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ORIGINAL ARTICLE

Apatinib suppresses the proliferation and apoptosis of gastric cancer cells *via* the PI3K/Akt signaling pathway

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Summary

Purpose: *To observe the mechanism of the effects of Apatinib on the proliferation and apoptosis of human gastric cancer (HGC-27) cells via the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway through in vitro cytology experiments.*

Methods: *The human gastric cancer HGC-27 cell line was taken as the research object, and LY294002, an inhibitor of the PI3K/Akt signaling pathway, as the positive control. The experimental methods were as follows: (1) The proliferation of HGC-27 cells inhibited by Apatinib and LY294002 were observed by MTT assay; (2) flow cytometry was performed to detect the apoptosis of cells after they were treated with drugs and the positive control; (3) different effects of varying concentrations of Apatinib on apoptosis-related genes and proteins, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and cysteine-aspartic acid protease (Caspase) 9, were detected via fluorescence quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting, and the effects of different concentrations of Apatinib on the protein expressions of PI3K, phosphorylated (p)-PI3K, Akt and p-Akt were detected by Western blotting.*

Results: *(1) MTT results showed that Apatinib could effectively inhibit the proliferation of HGC-27 cells in a dose-dependent manner. (2) Flow cytometry results showed that Apatinib could induce the apoptosis of HGC-27 cells. (3) The results of qRT-PCR and Western blotting demonstrated that Apatinib was capable of inducing the expression of the pro-apoptotic genes, Bax and Caspase 9, and inhibit the expression of the anti-apoptotic gene Bcl-2. The final results of Western blotting confirmed that Apatinib could decrease the protein expression levels of p-PI3K and p-Akt, thus inhibiting the phosphorylation of the PI3K/Akt pathway.*

Conclusions: *This study proves that Apatinib can effectively suppress the proliferation and induce the apoptosis of human gastric cancer HGC-27 cells, the mechanism of which is related to the inhibition of phosphorylation of the PI3K/Akt signaling pathway.*

Key words: *HGC-27 cell, PI3K/Akt signaling pathway, apatinib, LY294002, gastric cancer*

Introduction

Gastric cancer is one of the most common malignant tumors of the digestive system. There are more than 900,000 new cases every year, and the cancer was in the middle and advanced stage when found in 90% of the patients. It frequently occurs in Southeast Asia, and its mortality rate is second only to those of liver cancer and lung cancer [1]. According to the 2015 Annual Report of China's Cancer Registration published by the National Cancer Registration Center, gastric cancer is a se-

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rious threat to the life and health of people in the whole nation [2]. The formation of gastric cancer is a complicated process, and its occurrence and development involve interaction among various signal pathways, but the specific pathogenesis is still unclear. The literature has pointed out that the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway can affect the proliferation and apoptosis of gastric cancer [3,4].

PI3K/Akt is one of the important signaling pathways in cells. When activated abnormally, it can activate downstream signaling molecules, thus further affecting the occurrence and development of various malignant tumors, such as gastric cancer and lung cancer [5-7]. The literature has indicated that gastric cancer development is related to excessive cell proliferation and apoptosis inhibition, and that the PI3K/Akt signaling pathway can prevent cells from initiating programmed death [8]. It has also been pointed out that LY294002, as an inhibitor of the PI3K/Akt signaling pathway, can induce apoptosis and inhibit the proliferation of gastric cancer, colon cancer and other cancer cells [9-12]. Apatinib is a new oral targeted drug, which blocks the signal transduction pathway after the vascular endothelial growth factor (VEGF), binds to its corresponding receptors by inhibiting the activity of the VEGF receptor-2 (VEGFR-2) tyrosine kinase, thus effectively suppressing the formation of tumor blood vessels and playing an anti-tumor role [13-15].

In this study, the inhibitory effect of Apatinib on the proliferation and apoptosis of gastric cancer cells was observed through *in vitro* experiments, so as to further explore the molecular mechanism of action of Apatinib on gastric cancer and its signaling pathway, thus providing a theoretical basis for the research on it in the treatment of gastric cancer and potent theoretical support for later drug development.

Methods

Materials

The human gastric cancer HGC-27 cell line (Shanghai Fusheng Industrial Co., Ltd., Shanghai, China), Apatinib (Jiangsu Hengrui Pharm Co., Ltd., Nanjing, China), LY294002 (PI3K Inhibitor) (Sigma, St. Louis, MO, USA), the bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China), 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-diphenytetrazoliumro-mide (MTT) (Sigma, St. Louis, MO, USA), the Annexin V-fluorescein isothiocyante (FITC)/propidium iodide (PI) apoptosis detection kit (Jiangsu KeyGEN BioTECH Co., Ltd., Nanjing, China), Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Rockville, MD, USA), and rabbit anti-human primary antibodies [PI3K, phosphorylated (p)-PI3K, Akt, p-Akt, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), cleaved-cysteine-aspartic acid protease (Caspase) 9] and rabbit anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibodies (Beijing Transgen Biotech Co., Ltd., Beijing, China).

Cell culture

HGC-27 cells were cultured using RPMI-1640 medium with 10% fetal bovine serum (FBS) in an environment with 5% CO_2 at 37°C. The state of the cells was observed every day, and when the cells grew to 70-80% confluence, passage began. After the RMPI-1640 with 10% FBS was discarded, the cells were washed with phosphate-buffered saline (PBS) for 3 times, added with 0.25% trypsin until the bottom of the plate could be covered, and observed under a microscope. When the cells became round, the RPMI-1640 with 10% FBS culture solution was added to stop trypsin. Then the cells were blown and beaten repeatedly, mixed evenly, and inoculated into a new culture plate at a ratio of 1:4 for subculture. Subsequently, the cells in the logarithmic growth phase were taken for further experiments.

Detection of cell proliferation via MTT assay

Gastric cancer HGC-27 cells in the logarithmic growth phase were taken, digested with 0.25% trypsin, blown into cell suspension, mixed and counted. The cell suspension was then diluted to 2×10^4 cells/mL with the culture solution, and the cells were inoculated into a 96-well plate at a dose of 100 μL/well. Defining one as blank control, the wells around it were added with PBS at a dose of 200 μL/well, and were then placed in a cell incubator for standing. After the cells fully adhered to the wall, the prepared Apatinib at different concentrations (0, 0.5, 1.0, 1.5 and 2.0 μmol/L, respectively) was added to the test wells at 100 μL/well, and LY294002 at different concentrations (0.312, 0.625, 1.25, 2.5 and 5.0 μg/mL, respectively) was added in the positive control group, with 6 repeated wells each. The remaining test wells were used as the negative control group and added with RPMI-1640 at a dose of 100 μL/well. After standing for 48 h, 20 μL MTT was added to each well for further culture. After 4-h culture, the supernatant was discarded, 100 μL dimethyl sulfoxide (DMSO) was added to each well for reaction for 10 min, and the optical density (OD) value at the wavelength of 490 nm was read using a microplate reader. The experiments were repeated for three times. According to the experimental results, the inhibition rate was calculated as follows: Inhibition rate = $1-[(\text{average OD value}_{\text{experimental group}} - \text{OD value}_{\text{blank well}})/(\text{average OD value}_{\text{negative control group}} - \text{OD value}_{\text{blank well}})\times100\%]$.

Detection of the apoptosis rate of HGC-27 cells via flow cytometry

Gastric cancer HGC-27 cells in the logarithmic growth phase were taken, digested with 0.25% trypsin, blown into cell suspension, mixed and counted. The cell suspension was then diluted to 2×10^4 cells/mL with the culture solution, and then the cells were inoculated into

a 6-well cell culture plate at a dose of 100 µL/well and let stand in a cell incubator. After the cells fully adhered to the wall, 0.3 mL Apatinib at different concentrations (0.5, 1.0, 1.5 and 2.0 µmol/L) was added to each well, 0.3 mL RPMI-1640 was added to the negative control group, and 5 µg/mL LY294002 was added to the positive control group. After standing for 48 h, the supernatant in each well was collected into a centrifuge tube, and the cells were digested with 0.25% trypsin and collected into the corresponding centrifuge tube for centrifugation at 1,000 rpm for 5 min. After that, the supernatant was discarded, and the cells were washed with PBS 3 times. Then, 500 µL binding buffer containing 10 µL Annexin V-FITC (fluorescein isothiocyanate) was added to the precipitates of each cell, and the cells were resuspended evenly. Subsequently, the cells were added with 5 µL propidium iodide (PI) and placed in the dark for 15 min. Finally, the apoptosis rate of the cells was detected by flow cytometry.

Detection of the messenger ribonucleic acid (mRNA) expression in HGC-27 cells via qRT-PCR

The cultured HGC-27 cells were inoculated into a 6-well plate, and were divided into 4 groups, namely, blank control group (control group) and Apatinib treatment group (the concentrations were 0.5, 1.0, 1.5, and 2.0 µmol/L, respectively). After treatment with Apatinib for 48 h, the cells were collected. According to the instructions of TRIzol kit (Invitrogen, Carlsbad, CA, USA), the total RNA in each group of cells was extracted, whose concentration and purity needed to be detected, and the results were qualified when the absorbance $(A)_{260}/A_{280}$ was 1.8-2.0. Then, reverse transcription (RT) was performed with reference to the operation method provided in the instructions of the kit, and fluorescence real-time quantitative polymerase chain reaction (qPCR) detection kit was applied to detect the mRNA expression with each complementary deoxyribonucleic acid (cDNA) obtained by RT as a template. The detailed sequences of PCR primers used are shown in Table 1. PCR conditions: a total of 1 cycle of reaction at 94°C for 5 min, 5 cycles of reaction at 94°C for 30 s, 57°C for 30 s and 72°C for 30 s, and 1 cycle of reaction at 72°C for 5 min. After PCR amplification, the Ct value was read according to the amplification curve, and the relative level of mRNAs was calculated with GAPDH as the control gene according to the following formula: ΔCt (target gene) = Ct (target gene) - Ct (control gene), and ΔΔCt =ΔCt (target gene) -ΔCt (standard value). The relative expression level of the target gene was $2^{-\Delta\Delta Ct}$.

Detection of the protein expression in HGC-27 cells via Western blotting

Gastric cancer HGC-27 cells in the logarithmic growth phase were digested with 0.25% trypsin, blown into cell suspension, mixed and counted. The cell suspension was then diluted to 2×10^4 cells/mL with the culture solution, and then the cells were inoculated into a 6-well cell culture plate at a concentration of 2.7 mL/well and let stand in a cell incubator. Until the cells fully adhered to the wall, 0.3 mL Apatinib (0.5, 1, 1.5 and 2 µmol/L) was added to each well, and 0.3 mL RPMI-1 640 was added in the negative control group. After 48 h of culture, Western blotting was performed. All the cells were collected into a 1.5 mL Eppendorf (EP) tube, and 300 µL protein lysate and 10 µL protease inhibitors were added, respectively. After that, the cells were placed in ice water for 30 min of incubation, followed by centrifugation at 12,000 g for 15 min, and then the supernatant was collected to obtain the total protein. The BCA protein assay kit was then used to detect the concentration of the total protein extracted. Then, the expression level of the target protein was detected by Western blotting. Methods: 10 µL supernatant was sucked, evenly mixed with the loading buffer, and subjected to dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the membrane was transferred onto the prepared polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), let stand at room temperature and sealed for 1 h. Then, Akt, p-Akt, PI3K, p-PI3K, Bax, Bcl-2, Caspase 9 and GAPDH antibodies (diluted at 1: 1,000) were added to the membrane and placed at 4°C overnight. Subsequently, the membrane was washed with washing buffer 5 times, with 10 min/time, incubated with the HRP-labeled rabbit anti-mouse antibody (diluted at 1: 800) pretreated by shaking at room temperature for 1 h, and washed with buffer for three times. Finally, the color was developed with diaminobenzidine and photographed using the Fluorchem 9900 imaging system.

Table 1. RT-PCR primer sequences

Gene name	*Primer name*	*Primer sequence*
Bcl-2	Forward primer	5'-TGGGATGCCTTTGTGGAAC-3'
	Reverse primer	5'-CATATTTGTTTGGGGCAGGTC-3'
Bax	Forward primer	5'-TGCTACAGGGTTTCATCCAG-3'
	Reverse primer	5'-ATCCACATCAGCAATCATCC-3'
Caspase 9	Forward primer	5'-AGCCAGATGCTGTCCCATAC-3'
	Reverse primer	5'-CAGGAGACAAAACCTGGGAA-3'
GAPDH	Forward primer	5'-GGAAAGCTGTGGCGTGAT-3'
	Reverse primer	5'-AAGGTGGAAGAATGGGAGTT-3'

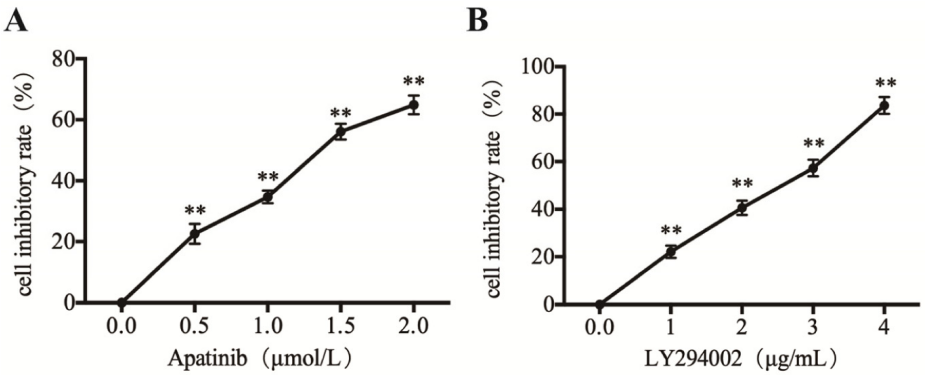

Figure 1. Effect of Apatinib and LY294002 on the inhibition rate on HGC-27 cells detected *via* MTT assay (**p<0.01).

Statistics

The data were statistically analyzed by SPSS 19.0 software (IBM, Armonk, NY, USA), expressed as mean±SD and detected using the t-test value. P<0.05 showed that the difference was significant, and p<0.01 suggested that the difference was extremely significant.

Results

Effect of apatinib on the proliferation of gastric cancer cells detected via MTT assay

MTT assay was used to detect the inhibitory effect of Apatinib on HGC-27 cell proliferation. LY294002 at different gradient concentrations (0, 0.625, 1.25, 2.5 and 5 μg/mL) was taken as the positive control. The results (Figure 1) revealed that Apatinib has an obvious inhibitory effect on gastric cancer HGC-27 cells in a dose-dependent manner (p<0.01). As shown in Figure 1B, LY294002 also inhibited the proliferation of HGC-27 cells in a dose-dependent manner (p<0.01). The above results indicated that Apatinib is similar to LY294002, both of which could significantly inhibit the proliferation of gastric cancer HGC-27 cells.

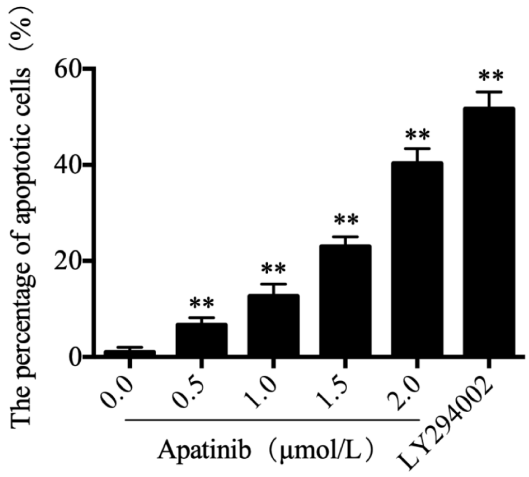

Figure 2. Effect of Apatinib at different concentrations on the apoptosis of HGC-27 cells detected *via* Annexin V-FITC/PI method. Compared with LY294002, Apatinib could also significantly promote the apoptosis of HGC-27 cells (**p<0.01).

Effect of Apatinib on the apoptosis of HGC-27 cells detected via flow cytometry

HGC-27 cells were treated with different concentrations of Apatinib and 5 μg/mL LY294002 for 48 h, and the apoptosis rate was measured by Annexin V-FITC/PI method. It was found that the apoptosis rate of HGC-27 cells was increased with the increase in the concentration of Apatinib (Figure 2), indicating that Apatinib could clearly induce apoptosis of HGC-27 cells in a dose-dependent manner.

Effect of Apatinib on the expression of apoptosis-related genes in HGC-27 cells

The results of qRT-PCR and Western blotting (Figures 3 and 4) manifested that the mRNA and protein expression levels of pro-apoptosis genes, Caspase 9 and Bax, in different-concentration of the Apatinib group were remarkably higher than those in blank control group (p<0.01). However, the mRNA and protein expression levels of the anti-apoptosis gene Bcl-2 was clearly lower than that in the blank control group (p<0.01).

Effect of Apatinib on the PI3K/Akt signaling pathway detected via Western blotting

In order to study whether the effects of Apatinib on the apoptosis and proliferation of HGC-27 cells are related to the PI3K/Akt signaling pathway, Western blotting was performed to detect the expression levels of the related proteins (PI3K, p-PI3K, Akt and p-Akt) in HGC-27 cells treated with different concentrations of Apatinib. According to the results (Figure 5), the expression levels of PI3K and Akt remained almost unchanged, while the expression levels of p-PI3K and p-Akt declined with the increase in the concentration of Apatinib. The

above results suggest that Apatinib could inhibit the expression of the PI3K/Akt signaling pathway by suppressing the phosphorylation of PI3K and Akt proteins while the total protein expression remained unchanged.

Discussion

Gastric cancer is one of the most common malignant tumors of the digestive tract, but there is no effective diagnostic method for early disease. At

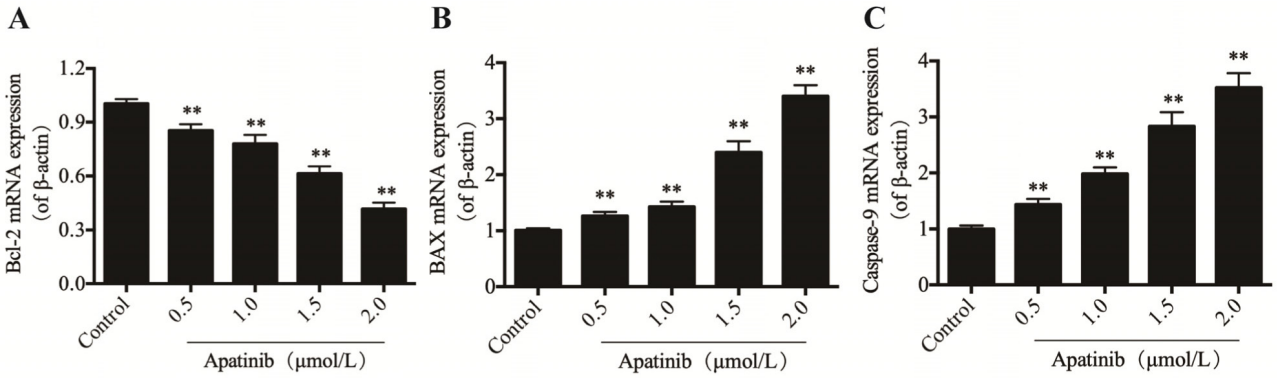

Figure 3. Effect of Apatinib on the mRNA expression of apoptosis-related genes in gastric cancer HGC-27 cells detected *via* qRT-PCR. Compared with those in blank control group, the mRNA expression levels of Bax and Caspase 9 in HGC-27 cells are significantly promoted, while the mRNA expression level of Bcl-2 is inhibited in the Apatinib group (**p<0.01).

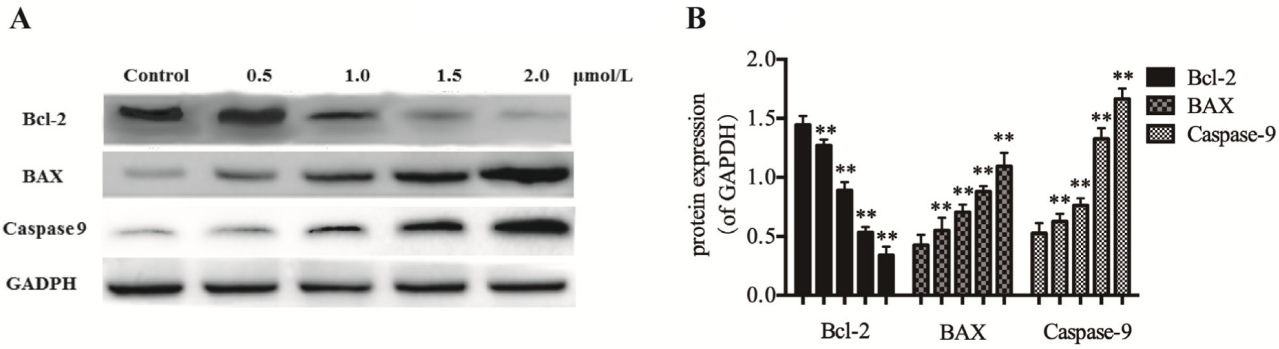

Figure 4. Effect of Apatinib on the expression of apoptosis-related proteins in gastric cancer HGC-27 cells detected *via* Western blotting. (A): Western blotting of the protein expressions of Bax, Caspase 9 and Bcl-2. (B): Gray scale analysis of the protein expressions of Bax, Caspase 9 and Bcl-2. Compared with those in the blank control group, the protein expressions of Bax and Caspase 9 in HGC-27 cells are notably promoted, but the protein expression of Bcl-2 is inhibited in the Apatinib group (**p<0.01).

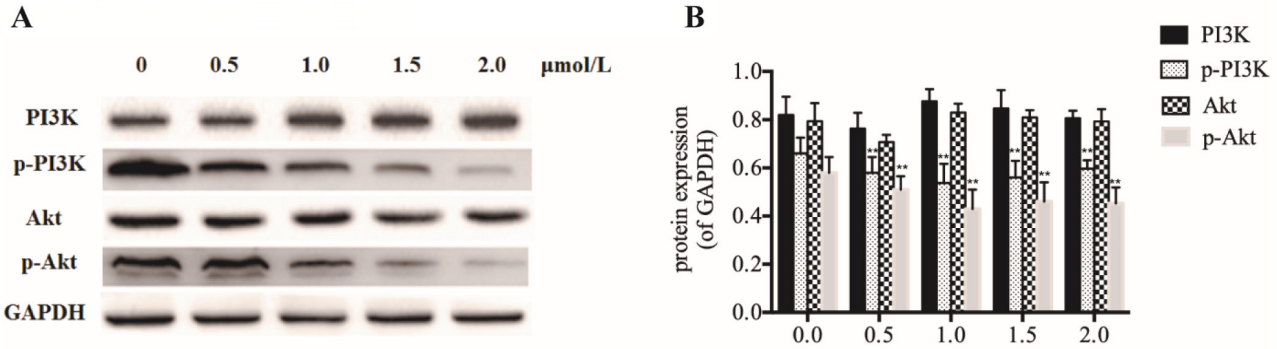

Figure 5. Effects of different concentrations of Apatinib on the expression levels of related proteins (PI3K, p-PI3K, Akt and p-Akt) in HGC-27 cells detected *via* Western blotting. (A): Western blotting of the protein expressions of PI3K, p-PI3K, Akt and p-Akt. (B): Analysis results of the expression levels of related proteins (PI3K, p-PI3K, Akt and p-Akt) in HGC-27 cells treated with different concentrations of Apatinib. With the increase in the concentration of Apatinib, the expression levels of PI3K and Akt remained unchanged, but the expression levels of p-PI3K and p-Akt decreased (**p<0.01).

present, the treatment of gastric cancer is mainly surgical resection and chemotherapy. Surgical resection can effectively remove the tumor, but there are some disadvantages such as large trauma and slow recovery. Moreover, due to lack of targeting therapies, these traditional chemicals will also cause damage to normal tissues and cells, creating more serious toxic side effects. Therefore, studying the causes and development mechanisms of gastric cancer at the molecular level and developing targeted drugs specifically targeting tumor target proteins and signal pathways have become the focus of current research. Some studies have pointed out that Apatinib has a certain inhibitory effect on the development of gastric cancer, non-small cell lung cancer, liver cancer, leukemia and other diseases, but its specific mechanism of action is not yet clear [16-21].

In this study, it was observed in MTT assay and flow cytometry that Apatinib significantly inhibited the growth of the gastric cancer HGC-27 cell line *in vitro* and induced cell apoptosis, and was also shown to affect the inhibition rate and apoptosis rate in a dose-dependent manner. The proliferation and growth of tumors depend on the regulation and activation of many signaling pathways, among which the growth and proliferation of some tumor cells are closely related to many signaling pathways associated with protein tyrosine kinases. Among them, the PI3K/Akt signaling pathway is the main downstream signaling pathway for protein tyrosine kinase activation, which will promote the proliferation and migration of tumor cells. The related research [17-20] has indicated that the PI3K/Akt signaling pathway is correlated with the occurrence, development, invasion and metastasis of many kinds of tumors, and has guiding effects on the grade of malignancy and prognosis of malignant tumors. In this study, the PI3K/Akt signaling pathway, demonstrated that the expression levels of p-PI3K and p-Akt decreased in a dose-dependent manner. The above results indicate that the phosphorylation levels of PI3K and Akt proteins will decrease with the increase in the concentration of Apatinib, which verifies that Apatinib inhibits the activity of the PI3K/Akt signaling pathway by blocking the phosphorylation of PI3K and Akt proteins.

Conclusions

This study found that Apatinib could effectively inhibit the proliferation of gastric cancer HGC-27 cells *in vitro*, induce their apoptosis, and suppress the activation of the PI3K/Akt signaling pathway-related proteins, p-PI3K and p-Akt. Therefore, this study provides a research basis for molecular targeted therapy of gastric cancer and also a solid theoretical foundation for the combined therapy with Apatinib and other drugs.

Conflict of interests

The authors declare no conflict of interests.

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