Eupafolin inhibits breast cancer cell proliferation and induces apoptosis by inhibiting the PI3K/Akt/mTOR pathway

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Abstract. Eupafolin is a flavonoid extracted from common sage. Previous studies have reported that Eupafolin has antioxidant, anti-inflammatory, and anti-tumor effects. However, its role in breast cancer remains unclear. The present study investigated the effects and underlying mechanism of action of Eupafolin using breast cancer cell lines. The effects of Eupafolin on breast cancer cell proliferation, migration, apoptosis and the cell cycle were determined. Cell viability and Transwell assays, reverse transcription-quantitative PCR, flow cytometry and western blot analysis were used in this study. The data showed that the proliferation, migration and invasion ability of EO771 cells treated with Eupafolin was significantly decreased, and the apoptosis rate was increased compared with that of the control. The protein levels of Bax and cleaved caspase 3 increased, whereas that of Bcl-2 decreased. In addition, Eupafolin treatment also caused the proliferation of breast cancer cells to be arrested at the G0/G1 phase. Furthermore, results from western blotting indicated that Eupafolin treatment decreased the protein levels of p-PI3K, p-Akt and p-mTOR. Taken together, the present findings demonstrate that Eupafolin has a significant inhibitory effect on the proliferation of EO771 cells, inhibits cell migration and invasion, and promotes cell apoptosis, thereby causing G0/G1 phase arrest, at least partially through the PI3K/Akt/mTOR signaling pathway. Therefore, the findings provide novel insights regarding the use of Eupafolin for the treatment of breast cancer.

Introduction

Breast cancer is one of the most dangerous invasive cancer in women that has a global prevalence (1). Although the overall mortality rate of patients with breast cancer has decreased, it is continuing to emerge as a major health issue in women worldwide. At present, surgical treatment is the best option. Chemotherapy and radiotherapy are used to inhibit the growth and spread of tumors, and after many years of medical advancement and improvement, both chemotherapy and radiotherapy have been shown to prolong the lives of patients (2). The proliferation and recurrence rates of breast cancer cells are very high, and some patients develop drug resistance, which may cause side effects. Therefore, identifying non-toxic and efficacious natural compounds for the treatment of breast cancer is of utmost importance.

Traditional Chinese medicine has been widely used in China. Due to its non-toxic effects and efficacy, it is often used in combination with other medicines. With the continuous progress in modern medicine, preventing and treating the recurrence and metastasis of breast cancer using genetic technology and molecular biology methods will become a trend in future breast cancer research. Eupafolin is a flavonoid, which has anti-inflammatory, anti-viral, anti-angiogenesis and anti-tumor activities (3). Angiogenesis is closely associated with tumor development and metastasis, and Eupafolin can inhibit the activation of VEGFR2 and its associated signaling pathways. The molecular mechanism of the anti-cancer effect of Eupafolin may be associated with the activation of caspase-3 (4), the downregulation of vascular endothelial growth factor (VEGF) (5), and inhibition of the Akt signaling pathway (6). However, the underlying mechanism of its anti-cancer effect in breast cancer remains unclear. Therefore, understanding the effect of Eupafolin on breast cancer and identifying the mechanism of action will help in the management of breast cancer.

Proteins involved in the PI3K/Akt/mTOR pathway are abnormally expressed in several tumors, thereby leading to the progression of breast cancer, gastric cancer, nasal cancer and pancreatic cancer, among others. This pathway is closely associated with tumor proliferation, autophagy...
and migration (7,8). Several studies show that targeting this pathway using drugs or drug combinations is effective in the treatment of tumors (9). Therefore, research on drugs targeting the PI3K/Akt/mTOR pathway may have great significance in the management of breast cancer.

Therefore, the aim of the present study was to investigate whether Eupafolin could inhibit the proliferation and apoptosis of breast cancer cells (EO771 cell line), and to identify its possible underlying mechanism of action. Experimental results showed that Eupafolin significantly inhibited the proliferation of EO771 cells by modulating the PI3K/Akt/mTOR pathway, causing G_{0}/G_{1} phase arrest, and promoting apoptosis.

**Materials and methods**

**Cell culture and processing.** The mouse breast cancer cell line, EO771, selected for the present study was obtained from Binsui Biotechnology Co., Ltd. EO771 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific Inc.), with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), and maintained in an atmosphere of 5% CO\textsubscript{2} in an incubator at 37°C. Eupafolin (purity ≥99%) was purchased from Yuanye Biotechnology. In the present study, Eupafolin was dissolved in dimethyl sulfoxide (DMSO; Beijing Solarbio Science & Technology Co., Ltd.) at different concentrations (0, 25, 50 and 100 μM). Subsequently, cells were treated with Eupafolin for different periods.

**Cell viability test.** Cell viability was determined using Cell Counting kit-8 (CCK-8; MedChemExpress). In brief, 5,000 EO771 cells were seeded into 96-well plates, treated with 0, 25, 50 and 100 μM Eupafolin, and incubated at 37°C for 24, 36 and 48 h. Then, 10 μl CCK-8 solution was added to each well and the cells were incubated for 1.5 h. A microplate reader was used to measure the absorbance at 450 nm (TECAN).

**Determination of apoptosis and cell cycle.** Apoptosis was measured according to the instructions provided with the Annexin-V/Fluorescein isothiocyanate (FITC) apoptosis detection kit (Ebisson). After drug treatment for 24 h, cells were digested with trypsin without EDTA, collected, centrifuged at 500 x g for 5 min at 37°C, resuspended in pre-cooled PBS, centrifuged at 500 x g for 5 min to discard the supernatant, resuspended in 200 μl 1X binding buffer; 5 μl sample was mixed with FITC-Annexin V. Then, 5 μl propidium iodide (PI) staining solution was added to 200 μl 1X binding buffer 5 min prior to detection. Flow cytometry was performed using FACS (Thermo Fisher Scientific, Inc.). For cell cycle analysis, the cells were harvested and fixed in 70% ethanol at 4°C overnight. Next, cells were resuspended in 500 μl 1X PI solution (Baihao) for 30 min at 37°C. Flow cytometry analysis was performed using FACS (Thermo Fisher Scientific, Inc.). The collected data were analyzed using ModFit LT (version 2.0; Verity Software House, Inc.) to determine cell cycle distribution.

**Cell scratch test.** A total of 10,000 EO771 cells was seeded into 6-well culture plates in DMEM containing 10% FBS, and placed in an incubator until the cell density was 90% or higher. Using a sterile 100-μl plastic pipette, a wound was created; the cell debris was removed by washing with PBS and images were captured using an inverted light microscope (Olympus Corporation) with a digital camera (magnification, x80) at 0 h. A total of 3 ml of FBS-free medium was added per well; then, 25, 50 and 100 μM Eupafolin was added for 24 h. Cells were washed with PBS and images were obtained using microscopy. Healing areas were analyzed using ImageJ software (version 1.51; National Institutes of Health). The experiment was performed at least in triplicate.

**Cell migration and invasion experiments.** A Transwell (Corning, Inc.) assay was performed to determine cell migration and invasion abilities. After Eupafolin treatment, cells to be tested in the logarithmic growth phase were digested, resuspended in serum-free medium, and adjusted to a density of (1-10)x10\textsuperscript{5} cells/ml. A volume of 500 μl DMEM containing 10% FBS was added to the lower chamber of each transwell; tweezers were used to place the cells in a new 24-well plate. A volume of 100 μl of the cell suspension was added to the upper chamber, and the transwells were placed in an incubator for 24 h. Next, the cells were removed and the medium was aspirated. In a new 24-well plate, 600 μl 4% paraformaldehyde was added. The transwell was placed at room temperature and cells were fixed for 20-30 min. The fixative solution was removed and cells were stained at 37°C using 0.2% crystal violet for 10 min. Then, the cells were washed three times with PBS to remove the unbound crystal violet. The upper surface of the chamber was gently wiped with a cotton swab. Excess crystal violet was removed prior to microscopy. After drying, five fields were randomly selected and the cells were observed and counted under an inverted light microscope with a digital camera (magnification, x80). The cell invasion test procedure that was used was similar to that of the cell migration assay, except that the upper chamber was covered with BD Matrigel™ Matrix (Corning, Inc.).

**Reverse transcription-quantitative PCR (RT-qPCR).** According to the instructions provided with the kit, TRIzol reagent (Ruan) was used to extract total RNA, and cDNA was generated using the FastKing RT kit (Tiangen Biotech Co., Ltd) with 2 μg RNA, according to the manufacturer's instructions. The primers used were obtained from NCBi. A real-time fluorescent quantitative PCR detection system (Eppendorf) was used to perform the RT-qPCR reactions using SYBR-Green (Tiangen Biotech Co., Ltd.) and a total of 20 μl reaction mixture. The 2^(-ΔΔCq) method (10) was used to analyze gene expression levels. Primer sequences were as follows: Matrix metalloprotease (MMP) forward, 5'-CTGAGGAGTGTGCTCATAG-3' and reverse, 5'-TGGTTGTCGCCAGTGATT-3'; MMP9 forward, 5'-CTTACCCGGCTAAACACCTT-3' and reverse, 5'-TTCCACCGCGCTAAAACACCT-3'; VEGFA forward, 5'-ATAGGAGAGATGAGCTTCC-3' and reverse, 5'-TCTGCTTACATCTGCTGCT-3'; β-actin forward, 5'-GGCAAGGCTGCCGACC-3' and reverse, 5'-GATCCATGCAATGCAGTG-3'; cyclin D1 forward, 5'-CTGGTGC TGCAGTGAAACCACAT-3' and reverse, 5'-TCTGATGCCGAGACCTT-3'; CDK4 forward, 5'-CGAGCTGTA GGCTGATGGAT-3' and reverse, 5'-CCAGGGCGCTTAA

**Category**

- **Cell culture and processing.**
- **Cell viability test.**
- **Determination of apoptosis and cell cycle.**
- **Cell scratch test.**
- **Cell migration and invasion experiments.**
- **Reverse transcription-quantitative PCR (RT-qPCR).**
GAAGCTGAGA-3' and CDK6 forward, 5'-AGCCCTGCTGTGAAGAAAA-3' and reverse, 5'-TAGACGGACGGACTTCCTGCG-3'. The qPCR reaction conditions were as follows: Initial denaturation for 15 min at 95˚C, followed by 40 cycles of denaturation for 10 sec at 95˚C, annealing for 30 sec at 60˚C and extension for 20 sec at 72˚C.

Western blotting. After treating the cells with Eupafolin for 24 h, EO771 cells were harvested, washed twice with PBS, and lysed on ice with RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min. Then, the BCA method was used to determine the protein concentration: 5X loading buffer was added and proteins were denatured at 95˚C by boiling in a water bath for 10 min. A total of 20 µg protein sample was added to each well, separated using 12% SDS-PAGE at 120 V, and then transferred to PVDF membranes (Thermo Fisher Scientific, Inc.). Skimmed milk powder (5%) was used to block the PVDF membranes for 1 h at room temperature. Next, the membranes were incubated with primary antibodies directed against Bcl-2 (cat. no. 4223), Bax (cat. no. 2772), cleaved caspase 3 (cat. no. 9661), PI3K (cat. no. 4257), p-PI3K (cat. no. 17366), Akt (cat. no. 4691), p-Akt (cat. no. 4060), Mtor (cat. no. 2983), p-mTOR (cat. no. 5536) and GAPDH (cat. no. 5174) (all 1:1,000; Cell Signaling Technology, Inc.) at 4˚C overnight. Subsequently, membranes were washed three times with PBS, then incubated with a corresponding horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. A0208; Beyotime Institute of Biotechnology) for 1.5 h at room temperature and washed five times with PBS. Then, protein bands were visualized using an enhanced chemiluminescence assay kit (Beyotime Institute of Biotechnology) and photographed using an imaging system (Tanon). Finally, data were analyzed using ImageJ software (version 1.8.0; National Institutes of Health).

Results

Eupafolin decreases the viability and proliferation of breast cancer cells. EO771 cells were treated with Eupafolin for 24, 36 and 48 h to study the effects of the compound (Fig. 1A) on cell proliferation. The results of the CCK-8 assay indicated that Eupafolin inhibited the viability of EO771 cells, and the inhibitory effect was proportional to the treatment time and dose (Fig. 1B).

Eupafolin inhibits the invasion and migration of breast cancer cells. The scratch test was used to determine the wound healing time of EO771 cells. Furthermore, a Transwell assay was performed to determine the ratio of invasion and migration of EO771 cells (Fig. 2A and B). Results showed that compared with the control cells, Eupafolin significantly decreased the migration and invasion of EO771 cells (Fig. 2C). MMP2, MMP9 and VEGF-A are positively associated with the migration ability of tumor cells and can be used as marker genes. Therefore, their expression following Eupafolin treatment was tested, and the results showed that Eupafolin inhibited MMP2, MMP9 and VEGF-A (Fig. 2D). Taken together, these results indicate that Eupafolin prevented further deterioration of breast cancer cells.

Eupafolin induces apoptosis of breast cancer cells. Next, whether Eupafolin could induce apoptosis in breast cancer cells was tested. Flow cytometry was used to analyze the apoptosis ratio of EO771 cells following treatment with Eupafolin. The results showed that compared with the control, 100 µM Eupafolin increased the apoptosis rate of EO771 cells by 18% (Fig. 3A). To further understand the specific mechanism of Eupafolin in causing apoptosis in EO771 cells, the expression of apoptosis-associated proteins was evaluated using western blotting. The protein levels of cleaved caspase 3 and Bax were increased, whereas Bcl-2 protein levels were decreased (Fig. 3B and C).

Eupafolin induces G_0/G_1 phase arrest in breast cancer cells. After staining with PI, cell cycle analysis of EO771 cells treated with various concentrations of Eupafolin was performed using flow cytometry. Eupafolin induced G_0/G_1 phase arrest in EO771 cells (Fig. 4A and B).
addition, RT-qPCR was used to determine the mRNA levels of cycle-associated genes. The results showed that Eupafolin inhibited the expression of cyclin D1, CDK4 and CDK6 mRNA (Fig. 4C).

Eupafolin downregulates the PI3K/Akt/mTOR pathway. The aforementioned data indicated that Eupafolin had an impact on the proliferation and apoptosis of EO771 cells. PI3K/Akt/mTOR was found to play an important role in the
occurrence of many tumors. Therefore, whether Eupafolin affected EO771 cells through this pathway was tested. Results from western blotting showed that Eupafolin significantly decreased protein levels of p-PI3K, p-Akt and p-mTOR. Taken together, these results indicate that Eupafolin affected the proliferation and apoptosis of EO771 cells through the PI3K/Akt/mTOR pathway (Fig. 5A and B).

Discussion

The GLOBOCAN 2018 Global Cancer Analysis Annual Report released by the World Health Organization in 2018 indicates that annually, 2.088 million new cases of breast cancer and 627,000 deaths occur worldwide (1). China’s incidence rate ranks 120 in the world (11). Although the total incidence of breast cancer in China is lower than that in other developed countries, the trend shows a gradual increase (1). Current breast cancer treatment strategies mainly focus on chemotherapy and radiotherapy; however, these treatment strategies almost inevitably have side effects, which have become the bottleneck of clinical tumor treatment (12). Since natural compounds have advantages of higher efficacy and lower toxicity, many natural compounds have been used clinically (13). Eupafolin is a natural compound extracted from plants. Previous studies have reported that Eupafolin has anti-inflammatory and anti-tumor effects (3). For example, Eupafolin can inhibit the viability of esophageal cancer cells in vivo and in vitro by targeting T-LAK, and by downregulating Mcl-1 and upregulating Mcl-1 (4). Bim enhances TRAIL-mediated apoptosis of renal cancer cells (14). In addition, Eupafolin can induce apoptosis in HeLa human cervical cancer cells by inhibiting the expression of associated apoptosis proteins (3). However, the role of Eupafolin in breast cancer and its possible underlying mechanism of action have not yet been elucidated. Therefore, the present study aimed to determine the effect of Eupafolin on breast cancer cells. Our results showed that Eupafolin treatment had a significant inhibitory effect on breast cancer cell proliferation, promoted cell apoptosis, and induced G0/G1 phase arrest. Therefore, the data indicated that Eupafolin could inhibit the proliferation of breast cancer cells and induce apoptosis thereof, via downregulating the PI3K/Akt/mTOR pathway.
The anti-breast cancer proliferation activity of Eupafolin is shown in Fig. 1. Within 48 h of treatment, the inhibitory effect of Eupafolin increased in a time- and dose-dependent manner. MMP degrades various protein components in the extracellular matrix, destroys the histological barrier of tumor cell invasion, and plays a key role in tumor invasion and metastasis (15). VEGF is abnormally expressed in liver cancer and plays an important role in liver cancer neovascularization and tumor growth (5). The treatment of cancer by targeting VEGF and its receptor, VEGFR, is a much-explored topic in drug research (16). After treatment with different concentrations of Eupafolin, the migration and invasion ability of breast cancer cells decreased significantly. Furthermore, the mRNA levels of associated genes were detected using RT-qPCR, and the present data showed that mRNA levels of MMP2, MMP9 and VEGFA were significantly decreased (Fig. 2). Caspase 3 is the most important terminal splicing enzyme in apoptosis and plays an important part in the cell-killing mechanism (17). In addition, Bcl-2 was one of the first members of the Bcl-2 protein family reported to regulate cell death. In cancer, Bcl-2
can prevent apoptosis of some cells and is highly expressed in cancer cells that occur in lymph nodes and other organs of the immune system (18). The balance between Bcl-2 and Bax protein determines cell survival or apoptosis. Flow cytometry results in the present study revealed that the apoptosis rate of cells increased significantly. Moreover, protein levels of the pro-apoptotic proteins, Bax and cleaved caspase 3, increased significantly, whereas protein levels of Bcl-2 decreased significantly after Eupafolin treatment (Fig. 3). The decrease or resistance of cell apoptosis often leads to further malignancy (19). Therefore, in some studies, it was shown that the apoptosis rate of tumor cells can be increased by activating or inhibiting various signaling pathways (20,21). Naringin has been shown to inhibit the PI3K/Akt/mTOR pathway and promote the apoptosis of thyroid cancer cells (22), whereas hyperoside-induced breast cancer cell apoptosis is achieved via the reactive oxygen species (ROS)-mediated NF-κB signaling pathway (23).

Several studies have shown that there are critically important phases in the cell cycle: G1 to S and G2 to M, which occur in a period of complex and active molecular level changes, which are easily affected by external conditions. If tumor growth needs to be suitably controlled, it can be achieved via two mechanisms. Cyclin-dependent protein
kinases are a group of serine/threonine protein kinases, which can drive the cell cycle. Each cyclin-dependent kinase (CDK) binds to a different type of cyclin to form a complex, which regulates the transition of cells from the G1 to the S phase, or from the G2 to the M phase and exit from the M phase. Increased expression of CDK and cyclin has been observed in most cancer cells; the increased activity of CDK may be a therapeutic target, can effectively inhibit the further progression of tumors, including breast cancer, non-small cell lung cancer, esophageal cancer, gastric cancer and liver cancer (32). Astragaloside IV upregulates Nrf2 through the PI3K/Akt/mTOR signaling pathway to regulate inflammatory and oxidative stress, thereby effectively inhibiting breast cancer cell metastasis (12). The combined use of compound Sophora flavescent and gefitinib can upregulate autophagy in lung cancer by inhibiting the PI3K/Akt/mTOR pathway (33). In addition, in animal models, the addition of Akt allosteric inhibitors and dual PI3K and mTOR inhibitors can inhibit the PI3K/Akt/mTOR pathway and inhibit the growth of esophageal cancer (34). These findings are consistent with the present results. In the present study, results from western blotting indicated that Eupafolin decreased the phosphorylation of PI3K, Akt, and mTOR proteins, but did not change their total protein levels (Fig. 5).

Taken together, the present findings showed that Eupafolin significantly inhibited the proliferation and migration of breast cancer cells, promoted apoptosis, and caused G_{1}/G_{S} phase arrest, which was achieved by inhibiting the PI3K/Akt/mTOR pathway (Fig. 6). Thus, these results provide a theoretical basis for the use of Eupafolin in subsequent clinical trials. However, further research is needed to identify whether Eupafolin inhibits the growth of breast cancer cells through other pathways and target genes.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

JW, XZ, JZ and YD conceptualized the study and performed experimental research. XZ, SH and HP generated and analyzed the data. JW, XZ, BY and QL performed data analysis and edited the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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