

Quercetin-3-methyl ether suppresses human breast cancer stem cell formation by inhibiting the Notch1 and PI3K/Akt signaling pathways

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Abstract. Breast cancer is a leading cause of mortality among women with cancer worldwide. Quercetin-3-methyl ether, a natural compound occurring in various plants, has been indicated to have potent anticancer activity. Breast cancer cell growth and survival were examined by CCK-8 and colony formation assay, whilst cell cycle and apoptosis were determined by flow cytometry. Cell invasion and migration were assessed by wound-healing assay and Transwell assay. Cancer stem cell formation was analyzed by mammosphere formation assay and related signaling pathways were detected by western blotting. In the present study, it was observed that treatment with quercetin-3-methyl ether significantly inhibited cell growth, induced apoptosis and cell cycle arrest at the G₂-M phase, and suppressed invasion and migration in human breast cancer cells, including the triple negative MDAMB-231 cell line, and the estrogen receptor-positive/progesterone receptor-positive/human epidermal growth factor receptor 2-negative MCF-7 and T47D cell lines. This compound also markedly suppressed the epithelial-mesenchymal transition process as evidenced by the upregulated expression of E-cadherin, and the concomitant downregulated expression of vimentin and MMP-2. Furthermore, it was demonstrated that quercetin-3-methyl ether treatment inhibited mammosphere formation and the expression of the stemness-related genes, SRY-box 2 and Nanog. Mechanistically, this compound decreased the expression of Notch1, and induced the phosphorylation of phosphoinositide 3-kinase (PI3K) and Akt. It also attenuated the human insulin growth factor 1-induced

phosphorylation of PI3K, Akt and glycogen synthase kinase β . Additionally, the combination of quercetin-3-methyl ether and a secretase inhibitor (DAPT) exhibited additive suppression of the expression of Notch1, PI3K, Akt and mammalian target of rapamycin and a more marked inhibitory effect on cell proliferation and colony formation compared with either drug alone. Treatment with quercetin-3-methyl ether alone markedly suppressed the levels of tri-methyl histone H3 (Lys27), but had no effect on the expression of enhancer of zeste homolog 2. Overall, these findings indicated that quercetin-3-methyl ether may be a potential therapeutic compound for the treatment of triple negative and hormone-sensitive breast cancer.

Introduction

Breast cancer is a common cause of mortality among types of cancer worldwide, and the majority of cases of breast cancer-associated mortality (90%) are caused by invasion and metastasis (1,2). Increasing data demonstrates the importance of the epithelial-mesenchymal transition (EMT) in breast cancer invasion and metastasis (1,3,4). EMT is a complex process in which epithelial cancer cells gain a mesenchymal phenotype and lose their epithelial features. In this process, the epithelial cells gain increased migratory and invasive properties to become mesenchymal cells through the EMT. In breast cancer, a cascade of EMT processes can lead to cell detachment, migration, invasion and colonization at secondary sites (1,3,4). Cancer stem-like cells (CSCs) are a unique population of cancer cells, which are associated with tumor initiation, progression, migration, invasion, resistance to chemotherapy and radiation therapy, and relapse (5). It has been reported that EMT results in the increase of CSC-like properties in various types of cancer, including breast cancer, whereas inhibiting the process of EMT may repress CSC formation (6-8). Therefore, targeted the suppression of EMT and CSCs is emerging as an attractive method for the curative treatment of several type of cancer.

The Notch signaling pathway is a highly conserved cell signaling pathway, which is important in cell proliferation, survival, apoptosis and differentiation, and in the modulation

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of EMT and CSC maintenance (9). The abnormal activation of Notch signaling has been found to be associated with tumor development and progression in breast cancer (10,11). In mammals, there are four different Notch receptors and five known ligands, which have been implicated in tumorigenesis (10). Increased expression levels of Notch receptors and their ligands in breast cancer tissues have been observed compared with levels in normal control tissues (11). Notch receptors are considered to be breast oncogenes partly due to the fact that the overexpression of Notch1 or Notch 4 can lead to the formation of spontaneous murine mammary tumors *in vivo* (12). Furthermore, the enhanced expression of Notch1 and/or its ligand in human mammary tumors has been correlated with poor patient survival rates (13), and Notch has been demonstrated to be important for the survival of CSCs (14). Therefore, Notch may be a potent therapeutic target in breast cancer. Crosstalk between the phosphoinositide 3-kinase (PI3K)/Akt pathway and Notch family members (Notch1 and 3) has been shown to be critical in cancer progression (15). The PI3K/Akt/mammalian target of rapamycin (mTOR) pathway has a crucial function in cell survival, proliferation, migration, invasion and apoptosis, and it commonly activated in mammary tumors. Enhanced PI3K/Akt activity has been associated with poor patient prognosis, and it is considered a key signaling pathway leading to resistance to standard therapies in breast cancer (16,17). It has been noted that the PI3K/Akt pathway can promote CSC activity (18), therefore, targeting this pathway may offer a potential strategy for overcoming resistance to conventional breast cancer therapies (19). Combined therapy, including the combination of endocrine and PI3K/Akt pathway inhibitors, has shown clinical benefit, and novel combination strategies are now being examined clinically for their efficacy in the treatment of patients with breast cancer.

Enhancer of zeste homolog 2 (EZH2), a histone-lysine N-methyltransferase enzyme, is a polycomb group protein involved in the regulation of cell proliferation, stem cell maintenance, differentiation and neoplastic cell transformation (20). The overexpression of EZH2, identified in aggressive and metastatic breast cancer, has been correlated with poor prognosis, and can predict patient survival rate as an independent biomarker (20,21). Therefore, an increased level of EZH2 is considered to indicate propensity for metastasis and poor outcome in patients with mammary tumors (21). EZH2 is of interest as a therapeutic target in triple negative breast cancer (TNBC) due to its overexpression in this form of the malignancy (22). EZH2-targeted therapy may be a novel and promising treatment strategy for patients with breast cancer.

Quercetin-3-methyl ether is a natural compound found in various plants, including *Allagopappus viscosissimus* (23), *Opuntia ficus-indica* var. *saboten* (24), *Lychnophora staavioides* (25), *Rhamnus species* (26), *Semecarpus anacardium* (27) and *Larrea divaricate* (28). Previous studies have demonstrated that quercetin-3-methyl ether acts as an anticarcinogenic flavonoid in a number of human cancer cell lines, including HL-60, A431, SK-OV-3, HeLa, and HOS cells (23), and that this compound induces apoptosis in lymphoma cells through nitrosative stress (28). Our previous study confirmed that quercetin-3-methyl ether had a potent inhibitory effect on skin carcinogenesis (29) and significantly

inhibited cell growth in human epidermal growth factor receptor 2 (HER2)-positive SK-BR-3 lapatinib-sensitive and -resistant breast cancer cells (30). However, whether this compound can influence TNBC and hormone-sensitive breast cancer cells remains to be fully elucidated. The present study showed that quercetin-3-methyl ether significantly inhibited cell proliferation through the induction of apoptosis and cell cycle arrest at the G₂-M phase, and suppresses cell invasion and migration in human TNBC and hormone-sensitive breast cancer cells, including the triple negative MDAMB-231 cell line and the estrogen receptor (ER)-positive/progesterone receptor (PR)-positive/HER2-negative MCF-7 and T47D cell lines. Quercetin-3-methyl ether was shown to exert these effects by inhibiting the EMT process and CSC formation through repression of the Notch1 and PI3K/Akt signaling pathways.

Materials and methods

Reagents. Quercetin-3-methyl ether, human insulin-like growth factor 1 (IGF-1) and DAPT were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from Gibco; Thermo Fisher Scientific, Inc. Antibodies against Cyclin B1 (cat. no. 4138), Cyclin-dependent kinase 1 (CDK1; cat. no. 2546), B-cell lymphoma (Bcl)-2 (cat. no. 2870), Bcl-extra large (Bcl-xl; cat. no. 2764), PI3K (cat. no. 3358), phosphorylated PI3K (cat. no. 3821), total Akt (cat. no. 4691S), phosphorylated Akt (Ser473; cat. no. 4060S), mTOR (cat. no. 2983), phosphorylated mTOR (cat. no. 5536), phosphorylated Glycogen synthase kinase β (GSK3 β ; cat. no. 5558), Notch1 (cat. no. 4380), EZH2 (cat. no. 5246S), tri-methyl-histone H3 (Lys27; cat. no. 9733), E-cadherin (cat. no. 3195), Vimentin (cat. no. 5741), Matrix metalloproteinase 2 (MMP2; cat. no. 4022), SRY-box 2 (SOX2; cat. no. 3579), Nanog (cat. no. 4903) and GAPDH (cat. no. 5174) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. Human breast cancer cells (MCF-7, T47D and MDA-MB-231) and human breast non-tumorigenic MCF-10A epithelial cells were obtained from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MCF-7 and MDA-MB-231 cells originate from human invasive adenocarcinoma, and T47D cells originate from human ductal carcinoma. MCF-7 and T47D cells are known to express estrogen and progesterone receptors, belong to the luminal A group, and have marginal metastatic potential. MDA-MB-231 cells belong to the triple-negative basal-like group and are highly metastatic (31). The MCF-7, T47D and MDA-MB-231 cells were cultured at 37°C in a 5% CO₂ incubator in DMEM containing 10% FBS and 1% penicillin/streptomycin. The MCF-10A cells were cultured in high-glucose DMEM medium with 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO₂ incubator.

Cell growth assay. To examine the effect of quercetin-3-methyl ether on breast cancer cell growth, the cells were seeded (3x10³ cells/well) in 96-well plates with 10% FBS/DMEM

and cultured at 37°C in a 5% CO₂ incubator. After 24 h, the cells were replenished with fresh medium and treated with quercetin-3-methyl ether (0-20 µM) and/or DAPT (10 µM). Following culturing for the indicated times (0-72 h), 10 µl of CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added into each well and the wells were incubated for 1 h at 37°C in a 5% CO₂ incubator. Finally, the absorbance was measured at 450 nm.

Cell cycle assay. The breast cancer cells were seeded (2x10⁵ cells/well) into six-well plates with 10% FBS/DMEM and were incubated overnight at 37°C in a 5% CO₂ incubator. The cells were then starved in serum-free medium for 24 h, treated with quercetin-3-methyl ether (0-20 µM) for 48 h, stained with propidium iodide and subjected to cell cycle analysis according to a previously described method (29,30).

Western blot analysis. The cancer cells (1x10⁶) were cultured overnight in a 10-cm dish, and starved in serum-free medium for 24 h. The starved cells were then treated with quercetin-3-methyl ether (0-20 µM) or DAPT (10 µM) for 48 h in culture medium containing 10% FBS. For the induction of phosphorylated PI3K and Akt, the cells were pretreated with quercetin-3-methyl ether for 2 h, and then exposed to 100 ng/ml IGF-1 for 20 min. The harvested cells were lysed with lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) on ice for 30 min. Following centrifugation at 12,000 x g for 15 min at 4°C, the supernatant was collected for further analysis. The total protein concentration of the cell lysates was determined with a dye-binding protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. The whole cell lysate proteins were subjected to western blot analysis according to previous protocols (29,30). Following denaturation, the proteins (50-100 µg the amount of total proteins varied depending on different targeted proteins) were separated via 8-15% SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with blocking buffer (5% non fat milk) for 1 h at room temperature and then incubated with dilutions of primary antibodies (1:1,000) in blocking buffer overnight at 4°C. Subsequently, the membranes were incubated with secondary goat anti-rabbit or goat-anti-mouse antibodies conjugated with horseradish peroxidase (1:2,000) in blocking buffer for 1 h at room temperature. Protein bands were visualized using an ECL detection kit (Bio-Rad Laboratories, Inc.). Quantity One 1-D Analysis software (Bio-Rad Laboratories, Inc.) was used for scanning and densitometric analysis.

Apoptosis assay. The breast cancer cells were seeded (2x10⁵ cells/well) into six-well plates with 10% FBS/DMEM and incubated overnight at 37°C in a 5% CO₂ incubator. The cells were then starved in serum-free medium for 24 h, followed by treatment with quercetin-3-methyl ether (0-20 µM) for 48 h. Annexin V/propidium iodide staining was used to analyze apoptotic cells according to the manufacturer's protocol (AD10-10; Dojindo Molecular Technologies, Inc.). The fluorescence intensity of the Annexin V/propidium iodide staining was detected by flow cytometry.

Scratch wound-healing assay. A total of 4x10⁵ cells were seeded into 12-well plates and cultured for 24 h, when an artificial wound was made with a P20 pipette tip in each well. Following washing of the cells three times with culture medium, fresh medium supplemented with quercetin-3-methyl ether (0-20 µM) was added. Images were captured under an inverted microscope equipped with a camera at different time points (24-72 h). The wound gap distance was quantitatively determined with Image J software (National Institutes of Health, Bethesda, MD, USA) and closure was determined as follows: Closure (%) = migrated cell surface area/total surface area x100.

Migration and invasion assays. Migration and invasion assays were performed using a Transwell chamber (8-µm pore size; Corning Incorporated, Corning, NY, USA). For the migration assays, 5x10⁴ cells in serum-free medium were placed into the upper chamber. For the invasion assays, the same number of cells in serum-free medium were placed into the upper chamber with an insert coated in Matrigel (BD Biosciences, San Jose, CA, USA). The lower compartments were filled with 10% FBS-medium, and quercetin-3-methyl ether (0-20 µM) was added to the upper and lower compartments at the same concentration. Following culture for 48 h, the cells in the upper chamber were removed, and the cells in the lower chamber were stained with crystal violet and counted in four views for each well using a light microscope (TS100, Nikon Corporation, Tokyo, Japan).

Mammosphere formation assay. Single-cell suspensions were cultured at a density of 2,000 cells per well in six-well Ultra-Low Attachment Plates (Corning Incorporated) with DMEM/F-12 medium containing 2% B27, 20 ng/ml EGF, 20 ng/ml bFGF, 10 ng/ml heparin, 0.4% bovine serum albumin (EMD Millipore) and 1% penicillin/streptomycin. After 3 days, the resulting spheroids were treated with quercetin-3-methyl ether (0-20 µM) for another 7 days, following which the mammospheres were counted under a light microscope (TS100, Nikon Corporation).

Colony formation assay. The cells were seeded at a density of 500 cells per well in 60-mm dishes with 10% FBS/DMEM and were incubated overnight at 37°C in a 5% CO₂ incubator. The cells were then supplied with fresh medium and treated with vehicle control (DMSO), quercetin-3-methyl ether (10 µM) and/or DAPT (10 µM). The medium, with or without drug, was replaced every 3 days. Following 8 days of incubation, the colonies were fixed with 10% formaldehyde for 15 min and stained with 1.0% crystal violet solution for 20 min. Finally, images were captured and colonies were counted.

Statistical analysis. As appropriate, data are expressed as the means ± standard deviation, and significant differences were evaluated using Student's t-test or one-way analysis of variance, with the post hoc test following the latter being Tukey's test. GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to process the data. P<0.05 was considered to indicate a statistically significant difference.

Results

Quercetin-3-methyl ether inhibits cell growth by inducing cell cycle arrest at the G₂-M phase and apoptosis in breast cancer cells. First, the effect of quercetin-3-methyl ether on the growth of MCF-10A, MCF-7 and T47D cells was examined. The results revealed that treatment with this compound (0-20 μ M) for 72 h had a marked inhibitory effect on the growth of breast cancer cell lines, but had no significant effect on the growth of the non-tumorigenic epithelial cells (MCF-10A; Fig. 1A). Subsequently, whether quercetin-3-methyl ether inhibited cancer cell proliferation through the regulation of cell cycle processes was determined. Flow cytometric analysis of cell cycle distribution indicated that treatment with 10 μ M quercetin-3-methyl ether for 48 h significantly increased the number of cancer cells at the G₂-M phase ($P < 0.05$; Fig. 1B). As the G₂-M phase is regulated primarily by Cyclin B1/CDK1, the protein expression of Cyclin B1 and CDK1 in quercetin-3-methyl ether-treated breast cancer cells was subsequently determined. The results revealed a marked decrease in the protein expression of these two genes in MCF-7 and T47D cells following treatment with quercetin-3-methyl ether for 48 h (Fig. 1C). To examine whether quercetin-3-methyl ether inhibited cell growth via the induction of cell death, the rate of apoptosis was detected in the two hormone-sensitive breast cancer cell lines (MCF-7 and T47D) treated with quercetin-3-methyl ether. The findings indicated that, following treatment for 48 h, 10 μ M quercetin-3-methyl ether significantly induced apoptosis in the two cell lines ($P < 0.05$; Fig. 1D). The Bcl-2 family is crucial in the regulation of apoptosis. Therefore, the effects of quercetin-3-methyl ether on the protein expression of Bcl-2 family members was also determined. The results demonstrated that the levels of the key anti-apoptotic proteins Bcl-2 and Bcl-x1 were downregulated in the two cell lines following treatment for 48 h (Fig. 1E). Collectively, these data suggested that quercetin-3-methyl ether inhibited breast cancer cell growth through inducing apoptosis and G₂-M phase cell cycle accumulation.

Quercetin-3-methyl ether suppresses the migration and invasion of breast cancer cells. The effects of quercetin-3-methyl ether on the migration and invasion of breast cancer cells were subsequently determined. A scratch wound-healing assay demonstrated that breast cancer cells (MCF-7 and MDA-MB-231) had high mobile capacities; whereas treatment with quercetin-3-methyl ether (0-20 μ M) for 24-72 h significantly decreased the motility of breast cancer cells into the wound gap in a dose- and time-dependent manner (Fig. 2A). Accordingly, a Transwell migration assay demonstrated that quercetin-3-methyl ether significantly inhibited breast cancer cell migration through the Transwell insert membrane (Fig. 2B). Following treatment with 10 μ M of quercetin-3-methyl ether for 48 h, the rates of cell migration in the MDA-MB-231 and MCF-7 cells were reduced to 38 and 26%, respectively ($P < 0.05$; Fig. 2B). Similarly, the Matrigel invasion assay revealed that quercetin-3-methyl ether treatment of the two cell lines dose-dependently reduced the number of invasive cells that migrated through the Matrigel base membrane from the upper to the lower chamber ($P < 0.05$; Fig. 2C). Notably, the cell invasion rates were reduced to

55% (MDA-MB-231 cells) and 43% (MCF-7 cells) following treatment with 10 μ M of quercetin-3-methyl ether for 48 h ($P < 0.001$). These data suggested that quercetin-3-methyl ether had marked inhibitory effects on the migratory and invasive capacities of breast cancer cells. In addition, as shown in the images in Fig. 2C, the cell number in the MCF-7 0 μ M group was higher than that in the MDA-MB-231 0 μ M group, however, a contrasting trend of cell number is shown in the bar graph when comparing the two cell lines. The reason for this was that cell number was counted in four views of each well using a microscope (total of four views), whereas the images shown in Fig. 2C were randomly selected from one of the four views (single view). Although the images suggest the cell number in the MCF-7 0 μ M group was markedly higher than that in the MDA-MB-231 0 μ M group, the image represents a single view and does not show the total number. However, the cell counting results shown in the bar graph represent the average values per well in three independent experiments. The reason for the change in the cell lines was that suppression of the migration and invasion in breast cancer cells was found. Therefore, MDA-MB-231 cells were selected to examine the effects of quercetin-3-methyl ether on cell invasion, metastasis and cancer stem cell formation due to the fact that this cell line is a TNBC line with high metastatic and malignant potential.

Quercetin-3-methyl ether inhibits EMT and CSC formation in breast cancer cells. EMT, an important program enabling epithelial cells to acquire a mesenchymal phenotype, characterized by the decreased expression of E-cadherin and the overexpression of Vimentin and cellular proteases, including MMP-2, is closely linked with the invasion and metastasis of breast cancer (1). The present study determined the effect of quercetin-3-methyl ether on the expression of EMT-related genes in breast cancer cells. The data indicated that treatment with quercetin-3-methyl ether led to a reduction in the expression of mesenchymal cell biomarkers, including Vimentin and MMP-2, and concomitantly, the overexpression of epithelial cell biomarker E-cadherin (Fig. 3A). As EMT may contribute to the increase of CSC-like properties, and CSCs have been implicated in cancer invasion and metastasis, the present study examined whether quercetin-3-methyl ether can suppress mammosphere formation and the expression of stemness regulatory genes, including SOX2 and Nanog, in breast cancer cells. The results demonstrated that quercetin-3-methyl ether effectively reduced the number of mammospheres ($P < 0.05$; Fig. 3B) and inhibited the protein expression of SOX2 and Nanog in a dose-dependent manner (Fig. 3C). These findings suggested that quercetin-3-methyl ether inhibited breast cancer invasion and metastasis, possibly by suppressing the intrinsic EMT process and CSC formation.

Quercetin-3-methyl ether downregulates Notch1, PI3K-AKT and EZH2 signals in breast cancer cells. EZH2, the catalytic subunit of polycomb repressive complex 2, can downregulate gene transcription through histone H3 trimethylation on lysine 27 (H3K27me3). It has been reported that EZH2 causes expansion of breast cancer stem cells through activation of the Notch and PI3K/Akt signaling pathways (32). Therefore, the present study determined changes in the levels of these proteins in the hormone-sensitive and TNBC cells in response

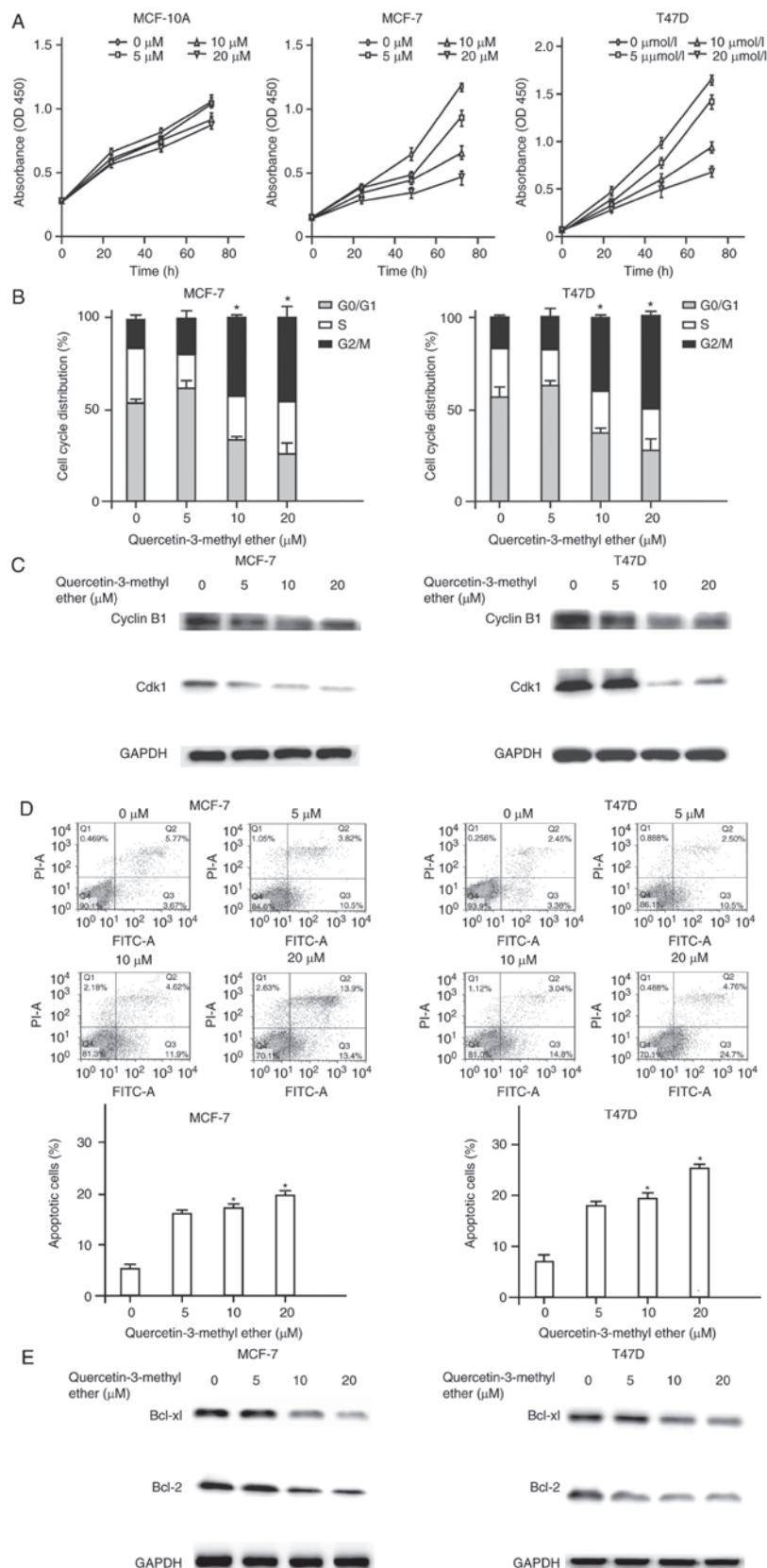


Figure 1. Quercetin-3-methyl ether leads to marked inhibition of cell growth by inducing G₂-M phase accumulation and apoptosis in breast cancer cells. (A) Quercetin-3-methyl ether inhibited the growth of breast cancer cells (MCF-7 and T47D), whereas no change was observed in non-tumorigenic epithelial cells (MCF-10A). Cells were treated with 0-20 μ M quercetin-3-methyl ether for 0-72 h. Cell growth was determined by an CCK-8 assay. Data are presented as the mean \pm SD. Cells were starved in serum-free medium for 24 h and then treated with quercetin-3-methyl ether (0-20 μ M) for 48 h. (B) Quercetin-3-methyl ether induced significant G₂/M arrest in breast cancer cells. Cell cycle analysis was performed using flow cytometry. Data are presented as the mean \pm SD. *P<0.05 quercetin-3-methyl ether, vs. DMSO (control). (C) Quercetin-3-methyl ether suppressed the expression of Cyclin B1 and CDK1 in breast cancer cells. The protein levels of Cyclin B1 and CDK1 were determined by western blot analysis. (D) Quercetin-3-methyl ether induced apoptosis of breast cancer cells. Apoptosis was detected by flow cytometry. Data are presented as the mean \pm SD. *P<0.05 quercetin-3-methyl ether, vs. DMSO. (E) Levels of Bcl-x1 and Bcl-2 were determined by western blot analysis. Treatment decreased the expression of Bcl-x1 and Bcl-2. CDK1, Cyclin-dependent kinase 1; Bcl-2, B-cell lymphoma 2; Bcl-x1, Bcl-extra large; SD, standard deviation.

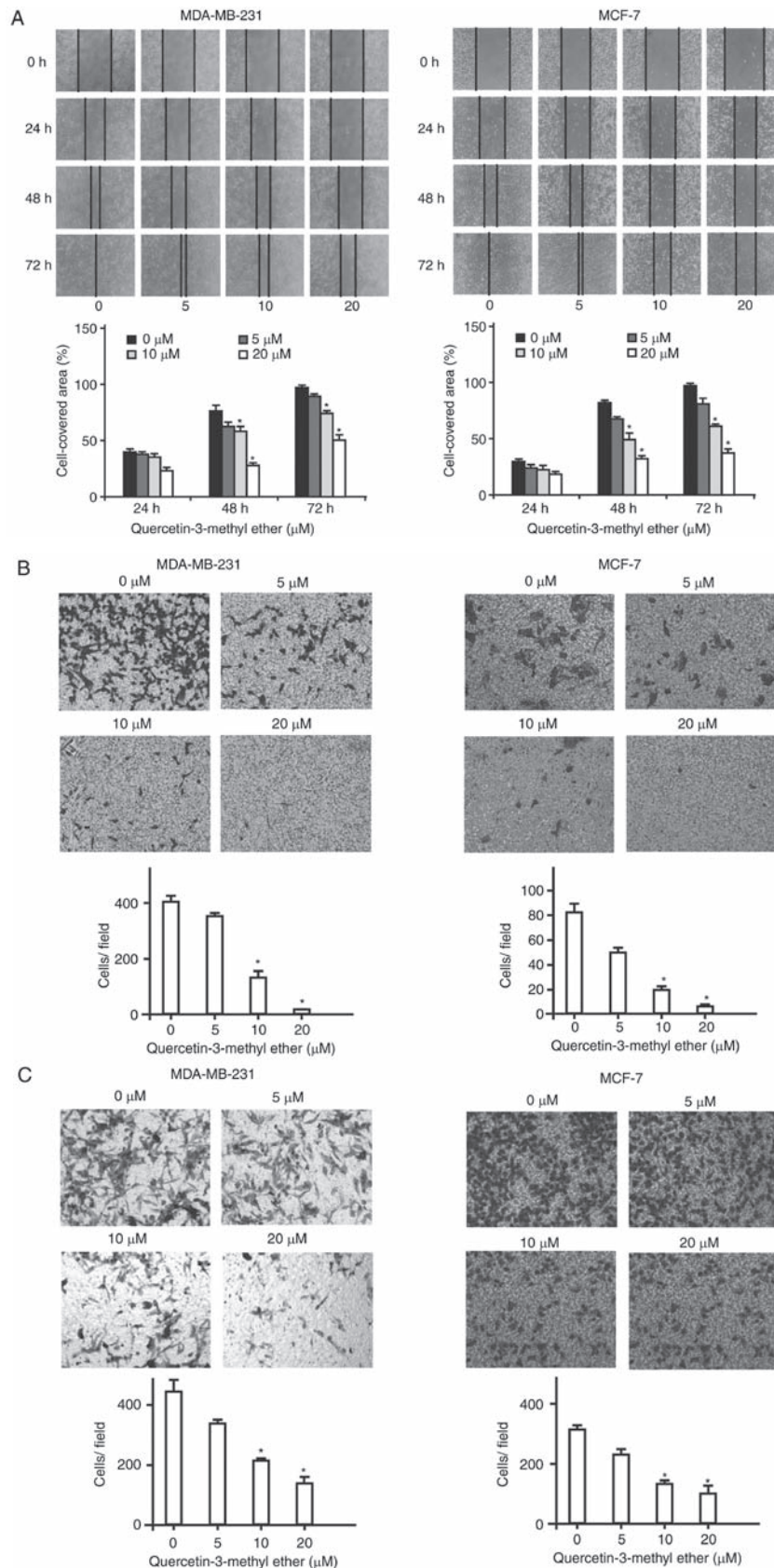


Figure 2. Quercetin-3-methyl ether significantly inhibits the migration and invasion of breast cancer cells. (A) Quercetin-3-methyl ether inhibited cell motility. Cells were treated with 0-20 μ M quercetin-3-methyl ether for 24-72 h. Cell motility was determined using a scratch wound-healing assay. Images were captured using a microscope (magnification, $\times 100$). Closure rate is shown as a percentage of wound closure. Data are presented as the mean \pm SD. * $P < 0.05$ quercetin3-methyl ether, vs. DMSO. (B) Quercetin-3-methyl ether inhibited cell migration. Cells were treated with 0-20 μ M quercetin-3-methyl ether for 48 h. Cell migration was examined using a Transwell assay. Cells that penetrated the lower compartment were stained with crystal violet. The cell numbers were counted under an inverted microscope (magnification, $\times 100$). Data are presented as the mean \pm SD. * $P < 0.05$ quercetin3-methyl ether, vs. DMSO. (C) Quercetin-3-methyl ether inhibited cell invasion. Cells were treated with 0-20 μ M quercetin-3-methyl ether for 48 h. Cell invasion was examined using the Matrigel Transwell chamber assay. Cells that invaded the lower chamber were stained with crystal violet. The cell numbers were counted under an inverted microscope (magnification, $\times 100$). Data are presented as the mean \pm SD. * $P < 0.05$ quercetin3-methyl ether, vs. DMSO. SD, standard deviation.

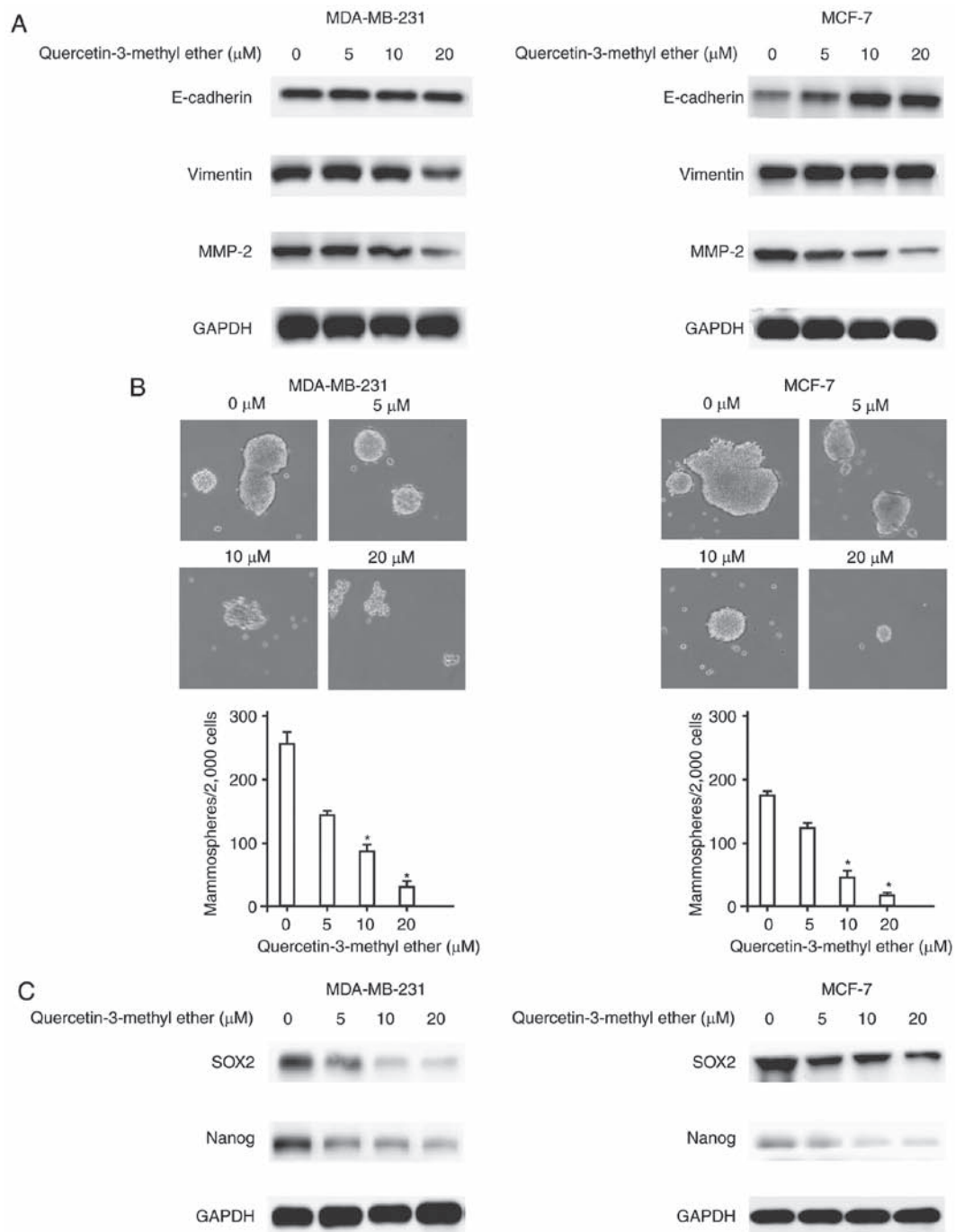


Figure 3. Quercetin-3-methyl ether markedly suppresses epithelial-mesenchymal transition and cancer stem-like cell formation in breast cancer cells. (A) Cells were starved in serum-free medium for 24 h and then treated with quercetin-3-methyl ether (0-20 μM) or DMSO (control) for 48 h. The protein levels of E-cadherin, vimentin and MMP-2 were determined by western blot analysis. (B) Quercetin-3-methyl ether suppressed mammosphere formation in breast cancer cells. Non-adherent spheroid breast cancer cells were treated with different doses of quercetin-3-methyl ether (0-20 μM) for 7 days. Images (magnification, x200) were captured and the numbers of spheres were counted under a microscope. Data are presented as the mean \pm standard deviation. * $P < 0.05$ quercetin-3-methyl ether, vs. DMSO. (C) Breast cancer cells were treated with different concentrations of quercetin-3-methyl ether for 48 h, and the protein expression levels of SOX2 and Nanog was detected by western blot analysis. MMP-2, matrix metalloproteinase 2; SOX2, SRY-box 2.

to quercetin-3-methyl ether treatment. The results indicated that quercetin-3-methyl ether markedly decreased the constitutive protein levels of phosphorylated PI3K, phosphorylated AKT and phosphorylated mTOR in MCF-7 and T47D cells (Fig. 4A). Furthermore, the compound suppressed the levels of IGF-1-induced phosphorylated PI3K, Akt and GSK3 β in hormone-sensitive cells (Fig. 4B). As quercetin-3-methyl ether had a significant repressive effect on the expression of

Notch1 (Fig. 4C), the combinational efficacy of this agent and the Notch signaling inhibitor DAPT (a γ -secretase complex inhibitor) was examined. The combination of these compounds exhibited an additive inhibitory effect on the protein expression of Notch1, PI3K, Akt and mTOR in MCF-7 and MDA-MB-231 cells (Fig. 4D). Furthermore, combining these two compounds had more marked inhibitory effects on breast cancer cell proliferation and colony formation (Fig. 4E and F). It was also found

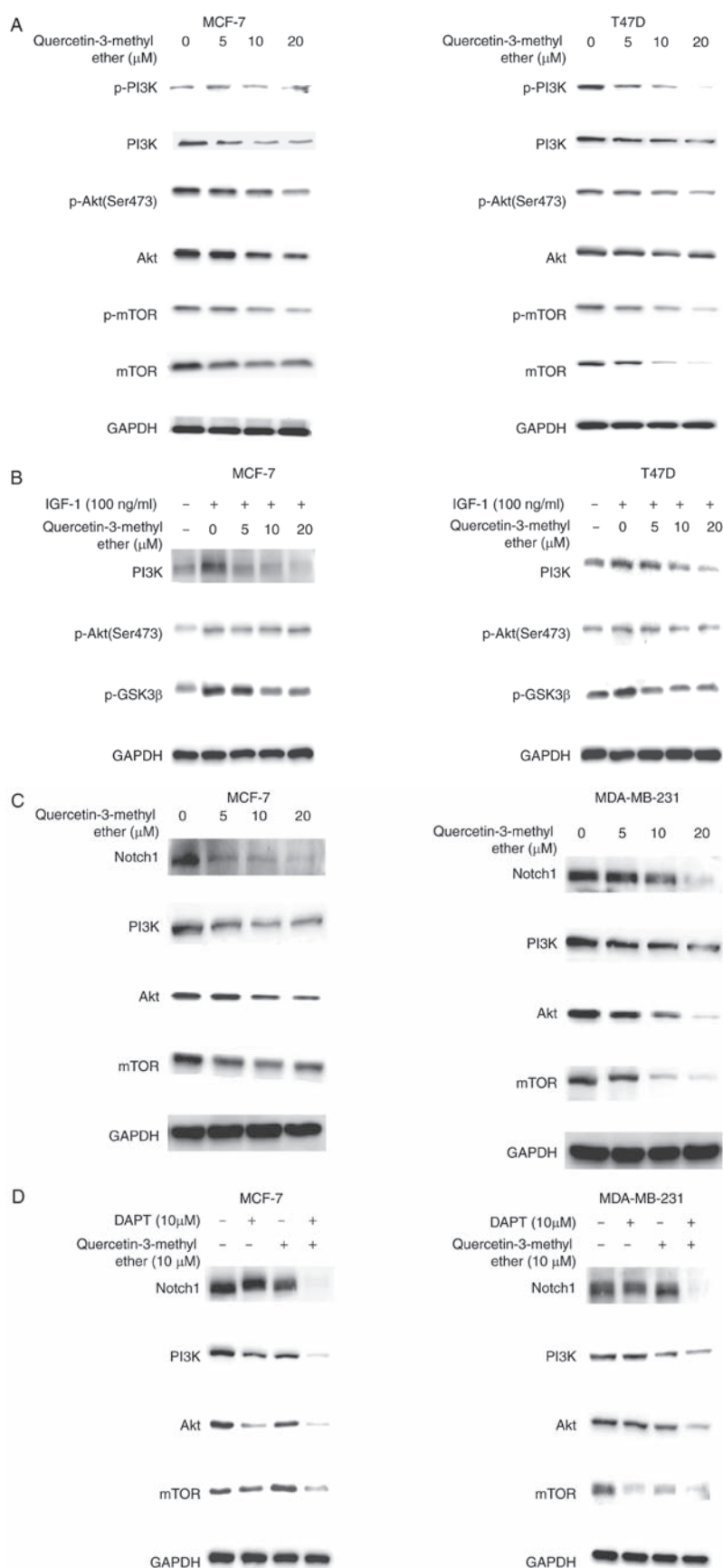


Figure 4. Quercetin-3-methyl ether downregulates Notch1, PI3K/Akt and EZH2 signals in breast cancer cells. Cells were starved in serum-free medium for 24 h and then treated with quercetin-3-methyl ether (0-20 μ M) for 48 h. The expression levels of (A) p-PI3K, total PI3K, p-Akt, total Akt, p-mTOR and total mTOR were detected by western blot analysis. (B) Cells were starved in serum-free medium for 24 h and then treated with quercetin-3-methyl ether (0-20 μ M) for 2 h prior to exposure to 100 ng/ml IGF-1 for 20 min. The expression levels of p-PI3K, p-Akt and p-GSK3 β were determined by western blot analysis. (C) Cells were starved in serum-free medium for 24 h and then treated with quercetin-3-methyl ether (0-20 μ M) for 48 h. The expression levels of Notch1, PI3K, Akt and mTOR were detected by western blot analysis. (D) Cells were treated with 10 μ M quercetin-3-methyl ether and/or 10 μ M DAPT for the indicated times and expression levels of Notch1, PI3K, Akt and mTOR were detected.

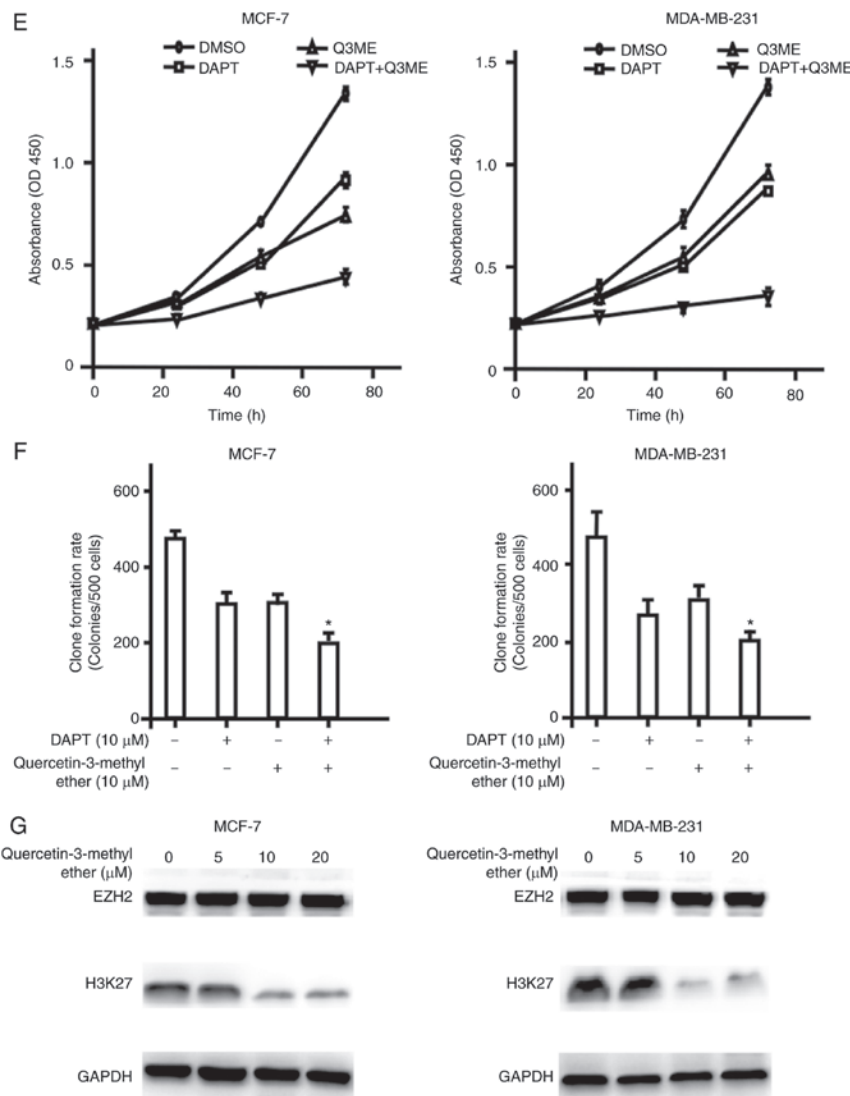


Figure 4. Continued. (E) Cell growth was evaluated with a CCK-8 assay and (F) colony formation was examined by a colony formation assay. The combination of quercetin-3-methyl ether and DAPT exhibited more marked inhibition of growth and colony formation capacity of MCF-7 and MDA-MB-231 cells. Data are presented as the mean \pm standard deviation. * $P < 0.05$, drug-treated, vs. DMSO-treated. (G) Expression of EZH2 and H3K27me3 were detected by western blot analysis. PI3K, mTOR, mammalian target of rapamycin; p-, phosphorylated; Q3ME, quercetin-3-methyl ether; IGF1, insulin-like growth factor 1; EZH2, enhancer of zeste homolog 2.

that quercetin-3-methyl ether inhibited H3K27me3 signals in a dose-dependent manner, but had no significant effect on the expression of EZH2 (Fig. 4G). These data suggested that quercetin-3-methyl ether suppressed EMT and CSC formation in the hormone-sensitive breast cancer cells and TNBC cells, possibly via the downregulation of H3K27me3 epigenetic marks and the Notch1 and PI3K/Akt pathways.

Discussion

The majority of cases of breast cancer-associated mortality are caused by highly metastatic disease, the relatively high rates of which have been attributed to the lack of effective treatments. Breast cancer is a common and complicated malignant disease characterized by a range of aberrations at the genomic and molecular levels, which manifest in dysregulated signaling pathways involved in the development, progression and metastasis of the cancer. Therefore, the identification of

novel agents that specifically target the metastatic properties of cancer cells is considered important for the effective clinical control of this malignancy. There are three main subtypes of breast cancer based on the primary biomarkers: Luminal tumors (ER- and PR-positive), HER-2-positive tumors and TNBCs (negative for all three markers). These cancer subtypes have distinct properties and prognoses. The luminal type is well differentiated, whereas the HER-2-positive and TNBC types are poorly differentiated. Our previous study demonstrated that quercetin-3-methyl ether potently inhibited anchorage-dependent or -independent growth of HER-2-positive human breast cancer cells (SK-BR-3) sensitive or resistant to lapatinib treatment (30). In the present study, it was demonstrated that quercetin-3-methyl ether exhibited inhibitory effects on the proliferation, migration and invasion of cells, and simultaneously induced apoptosis in luminal tumor cells (MCF-7 and T47D) and TNBC cells (MDA-MB-231).

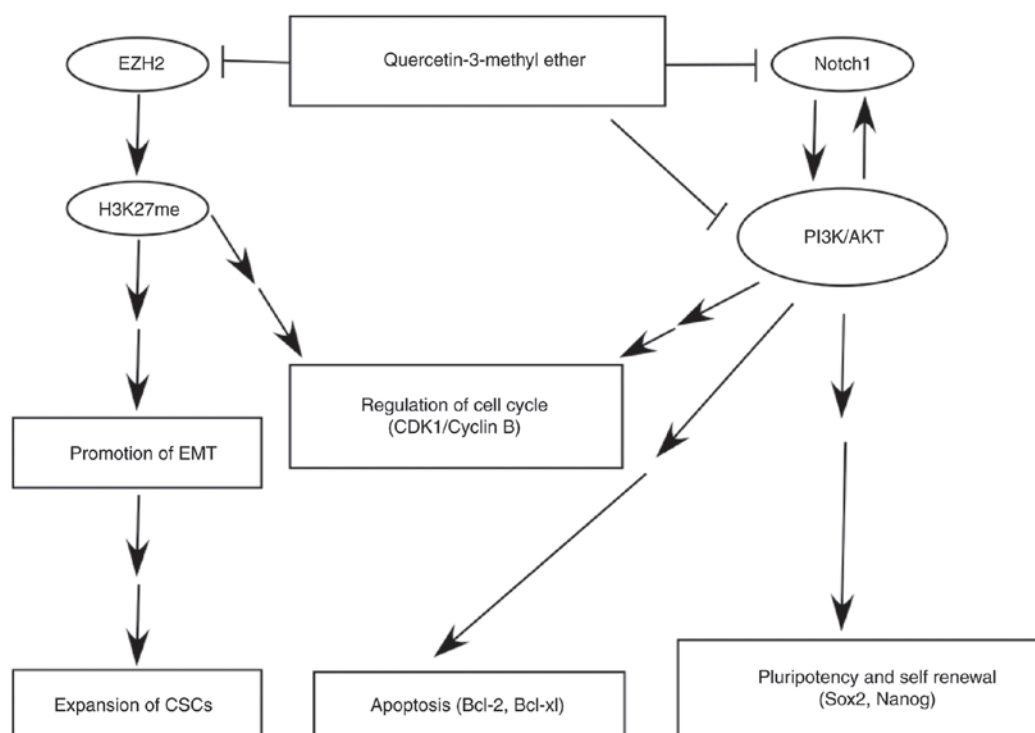


Figure 5. Simplified diagram of the proposed anticancer mechanism of quercetin-3-methyl ether in breast cancer based on the results of the present study. Quercetin-3-methyl ether inhibits Notch1, PI3K/Akt and EZH2 signaling pathways, which results in suppression of EMT and CSC formation, and induction of cell cycle arrest and apoptosis. Ultimately, cancer cell proliferation, migration and invasion are repressed by quercetin-3-methyl ether. PI3K, EZH2, enhancer of zeste homolog 2; CDK1, cyclin-dependent kinase 1; Bcl-2, B-cell lymphoma 2; Bcl-xl, Bcl-extra large; Sox2, SRY-box 2; EMT, epithelial-mesenchymal transition; CSCs, cancer stem-like cells.

EMT is frequently abnormally activated during cancer invasion and metastasis. EMT is a reversible molecular process involving the loss of epithelial markers, including E-cadherin, and increased expression of mesenchymal markers, including Vimentin. It has been reported that breast cancer cells typically exhibit an EMT phenotype, characterized by the high expression of EMT-regulatory transcription factors and mesenchymal markers and downregulation of epithelial markers (33,34). Upon the downregulation of E-cadherin, epithelial cells gain fibroblastic properties that enable them to dissociate from the epithelium and exhibit enhanced migratory capabilities. The findings of the present study indicated that quercetin-3-methyl ether decreased the expression of Vimentin and increased the expression of E-cadherin, suggesting an inhibitory effect of EMT in breast cancer cells. In addition, EMT is key in CSC formation. Previous clinical data have demonstrated an association between the proportion of CSCs and poor prognosis in patients with breast cancer. An improved understanding of CSCs is expected to have important implications for cancer prevention and therapy. In particular, inhibiting EMT and CSC formation is an attractive strategy for cancer prevention and management. The present study demonstrated that quercetin-3-methyl ether may effectively inhibited EMT and CSC formation, and consequently, reduced the migration and invasion capacities of breast cancer cells. These results offer novel insight with potential clinical applications into the anticancer mechanisms of flavonoids.

The PI3K/Akt/mTOR pathway has a key regulatory function in cell survival, proliferation, migration, metabolism, angiogenesis and apoptosis. It is the most frequently dysregulated

pathway and may be of importance as a possible therapeutic target in breast cancer. Overactivation of this pathway has been linked with increased cell growth and, clinically, poor prognosis in various types of cancer (16,17,19). The results of the present study demonstrated that quercetin-3-methyl ether significantly suppressed the constitutive activation of the PI3K/Akt/mTOR signaling pathway and the IGF-1-induced phosphorylation of PI3K, Akt and GSK-3 β in breast cancer cells. Increasing data indicates that crosstalk between the PI3K/Akt pathway and Notch signaling is important in cancer. Notably, Notch may regulate the Akt pathway in normal and breast cancer cells. In previous studies, Notch signaling triggered an autocrine signaling loop, which activated Akt, in breast epithelial cells, and the suppression of Notch reduced the activity of Akt in breast tumor cells (35,36). Additionally, the increased expression of Notch1 in breast cancer has been significantly correlated with metastasis, EMT and CSC formation. Previous studies have indicated that inhibiting cell growth, migration and invasion, and inducing apoptosis by the inhibition of Notch1 may be partly achieved by inactivating AKT signaling (35). The present data demonstrated that treatment with quercetin-3-methyl ether led to a decrease in the expression of Notch1, PI3K, Akt and mTOR in TMBC and hormone-sensitive breast cancer cell lines, and that the combination of this compound with the Notch signaling inhibitor DAPT had an additive inhibitory effect on the expression of these proteins.

EZH2 protein is overexpressed in patients with aggressive breast tumors and may be a significant biomarker of recurrence and metastasis in breast cancer. Previous studies have

indicated that EZH2 serves a function in self-renewal of breast CSCs, and is involved in EMT and cell invasion. Furthermore, it has been reported that EZH2 contributes to the expansion of breast CSCs via Notch signaling activation (32), and that overexpressed EZH2 protein is associated with an increased level of phosphorylated Akt (Ser473) in invasive breast cancer. The present study identified that quercetin-3-methyl ether reduced the expression of H3K27me3, Notch1, PI3K, Akt and mTOR, and inhibited the Notch1 and PI3K/Akt signaling pathways in breast cancer cells. These data suggested that quercetin-3-methyl ether repressed EMT and CSCs, possibly through inhibition of the EZH2, Notch1 and PI3K/Akt signaling pathways. Taken together, quercetin-3-methyl ether considerably decreased H3K27 methylation, possibly by inhibiting EZH2 methyltransferase activity, which led to the repression of EMT promotion, CSC expansion and cell cycle dysregulation (Fig. 5). This agent also inhibited Notch1 and PI3K/Akt signaling, which resulted in the downregulation of protein markers associated with cell cycle, apoptosis, stem cell pluripotency, and self-renewal, including CDK1, Cyclin B1, Bcl-x1, Bcl-2, Sox2 and Nanog (Fig. 5).

In conclusion, the present study demonstrated that quercetin-3-methyl ether potently inhibited migration and invasion in TNBC and hormone-sensitive breast cancer cells; this may have occurred partly through the suppression of EMT and CSC induction, an effect mediated via inhibition of the Notch1, PI3K/Akt and EZH2 signaling pathways. These findings suggest that quercetin-3-methyl ether may be a potential chemopreventive and therapeutic drug for the targeted eradication of CSCs in breast cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Conception and design: LC, TL, KZ and JL; Development of methodology: LC, BL, JZ, CL and JL; Acquisition of data: LC, YY and ZY; Analysis and interpretation of data: LC, YY, ZY and JL; Writing, review, and/or revision of the manuscript: LC and JL; Administrative, technical, or material support: BL, JZ and CL; Study supervision: KZ and JL.

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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