Research Article

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Folate-targeted PTEN/AKT/P53 signaling pathway promotes apoptosis in breast cancer cells

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Abstract: Objective Folate deficiency is closely related to the occurrence of human tumors and plays an important role in cell growth, differentiation, repair, and host defense. We studied the effects of folic acid on the apoptosis of breast cancer cells (MDA-MB-231) and on the activity of the PTEN/AKT/P53 signaling pathway in breast cancer cells.

Methods Breast cancer cells (MDA-MB-231) were treated with folate alone or in combination with a PTEN specific inhibitor, SF1670. Cell viability was detected by a MTT assay, and the expression levels of apoptosis-related proteins and PTEN/AKT/P53 signaling pathway were detected via Western blot analysis. Rate of apoptosis was measured via cytometry.

Results Folic acid inhibited the cell viability of MDA-MB-231 cells and the expressions of Bcl-2 and p-AKT proteins and upregulate the expression of Bax, PTEN, and P53 proteins, thereby inducing apoptosis in these cells. SF1670 treatment inhibited the expressions of Bcl-2 and p-AKT protein and upregulate Bax, PTEN, and P53 protein expression.

Conclusion Folic acid has cytotoxic effects on MDA-MB-231 cells and can induce apoptosis by targeting the PTEN/AKT/P53 signaling pathway.

Keywords: folic acid; breast cancer cell; apoptosis; PTEN; AKT/P53 signaling pathway.

Breast cancer is a malignant tumor that seriously threatens the physical and mental health of women around the world. It occurs in the mammary gland epithelial tissue,

which cells easily metastasize to vital organs, such as the lungs, bones, and brain, thus directly threatening the lives of patients [1-3]. According to statistics from the National Cancer Registry, 4.292 million new cases of breast cancer were recorded in China in 2015, ranking first in the incidence of female malignancies, which account for approximately 15% of new cases of tumors throughout the year [4, 5]. Clinically, with the introduction of new chemotherapy drugs and the emergence of new chemotherapy regimens, the five-year survival rate of patients with breast cancer has increased by approximately 86%. However, the acquisition of acquired drug resistance is an important factor that leads to poor prognosis and even death in patients with breast cancer during chemotherapy. Therefore, new therapeutic drugs and targets need to be studied [6].

Folic acid, also known as vitamin B9, is a water-soluble vitamin. The human body cannot synthesize folic acid, which must be obtained from the diet (vegetables and fruits). Folic acid deficiency develops if vegetables and fruits are not adequately ingested. Folate deficiency may be involved in the development of human tumors. Folic acid plays an important role in cell growth, differentiation, repair and host defense. As well as being essential for the synthesis of purines and pyrimidines and the conversion of deoxyuridine (dUMP) to deoxythymidylate (dTTP) and DNA methylation [7, 8]. A lack of folic acid leads to a paradigm shift in DNA methylation, DNA and even chromosome breaks; folate deficiency has a mutagenic effect and is closely related to tumorigenesis [9, 10]. A study including 367,993 women aged between 35-70 found that a folic acid supplement could reduce the risk of breast cancer in premenopausal women with estrogen receptor- and progesterone receptor-negative (ER-, PR-) tumors. The risk of breast cancer is 14% lower in people who are accustomed to low-folate diets [11].

In vivo, phosphatase and tensin homolog (PTEN) can trigger a series of cascades by antagonizing the activity of phosphorylases, such as tyrosine kinase, and affecting cell survival, proliferation, and metabolism, thereby inhibiting tumor development [12-15]. The PTEN deleted on chromosome 10 is located at chromosome 10 q23.3 and

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consists of nine exons, which encodes a protein consisting of 403 amino acids with phosphatase activity; it is a tumor suppressor that is needed for maintaining normal cell survival [16-18]. As a tumor suppressor gene, PTEN plays a key role in the regulation of the tyrosine kinase receptor (RTK)/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB, also known as Akt) signaling pathway. The PI3K/Akt signaling pathway is over-activated in many types of human cancers, and the targeted regulatory factors of this pathway have enormous potential value. Cytoplasmic PTEN may be involved in the regulation of PI3K/Akt signaling pathway activation and inhibition of tumorigenesis. In 2019, studies have reported that the PTEN/Akt/P53 signaling pathway plays a key regulatory role in the proliferation of breast cancer cells [19, 20]. In this study MDA-MB-231 cells were treated with folic acid to analyze the inhibitory effect of folic acid on PTEN/AKT/P53 signaling pathway in breast cancer cell apoptosis.

Materials and methods

Main reagents and instruments

DMEM medium (Corning, USA); fetal bovine serum, double antibody, trypsin, DPBS (Gibco, USA); SF1670 (Abcam, USA); total protein extraction kit, protein concentration determination kit, ECL luminescence kit (Jiangsu Kaiji Biotechnology Co., Ltd.); BCL-2, BAX, PTEN, p-AKT, AKT, P53, β -actin primary antibody, secondary antibody against rabbit, secondary antibody against mouse (American R&D Systems); Death kit, MTT cell proliferation and cytotoxicity kit, cell cycle and apoptosis detection kit (Shanghai Biyuntian); CO₂ constant temperature incubator, real-time PCR instrument, microplate reader (American Thermo); and protein Electrophoresis instrument, film transfer instrument (Bio-Rad, USA).

Cell culture

Human breast cancer cells (MDA-MB-231 cells) were cultured in DMEM medium containing 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin and cultured at 37 °C in an incubator with 5% CO₂.

Cell processing

For the folic acid concentration gradient treatment, MDA-MB-231 cells were treated with folic acid at concentrations of 0, 1, 5, 10, and 20 μ M for 24 h.

For the PTEN inhibitor (SF1670) combined with folic acid treatment, MDA-MB-231 cells were uniformly seeded into a six-well plate, cultured for 8–12 h overnight, pre-treated with 1 μ M SF1670 for 2 h, and supplemented with 20 μ M folic acid in the culture solution for 24 h.

Flow cytometry detection

MDA-MB-231 cells in logarithmic growth phase were uniformly seeded into six-well plates. After cell confluence to 80%, the cells were treated with 0, 1, 5, 10, and 20 μ M folic acid for 24 h. Following the instructions, Annexin V and PI were added to the solution, mixed, and incubated for 15 min at room temperature in the dark. Flow cytometry was used to detect the rate of , and the percentage of apoptosis was analyzed using the Flowjo software.

Western Blot test

The treated cells were washed with pre-cooled PBS, and the processed samples were lysed using the protein collection system. Equal amounts of proteins were separated via 12% SDS-PAGE and then transferred to the PVDF membrane by using the wet transfer method. A 5% skim milk powder was used for blocking at room temperature for 1 h. The primary antibody was incubated at 4 °C overnight. TBST was used for cleaning every 10 min in three parallel treatments. The secondary antibody was incubated for 1 h at room temperature, washed thrice with TBST, and then developed.

Cell proliferation assay

MDA-MB-231 cells in logarithmic growth phase were inoculated into 96-well plates at 2×10^4 per well. The final concentrations of folic acid were 0, 1, 5, 10, and 20 μ M. Three replicate measurements were obtained. Subsequent proliferation assays were performed according to the MTT instructions.

Figure 1: Folic Acid treatment can inhibit the proliferation of MDA-MB-231 cells and the expression level of Bcl-2 and up-regulate the expression level of Bax. A)MDA-MB-231 cells were treated with folic acid at different concentrations (0 μM, 1 μM, 5 μM, 10 μM, 20 μM) for 24 h, and cell viability was measured by MTT (P<0.05). B) After 24 h of folic acid treatment, the protein expression levels of Bcl-2 and Bax in MDA-MB-231 cells were detected by Western blot, and β-Actin was used as an internal control.

Table 1: The apoptosis rate of cells increased with the increase of folic acid concentration.

Group	N	Apoptotic rate
0 μM	3	2.36±0.1075
1 μM	3	11.56±0.397*
5 μM	3	20.99±1.444**
10 μM	3	30.67±2.032**
20 μM	3	37.98±1.563**

Statistical analysis

Data analysis was performed using the GraphPad Prism 5.0 software, and multiple statistical analysis was performed using two-tailed unpaired t-test and Tukey post-test. P < 0.05 was considered significant.

Results and analysis

Effects of folic acid treatment on the activity and apoptosis of MDA-MB-231 breast cancer cell

MDA-MB-231 breast cancer cells were treated with 0, 1, 5, 10, and 20 μM folic acid for 24 h. The results of MTT assay showed that folic acid had an inhibitory effect on the cell viability of MDA-MB-231 cells, and the inhibitory effect increased with increasing folic acid concentration (P<0.05) (Fig.1 A). The expression levels of apoptosis-related proteins BCL-2 and BAX were detected via Western blot analysis. The expression of BCL-2 protein in MDA-MB-231 cells decreased with increasing folic acid concentration, whereas the expression level of Bax protein increased in a concentration gradient (Fig.1 B-D). The rate of apoptosis was detected via flow cytometry. The rate of apoptosis of cells increased with increasing folic acid concentration (Table 1).

Effect of folic acid treatment on PTEN/Akt/ P53 signaling pathway in breast cancer cells

MDA-MB-231 breast cancer cells were treated with 0, 1, 5, 10, and 20 μM folic acid for 24 h. Western blot analysis results show that folic acid can significantly upregulate the PTEN and P53 in MDA-MB-231 breast cancer cells and inhibit the phosphorylation level of AKT (Fig. 2 A-D).

Figure 2: Folic Acid up-regulates the expression level of PTEN and P53, and inhibits the expression level of p-AKT in MDA-MB-231 cells. After treatment with MDA-MB-231 breast cancer cells at different concentrations (0 μM, 2.5 μM, 5 μM, 10 μM, 20 μM) for 24 h, the expression levels of PTEN, p-AKT, AKT and P53 protein were detected by Western blot, and β-Actin was used as internal reference control.

Effect of folic acid combined with PTEN inhibitor (SF1670) on the apoptosis of breast cancer cells

To confirm the role of PTEN in folate-induced apoptosis of MDA-MB-231 breast cancer cells, we pretreated MDA-MB-231 cells with 1 μM PTEN inhibitor (SF1670) for 2 h, followed by 20 μM folic acid for 24 h. The expression of apoptosis-related protein BCL-2 and BAX was detected via Western analysis. The pretreatment of PTEN inhibitor (SF1670) reversed the inhibitory effect of folic acid on BCL-2 protein expression in MDA-MB-231 cells and the upregulation effect of BAX protein expression levels. Hence, the downregulation of PTEN expression significantly inhibited folate-induced apoptosis in breast cancer cells MDA-MB-231 (Fig. 3 A-C).

Effects of folic acid combined with PTEN inhibitor (SF1670) on PTEN/Akt/P53 signaling pathway in breast cancer cells

Over-activation of the PI3K/Akt signaling pathway is widespread in human cancer types, and is regulated by cytoplasmic. To confirm the role of PTEN/Akt/P53 in folate-induced apoptosis of breast cancer cells, MDA-MB-231 cells were pretreated with 1 μM PTEN inhibitor (SF1670) for 2 h. Folic acid was added to the culture medium for 24 h. The expression levels of PTEN, p-AKT, AKT, and P53 were detected via Western blot analysis. The PTEN inhibitor (SF1670) reversed the upregulation of PTEN and P53 in MDA-MB-231 cells and the downregulation of p-AKT expression levels by folic acid. Hence, the PTEN/Akt/P53 signaling pathway is involved in folate-induced apoptosis in breast cancer cells (Fig. 4 A-D).

Discussion

Breast cancer is the “first killer” that has threatened the health of women around the world. In recent years, it has occurred in young people and is the number one cause of death in women in Western developed countries [21]. The cause of breast cancer is very complicated. The abnormal expression of oncogenes or tumor suppressor genes is closely related to the occurrence and development of breast cancer. Therefore, the search for new and effective molecular markers or targets is essential for the diagnosis and treatment of breast cancer [22]. Folic acid, also known as pteroyl glutamic acid, consists of three parts,

Figure 3: SF1670 inhibits the up-regulated expression of Bax and down-regulated expression of Bcl-2 by folic acid in MDA-MB-231 cells. After pre-treatment of MDA-MB-231 cells with 1 μM SF1670 for 2 h, the cells were supplemented with 20 μM folic acid for 24 h, and the protein expression levels of Bcl-2 and Bax were detected by Western blot. β-Actin was used as an internal control.

namely, acridine nucleus, *p*-aminobenzoic acid, and glutamic acid. Mammals can only absorb exogenous folic acid through the gut and cannot synthesize folic acid by themselves [23]. Epidemiological investigations have shown that the lack of folic acid can lead to the occurrence of human tumors, such as breast, ovarian, colorectal, and pancreatic cancer. [24-29].

In 1972, Kerr and Wyllie proposed the concept of apoptosis, which is an active process involving the activation, expression, and regulation of a range of genes. Apoptosis is not a phenomenon of autologous injury under pathological conditions, but a death process that is actively sought for enhanced adaptation to the living environment; hence, it is also called programmed cell death (PCD)

Figure 4: SF1670 inhibits the up-regulated expression of PTEN and P53, and down-regulated expression of p-AKT by folic acid in MDA-MB-231 cells. After pretreatment of MDA-MB-231 cells with 1 μM SF1670 for 2 h, 20 μM folic acid was added to the culture medium for 24 h. The protein expression levels of PTEN, p-AKT, AKT and P53 were detected by Western blot, and β-Actin was used as an internal control.

[30]. However, the deregulation of the apoptotic process may be directly or indirectly related to the occurrence of many diseases, such as tumors and autoimmune diseases [31, 32]. Cancer treatments rely on a variety of cytotoxic radiotherapy and chemotherapy, which have a certain therapeutic effect on many hematological malignancies and some solid tumors. Chemotherapy drugs can induce apoptosis in vitro and in vivo. We showed that folic acid can inhibit the activity of breast cancer cell MDA-MB-231 and the expression of Bcl-2 and Bax, thereby promoting the apoptosis of MDA-MB-231 cells.

PTEN was discovered in 1997 on the chromosome 10 region, where human late tumor-associated gene loss is often found. The subcellular localization of PTEN is crucial for its function in normal cells and enable its anti-cancer effect. The inhibitory effect of PTEN on tumors is achieved by inhibiting PI3K activity. The PI3K effector, AKT, is activated when PTEN is mutated and inactivated, which leads to further tumorigenesis and tumor development [33-36]. The PTEN/AKT signaling pathway plays an important role in cell proliferation, growth, apoptosis, cell cycle regulation, and angiogenesis [37, 38]. The PI3K/Akt signaling pathway directly promotes the expression of Bcl-2 and inhibits the expression of the apoptotic protein Bax, thereby inhibiting apoptosis [39-41]. Weijun Wan *et al.* have shown that liver X receptor agonist-induced up-regulation of lncRNA can target PTEN/AKT/P53 signaling pathway and inhibits breast cancer cell proliferation. The expression levels of PTEN and P53 in MDA-MB-231 cells increased with increasing folic acid concentration, whereas the phosphorylation level of AKT protein decreased. The results of this experiment suggest that folic acid may promote the expression of Bcl-2 and inhibit the expression of Bax through PTEN/AKT/P53 signaling pathway, thereby promoting the apoptosis of MDA-MB-231 cells.

In conclusion, folic acid can inhibit the cell viability of breast cancer cell MDA-MB-231, induce apoptosis, decrease Bcl-2 expression, and promote Bax expression by targeting the PTEN/AKT/P53 signaling pathway. These results provide experimental evidence for the application of folic acid as an antitumor drug in the treatment of breast cancer cells.

Conflict of interest: Authors state no conflict of interest.

Data Availability Statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

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