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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 upregulated 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 upregulated 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.
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); CO\textsubscript{2}, constant temperature incubator, real-time PCR instrument, microplate reader (American Thermo); and protein Electrophoresis instrument, film transfer instrument (Bio-Rad, USA).

**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 μM 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 μM SF1670 for 2 h, and supplemented with 20 μM 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 μM 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\textsuperscript{4} per well. The final concentrations of folic acid were 0, 1, 5, 10, and 20 μM. Three replicate measurements were obtained. Subsequent proliferation assays were performed according to the MTT instructions.
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).

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**  |

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).
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,
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 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 \( \mu \)M SF1670 for 2 h, the cells were supplemented with 20 \( \mu \)M folic acid for 24 h, and the protein expression levels of Bcl-2 and Bax were detected by Western blot. \( \beta \)-Actin was used as an internal control.

**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 \( \mu \)M SF1670 for 2 h, 20 \( \mu \)M 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 \( \beta \)-Actin was used as an internal control.
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