Naringin suppressing the PI3K/Akt signaling pathway as a drug target in BIU87 cells

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Abstract

Background: Flavonoids are regarded as potential anti-cancer drugs due to their toxicity and anti-proliferation functions. Naringin, which is a kind of flavanone compounds targeting anti-tumor treatment, has been focused on reliving several types of cancers, besides of bladder cancer. This paper was aimed to investigate whether and how naringin induced bladder cancer cells.

Method: MTT, cell-cycle, transwell and cell apoptosis were used to assess the cellular responses. Molecular expressions and the signaling pathway of naringin inducing BIU87 cells were evaluated by western blot and real-time quantitative PCR.

Results: It was found that naringin effectively suppressed proliferation and invasion of BIU87 cells by downregulating the expression of PI3K and its downstream pathway. The corresponding proteins related to viability and motility, including CDK2, Bcl-2 and Fascin, were inhibited.

Conclusion: It can be concluded that naringin targeting to PI3K in bladder tumor cells expressed abundantly and inhibited the growth and invasive ability of tumor cells.

Background

Bladder cancer, which is a kind of genitourinary malignant disease, accounts for nearly 4.5% of the worldwide cancer burden and has been the fifth most common cancer [1–3]. By now, the therapeutic effect of bladder cancer is not ideal because of its complicated biological behavior. Although the chemoprevention in clinic is widespread, urothelial cancer still recurs frequently, even if the primary cancer has been completely removed [4]. Nowadays, numerous of tumor suppressor genes, which have been implicated in inducing bladder carcinogenesis, are regarded as novel targets for therapy or prevention this disease[5].

The phosphatidylinositol 3-kinase (PI3K) signaling pathway plays a vital role in cancer cells growth, proliferation, survival and metabolism. Akt occupies a key regulatory node in the PI3K pathway, below which the pathway branches significantly influence a wide range of cellular processes that promote cell cycle progression, cell growth, energy metabolism and resistance to apoptosis by inhibiting proapoptotic Bcl-2 family members[6, 7]. Suppression of the PI3K/Akt leads to the blockade of cell proliferation, and it demonstrates the cellar responses of diminishing cells cycle progression, promoting cells death during cancer development [8]. Accordingly, using the PI3K inhibitors for treatment the bladder cancer can be considered to be a promising method to decrease the tumor suppressive activity [9].

Naringin (4', 5, 7-trihydroxy-flavonone-7-rhamnoglucoside) is a citrus flavonoid, which can be found in the pericarp citrus and sour flavor fruits that have been proven no side effects to human bodies [10, 11]. The bioactivities of naringin and its potential pharmacological properties for treating diseases, including cardiovascular health, hyperglycaemia, and Alzheimer's disease [12], and so on, appear to be useful to

relieve pains. Recently, naringin was shown anti-cancer activity due to its antioxidant, anti-free radicals, inhibition of proliferation and inducing apoptosis of cancer cells [13]. It was reported that naringin could decrease the expression of TNF-α and inhibit the growth of W256 cells significantly [14]. Furthermore, naringin also played a protective role in liver and decreased the occurrence of liver cancer [15]. Until now, however, the relationship between naringin and inhibitory of bladder cancer has not been well understood. According to the previous studies, we hypothesized that naringin may suppress the bladder cancer due to regulating the corresponding signaling pathways. To assess the hypothesis, the present work investigated the effect of naringin against the proliferation and migration/invasion of bladder cancer cells, expecting to provide more supporting evidences for the application of naringin in bladder cancer therapies in clinic.

Methods

Cells culture

Human bladder cancer cell lines (BIU87) and human embryonic kidney 293 cell lines (HEK293), which were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China, were cultured in DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) in an incubator at 37℃.

MTT assays

The MTT assay was performed on manufacturer's specifications strictly. 100 μL cell suspensions at a concentration of 1×10^5 were added in 96-well plates. After attachment, the medium was replaced with DMEM that containing different concentrations of naringin (10 μmol/L, 20 μmol/L, 30 μmol/L). For incubating for 12, 24, 36, 48 and 60 h, respectively, 10 μL modified tetrazolium salt-3-(4–5 dimethylthiozol-2yl)-2-5diphenyl-tetrazolium bromide (MTT, Sigma, USA) solution (5 mg/mL in PBS) was added. For incubating 4 h, 150 μL DMSO was to dissolve the crystals. The optical density at 490 nm was measured by micro-plate reader (Bio-rad, USA). The relative growth rate (RGR) was calculated as follows.

$$RGR\ (\%)=(OD_{treatment}/OD_{control}) \times 100\%$$

where $OD_{treatment}$ is the optical density of naringin treatment group, and $OD_{control}$ is the optical density of control group.

Cell-cycle assay

BIU87 cells were seeded in 6-well plates and incubated overnight for attachment. Then, medium was replaced with an indicated concentration of naringin. After incubation for 24 h, cells were washed with PBS twice and re-suspended in a staining buffer solution, and then were analyzed with FACS Vantage flow cytometer (Becton Dickinson and Co., USA).

Cell migration and invasion assay

Migration and invasion assays were performed according to the instruction of modified boyden chambers, with the parameters of membrane that 6.5 mm in diameter, 8 μm pore size, Matrigel 100 μg/cm^2. 100 μL DMEM supplemented with 0.1% bovine serum albumin containing 1×10^5 cells were added into the upper insert. The inserts were then transferred to wells of a 24-well plate filled with 600 μL DMEM containing 10% serum and indicated concentrations of naringin. After incubating for 18 h, non-invaded cells were removed, and the invaded cells were fixed with 95% ethanol, stained with trypan blue, and imaged at 5 random locations.

Cell apoptosis

Cells exposed to naringin at an indicated concentration for 24 h were harvested, washed twice with ice-cold PBS, then re-suspended in the dark with Annexin V-FITC and PI buffer (Sigma, USA). The cells and the corresponding apoptosis rate was analyzed by flow cytometry and Mod Fit LT software.

Hochest 33258 staining

BIU87 cells were treated as the same method as cell apoptosis, and then fixed with 4% polyoxymethylene. After washing, 10 μg/mL hochest 33258 (Merck, Germany) were added to the wells. After incubating at room temperature on a shaker for 30 min out of light, cells were observed with fluorescence microscope after washing with PBS and drying in air.

Reverse transcription and quantitative real-time PCR

Cells were treated using the same protocol as that for cell apoptosis described above. Total RNA was isolated using Trizol reagent (Invitrogen, USA) and was reversed and transcribed into complementary DNA (cDNA) using a RT reaction kit (Promega, USA). Gene expression was quantified by using Mx 3000P real-time PCR system (Applied Biosystems, USA) and SYBR Premix Ex Taq (TaKaRa, Japan). The expression levels were normalized to the housekeeping gene GAPDH. The primer for selected genes is listed in Table 1.

Table 1
Sequences of primer-pairs

Name	Forward primer (5'->3')	Reverse primer (5'->3')
Cyclin E	CCATCATGCCGAGGGAGC	TTTGCCCAGCTCAGTACAGG
CDK2	GCCATTCTCATCGGGTCCTC	ATTTGCAGCCCAGGAGGATT
Bcl-2	GGTGAACTGGGGGAGGATTG	GGCAGGCATGTTGACTTCAC
Bax	AGCTGAGCGAGTGTCTCAAG	GTCCAATGTCCAGCCCATGA
PI3K	GGACCCGATGCGGTTAGAG	ATCAAGTGGATGCCCCACAG
FASCIN	TCTGGGAGTACTAGGGCCAC	CCAGTTTGAAAGGCAAGGGC
GAPDH	GAGAAGGCTGGGGCTCATTT	AGTGATGGCATGGACTGTGG

Western blotting analyses

Cells were treated with the same cells apoptosis method mentioned above and harvested on the ice. The cells were prepared for western blot analysis as described previously [16]. The antibodies used for western blotting analysis are PI3K, Fascin, Cyclin E, CDK2 (Bioworld, USA), Akt, phospho-Akt (Ser473) (Santa Cruz, USA), Bax and Bcl-2 (Abcam, USA).

Statistical analysis

Each experiment was repeated at least three times. Data were presented as the mean ± standard (SD). Difference between groups were analyzed by one-way analysis of variance (ANOVA) by SPSS 18.0 software. Significant difference was shown as $p < 0.05$.

Results

To evaluate the inhibitory effect of naringin against growth of BIU87 and HEK293 cells, the dose-response study with varying concentrations of naringin (0, 10, 20 and 30 μmol/L) was performed. As shown in Fig. 1 (b), the proliferation of BIU87 cells exposure to naringin was inhibited in a concentration dependent manner at any time point, which was compared with that exposure to DMSO. However, different treatments of naringin toward HEK293 cells were not exhibited markedly influence on cells viabilities. In order to further investigate the naringin inhibiting the growth of bladder cancer cells, cycle analysis of BIU87 cells exposed to naringin for 24 h was performed. It was shown that naringin exhibited the ability of inducing a dose-dependent increase in the percentage of cells in G_1 phase and a corresponding decrease in S phase compared to control, which provided more supporting evidence that naringin blocked BIU87 cells at the G_1 phase of the cell cycle, as depicted in Fig. 1 (c). In order to demonstrate the blocking performance on transformation of BIU87 cells from G1 phase to S phase in the cell cycle, western blotting analysis and PCR were used to examine the expression of Cyclin E and CDK2, which are the key

regulators in the G_1 phase. The expression levels of Cyclin E and CDK2 in BIU87 cells that treated with 20 or 30 μmol/L naringin for 24 h were decreased, which were shown in Fig. 1 (d) and Fig. 1 (e). It can be concluded that naringin arrested cells at G1 phase and then suppressed cells growth via down-regulated Cyclin E and CDK2.

Hochest 33258 was used to stain the apoptosis of BIU87 cells induced by naringin. Figure 2 (a) shows the naringin-induced apoptotic nucleus of BIU87 cells. It can be observed that the condensed chromatin in naringin treated cells was increased with higher concentration of naringin. Figure 2 (b) depicts the specific values of early apoptosis rates and the scatter plots of BIU87 cells detected by flow cytometry. It was shown that the naringin-induced BIU87 cells exhibited a slightly higher apoptosis level than that control cells incubated with DMSO. Western blot analysis showed that Bax was up regulated and Bcl-2 was down regulated in dose-dependent manner, as plotted in Fig. 2 (c). Furthermore, the Bax and Bcl-2 gene expressions in BIU87 cells were up and down regulated at mRNA level with a significant difference after exposing to 20 and 30 μmol/L naringin (Fig. 2(d)).

Transwell assay (with or without matrigel) was used to evaluate the inhibitory effect of naringin on HEK293 and BIU87 cells. Figure 3 (a) and Fig. 3 (b) show that naringin significantly decreased the tendency of invasion and migration ability of BIU87 cells with an increase of concentration of naringin, whereas that has no remarkable influence on normal HEK293 cells. Figure 3 (c) and Fig. 3 (d) plot the western blot analysis and RT-PCR results of Fascin expression at protein and mRNA levels, respectively, which showed that BIU87 cells exposure to naringin (20 or 30 μmol/L) for 24 h was decreased dramatically.

PI3K/Akt pathway is frequently altered in majority of human cancers, indicating that PI3K might be targeted to influence bladder cancer cells proliferation and migration after inducing with naringin. Accordingly, the PI3K pathway in BIU87 cells was evaluated. As demonstrated in Fig. 4 (a-b), naringin at 20 μmol/L markedly inhibited the expression of PI3K in protein and mRNA levels. The cancer-related cytokines PI3K and $Akt^{p-Ser473}$ were obviously negatively regulated after treatment with naringin, whereas the expression of Akt was almost no changing. Furthermore, it can be found from Fig. 4 (c-f), the levels of downstream proteins including CDK2, Cyclin E, Bcl-2 and Fascin were all suppressed by naringin, demonstrating that naringin could effectively restrain proliferation and invasion of BIU87 cells by regulating PI3K/Akt pathway.

Discussion

Inhibition of PI3K signaling, which is considered as the vital regulator of cancer cells, can diminish cells proliferation, and in some circumstances, promote cells death. Significant efforts on generating and evaluating inhibitors of the PI3K pathway have been made, meanwhile, cancer patients are necessary to define PI3K as a promising target for curing this disease [17, 18]. The most advanced PI3K pathway inhibitors are rapamycin analogs that specifically inhibit mTORC1[19, 20]. The relationship between the

PI3K-AKT-mTOR pathway and cancer development, which induces the genetic variations as a result of bladder cancer risk, have been established [7, 21].

Recently, flavonoids isolated from herbs have attracted many researchers interest because of their anticancer effects on various cancer cells. Naringin, which is mainly recognized as blocking PI3K/Akt pathway, appears through cell cycle arrest and apoptosis and finally interrupts cells growth [22–24]. It was reported that naringin could inhibit breast cancer, lung cancer and cervical cancer, and so on [25–27]. Consistently, we confirmed the antitumor potential of naringin on bladder cancer and focused on how naringin induced PI3K/Akt pathway in bladder cancer cells.

As it knows, the PI3K/Akt mediates cellular processes as a fundamental signaling pathway. In the present study, naringin could effect on suppressing the proliferation, migration and invasion of BIU87 cells with an increase of concentration compared with untreated control cells. Viability of BIU87 cells was inhibited, accompanied with the induction of G1 cell cycle-related proteins, such as CDK2 and cyclin E, which regulated the cell cycle and apoptosis. Usually, cell cycle aperiodicity is a classic feature of tumor cells [28]. BIU87 cells treated with naringin accumulated mostly in the G1 phase in a dose-dependent manner, whereas that distributed less in the S phase, indicating that the growth of BIU87 cells were interrupted by blocking G1/S phase progression. Furthermore, downregulating the protein levels of CDK2 and cyclin E, regulating the ratio of G1 phase and S phase, provided molecular evidence for anti-proliferation [29].

Apoptosis is programmed cell death [30]. Typical pro-apoptotic proteins, such as Bax, translocate to the mitochondrial membrane and make the mitochondrial outer membrane status by the membrane permeable. On the contrary, Bcl-2 is an anti-apoptotic protein, which maintains the mitochondrial membrane status [24, 31]. Consequently, the equilibrium of the expression levels of pro- and anti-apoptotic proteins is a key factor of cells apoptosis [32]. It was found that naringin promoted BIU87 cells apoptosis through downregulating Bcl-2 level and whereas that through increasing Bax expression. The Bax/Bcl-2 ratio of naringin treated cells was higher than that of control, which promoted apoptosis, especially early apoptosis.

Moreover, cells proliferation, migration and invasion of BIU87 stimulated by naringin were also inhibited. In general, fascin is overexpressed in tumor tissues and plays a role in tumor cells invasion and metastasis [32]. It was reported that fascin was correlated with breast cancer patients who received chemotherapy, which was mainly mediated via PI3K/Akt pathway [34]. The present motility assays demonstrated that naringin suppressed the migration and invasion of BIU87, at the same time down-regulated the fascin. So it can be inferred that naringin modulated PI3K/Akt/ fascin pathway by fascin targeting inhibition of cell invasion and metastasis.

The results corresponding to PI3K provide a supplement for anti-cancer potential of naringin. What's more, a specific mechanism that naringin targets to PI3K and its downstream signaling pathways mediating BIU87 behaviors, including negatively regulating tumor cells survival and invasion, has been established. The present results have widened the application of PI3K inhibitors and might attracted the

researchers' attention on targeting therapy of bladder cancer. Nevertheless, only a preliminary study on naringin and its effect on BIU87 cells were performed. Further studies should be focused on addressing how naringin plays an essential role and how to regulate PI3K pathway in vivo, providing more evidences for this natural anti-cancer candidate drug in clinic.

Conclusions

This study investigated naringin suppressed the proliferation of BIU87 cells with blocking G1/S phase and inhibiting the expression of CDK2 and Cyclin E. The mechanism of naringin inhibiting BIU87 cells migration and invasion, and inducing apoptosis were due to regulating PI3K/Akt pathway. Thus, these results suggest that naringin can exert antitumor effect on BIU87 cells and can be potentially used as a candidate therapy of bladder cancer in clinic.

Declarations

List of abbreviations

MTT= modified tetrazolium salt-3-(4-5 dimethylthiozol-2yl)-2-5diphenyl-tetrazolium bromide; BIU87= human bladder cancer cell lines; PI3K= phosphatidylinositol 3-kinase; CDK2= cyclin-dependent kinases 2; Bcl-2= B-cell lymphoma-2; Akt= protein kinase B; TNF-α= tumor necrosis factor-α; HEK293= human embryonic kidney 293 cell lines; DMEM= dulbecco modified eagle medium; FBS= fetal bovine serum; DMSO= dimethyl sulfoxide; PBS= phosphate buffer saline; SDS-PAGE= sodium dodecylsulphate polyacrylamide gel electrophoresis; PVDF= polyvinylidene difluoride; ECL= enhanced chemiluminescence reagent.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The materials from this study are available from the corresponding author on reasonable request.

Competing interests

The authors declared no competing interests. There was no research involving human participants and animals study. All authors reviewed the manuscript and agreed to publish this paper.

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Authors' contributions

JZ Li: Project development, Data analysis, Manuscript writing. J Zhao: Project development, Data analysis, Manuscript editing. HY Zhang: Data analysis. MX Liu: Data management. X Li: Project development. All authors have read and approved the manuscript.

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Figures

FIG 1

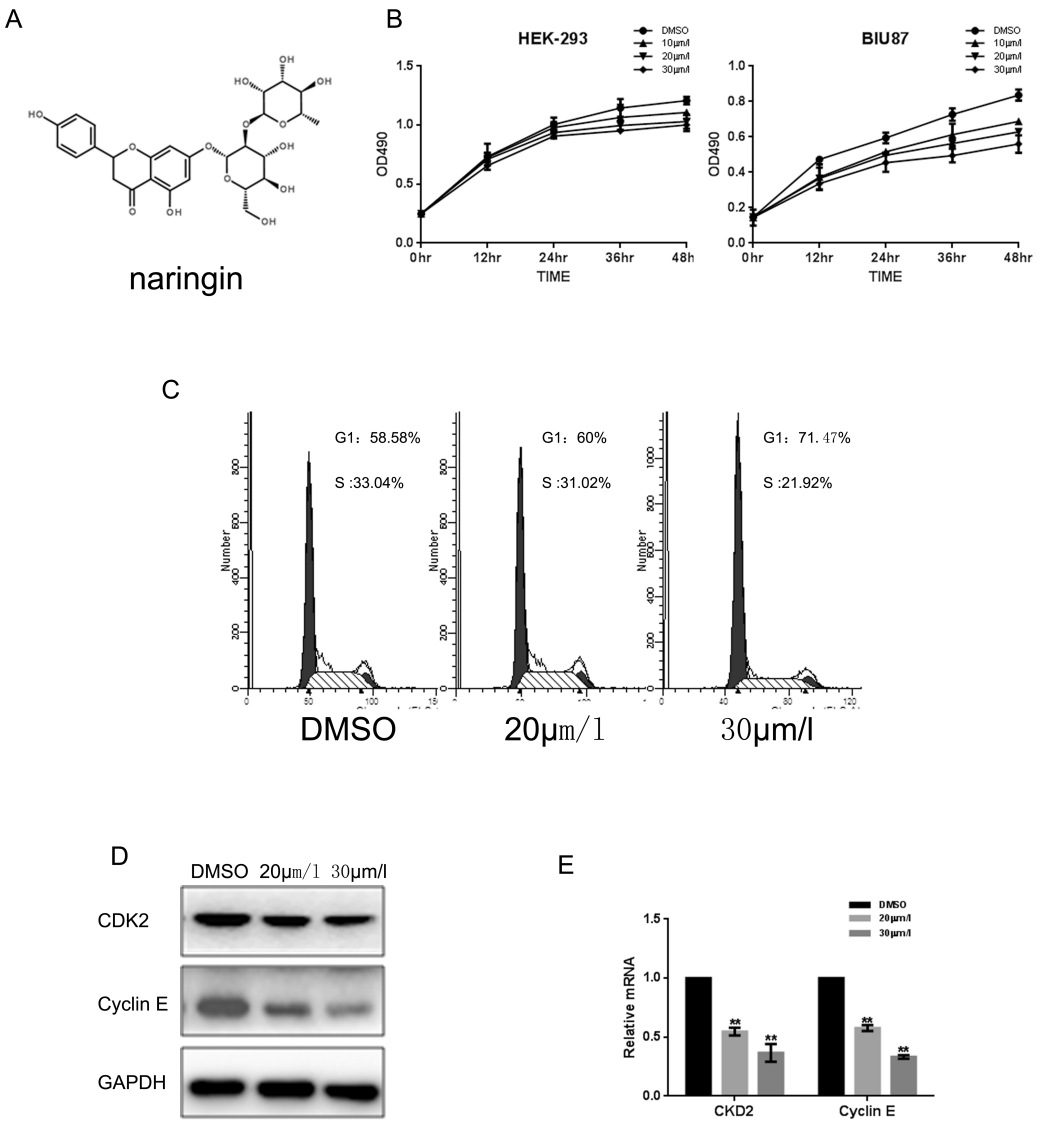

Figure 1

Naringin inhibits the proliferation of bladder cancer cells. (a) The structure of naringin, (b) proliferation analyses of BIU87 and HEK293 cells detected by MTT, (c) cell cycles of BIU87 cells performed by FACS vantage flow cytometer, (d) western blotting analysis of Cyclin E and CDK2 related protein expressions, (e) real-time quantitative PCR analysis of Cyclin E and CDK2 related gene expressions. BIU87 and HEK293 cells were cultured in the plates and treated with 20 mmol/L DMSO (referred to as control group),

or in the presence of indicated concentrations of naringin (10 μmol/L, 20 μmol/L or 30 μmol/L) for 24 h, respectively. MTT solution was added into the wells and measured at 490 nm. Cells were harvested and lystaed for western blotting analysis. Anti-GAPDH antibody was used for the evaluation of equal protein loading on the blot. For PCR test, the relative mRNA levels were expressed as fold from housekeeping gene (GAPDH) and then calculated relative to control (DMSO treatment group was as referred). Data were represented as mean ± SD. All experiments were repeated three times. *p<0.05.

FIG 2

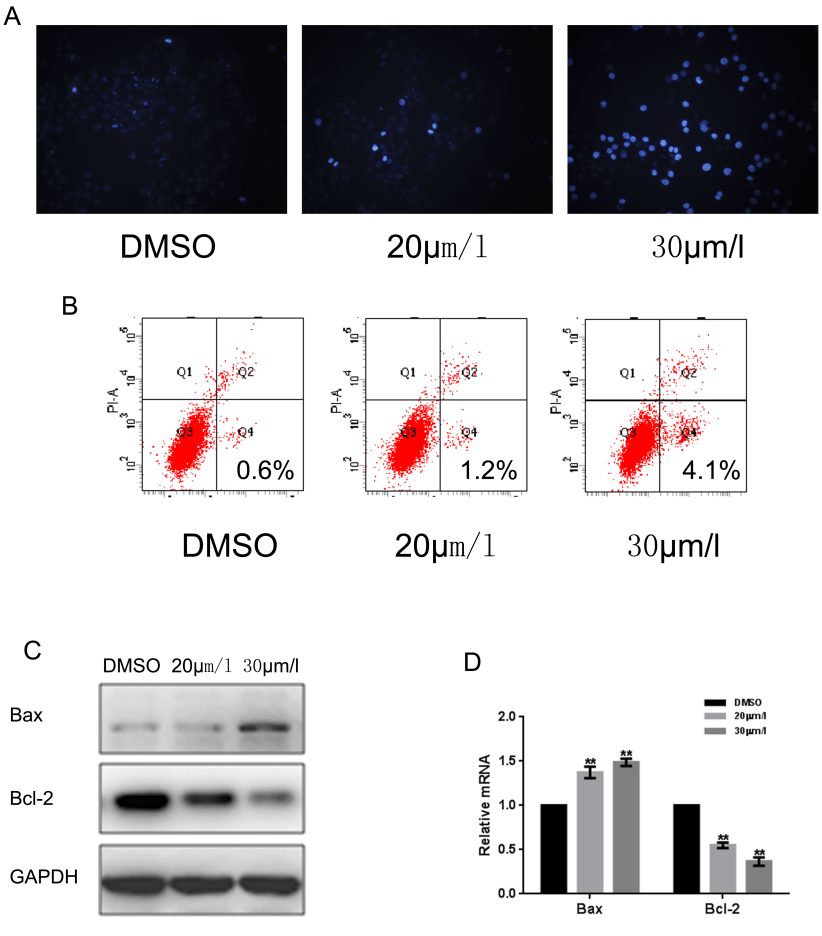

Figure 2

Naringin induces the apoptosis of bladder cancer cells. (a) Photomicrographs of BIU87 cells staining with Hochest33258 in the presence of DMSO or naringin for 24 h, (b) flow cytometric analysis of apoptosis of BIU87 cells, (c) western blotting analysis of Bax and Bcl-2 related protein expressions, (d) real-time quantitative PCR analysis of Bax and Bcl-2 related gene expressions. BIU87 cells were treated with 20 mmol/L DMSO (referred to as control group), or in the presence of indicated concentrations of naringin (10 μmol/L, 20 μmol/L or 30 μmol/L) for 24 h, respectively. Anti-GAPDH antibody was used for the evaluation of equal protein loading on the blot. The relative mRNA levels were expressed as fold from housekeeping gene (GAPDH) and then calculated relative to control (DMSO treatment group was as referred). Data were represented as mean ± SD. All experiments were repeated three times. *$p<0.05$.

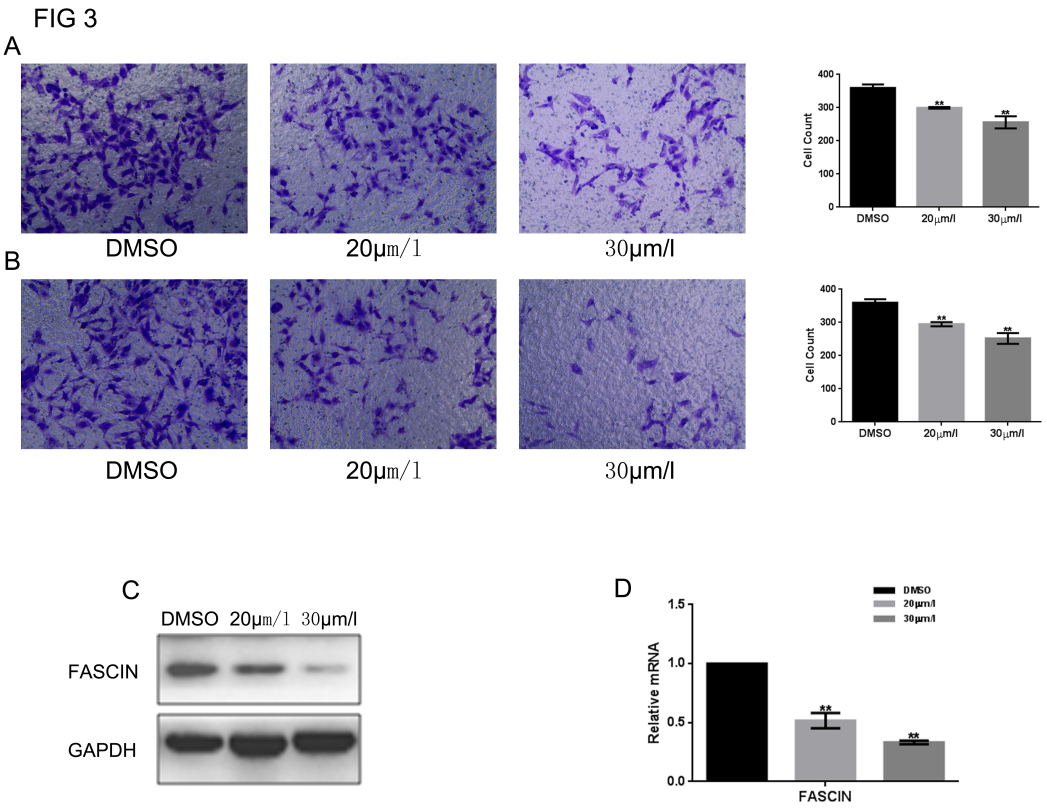

Figure 3

Naringin represses the migratory and invasive potential of bladder cancer cells. (a) Stained migratory cells and numbers of BIU87 cells through the membrane for 24 h after DMOS or indicated concentrations of naringin (20 μmol/L or 30 μmol/L), (b) Stained invasive cells and numbers of BIU87 cells through the membrane coated with matrigel for 24 h after DMOS or indicated concentrations of naringin (20 μmol/L or 30 μmol/L), (c) western blotting analysis of Fascin, (d) mRNA expression of Fascin detected by PCR.

Cells were treated with DMOS or naringin for 24 h and harvested for western blotting and PCR test. Results were represented as mean ± SD. All experiments were performed in triplicate. *p<0.05.

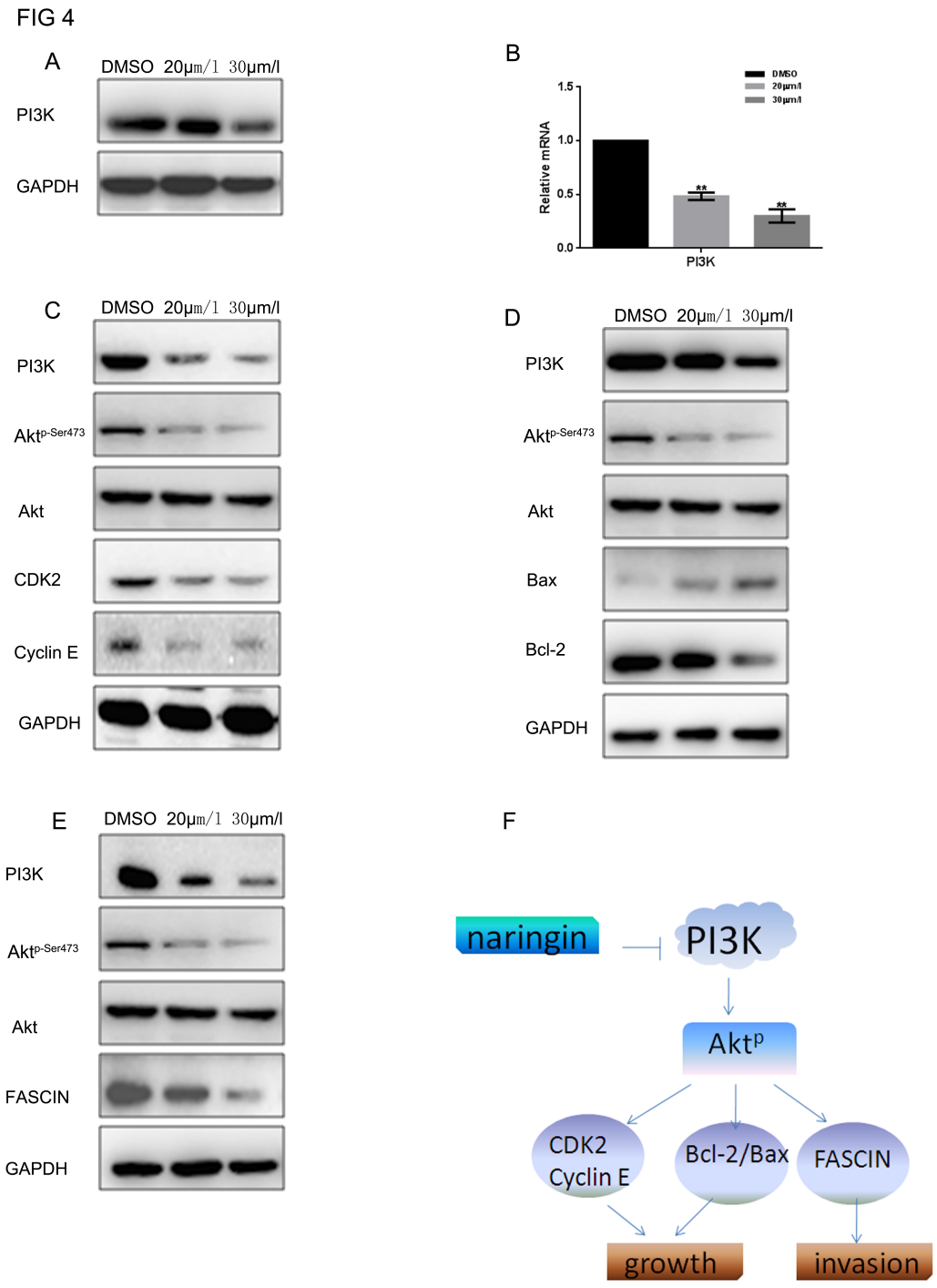

Figure 4

Naringin regulates PI3K/Akt pathway of bladder cancer cells. (a) Western blotting results of PI3K, (b) mRNA expression of PI3K, (c) expression of PI3K, Cyclin E, CDK2, Akt, and Aktp-Ser473 detected by western blot, (d) expression of PI3K, Bax, Bcl-2, Akt, and Aktp-Ser473 detected by western blot, (e)

expression of PI3K, Akt, Aktp-Ser473 and Fascin detected by western blot, (f) schematic of naringin regulating BIU87 cells proliferation and invasion through inhibiting PI3K/Akt pathway. Cells were treated with DMSO or naringin for 24 h and harvested for western blotting and PCR test. Results were represented as mean ± SD. All experiments were performed in triplicate. *$p<0.05$.