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Original Article

Baicalein and U0126 suppress human breast cancer cell line MCF-7 through regulating MAPK signaling pathway

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Abstract: Backgroud: Baicalein is a member of active falvonoids compounds with anti-bacterial, anti-inflammatory, and anti-infection effect. The aim of the present study was to explore the inhibition effect of baicalein on human breast cancer cell line MCF-7 and the related mechanism. Methods: MCF-7 cells were treated by different concentrations of baicalein with or without U0126. Cell viability was tested by CCK8. Cell cycle was determined by flow cytometry. Cell migration was measured by wound healing assay. Cell apoptosis was detected by TUNEL and Annexin V/PI. The mRNA and protein levels of apoptosis related genes were detected by real time PCR and Western blot. Results: CCK8 assay revealed that baicalein suppressed MCF-7 cell viability in a dose-dependent manner. Baicalein induced MCF-7 cell apoptosis, and the effect was enhanced by U0126 ($P < 0.05$). Flow cytometry results showed baicalein restrained MCF-7 cells in G_0~G_1 phase. Wound healing assay demonstrated that baicalein obviously inhibited MCF-7 cells migration compared with control ($P < 0.05$). Baicalein significantly downregulated mRNA and protein levels of ERK1/2, GSK-3β, and p38 ($P < 0.05$). Conclusion: Baicalein inhibited MCF-7 cell proliferation, restrain cell cycle in G_0~G_1 phase, induced cell apoptosis, and suppressed cell migration in a dose-dependent manner. Baicalein played its role by regulating ERK expression. It can be used for breast cancer treatment.

Keywords: Baicalein, breast cancer, U0126, MAPK, apoptosis

Introduction

Breast cancer is a type of malignant tumor which occurs in the mammary gland epithelium and seriously threats to female health [1]. The cell proliferation, apoptosis, and migration of breast cancer are regulated by various signaling pathways, such as extracellular signal-regulated kinase (ERK), p38, and GSK-3β [2]. ERK is an important member of mitogen-activated protein kinase (MAPK) family. There are three main target molecules in ERK signaling pathway, such as Ras and its downstream Raf kinase, ERK1/2, and MEK1/2. EKR1/2 can be phosphorylated by numerous protein kinases and enter nucleus to promote cell entering S phase, leading to abnormal cell proliferation, apoptosis, and malignant metastasis [3]. P38 can regulate multiple cancer cell behaviors, such as cell cycle, apoptosis, epithelial mesenchymal transition, and drug resistance [4]. GSK-3β is a kind of serine/threonine protein kinase that can regulate cancer cell proliferation, differentiation, and apoptosis. It results in phosphorylation the serine/threonine residue of β-catenin, leading to its degradation [5].

Baicalein is a member of active falvonoids compounds of scutellaria baicalensis with anti-bacterial, anti-inflammatory, and anti-infection effect. It is mainly used in the treatment of paralysis after cerebrovascular disease as it can improve brain circulation, increase cerebral blood flow, and antiplatelet aggregation [6]. At present, some research reports indicated that baicalein inhibits a small number of cancer cells metastasis, such as liver cancer, prostate cancer, and bladder cancer. However, its role in

breast cancer has not been fully elucidated [7]. Furthermore, baicalein is reported to inhibit MAPK signaling pathway [8]. U0126 is a specific inhibitor of MEK1/2 that suppresses various tumors proliferation and metastasis. This study was aimed to investigate whether baicalein and U0126 play a synergistic effect in treatment of breast cancer.

Materials and methods

Cell line

Human breast cancer cell line (MCF-7 cell) was purchased from the Chinese academy of sciences, Shanghai insitute of biochemistry and cell biology cell bank. The MCF-7 cells were maintained in DMEM containing 10% fetal calf serum and cultured at 37°C and 5% CO_2. The cells were digested by 0.25% trypsin for passage.

CCK8 assay

Cell proliferation was detected using Cell counting kit-8 (Biotend). A total of 5×10^3 MCF-7 cells in 100 μl medium were seeded in 96-well plate. Different concentrations of baicalein (Sigma, USA) or U0126 (Alexis) were used to treat cells for 4 h with three replicates. Then 10 μl CCK8 solution was added to each well and incubated for 4 h. Finally, the plate was read at 450 nm to draw the curve.

Cell cycle detection

Cell cycle was tested using a cell cycle detection kit (Keygentec). MCF-7 cells were seeded in a 12-well plate and treated with baicalein or U0126. The cells were collected and washed with PBS for twice. Next, the cells were added to 1 ml 70% precooled ethanol at 4°C overnight. Then the cells were washed by PBS and added with 100 mg/L RNase at 37°C for 30 min. After stained by 50 mg/L PI at 4°C avoid of light for 30 min, the cells were detected on flow cytometry with the excitation wavelength at 488 nm. The primary result was analyzed by cell cycle matching software. All the experiments were repeated for three times.

TUNEL assay

MCF-7 cells were washed by PBS for twice and then fixed in 4% PFA at room temperature for 10 min. Next, the cells were treated by 3% hydrogen peroxide for 10 min to inhibit endogenous catalase. After incubated with protease at room temperature for 30 min, the cells were washed by PBS for twice. Then the cells were added with 50 μl TUNEL and incubated at room temperature for 60 min. At last, the cells were washed by PBS for three times and observed under microscope (Leica) to analyze the result.

Annexin V/PI assay

Cell apoptosis was detected using an Annexin V/PI assay kit (BD). After treated with baicalein or U0126, MCF-7 cells were collected and washed by PBS for twice. A total of 1×10^6 cells were resuspended in 400 μl 1× binding buffer and added with 5 μl Annexin V-FITC avoid of light for 15 min. Then the cells were added with 10 μl PI and incubated avoid of light for 5 min. The cells were then tested on flow cytometry. The results were analyzed by Cell Quest software. All the experiments were repeated for three times.

Wound healing assay

A total of 6×10^6 MCF-7 cells were seeded in 24-well plate for 24 h to form monolayer cell. Then the cells were scratched by sterile toothpick to form a straight line. After washed by PBS, the cells were treated by baicalein or U0126. At last, the plate was observed under the microscope at different time point to calculate the line width.

Real time PCR

Total RNA was extracted using Trizol reagent (Invitrogen) and reverse transcripted to cDNA using K1622 kit (Thermo Fermentas). The primers were designed by Primer 6.0. Real time PCR was applied to test target gene expression. Reaction condition: 55°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was used as internal reference. $2^{-\Delta Ct}$ was applied to calculate relative expression level. The primers sequences were as follows: ERK1/2, forward, 5'-AAT CAC ACG GTA GAC ACT GAA ATG CC-3', reverse, 5'-CAT CAT CCC ATC TAA AAT GTC CCC TG-3'. Bax, forward, 5'-CATATAACCCCGTCAACGCAG-3', reverse, 5'-GCAGCCGCCACAAACATAC-3'. Bcl-2, forward, 5'-GTCTTCGCTGCGGAGATCAT-3', reverse, 5'-CATTCCGATATACGCTGGGA-3'. Cyclin D1, forward, 5'-GCTGCGAAGTGGAAACCATC-3', rever-

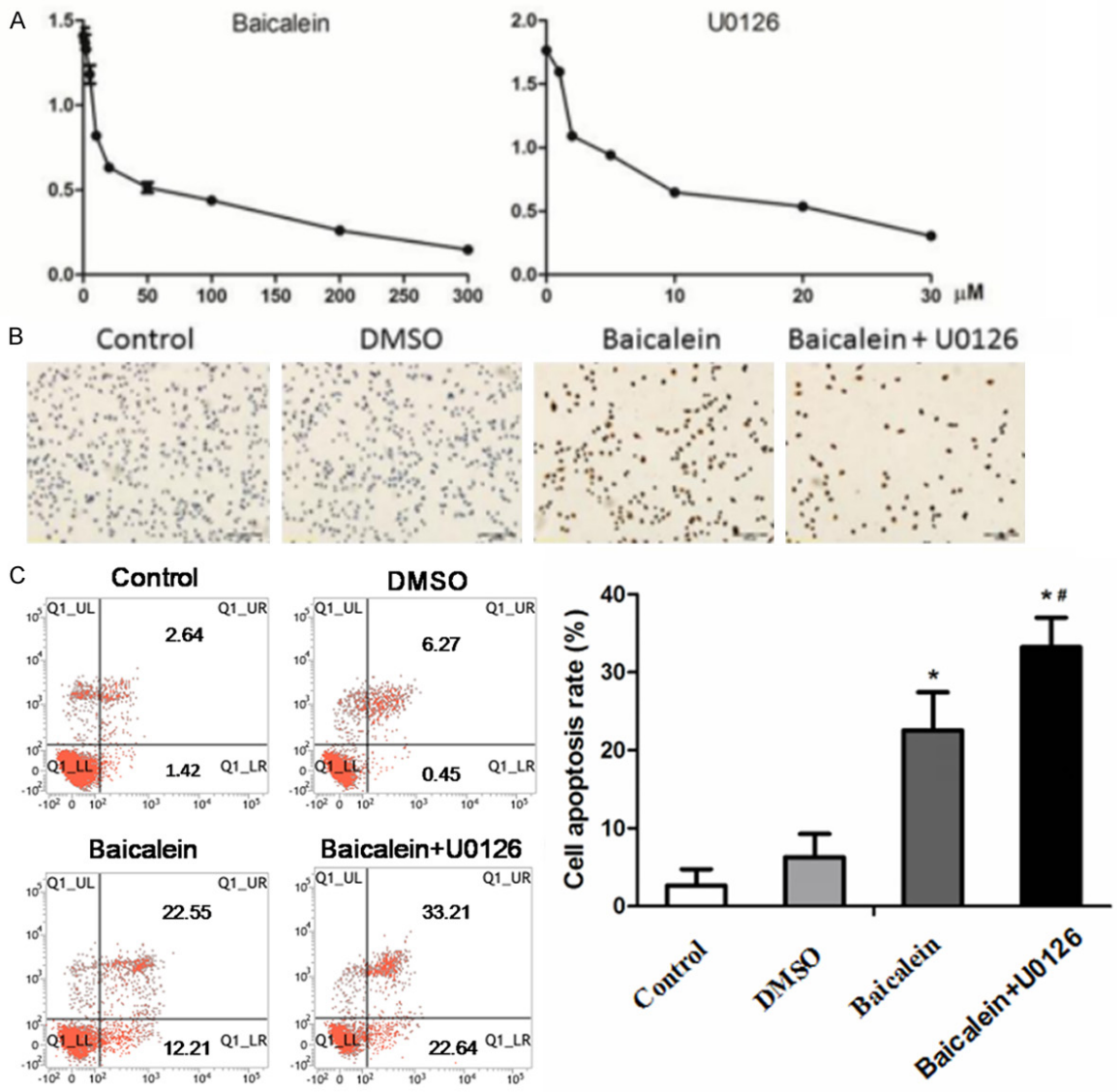

Figure 1. Baicalein suppressed MCF-7 cell proliferation and induced cell apoptosis. A. MCF-7 cell viability detected by CCK-8. B. MCF-7 cell apoptosis detected by TUNEL. C. MCF-7 cell apoptosis tested by Annexin V/PI double staining. *P < 0.05 vs. Control group; #P < 0.05 vs. Baicalein group.

se, 5'-CCTCCTTCTGCACACATTTGAA-3'. GSK-3β, forward, 5'-GGCAGCATGAAAGTTAGCAGA-3', reverse, 5'-GGCGACCAGTTCTCCTGAATC-3'. p38, forward, 5'-AACATCCTGTCGTCGCCTTAC-3', reverse, 5'-ACGTGCGTGACCTTAAAGTAGA-3'. GAPDH, forward, 5'-GCACCGTCAAGGCTGAGAAC-3', reverse, 5'-TGGTGAAGACGCCAGTGGA-3'.

Western blot

MCF-7 cells were cracked on ice for 15-30 min after added with protease inhibitor and lysis to extract protein. After centrifuged at 12,000 r/min for 15 min, the supernatant was moved to a new Ep tube. The protein was separated by 10% SDS-PAGE and transferred to PVDF membrane. After blocked by 5% skim milk for 1.5 h, the membrane was incubated in anti-p-p38 (Cell Signaling), p-GSK-3β (Cell Signaling) p-ERK1/2 (Santa Cruz), anti-Bax (BD), anti-Bcl-2 (Calbiochem), or anti-GAPDH (Shanghai Shenneng Bocai co., LTD) for 2 h. Then the membrane was incubated with secondary antibody (Beijing Xihuayi science and technology co., LTD) for 30 min and washed by PBST. Finally, the membrane was developed by DAB and scanned. Protein image processing system and Quantity one software were used for data

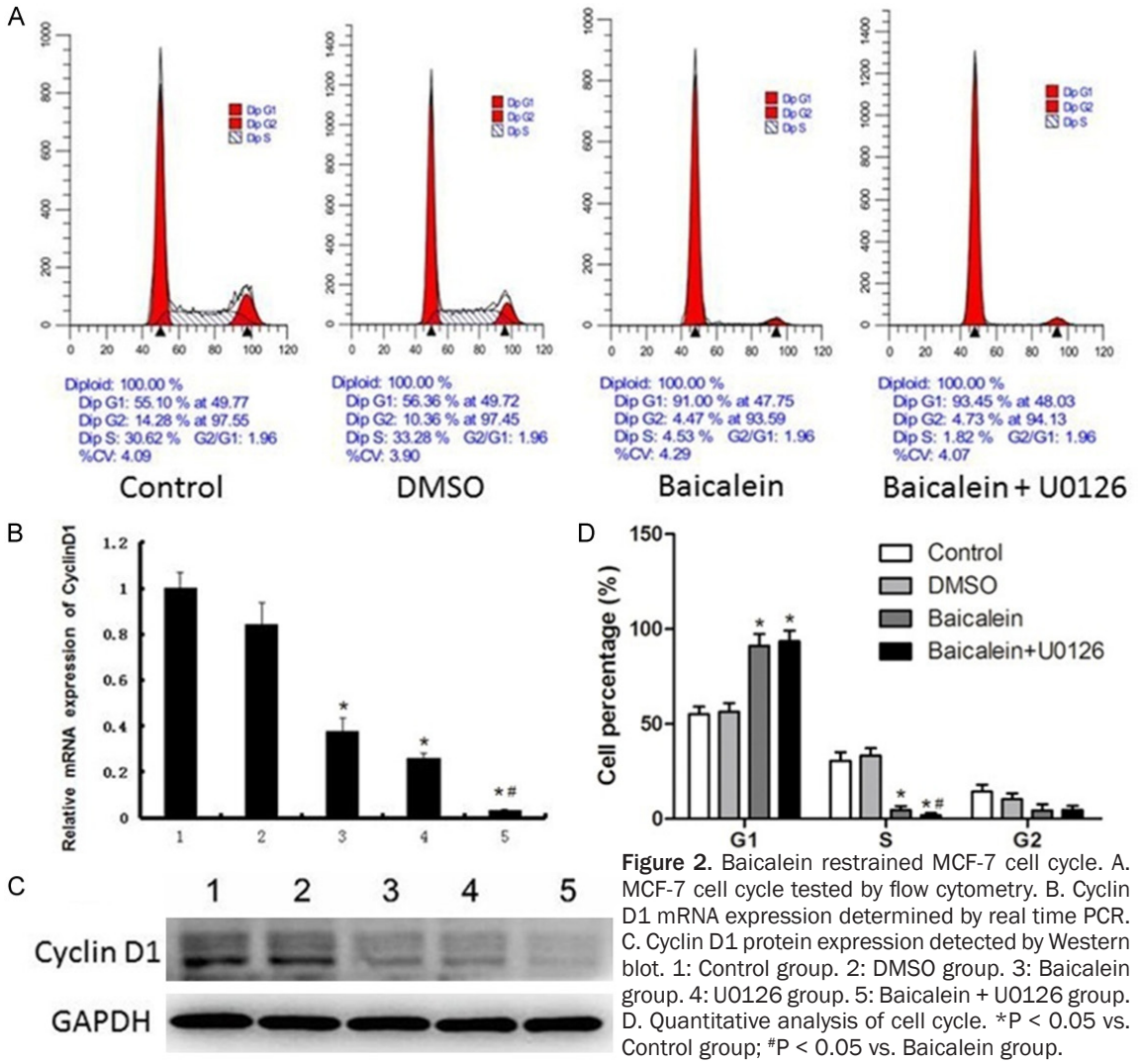

Figure 2. Baicalein restrained MCF-7 cell cycle. A. MCF-7 cell cycle tested by flow cytometry. B. Cyclin D1 mRNA expression determined by real time PCR. C. Cyclin D1 protein expression detected by Western blot. 1: Control group. 2: DMSO group. 3: Baicalein group. 4: U0126 group. 5: Baicalein + U0126 group. D. Quantitative analysis of cell cycle. *P < 0.05 vs. Control group; #P < 0.05 vs. Baicalein group.

analysis. All experiments were repeated for three times.

Statistical analysis

All the data was presented as mean ± standard deviation and analyzed by SPSS19.0 software. T-test or one-way ANOVA was applied for data comparison. P < 0.05 was considered as statistical difference.

Results

Baicalein suppressed MCF-7 cell proliferation and induced cell apoptosis

To explore the inhibitory effect of baicalein on MCF-7 cells, different concentrations of baicalein (1 μM, 2 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM, 200 μM, and 300 μM) was used to treat MCF-7 cells for 24 h. CCK8 results showed that cell viability was gradually decreased following baicalein dose elevation. Meanwhile, we also tested the effect of MEK inhibitor U0126 on MCF-7 cells. CCK8 results also demonstrated that MCF-7 cell viability declined treated by U0126 (**Figure 1A**). It indicated that both baicalein and U0126 can suppress MCF-7 cell proliferation.

We further explored the effect of baicalein on cell apoptosis. We treated MCF-7 cells with 20 μM baicaleina and 10 μM U0126. TUNEL assay revealed that baicalein caused cell apoptosis, while U0126 aggravated such effect (**Figure 1B**).

Next, Annexin V/PI staining was used to test which phase of cell apoptosis was baicalein mainly act on. The results presented that 20

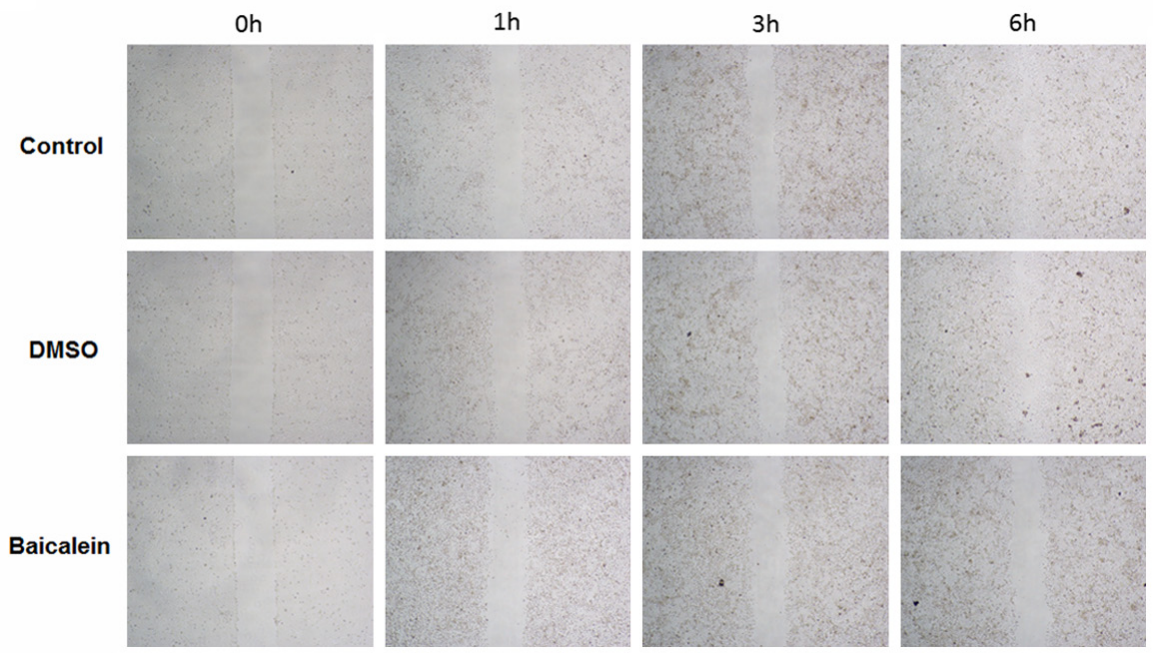

Figure 3. Baicalein inhibited MCF-7 migration. MCF-7 cell migration ability determined by wound healing assay.

μM baicalein increased both early and late phases of apoptosis, and U0126 enhanced the effect (**Figure 1C**).

Baicalein restrained MCF-7 cell cycle

As ERK can regulate cell cycle, we tried to clarify the effect of baicalein on cell cycle. Baicalein (20 μM) was added to MCF-7 cells and incubated for 24 h. The results of flow cytometry showed that compared with control group, cell content was obviously increased in G_0~G_1 phase, and reduced in S phase. And there is no significant change in G_2~M phase between control group and baicalein group (**Figure 2A** and **2D**). U0126 markedly declined cell content in S phase compared with single baicalein group (**Figure 2A** and **2D**).

We also detected the expression of cyclin D1 in MCF-7 cells treated by baicalein, which is an important protein of cell cycle. Both RT-PCR and Western blot revealed that cyclin D1 mRNA and protein levels were down-regulated after 20 μM baicalein treatment for 24 h (**Figure 2B** and **2C**). Inhibiting ERK1/2 further declined cyclin D1 expression.

Baicalein inhibited MCF-7 migration

Tumor metastasis is an important factor that leads to poor prognosis. MCF-7 cells were treated with 20 μM baicalein for 1 h, 3 h, and 6 h. Wound healing assay demonstrated that the scratch width in baicalein group was obviously larger than that in control group, suggesting that baicalein restrained MCF-7 cell migration (**Figure 3**).

Baicalein affected MAPK signaling pathway expression in MCF-7 cells

Previous study reported that baicalein can regulate ERK signaling pathway. The effect of baicalein on ERK signaling pathway and the expression of related proteins in MCF-7 cells was detected. Real time PCR results showed that baicalein obviously decreased the mRNA levels of ERK1/2, GSK-3β, and p38 in MCF-7 cells. Moreover, it significantly elevated mRNA expression of pro-apoptotic factor Bax, and down-regulated mRNA level of apoptosis suppression gene Bcl-2 (**Figure 4A**).

Furthermore, we examined expression of related protein in ERK signaling pathway. Western blot revealed that phosphorylation levels of ERK1/2, GSK-3β, and p38 were markedly declined in MCF-7 cells treated by baicalein. The protein level change of Bax and Bcl-2 were also presented similar trend with change of mRNA expression. Treatment of baicaleinand U0126 suppressed ERK signaling pathway

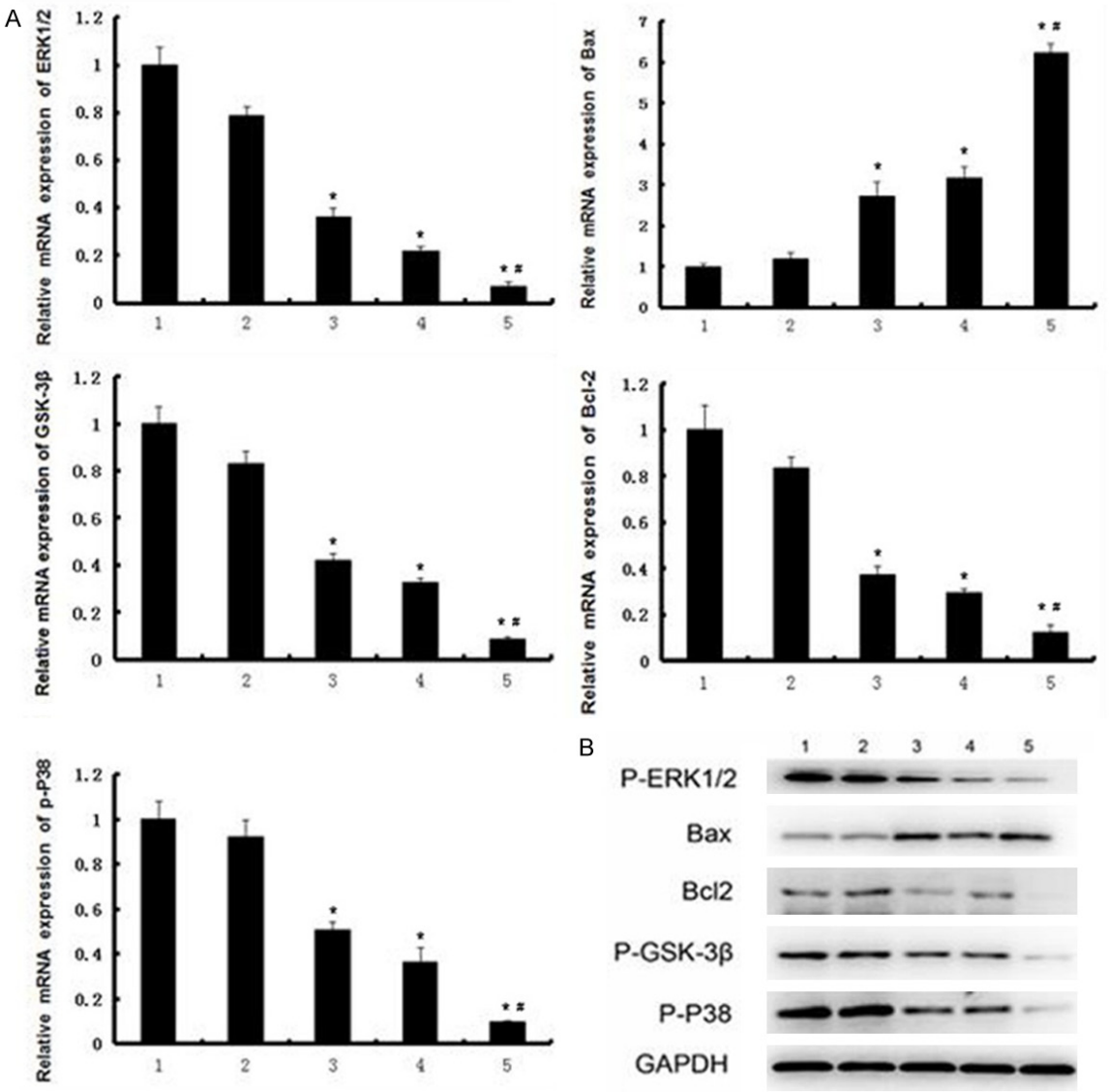

Figure 4. Baicalein affected MAPK signaling pathway expression in MCF-7 cells. A. ERK1/2, p38, GSK-3β, Bax, and Bcl-2 mRNA expression tested by real time PCR. B. ERK1/2, p38, GSK-3β, Bax, and Bcl-2 protein expression detected by Western blot. 1: Control group. 2: DMSO group. 3: Baicalein group. 4: U0126 group. 5: Baicalein + U0126 group. *P < 0.05 vs. Control group; #P < 0.05 vs. Baicalein group.

related proteins expression, compared with single baicalein treatment (**Figure 4B**).

Discussion

Breast cancer is one of the most common malignant tumors that threats to female health. As the highest malignant tumor incidence in female, breast cancer accounts for 30% of the morbidity and 20% of mortality [9]. Moreover, its prevalence rate shows rising trend following environment and dietary habit changes. For instance, menarche in advance, delay menopause, infertility, or later childbirth will increase the incidence of breast cancer [10]. Therefore, effective controlling of breast cancer cell proliferation and metastasis is of great significance for cancer therapy.

Flavonoids are the metabolites of polyphenols plant, mainly including isoflavones flavone, flavonols, flavanone, and isoflavanone, et al. [11]. Flavonoids possess a variety of biological activities, such as anti-inflammation, anti-virus, anti-oxidation, anti-aging, anti-tumor, and immune regulation [12]. Baicalein is an effective compo-

nent extracted from scutellaria root, which can be used for the treatment of liver cancer, prostate cancer, and bladder cancer. However, there is still lack of reports about the relationship between baicalein and breast cancer. Thus, clarifying the molecular mechanism of baicalein in breast cancer has profound clinical significance [13]. Our results revealed that baicalein suppressed breast cancer cell proliferation and induced cell apoptosis in adose dependence manner, suggesting that baicalein might be an important agent for the treatment of breast cancer.

U0126 is a high selective inhibitor of MEK1/2 that regulates the activity of MAPK signaling pathway [9]. Previous reports confirmed that ERK, GSK-3β, JNK, and p38 signaling pathways have the ability to promote cell growth and proliferation of breast cancer cells, and enhance drug resistance of breast cancer [14]. Our results showed that baicalein or U0126 significantly down-regulated phosphorylation levels of ERK, GSK-3β, p38, and JNK to suppress related pathways. Combined application of baicalein and U0126 presented synergistic inhibitory effect, resulting in more obvious restrain effect on breast cancer cells. Therefore, baicalein and U0126 suppressed cell proliferation by blocking cell growth signaling pathway.

Induction of cancer cell apoptosis is a common strategy for breast cancer therapy [15]. Bcl-2 family was proved to regulate cell apoptosis through mitochondrial pathway, of which Bax is a pro-apoptotic protein and Bcl-2 is an anti-apoptotic protein. Baicalein elevated the ratio of Bax and Bcl-2, leading to the pore formation on outer membrane of mitochondria and to the activation of caspase. Combined application of baicalein and U0126 further augmented such effect, indicating that baicalein and U0126 possess synergistic interaction [16].

Cancer cell metastasis is an important reason of high fatality rate. Breast cancer is most likely to transfer to bone, lung, pleura, liver, and brain [17]. Wound healing assay is usually used to test the effect of drug on cancer cell migration *in vitro*. Our results showed that baicalein obviously inhibited MCF-7 cells migration, suggesting that baicalein may play a critical role in controlling breast cancer progression.

It has been reported that phosphorylation level of ERK1/2 significantly enhances in high metastatic tendency breast cancer cells and tissue [18]. It is important for cell proliferation by promoting cells from G1 phase to S phase [19]. GSK-3β is an important factor in Wnt signaling pathway which has the ability to activate Bax, c-jun, caspase, and cyclin D1 [20]. P38 also plays a critical role in G1/S and G2/M checkpoint. Our results showed that baicalein restrained MCF-7 cells in G_0~G_1 phase, U0126 addition further declined cell content in S phase. Moreover, as an important regulator of cell cycle, cyclin D1 expression obviously decreased in MCF-7 cells treated by baicalein or U0126. All of these results implied that baicalein affected breast cancer cell cycle through regulating ERK1/2 and GSK-3β.

Conclusion

Baicalein induced breast cancer cell apoptosis, block cell cycle, and inhibit cell migration through regulating ERK, GSK-3β, and p38 signaling pathway. It also presented synergistic effect with MEK1/2 inhibitor U0126, which provide new strategy and theoretical basis for breast cancer treatment in clinic.

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Disclosure of conflict of interest

None.

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