limited by renal, neurological, and gastrointestinal toxicity [11]. Thus, the development of novel targeted therapies for this aggressive type of breast cancer is of paramount importance.

Apatinib, a novel vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor, has potential antiangiogenic and antineoplastic activities [3,12]. Previous study indicated that VEGF and VEGFRs play

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https://doi.org/10.1016/j.prp.2019.04.014
Received 30 January 2019; Received in revised form 11 April 2019; Accepted 26 April 2019

an important role in angiogenesis of breast and other cancers [13]. Apatinib could inhibit VEGF-induced endothelial cell proliferation and migration [14]. Recently, apatinib exerts satisfying efficacy on various types of cancers such as lung cancer, nasopharyngeal carcinoma and hepatocellular carcinoma [15–17]. Previous study also indicated that apatinib exhibited substantial antitumor activity in patients with TNBC [3]. Therefore, this study aimed to investigate the anti-tumor effects of apatinib in combination with cisplatin on TNBC, in order to provide new ideas and methods for the treatment of TNBC.

2. Materials and methods

2.1. Clinical samples

Sixty clinical specimens including TNBC and matched tumor-adjacent tissues were obtained from patients, who underwent modified radical mastectomy for breast cancer. Patients who received preoperative immunotherapy, chemotherapy or radiotherapy were excluded. Prior to the collection and use of clinical specimens, each patient signed an informed consent form. The agreement on clinical specimens involved in this study was licensed by the research ethics committee of The First Affiliated Hospital of Bengbu Medical College.

2.2. Immunohistochemistry (IHC)

TNBC tissues were cut into 4 μm slices, and embedded in paraffin. Later on, sections were incubated with VEGFR-2 primary antibody (ab2349, Abcam, Cambridge, MA, USA) overnight at 4 °C. Then, the slides were incubated with peroxidase combined secondary antibody (ab7090, Abcam). Then, DAB solution was performed according to the manufacturer's specification. VEGFR-2 staining was evaluated under a light microscope at 400 magnification. Staining intensity was scored manually: no staining = 0, weak staining = 1, moderate staining = 2, and strong staining = 3. Tumor cells in five fields were randomly selected and scored to get the average percentage of positively stained cells (0–100%). 0–5% scored 0; 6%–35% scored 1; 36%–70% scored 2; more than 70% scored 3. The final IHC score was designated as low or high expression group using the percent of positive cell score × staining intensity score as follows: low expression was defined as a total score < 4 and high expression with a total score ≥ 4.

2.3. Cell culture

Human TNBC cell line MDA-MB-231 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DEME, HyClone, Logan, UT, USA) supplemented with 10% fatal bovine serum (FBS, HyClone, Logan, UT, USA) at 37 °C in a humidified incubator with 5% CO_2.

2.4. CCK-8 assay

The cell viability was determined by cell counting kit-8 assay (CCK-8, Dojindo, Kumamoto, Japan). MDA-MB-231 cells (2×10^3 cells per well) were plated into 96-well plates and then treated as following: Cisplatin (0, 5, 10, 20 or 50 μM); Apatinib (0, 5, 10, 20 or 50 μM); Cisplatin (5 μM) and Apatinib (0, 5, 10, 20 or 50 μM). The cells were cultured for 72 h. After that, 10 μL CCK-8 reagent was plated into the each well and incubated with the plates for another 2 h at 37 °C. The absorbance of cells at a wavelength of 450 nm was measured with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Immunofluorescence

MDA-MB-231 cells were plated onto 24-well plates overnight and treated with cisplatin (5 μM) or/and apatinib (10 μM) for 72 h. Briefly, incubated cells were prefixed in 4% formaldehyde for 10 min, permeabilized in 100% methanol for 20 min. Next, cells were incubated with primary antibodies at 4 °C overnight: anti-Ki67 (Abcam; ab15580) (1:1000), DAPI (Abcam; ab104139). Subsequently, cells were incubated with secondary antibodies (1:5000, Abcam; ab150080) for 1 h. The samples were observed using a Nikon Eclipse TE2000 fluorescent microscope (Japan) with Q-Capture software.

2.6. Flow cytometric analysis of cell apoptosis

MDA-MB-231 cells were plated onto 6-well plates overnight and treated with cisplatin (5 μM) or/and apatinib (10 μM) for 72 h. The harvested cells were fixed in ethanol (70%, v/v) and washed twice with ice-cold PBS. After that, 5 μL FITC-conjugated annexin V and 5 μL propidium (PI) were added in the situation. Then, incubated for 15 min in the dark at room temperature. Following incubation, the cells were measured using flow-cytometer (BD, Franklin Lake, NJ, USA) with WinMDI 2.9 software.

2.7. Western blotting

MDA-MB-231 cells were plated onto 6-well plates overnight and treated with cisplatin (5 μM) or/and apatinib (10 μM) for 72 h. Proteins concentration were detected by Bradford Protein Assay Kit (Beyotime, Shanghai, China). Then, equivalent amounts of proteins were separated using 10% SDS-PAGE electrophoresis. Later on, the gels were transferred onto PVDF membranes (Thermo Fisher Scientific) in 2 h. After that, the PVDF membranes were blocked with 5% defatted milk in TBST at room temperature for 1 h. Then the membranes were incubated with primary antibodies overnight: anti-Bax (Abcam; ab32503) (1:1000), anti-active caspase 3 (Abcam; ab2302) (1:1000), anti-Bcl-2 (Abcam; ab196459) (1:1000), anti-β-actin (Abcam; ab8227) (1:1000), anti-p-VEGR2 (Abcam; ab5473) (1:1000), anti-p-Akt (Abcam; ab38449) (1:1000), anti-p-mTOR (Abcam; ab84400) (1:1000). After washing, the membranes were incubated with secondary antibody (1:5000, Abcam; ab7090) at room temperature for 1 h. Finally, the PVDF membranes were incubated with electro chemiluminescence (ECL) reagent (Santa Cruz Biotechnology) to measure the chemiluminescence intensity. The relative expression of protein was quantified by normalizing to β-actin.

2.8. Transwell migration and invasion assays

For migration assay, MDA-MB-231 cells were incubated in serum-free DMEM for 24 h. Cells (2.5×10^4 cells) were then seeded onto filters (8 mm pore size) in transwell inserts (Corning, New York, NY, USA) in filter and treated with cisplatin (5 μM) or/and apatinib (10 μM) for 72 h. The lower chamber of each well was added with DMEM containing with 10% FBS. After incubation, cells on the upper surface were removed with a cotton swab, and cells that had migrated to the lower surface stained with a solution containing 50% isopropanol and 0.5% crystal violet. The numbers of migrated cells at least 5 random fields from each membrane was counted. For invasion assay, identical methods were performed as those described in the migration assay except that the upper chamber is pre-treated with 100 μL of Matrigel.

2.9. Statistical analysis

The SPSS19.0 software (Chicago, IL, USA) was used for statistical analyses. Data were analyzed by one-way analysis of variance with the Bonferroni multiple comparison test. Comparison between two groups was performed using Student's t-tests. Data were represented as mean ± S.D. $P < 0.05$ was regarded as statistically very significant.

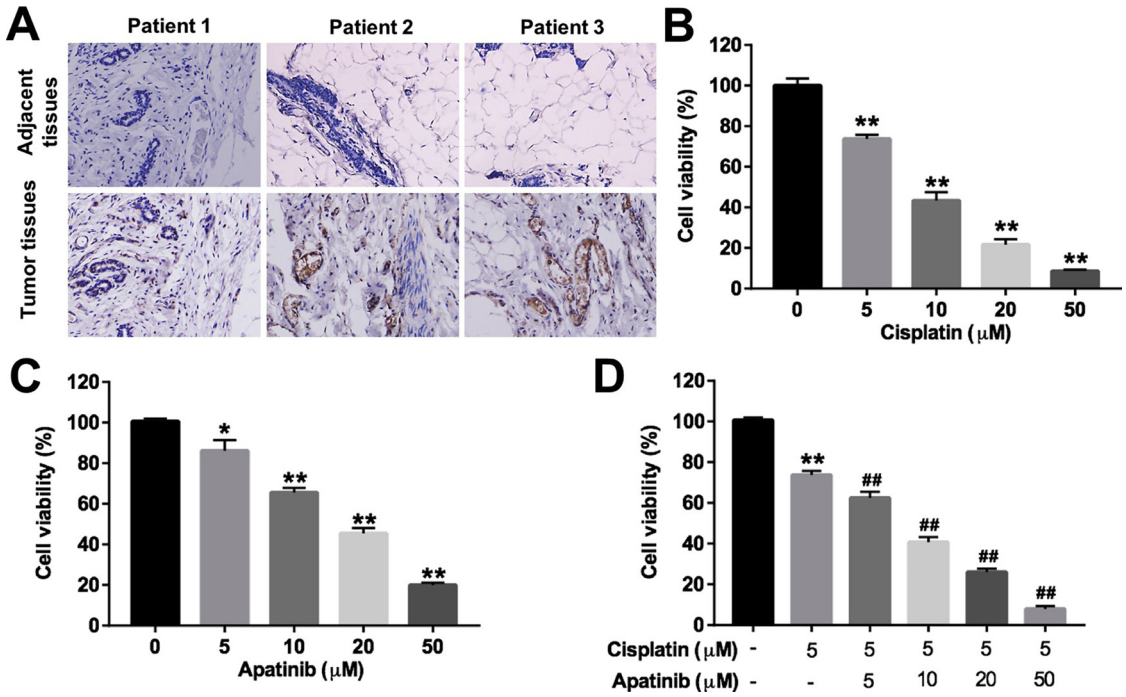

Fig. 1. The expression of VEGFR2 were upregulated in TNBC. (A) Representative IHC staining (×200); VEGFR2 immunohistochemical staining in TNBC tissues and adjacent tissues. **(B)** Cell viability of MDA-MB-231 after treating with cisplatin (0, 5, 10, 20 or 50 μM) for 72 h. **(C)** Cell viability of MDA-MB-231 after treating with apatinib (0, 5, 10, 20 or 50 μM) for 72 h. **(D)** Cell viability of MDA-MB-231 after treating with cisplatin (5 μM) and apatinib (0, 5, 10, 20 or 50 μM) for 72 h. *P < 0.05, **P < 0.01, compared with 0 μM group; ##P < 0.01, compared with 5 μM cisplatin treatment group.

3. Results

3.1. The expression of VEGFR2 was upregulated in TNBC

To explore the expression of VEGFR2 in TNBC, 60 cases of TNBC samples and adjacent samples were analyzed using IHC. As shown in Fig. 1A, the level of VEGFR2 was significantly increased in tumor tissues compared with that in adjacent tissues. In addition, according to the proportion of positive cells and staining intensity, the TNBC samples were divided into high or low VEGFR2 expression group (Table 1). Moreover, VEGFR2 expression correlated with clinic-pathological parameters including tumor size, nuclear grade, LN metastasis, TNM stage and Ki-67 (Table 1). These data indicated that the upregulation of VEGFR2 was observed in TNBC, which was associated with poor prognosis of patients.

3.2. Apatinib enhanced the anti-proliferation effect of cisplatin on MDA-MB-231 cells

CCK-8 assay was used to assess the anti-proliferation effect of cisplatin or/and apatinib on MDA-MB-231 cells. Both cisplatin (range from 5 to 50 μM) and apatinib (range from 5 to 50 μM) inhibited the proliferation of MDA-MB-231 cells in a dose-dependent manner, respectively (Fig. 1B and 1C). In addition, apatinib (5, 10, 20 or 50 μM) markedly enhanced the anti-proliferative effect of cisplatin on MDA-MB-231 cells (Fig. 1D). Since, combination of cisplatin (5 μM) with apatinib (10 μM) treatment induced 50% inhibition of cell growth (Fig. 1D), cisplatin (5 μM) and apatinib (10 μM) were utilized in the following experiments. Furthermore, the immunofluorescence assay indicated cisplatin significantly inhibited the proliferation of MDA-MB-231 cells. The anti-proliferation effect of cisplatin was notably increased in the presence of apatinib, compared with cisplatin alone treatment (Fig. 2A and B). These results suggested that apatinib could enhance the anti-proliferation effect of cisplatin on MDA-MB-231 cells.

Table 1
The association between the expression of VEGFR-2 and the clinicopathological characteristics in patients with TNBC.

Clinicopathologic factors	VEGFR-2 low (n = 17)	VEGFR-2 high (n = 43)	P
Age			
≤ 50 years	9 (52.94)	25 (58.14)	> 0.05
> 50 years	8 (47.06)	18 (41.86)	
Tumor size			
≤ 2cm	13 (76.47)	14 (32.56)	< 0.05
> 2cm	4 (23.53)	29 (67.44)	
Nuclear grade			
1	12 (70.59)	3 (6.98)	< 0.05
2	4 (23.53)	17 (39.53)	
3	1 (5.88)	23 (53.49)	
LN metastasis			< 0.05
negative	11 (64.71)	13 (30.23)	
positive	6 (35.29)	30 (69.77)	
TNM stage			< 0.05
I	10 (58.82)	2 (4.65)	
II	6 (35.29)	18 (41.86)	
III	1 (5.88)	23 (53.49)	
Ki-67 (%)			< 0.05
negative	10 (58.82)	12 (39.53)	
positive	7 (41.18)	31 (60.47)	

3.3. Apatinib enhanced the pro-apoptotic effect of cisplatin on MDA-MB-231 cells

In order to investigate the effects of cisplatin or/and apatinib on the apoptosis of MDA-MB-231 cells, flow cytometry was used. As indicated in Fig. 3A and B, cisplatin-induced cell apoptosis was markedly enhanced by the treatment of apatinib, compared with cisplatin alone treatment. In addition, western blotting was used to evaluate the expressions of apoptosis-related proteins Bax, Bcl-2 and active caspase-3.

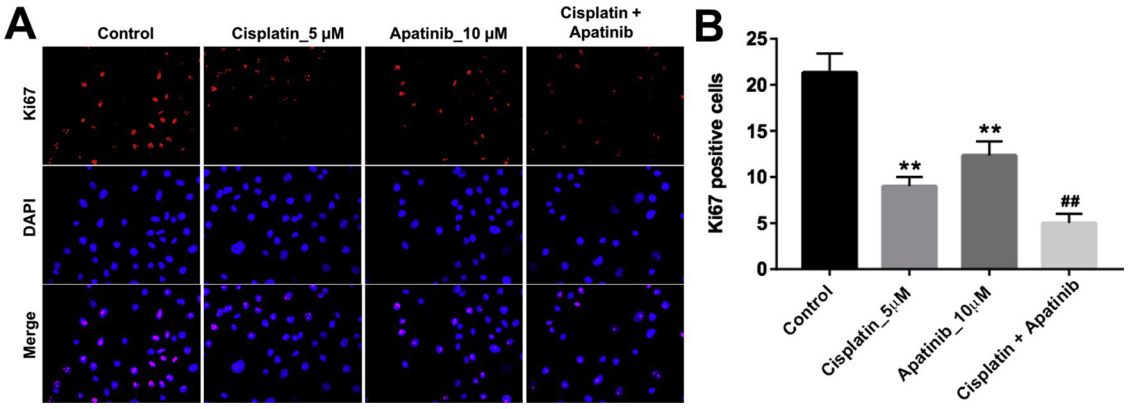

Fig. 2. Apatinib enhanced the anti-proliferation effect of cisplatin on MDA-MB-231 cells. MDA-MB-231 cells were treated with cisplatin (5 μM) or/and apatinib (10 μM) for 72 h. **(A)** The proliferation of cells were detected with Ki67 staining. **(B)** Relative fluorescence expression levels were quantified by Ki67 and DAPI staining. **P < 0.01, compared with control group; ##P < 0.01, compared with 5 μM cisplatin treatment group.

The results showed cisplatin significantly increased the expressions of Bax and active caspase 3 in cells, while decreased the expression of Bcl-2. As expected, the levels of Bax and active caspase 3 were further increased, and the expression of Bcl-2 was further decreased in the presence of apatinib (Fig. 3C–F). These data indicated that apatinib enhanced the pro-apoptotic effect of cisplatin on MDA-MB-231 cells.

3.4. Apatinib enhanced the inhibitory effects of cisplatin on the migration and invasion of MDA-MB-231 cells

Previous study demonstrated that TNBC is a severe aggressive tumor [2]. Therefore, we decided to investigate whether apatinib could enhance the anti-migratory and anti-invasive effects of cisplatin in MDA-MB-231 cells. As illustrated in Fig. 4A–D, cisplatin significantly inhibited cell migration and invasion. As expected, the anti-migratory and anti-invasive effects of cisplatin was markedly enhanced by the treatment of apatinib, compared with cisplatin alone treatment. These data illustrated that apatinib enhanced the inhibitory effects of cisplatin on the migration and invasion of MDA-MB-231 cells.

3.5. Apatinib enhanced the anti-tumor effect of cisplatin on MDA-MB-231 cells via inhibiting VEGFR2-Akt-mTOR pathway

The data reported above indicated that the expression of VEGFR2 were upregulated in patients with TNBC. Therefore, we further investigated whether combination of cisplatin with apatinib could affect the fate of TNBC cells via regulating VEGFR2-Akt-mTOR pathway. The data showed combination of cisplatin with apatinib decreased the expressions of p-VEGFR2, compared with the cisplatin alone treatment group (Fig. 5A and B). In addition, as indicated in Fig. 5A, C and D, the

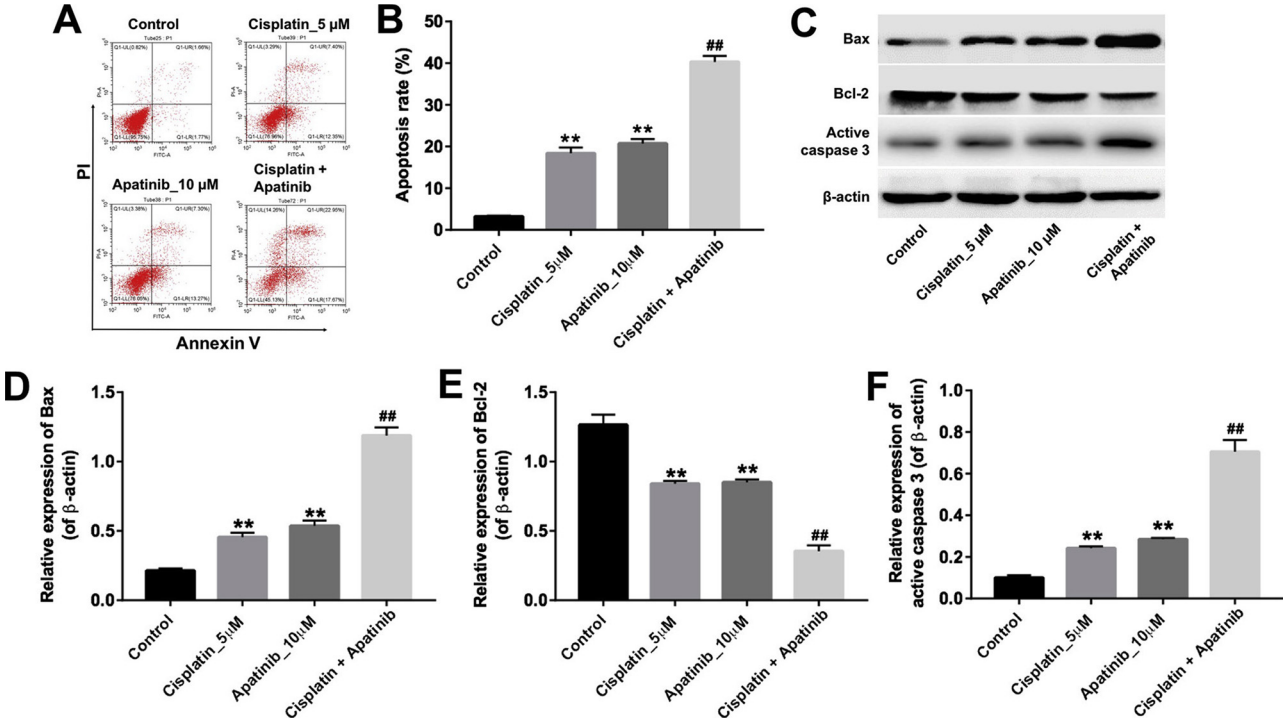

Fig. 3. Apatinib enhanced the pro-apoptotic effect of cisplatin on MDA-MB-231 cells. MDA-MB-231 cells were treated with cisplatin (5 μM) or/and apatinib (10 μM) for 72 h. **(A)** Apoptotic cells were measured with Annexin V and PI double staining. **(B)** The apoptosis cell rates were calculated. **(C)** The expressions of Bax, Bcl-2 and active caspase 3 were analyzed by western blotting in MDA-MB-231 cells. **(D)** The relative expression of Bax was quantified. **(E)** The relative expression of Bcl-2 was quantified. **(F)** The relative expression of active caspase 3 was quantified. **P < 0.01, compared with control group; ##P < 0.01, compared with 5 μM cisplatin treatment group.

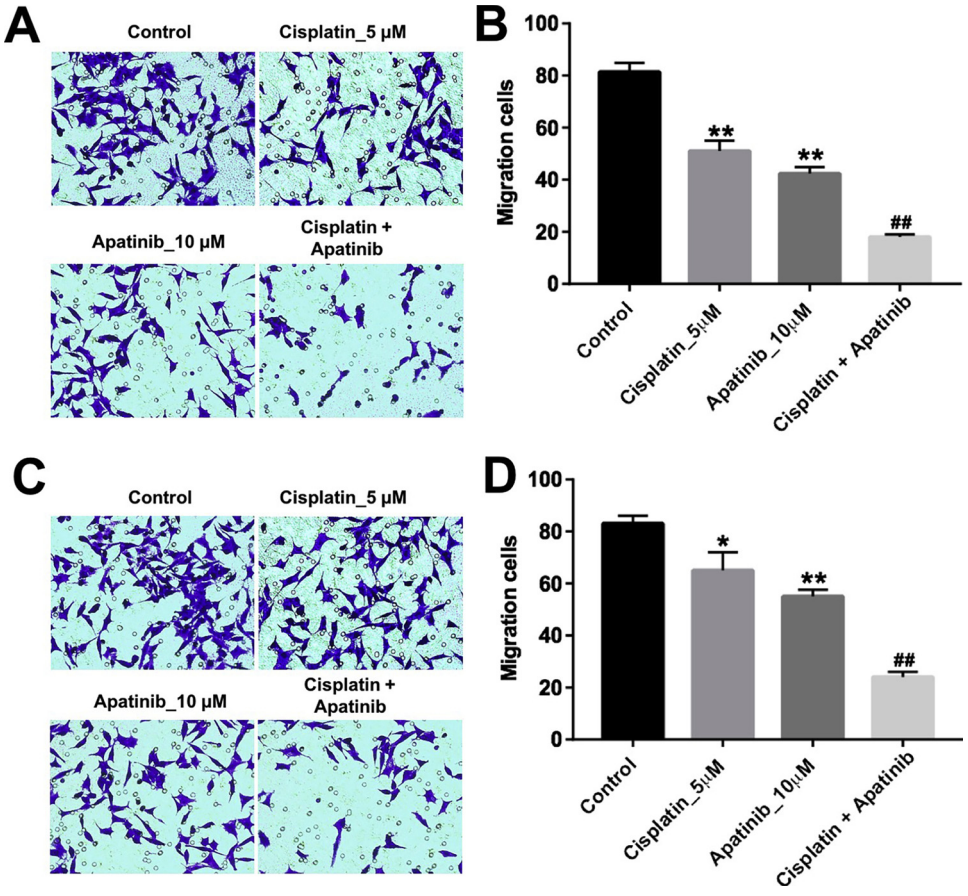

Fig. 4. Apatinib enhanced the inhibitory effects of cisplatin on the migration and invasion of MDA-MB-231 cells. MDA-MB-231 cells were treated with cisplatin (5 μM) or/and apatinib (10 μM) for 72 h. **(A)** Transwell migration assay was performed to determine the migratory capacity of MDA-MB-231 cells. **(B)** The migratory cells in each group were quantified. **(C)** Transwell invasion assay was performed to determine the invasive capacity of MDA-MB-231 cells. **(D)** The invasion cells in each group were quantified. *P < 0.05, **P < 0.01, compared with control group; ##P < 0.01, compared with 5 μM cisplatin treatment group.

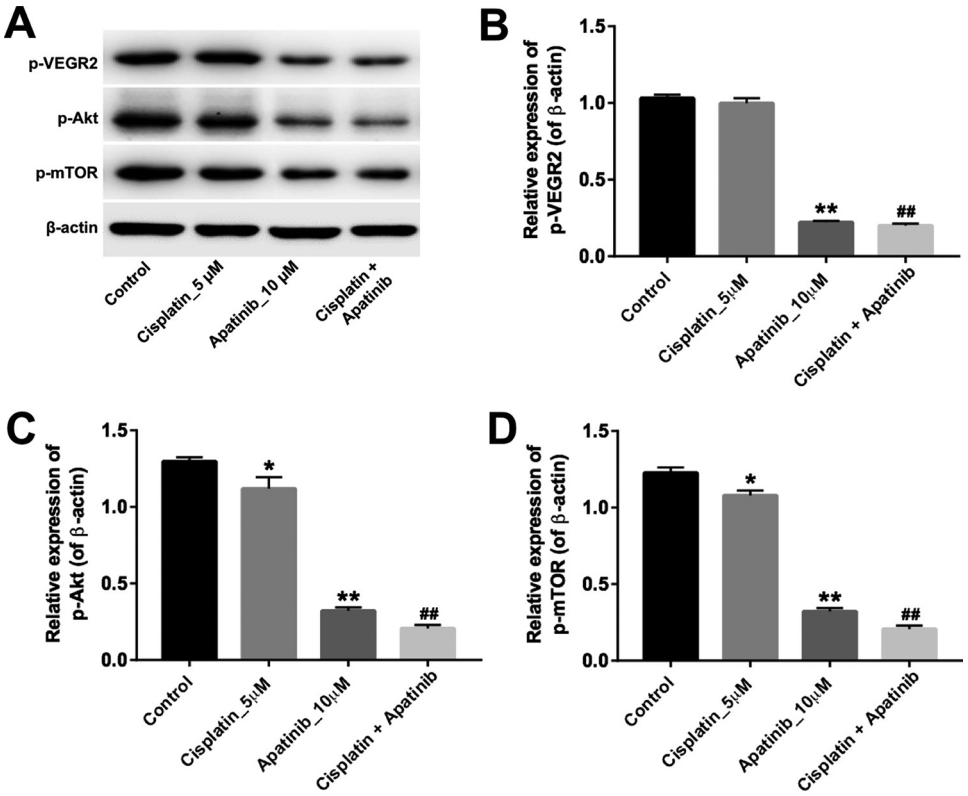

Fig. 5. Apatinib enhanced the anti-tumor effect of cisplatin on MDA-MB-231 cells via inhibiting VEGFR2-Akt-mTOR pathway. MDA-MB-231 cells were treated with cisplatin (5 μM) or/and apatinib (10 μM) for 72 h. **(A)** The expressions of p-VEGFR2, p-Akt and p-mTOR in MDA-MB-231 cells were detected by western blotting. **(B)** The relative expression of p-VEGFR2 was quantified. **(C)** The relative expression of p-Akt was quantified. **(D)** The relative expression of p-mTOR was quantified. *P < 0.05, **P < 0.01, compared with control group; ##P < 0.01, compared with 5 μM cisplatin treatment group.

expressions of p-Akt and p-mTOR were significantly decreased by cisplatin, which were further reduced in the presence of apatinib. These data suggested that apatinib enhanced the anti-tumor effect of cisplatin on MDA-MB-231 cells via inhibiting VEGFR2-Akt-mTOR pathway.

4. Discussion

Many studies have suggested that migration, invasion and metastasis are considered as the primary causes of cancer-related mortality [18]. TNBC always exhibits a high malignancy, which immensely lead to patient morbidity and mortality [19]. Angiogenesis is important for progression of malignancies; vascular endothelial growth factor (VEGF) is a main mediator of blood vessel biology [20]. VEGF plays its pro-angiogenic effects through combining and activating VEGFR2 [21]. In this study, we found that the expression of VEGFR2 was higher in patients with TNBC. According to the results of clinicopathological characteristics, the high VEGFR-2 expression in 60 cases of patients with TNBC was 71.67% (43/60). In addition, the study of the association between VEGFR-2 and the clinicopathological factors of TNBC showed that the expression of VEGFR-2 was not related to the age of the patients. However, it is related to tumor size, nuclear grade, LN metastasis, TNM stage and Ki-67. These data showed that VEGFR-2 is a biomarker for TNBC.

Previous studies indicated that apatinib could inhibit metastasis and tumorigenesis in patients with TNBC [3,22,23]. In this study, apatinib enhanced the pro-apoptotic, anti-migratory and anti-invasive effects of cisplatin on MDA-MB-231 cells. In addition, apatinib is an anti-angiogenic drug and a small-molecule inhibitor of VEGFR2, which could markedly inhibit VEGF signaling pathway [24,25]. Moreover, apatinib induced cell apoptosis and inhibited cell proliferation via blocking VEGF pathway [26,27]. Prior study also demonstrated that apatinib induce apoptosis in intrahepatic cholangiocarcinoma via inhibiting VEGF signaling [27]. Thus, we hypothesized that apatinib enhance the pro-apoptotic, anti-migratory and anti-invasive effects of cisplatin in TNBC through VEGFR2 signaling. Our results indicated that cisplatin has no effect on the expression of p-VEGFR2, while combination cisplatin with apatinib significantly decreased the expressions of p-VEGFR2. Therefore, we supposed that apatinib could enhance the anti-tumor effect of cisplatin via VEGFR2. Whether other factors are involved in this process needs to be clarified.

Angiogenesis and PI3K/Akt/mTOR pathway are the major molecular targets for the treatment of breast cancer [28]. Hong et al illustrated that VEGF activated VEGFR2 and initiated a PI3K-AKT-mTOR pathway, which exhibited an anti-apoptotic effect [27]. Inhibition of angiogenesis through the blocking of the VEGFR2-Akt-mTOR signaling pathway has exerted as a potential method in anti-tumor therapy [29,30]. In this study, we also found that cisplatin decreased the expressions of p-Akt and p-mTOR, while the levels of these proteins were further reduced in the presence of apatinib. Therefore, the possible mechanism was that apatinib decreased the VEGF-mediated Akt/mTOR signaling activity. Thus, we infer that intracellular VEGFR2 inhibitors, apatinib, could enhance the anti-tumor effect of cisplatin via VEGFR2-Akt-mTOR signaling pathway.

5. Conclusion

In conclusion, apatinib enhanced the anti-tumor effects of cisplatin on MDA-MB-231 cells via inhibition of VEGFR2. The results suggested that the combination of apatinib with cisplatin may serve as a potential method in the treatment of patients with TNBC.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The present study was supported by a grant from Clinical research of general medicine of China (grant no. 2016QK013). The authors thank Dr liuhao (Bengbu Medical College) for his helpful discussions.

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