Icariin induces apoptosis in breast cancer MCF-7 cells by regulating the MELK mediated PI3K/AKT signaling pathway

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Objective: To investigate the mechanism of icariin in promoting apoptosis of breast cancer cells by regulating the Phosphatidylinositol kinase (PI3K) PI3K/AKT signaling pathway mediated by Maternal embryonic leucine zipper kinase (MELK).

Methods: Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting were used to detect the expression of MELK. small interfering RNA (siRNA)-MELK cell transfection technology was used to detect the correlation between MELK and PI3K/AKT. Different icariin concentrations on proliferation, migration and apoptosis of breast cancer cells were detected by flow cytometry, CCK-8, and Transwell. Western blot was used to detect the effects of icariin on MELK expression, AKT, cyclin, and apoptosis-related proteins.

Results: Compared with normal mammary epithelial cells, the expression of MELK in breast cancer cells was significantly increased (p < 0.01). si-MELK decreased the expression of p-AKT, increased the expression of epithelial-mesenchymal transition (EMT)-related protein E-cadherin, and decreased the expression of N-cadherin and vimentin. Compared with the control group, icariin solution group showed a decrease in cell proliferation ability, a significant increase in cell apoptosis and a decrease in cell migration ability (p < 0.05). Icariin could induce G2/M arrest and inhibit the growth of breast cancer cells. Conclusion: Icariin can inhibit the expression of MELK, inhibit the PI3K/AKT signaling pathway to a certain extent, and further has a therapeutic effect on breast cancer.

Keywords
Icariin, Breast cancer, MELK, PI3K/AKT signaling pathway

1. Introduction
With the in-depth understanding of breast cancer-related genes and molecular mechanisms, the regulation of breast cancer cell apoptosis and proliferation by signaling pathways has become a current research focus. The PI3K/AKT signaling pathway plays an essential regulatory function in cell survival, proliferation, metastasis, metabolism, apoptosis. It is abnormally activated in many human tumors and plays a vital role in breast cancer cell apoptosis, breast cancer tumor growth, and metastasis [1].

Maternal leucine zipper kinase (MELK)/murine serine-threonine kinase 38 (MPK38) is a member of the AMP-associated serine-threonine kinase family. It has been found to be involved in the regulation of many cellular events, including cell proliferation, apoptosis, and metabolism, in part through phosphorylation of signaling molecules [2]. Under normal physiological conditions, the activity of Melk is tightly regulated by stress signals, which inhibit tumor cell proliferation, promote apoptosis, and inhibit tumorigenesis and development. Conversely, high MELK expression drives tumorigenesis. Indeed, studies have found elevated MELK expression levels in breast cancer compared to normal breast tissue [3–5]. Pitner MK discovery to target MELK may provide new therapeutic opportunities for triple negative breast cancer (TNBC) and other cancers [6]. However, there is not enough knowledge about MELK related inhibitors and the downstream substrates of Melk. Addressing these questions is currently the first step in identifying Melk for the treatment of breast cancer.

A recent study showed that MELK promotes apoptosis and cell growth arrest by stimulating TGF-β-mediated signaling. Smad proteins (Smad2, -3, -4, and -7) bind directly to the Melk kinase domain. Interactions between MELK and Smad2, -3 and -4 are significantly increased by TGF-β or ASK1 signaling, which is reduced when MELK binds to Smad7 [7]. Furthermore, MELK phosphorylates p53 at Ser15 residues, leading to p53-mediated cell cycle arrest and enhanced apoptosis by stimulating nuclear translocation of p53 [8]. Li et al. [9] found that MELK expression was increased in both gastric cancer cell lines and gastric cancer tissues. The expression of MELK can promote epithelial-mesenchymal transition (EMT) of gastric cancer cells and activate AKT to induce EMT by participating in the PI3K/AKT signaling pathway.
signaling cascade. This process leads to metastasis and poor prognosis of primary gastric cancer. Also, decreasing MELK expression can increase the arrest of G2/M cells in the cell cycle, promote apoptosis, and inhibit gastric cancer growth in vivo and in vitro [9]. Studies have shown that the expression of MELK is increased in breast cancer tissues, which is also associated with poor prognosis of breast cancer, possibly due to the involvement of MELK in the Bcl-G signaling pathway, which plays an anti-apoptotic role in breast cancer [10]. In view of tumor stem cell theory and expression characteristics of MELK, MELK may be an important target for breast cancer therapy [11]. The mechanism diagram is shown in Fig. 1.

HerbaEpimedii, belonging to the Epimedium genus in the Berberidaceae family, is a commonly used traditional Chinese medicine (TCM) that has many effects including improving immunity [12], improving cardiovascular blood flow [13], improving osteoporosis [14], promoting hematopoiesis [15], regulating endocrine secretion [16], antioxidant [17], and antitumor effects [14, 18, 19]. Icarin (ICA), the main active component of Epimedium, has been reported to inhibit the growth of breast cancer MDA-MB-453 and MCF7 cells by inducing G2/M phase arrest [20].

Whether MELK can exert an anti-apoptotic effect through PI3K/AKT in breast cancer has not been reported before. Whether icariin can inhibit this signaling pathway has not been studied in depth. In this experiment, we explored whether MELK could play an anti-apoptotic role through PI3K/AKT in breast cancer and further clarified whether icariin could promote apoptosis by inhibiting this pathway.

2. Methods

2.1 Culture of experimental cell lines

Human breast cancer cell line MCF-7, derived from American Type Culture Collection (ATCC) cell bank, Cells were cultured in RPMI1640 medium containing 10% fetal bovine serum + 1% penicillin-streptomycin and placed in incubator at 37 °C and 5% CO2. Cells were in logarithmic growth phase and passaged when the fusion rate reached 90%.

2.2 MELK activates AKT phosphorylation

The experiment was divided into three groups: MELK siRNA group, LY294002 inhibitor group, and control group. MELK siRNA (Shanghai Jima biopharmaceutical Co., Ltd, Shanghai, China) was used to interfere with MELK expression in the experimental group. Human breast cancer cell line MCF-7 was cultured, and after trypsinization, the cells were adjusted to contain $2 \times 10^5$ cells per milliliter of cell suspension and seeded into 6-well cell culture plates (ThermoFisher, Waltham, MA, USA). Cell transfection was performed when the degree of cell fusion was 60% under an inverted microscope (CKX53, OLYMPUS, Tokyo, Japan). Dilute 10 µL of 20 µmol/L siRNA (MELK-siRNA, siRNA-NC) storage solution (V1) with 150 µL of 1×riboFECTTM CP buffer p (ThermoFisher, Waltham, MA, USA), gently mix and incubate for 5 min at room temperature; add V2 mixture to 15 µL
riboFECT\textsuperscript{TM} CP reagent (Guangzhou sharp Biotechnology Co., Ltd, Guangzhou, China), gently blow and mix, incubate for 15 min at room temperature; and mix riboFECT\textsuperscript{TM} CP mixture was added to 1825 \(\mu\)L cell culture medium (V2) and gently mixed; the V2 mixture was placed in a 37 °C, CO\textsubscript{2} incubator for 24 h for subsequent experiments. The expression levels of AKT phosphorylation and EMT-related proteins E-cadherin, N-cadherin, and vimentin were detected by Western blotting, and GAPDH was the internal reference. After cells were treated with the PI3K inhibitor LY294002 (GLPBIO, Montclair, CA, USA) in the LY294002k group, the phosphorylation of AKT and the expression levels of EMT-related proteins E-cadherin, N-cadherin, and vimentin were detected by Western blotting.

2.3 Preparation of icariin solution

A small amount of DMSO (Sigma, San Francisco, CA, USA) was added to 10 mg of icariin to help dissolution. Then 10 \(\mu\)L of serum-free DMEM high glucose medium (ThermoFisher, Waltham, MA, USA) was added. The bacteria were filtered and sterilized with a 0.22 \(\mu\)m diameter filter. The 1 mg/mL stock solution was prepared and stored in the refrigerator at –20 \(\degree\)C for standby. The mother liquor was diluted to 25 \(\mu\)g/mL, 50 \(\mu\)g/mL, 100 \(\mu\)g/mL and 200 \(\mu\)g/mL with 10% PBS DMEM (Procell Biotechnology Co., Ltd, Wuhan, Hubei, China) high glucose medium. After cell adhesion, 200 \(\mu\)L icariin (200, 100, 50, 25 \(\mu\)g/mL) was added into the experimental group. The blank control group was added with the same volume of medium (containing the same concentration of DMSO).

2.4 Detection of MCF-7 cell proliferation by CCK-8 assay

Different groups of cells were seeded in 96-well plates (ThermoFisher, Waltham, MA, USA), cultured according to groups for 24, 48 and 72 h, and incubated at 37 °C for 4 h with 10 \(\mu\)L CCK-8 (Beyotime, Shanghai, China) per 100 \(\mu\)L of culture medium; the absorbance (OD value) at 450 nm wavelength was measured with a microplate reader (Multi-Mark, Labsystems, Helsinki, Finland). The cells were incubated to the logarithmic phase, total RNA was extracted by trizol (ThermoFisher, Waltham, MA, USA), and 1 \(\mu\)g of mRNA was taken as the template, and cDNA was synthesized by reverse transcription. Specific primers (Kinsray biotech Co, Nanjing, Jiangsu, China) were added for qRT-PCR, and three parallel holes were set in each group. The primer sequences were as follows, MELK 5'-TCTCCCACTAGGATTTCCTTT-3', downstream primers: 5'-TGATCAGGATGTGTTCAATAGA-3', and the length of amplified product was 121 bp. Internal reference GAPDH upstream primer: 5'-CAGGAGCCATTGCTGATGAT-3', downstream primers: 5'-GAAGGCTGGGGTCCTATT-3', and the length of the amplified product was 196 bp.

2.5 Detection of cell cycle distribution by flow cytometry

Cells in each group were cultured for 48 h, treated with 0.25% trypsin (ThermoFisher, Waltham, MA, USA) for 24 h, and rinsed three times with phosphate buffer (PBS) (Procell Biotechnology Co., Ltd, Wuhan, Hubei, China). Each sample was added with 0.5 mL of propylene sodium iodide staining solution, gently dried and mixed, so that the precipitated cells were resuspended, placed in the dark at 37 °C for 30 min, immediately detected by flow cytometry (CytoFLEX, Beckman Coulter, Inc., Carlsbad, CA, USA), and the cell cycle was analyzed using ModFit LT V4.1.7 (Verity Software House, Inc., Topsham, ME, USA).

2.6 Detection of apoptosis rate by Annexin V/PI staining

Cells in each group were cultured for 48 h and treated with 0.25% trypsin for 24 h. Rinse 3 times with PBS. The cells were gently resuspended by adding 195 \(\mu\)L of Annexin V-FITC binding solution (Dojindo, Kyushu, Japan), and then 5 \(\mu\)L of Annexin V-FITC was added to mix gently. Finally, 10 \(\mu\)L of propidium iodide (PI) staining solution (Dojindo, Kyushu, Japan) was added to mix evenly. After mixing, the cells were placed at room temperature for 15 min in the absence of light (cells were resuspended three times during placement), then placed in an ice bath; the apoptosis rate was detected by flow cytometry. Cell survival rate = OD value of drug-added group/OD value of the control group × 100%.

2.7 Transwell detection of cell migration

The Transwell chamber was placed in a 24-well plate, 50 \(\mu\)L of Matrigel gel was sucked into the precooled Transwell upper chamber, and the cell concentration was diluted to 5 \(\times\)10⁵/mL. 200 \(\mu\)L of cell suspension was added to the upper chamber, while serum-free medium (control group) and ICA containing 25 \(\mu\)g/mL, 50 \(\mu\)g/mL and 100 \(\mu\)g/mL were added to the lower chamber, and 500 \(\mu\)L DMEM medium containing 10% FBS were added to the lower chamber. The chamber was placed in 1% crystal violet dye solution (Solarbio, Beijing, China), removed after 30 min at room temperature, and rinsed twice with PBS. Photographs were taken under a 100-fold microscope (CX33, OLYMPUS, Tokyo, Japan), and the number of cells in the visual field was recorded. Five different visual fields were taken from each chamber for average, and all experiments were repeated three times.

2.8 Real-time fluorescence quantitative PCR (RT-PCR)

After incubation to the logarithmic phase, total RNA was extracted by trizol (ThermoFisher, Waltham, MA, USA), and 1 \(\mu\)g of mRNA was taken as the template, and cDNA was synthesized by reverse transcription. Specific primers (Kinsray biotech Co, Nanjing, Jiangsu, China) were added for qRT-PCR, and three parallel holes were set in each group. The primer sequences were as follows, MELK upstream primer: 5'-TCTCCCACTAGGATTTCCTTT-3', downstream primers: 5'-TGATCAGGATGTGTTCAATAGA-3', and the length of amplified product was 196 bp. Internal reference GAPDH upstream primer: 5'-CAGGAGCCATTGCTGATGAT-3', downstream primers: 5'-GAAGGCTGGGGTCCTATT-3', and the length of the amplified product was 121 bp. The cycle parameters were as follows: 45 cycles were conducted after maintaining 94 °C for 30 s, 5 s at 94 °C per cycle, 15 s at 55 °C, and 10 s at 72 °C per cycle. The MELK mRNA expression was analyzed by dissociation curve analysis, and 2^-ΔΔCt analyzed the data.

2.9 Western blot experiment

Total cellular proteins were extracted and mixed with the loading buffer after protein quantification; after SDS-PAGE electrophoresis, the proteins were transferred to (polyvinylidene fluoride) PVDF membranes. After blocking, Rabbit anti-MELK (1:1000, PeproTech, USA), p-AKT (1:1000, Sangon Biotech,Shanghai, China), E-cadherin, N-cadherin, vimentin, caspase 3, caspase 9, cyclin D1, Bax, Bcl-2 (1:1000, Beyotime, Shanghai, China), Glyceraldehyde-3-Phosphate
Dehydrogenase (GAPDH) monoclonal antibodies (1:2000) were used as primary antibodies and incubated with membranes at 4 °C overnight. Then the goat antirabbit (1:2000, ZSGB-BIO, Beijing, China) was used, and the corresponding protein expression was detected by the ECL chemiluminescence kit (SK6668-100, Sangon Biotech, Shanghai, China).

2.10 Statistical analysis

Statistical analysis of data was performed using software SPSS19.0 (IBM, Armonk, NY, USA). All experiments in this research were performed in triplicate. Values were expressed as mean ± standard deviation (SD). Statistical analysis was performed using student’s t-test. p-values among the groups were calculated using one-way analysis of variance (ANOVA). p < 0.05 was considered to be statistically significant.

3. Results

3.1 Differential expression of MELK in breast cancer cells and normal breast cells

The expression of MELK mRNA in breast cancer cells was significantly higher than that in normal breast epithelial cells (p < 0.01). Western blot showed the same results in the expression of Melk protein in MCF-10A cells and MCF-7 cells (Fig. 2A,B).

3.2 Regulation of MELK on AKT signaling pathway

Compared with the control group, the phosphorylation level of the AKT in si-MELK group and LY294002 group decreased (p < 0.05), but there was no significant change in AKT expression; and the expression of E-cadherin increased, the expression of N-cadherin increased and vimentin decreased (p < 0.05) (Fig. 2C,D).

3.3 Effect of icariin on the cell proliferation

Cell viability test after different concentrations of icariin treatment for 72 h showed that the higher the concentration of icariin, the lower the cell proliferation efficiency. When the concentration of icariin was 25 µg/mL, there was a significant difference compared with the control group (p < 0.05); when the concentration of icariin was greater than or equal to 50 µg/mL, there was a significant difference compared with 25 µg/mL (p < 0.05); this indicated that icariin could inhibit the proliferation of breast cancer cells (Fig. 3A).
3.4 Effect of icariin on cell cycle of breast cancer MCF-7

Cells were treated with different concentrations of icariin, and the results showed that compared with the control group, the cell cycle in the experimental group was blocked to different degrees, with 25 µg/mL, 100 µg/mL and 200 µg/mL cell cycle blocked significantly \( p < 0.05 \), mainly in G2/M phase (Fig. 3B). In addition, cyclin, compared with the control group, was significantly decreased in the icariin-added group, and the lowest expression was observed at the concentration of icariin of 100 µg/mL (Fig. 3C,D).

3.5 Effect of different concentration of icariin on apoptosis by flow cytometry

Flow cytometry was used to detect the difference of apoptosis in different groups. Compared with the control group, the apoptotic rate in the experimental group increased significantly \( p < 0.01 \), and with the increase of icariin concentration, the apoptotic rate increased. The apoptotic rate was the highest when the drug concentration was 100 µg/mL but decreased when the drug concentration was 200 µg/mL (Fig. 3E). The expression of apoptosis-related proteins was as follows: Bcl-2 expression decreased, Bax protein expression increased, Caspase-9 and cleaved-Caspase3 expression increased in the experimental group with icariin \( p < 0.05 \) (Fig. 3F).

3.6 Cell migration results

Compared with the control group, the cell migration rate in the experimental group was significantly reduced, and with the increase of drug concentration, the cell migration rate decreased. When the concentration was 200 µg/mL, the cell migration rate was the lowest (Fig. 4).
3.7 Results of apoptosis-related protein detection by Western Blot

Compared with the control group, the expression of MELK in the experimental group was decreased, and the lowest expression was observed when the concentration of icariin was 100 µg/mL; the level of p-AKT was also decreased (p < 0.05). These results all suggest that icariin may promote the apoptosis of breast cancer cells through the PI3k signaling pathway (Fig. 5).
Fig. 5. Effect of icariin on the expression of MELK and p-AKT. *p < 0.05, **p < 0.01.

Table 1. Research progress of PI3K/AKT, icariin and MELK in the occurrence and development of cancer.

| Author(s) | Primary coverage | Refs. |
|-----------|------------------|-------|
| Liu Z     | 3-BrPA induces apoptosis of breast cancer MDA-MB-231 cells by inhibiting PI3K/AKT signaling pathway | [38] |
| Dey JH    | Targeting fibroblast growth factor receptor can block PI3K/AKT signal transduction, induce apoptosis, and prevent the growth and metastasis of breast cancer | [1] |
| Cidado J  | Targeting PI3K/AKT signaling/mTOR pathway for breast cancer tumor therapy | [39] |
| Burris HA, Saini KS, Ghayad SE, Pande S | Down regulation of PI3K/AKT in the prevention of breast cancer | [40–43] |
| Li W      | Effects of tanshinone I (Tan I) on the tumorigenicity of MCF-7 and MDA-MB-453 human breast cancer cells via the PI3K/AKT signaling pathway | [44] |
| Gu Z      | Inhibitory effects of icariin on the growth and metastasis of k562 human esophageal cancer cells via PI3K/AKT and STAT3 pathways | [36] |
| Lei F     | ICA mediates miR-625-3p in sw579 and TPC1 cells to inactivate PI3K/Akt and MEK/ERK signaling pathways in the treatment of thyroid cancer | [45] |
| Liu G     | MELK promotes the progression of colorectal cancer by activating FAK/SRC pathway | [46] |
| Xu Q      | MELK promotes endometrial cancer progression by activating the mTOR signaling pathway | [47] |
| Seong H, Manoharan R, Beullens M | MELK can affect the development of tumor through Smad/TGF /p53 signaling pathway | [8, 48, 49] |

4. Discussion

Icariin, a flavonoid extracted from Epimedium, has attracted more and more attention in recent years for its antitumor activity. Previous studies have shown that icariin can induce apoptosis of a variety of tumor cells, and reduce the invasion and migration of tumor cells, thus achieving anti-tumor effects [21, 22]. Bu YK et al. [23] found that icariin could promote apoptosis of human colon cancer cells in vivo and in vitro; their studies provided evidence that icariin sensitized tumor cells to TRAIL-induced apoptosis through reactive oxygen species (ROS), extracellular regulated protein kinases (ERK) and C/EBP homologous protein (CHOP)-mediated upregulation of DR5 and DR4. Ji X et al. [24] showed that icariin had no significant inhibitory effect on esophageal cancer cell proliferation in vitro, but could induce apoptosis in vivo through Fas expression and secretion of Fas-L and IFN-γ, thus playing an anti-esophageal cancer role. We found that icariin significantly inhibited the proliferation of breast cancer cells and promoted their apoptosis, and the effect was more significant with the increase of drug concentration. The results are similar to those of Guo et al. [25].

MELK is expressed in the thymus, spleen, and developing tissues, such as the adult germ line and adult neural progenitor cells, but is almost undetectable in the kidney, liver, and muscle of adult tissues [26]. High expression of MELK in Breast cancer has been found to be associated with radiation resistance and poor prognosis in breast tumor models in vitro and in vivo. In this experiment, the expression of MELK in breast cancer cells was significantly increased compared with normal breast epithelial cells, and MELK was involved in breast cancer. This result is similar to that of Meng et al. [27]. Their experimental results demonstrated that MELK interacted with Bcl-G, a proapoptotic member of the Bcl-2 family, and participated in breast cancer development. However, the experiments of Giuliano CJ [28] and A Lin et al. [29] had different results. They used Crip-Cas9 gene edit-
ing technology to knock out the MELK gene and found that it had no effect on the proliferation of cancer cells. The results showed that MELK had no significant correlation with the influence of tumor development.

The PI3K/AKT signaling pathway plays a vital role in developing breast cancer [30, 31]. Various studies have shown that down-regulation of PI3K/AKT can effectively control the proliferation or promote apoptosis of breast cancer cells [32–35]. Zhen et al. [36] demonstrated that icariin can inhibit the migration of esophageal cancer cells by inhibiting PI3K/AKT and STAT3 pathway. In this study, the results showed that the use of icariin could reduce the phosphorylation of AKT, further affecting the proliferation and apoptosis of breast cancer cells. In breast cancer, MELK is associated with multiple signaling pathways [37]. However, there are few reports on the correlation between MELK and PI3K/AKT signaling pathways. We also found that MELK could promote AKT phosphorylation, but had little effect on AKT expression. In Table 1 (Ref. [1, 8, 36, 38–49]), we listed some research progress of PI3K/AKT, icariin and MELK in cancer occurrence and development.

EMT is an effective way for epithelial cells to acquire migration ability and has become an essential way for epithelial cell carcinoma to infiltrate and metastasize. The occurrence of EMT is accompanied by changes in markers, mainly including epithelial E-cadherin, N-cadherin, and vimentin [50, 51]. If E-cadherin expression is decreased, the migration ability of cells is enhanced. If the expression of interstitial marker protein and vimentin is increased, the migration ability of cells is enhanced. In this experiment, MELK can promote the expression of N-cadherin and vimentin and can inhibit E-cadherin expression, which also reflects from the side that MELK gene activation can promote the occurrence of breast cancer.

5. Conclusions

In conclusion, icariin has been reported to inhibit the proliferation of human breast cancer cells to some extent, but its mechanism needs further study. The results showed that icariin could further promote cell apoptosis by inhibiting the expression of Melk and decreasing the expression of p-AKT and cyclin. However, there are still some limitations in this experiment. Other types of breast cancer cells have not been explored, and the PI3K/AKT signaling pathway needs to be further studied.

Abbreviations

MCF-7 cells, Human breast adenocarcinoma cell line; MELK, Maternal embryonic leucine zipper kinase; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; CCK-8, Cell Counting Kit-8; EMT, epithelial-mesenchymal transition; ATCC, American Type Culture Collection; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B.

Author contributions

YZ and DQ conceived and designed the experiments; LX performed the experiments; TC analyzed the data; ZHY contributed reagents and materials; DQ and TTD wrote the paper. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

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Conflict of interest

The authors declare no conflict of interest.

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