Original Article
Quercetin induces bladder cancer cells apoptosis by activation of AMPK signaling pathway

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Abstract: Quercetin, a natural existing polyphenol compound, has shown anticancer capacity for liver, breast, nasopharyngeal and prostate carcinoma but has not been clinically approved yet. This might be due to lack of clear mechanistic picture. Bladder cancer is one of the most common cancers of the urinary tract in the world. In China, bladder cancer has the highest rate of incidence out of all malignancies of the urinary system. The anticancer application of quercetin on bladder cancer has not been investigated either. This study was aimed to examine the mechanisms of quercetin on inhibition of bladder cancer. First, two human and one murine bladder cancer cell lines were tested in vitro for inhibitory sensitivity by MTT and cologenic assays. Second, AMPK pathway including 4E-BP1 and S6K were examined by western blot. Quercetin induces apoptosis and inhibits migration. We are the first to show that quercetin displays potent inhibition on bladder cancer cells via activation of AMPK pathway.

Keywords: Quercetin, bladder cancer, AMPK

Introduction

Bladder cancer is one of the most frequent cancers of the urinary tract, it accounted for about 74,000 new cases and 16,000 deaths in the United States in 2015 [1]. Even though transurethral resection has served as the standard treatment, recurrence and metastasis are often seen in clinic [2]. The commonest way to prevent recurrence and progression is supplemented with intravesical chemotherapy or immunosuppressive agents [3, 4]. Despite such approaches, outcomes have changed very little for the past three decades [5], yet recurrence and metastasis still tend to occur. Therefore, in addition to current treatment, novel therapies, such as targeted therapy which has made great progress in cancer treatment [6-8] may be an important strategy for treatment of bladder cancer. To characterize efficient targets, a number of large-scale molecular studies have been conducted in bladder cancer [9-12], including AMPK [13]. AMPK plays an important role in regulating cellular metabolism, preserving cellular energy homeostasis, and is involved in many other cellular processes, including cell apoptosis [14, 15]. Recently, compelling evidence shows that AMPK actually plays a critical role in tumor survival and growth [16, 17]. Furthermore, AMPK has been considered as a potential therapeutic target for the treatment of cancer [18].

Quercetin, a plant-derived aglycone form of flavonoid glycosides, has been used as a nutritional supplement and may prevent occurrence of various diseases, including cancer. However, its therapeutic effect is unclear and molecular mechanisms of quercetin on cancer treatment have largely not been explored yet. Couple of reports demonstrates that NF-kappaB is a key inflammatory modulator for quercetin to kill cancer cells including melanoma and lung cancer. [19-23] and AMPK pathway may get involved in the anticancer event on various tumors [24, 25]. However, whether this is true in bladder cancer is unknown. Thus, we use the two human and one murine bladder cancer cell
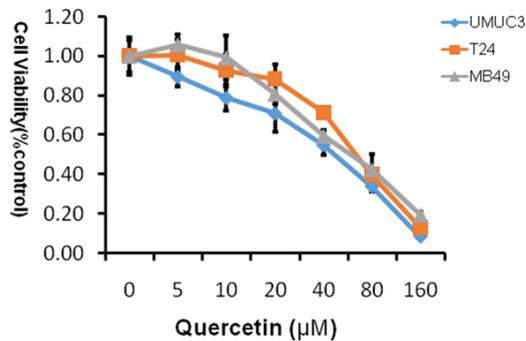
Figure 1. MTT proliferation assays. Treatment with quercetin on cell proliferation of human bladder cancer cell lines T24, UMUC3 and murine bladder cancer cell line MB49. Cell viability was assessed with 48 hour quercetin treatment at concentrations ranging from 0 to 160 μM using a tetrazolium-based assay. Results are presented as the median of 3 independent experiments.

Table 1. Inhibitory concentration 50% (IC50) of quercetin

Cells	MB49	T24	UMUC3
IC50 (μM)	58.63	64.02	42.29

lines to examine the killing effect of quercetin and the underlying mechanisms.

Materials and methods

Quercetin purchased from Aladdin chemistry Co. Ltd and Adamas Reagent Co. Ltd was dissolved in DMSO to prepare the stock solution of 50 mM. Antibodies for the protein characteristics were against total p70S6 kinase, phosphor-p70 S6 kinase (Thr389), total AMPK, phosphor-AMPK (Thr172), total 4E-BP1, phosphor-4EBP1, NF-κB p65, NF-κB and β-actin were purchased from Cell Signaling Technology. Compound C was from Selleckchem (Houston, Texas, USA).

Cell lines and culture conditions

Bladder cancer cell lines provided by Dr. P. Guo (Institute of Urology, Xi'an Jiaotong University, Xi'an, Shaanxi, P. R. China) was cultured in DMEM supplemented (Hyclone, Logan, UT, USA) with 10% of FBS (Hyclone, Logan, UT, USA) and 1% of penicillin-streptomycin at 37°C, in humidified air containing 5% of CO_2.

Cell viability assay

Cell viability was assessed using a tetrazolium-based assay. Cells were seeded at 6×10^3 per

well in 96-well culture plates and incubated in medium containing 10% FBS. Different seeding densities were optimized at the beginning of the experiments. After 24 hours, cells were treated with different concentrations of quercetin (0 μM, 5 μM, 10 μM, 20 μM, 40 μM, 80 μM, 160 μM) and incubated for 48 hours. 50 μl of MTT tetrazolium salt (Sigma) dissolved in Hank's balanced solution at a concentration of 2 mg/ml was added to each well with indicated treatment and incubated in CO_2 incubator for 5 hours. Finally, the medium was aspirated from each well and 150 μl of DMSO (Sigma) was added to dissolve formazan crystals and the absorbance of each well was obtained using a Dynatech MR5000 plate reader at a test wavelength of 490 nm with a reference wavelength of 630 nm.

Clonogenic assay

Clonogenic survival was defined as the ability of the cells to maintain their clonogenic capacity and to form colonies. 8×10^3 cells were seeded into 24-well dishes in 0.5 ml of medium for 24 hours. Cells were further treated with different concentrations of quercetin (0 μM, 5 μM, 10 μM, 20 μM, 40 μM, 80 μM), and then maintained for 6-8 days in a CO_2 incubator. Finally, the cells were fixed with 10% formaldehyde solution and then stained with 0.1% crystal violet, each experiment was performed in triplicate and colony numbers calculated through spectral scanning to determine the maximum absorption wavelength as 550 nm and measured its absorbance through area scanning. Statistical analysis was prepared with the SPSS software program. The ANOVA (analysis of variance) was used to compare colony numbers and determine the significance of these differences.

Assessment of apoptosis

Apoptosis was detected by flow cytometry via the examination of altered plasma membrane phospholipid packing by lipophilic dye Annexin V as described elsewhere. Treated cells were harvested by trypsin, washed twice with PBS, and then suspended in binding buffer at a concentration of 1×10^6 cells/mL according to the manufacturer's instruction. Thereafter, 5 μl of Annexin V-FITC and 5 μlM of propidium iodide were added into 100 μl of cell suspension and incubated for 30 minutes at room temperature in the dark. After adding 400 μl of binding buf-
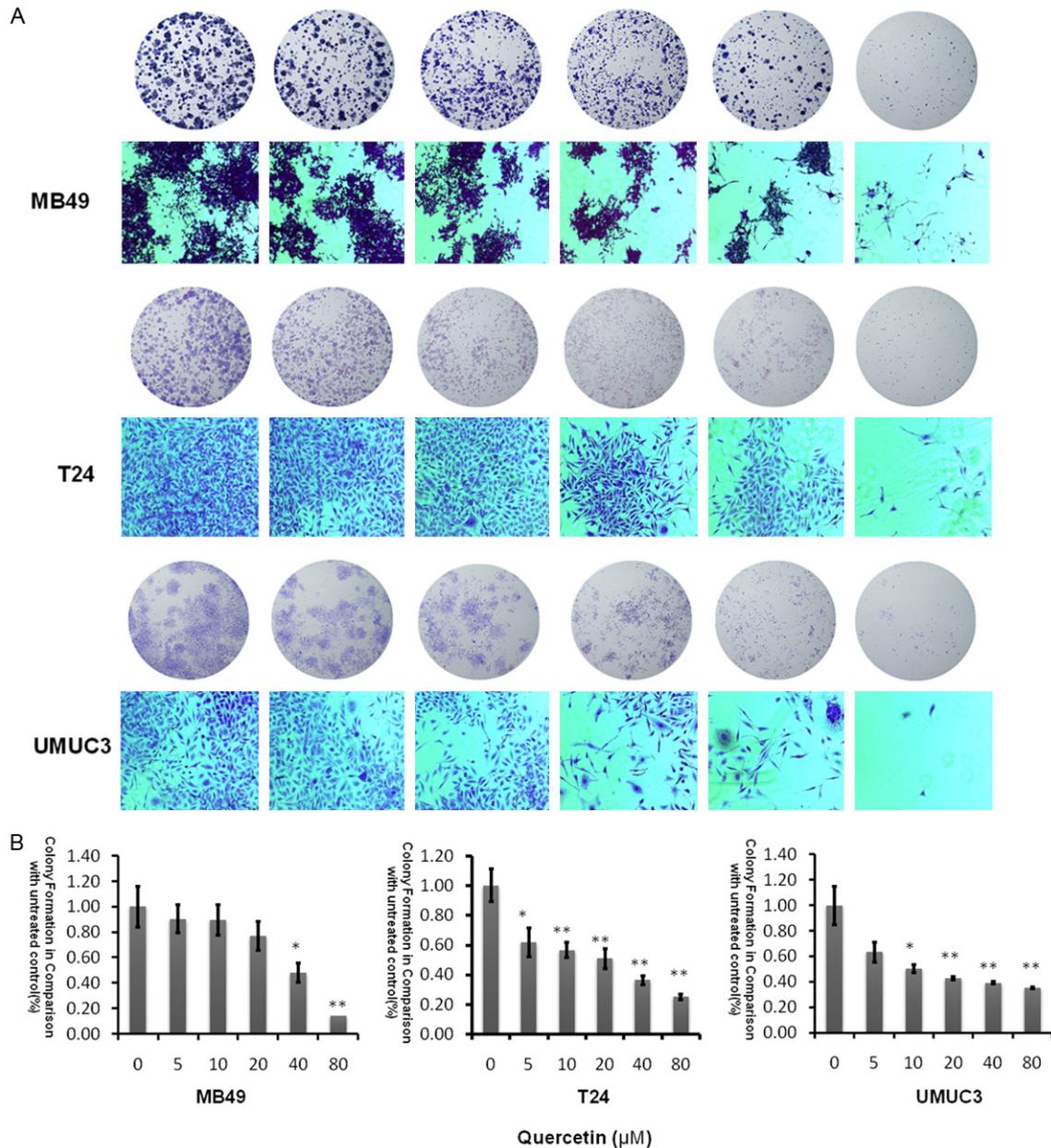
Figure 2. Evaluation of colony suppression of quercetin on bladder cancer cell lines. A. Clonogenic assay was assessed after 7 day quercetin treatment at various concentrations (0-80 µM) and pictures of migration on an inverted microscope with ×10 magnification. B. Bar chart shows quantitative data of average of 3 independent experiments (*P<0.05, **P<0.01 compared with control). Quantitative data were determined by solubilization of crystal violet and spectrophotometric reading at O D 550 nm. Results are presented as the median of 3 independent experiments (Comparison was drawn by the t-test (two-tailed). Data represent means ± SD; *P<0.05).

fer, labeled cells were counted by flow cytometry within 1 h. All early apoptotic cells (Annexin V-positive, propidium iodide-negative), necrotic/late apoptotic cells (double positive), as well as living cells (double negative) were detected by FACSC alibur flow cytometer and subsequently analyzed by Cell Quest software

(Becton Dickinson). Argon laser excitation wavelength was 488 nm, whereas emission data were acquired at wavelength 530 nm (FL-1 channel) for fluorescein isothiocyanate (FITC) and 670 nm (FL-3 c3 channel) for propidium iodide. At the same time, we analyzed it by fluorescence microscopy by seeding a total of
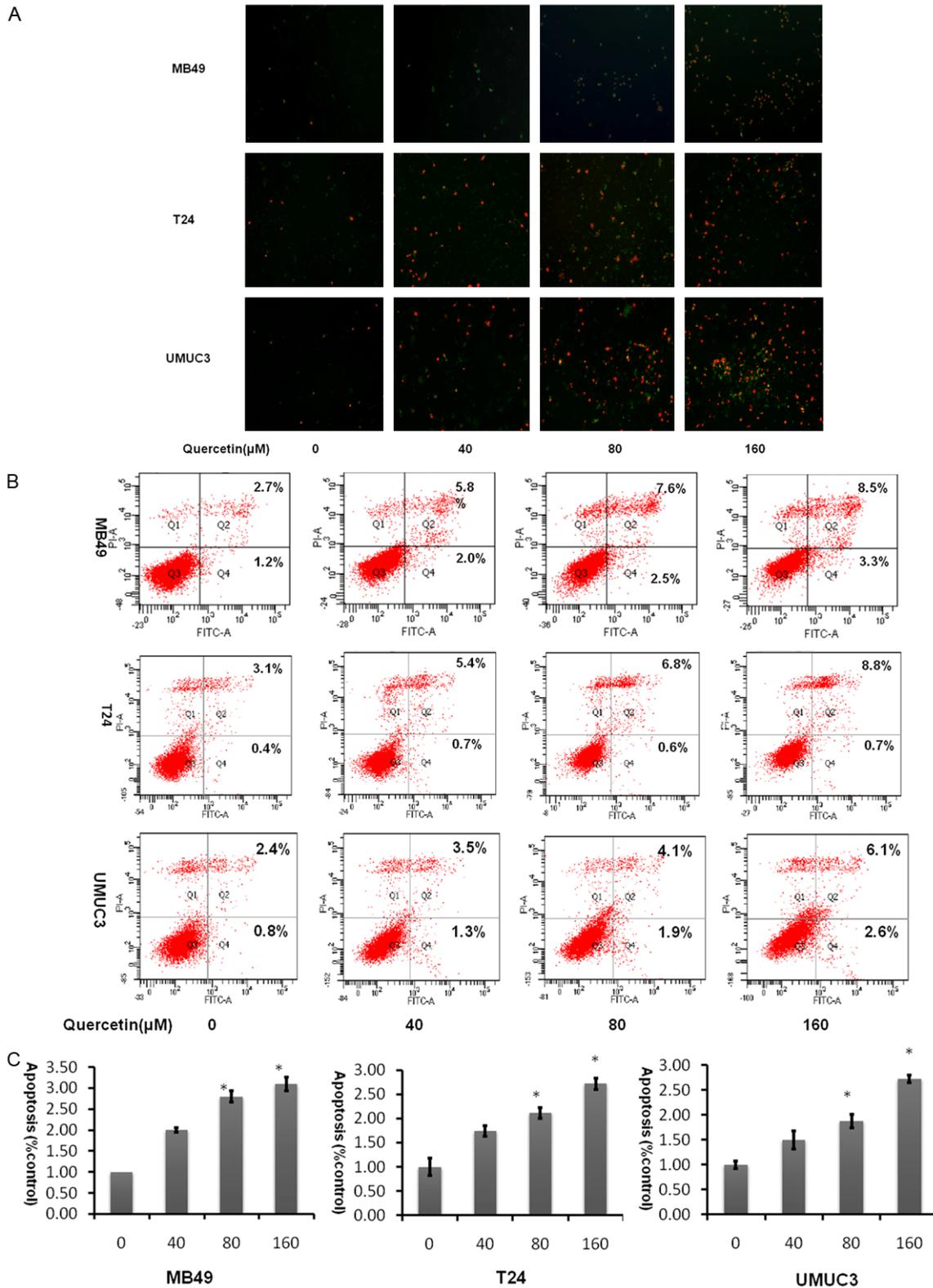
Figure 3. Quercetin induces apoptosis in bladder cancer cell lines in a dose dependent manner. A. Analysis of FITC and PI staining by fluorescent microscopic. B. Representative flow cytometry scatter plots of propidium iodide (PI) (Y axis) vs Annexin V-fluorescein isothiocyanate (FITC) (X axis). C. Bar chart shows quantitative data of average of 3 independent flow cytometry experiments (*P<0.05, **P<0.01 compared with control).
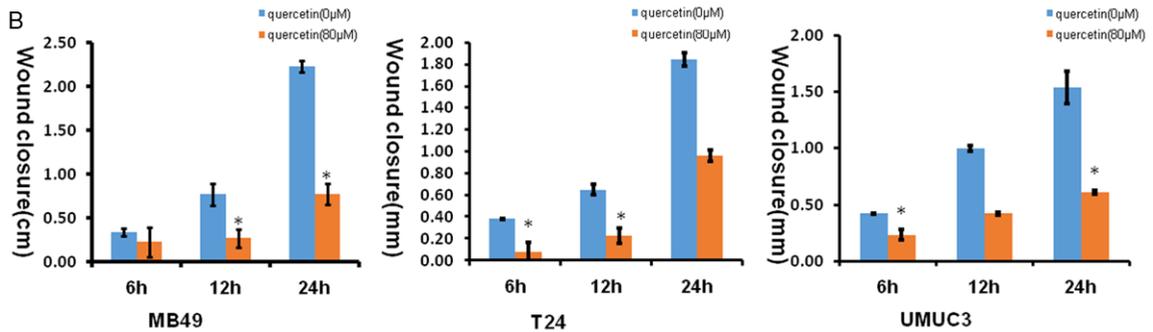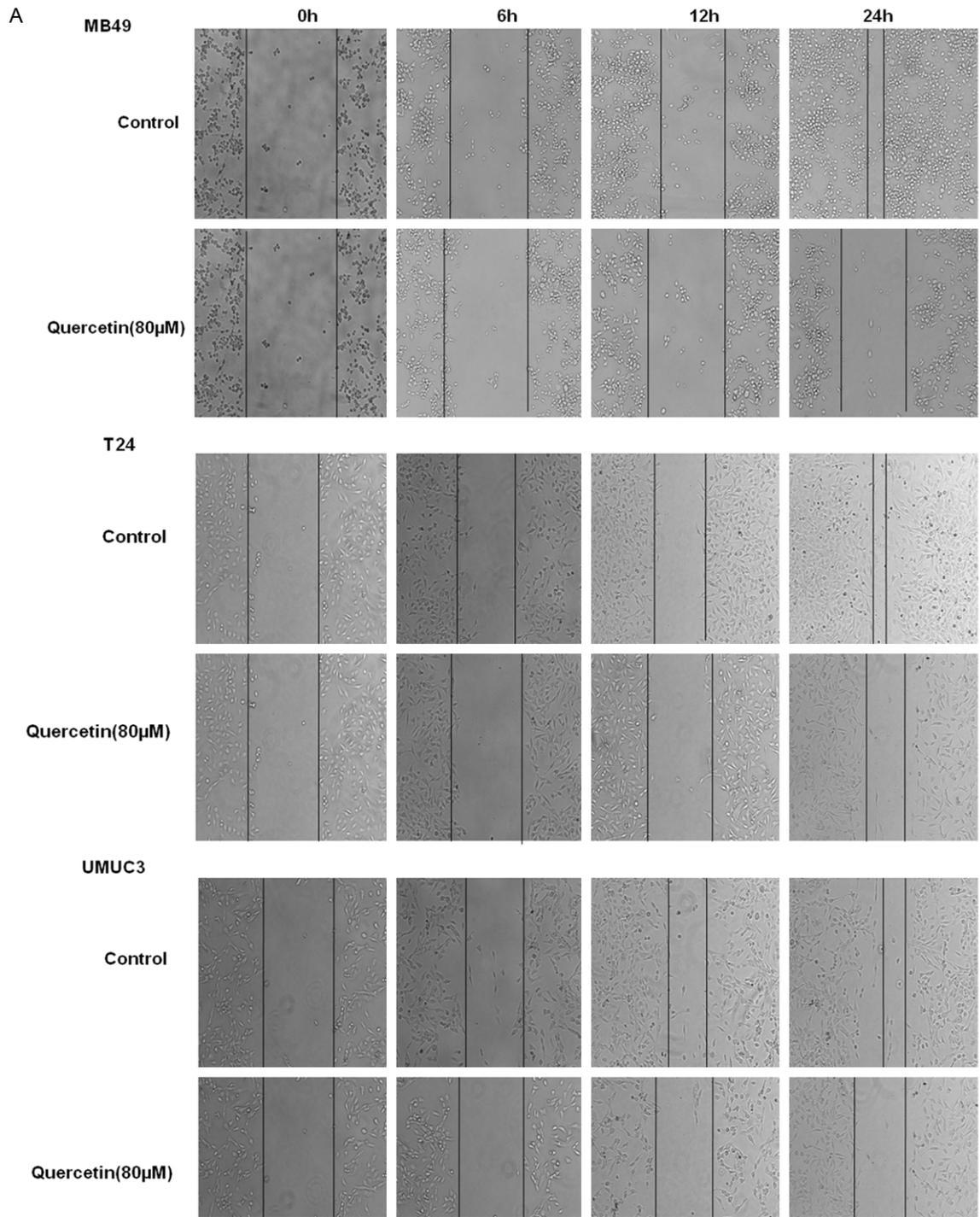
Figure 4. Quercetin showed impaired migration in wound healing assays. A. Images showed the gap of the scratched region of the different group cells; B. Bar chart shows wound closure distances data of average of 3 independent wound healing assays experiments (*P<0.05, **P<0.01 compared with control).
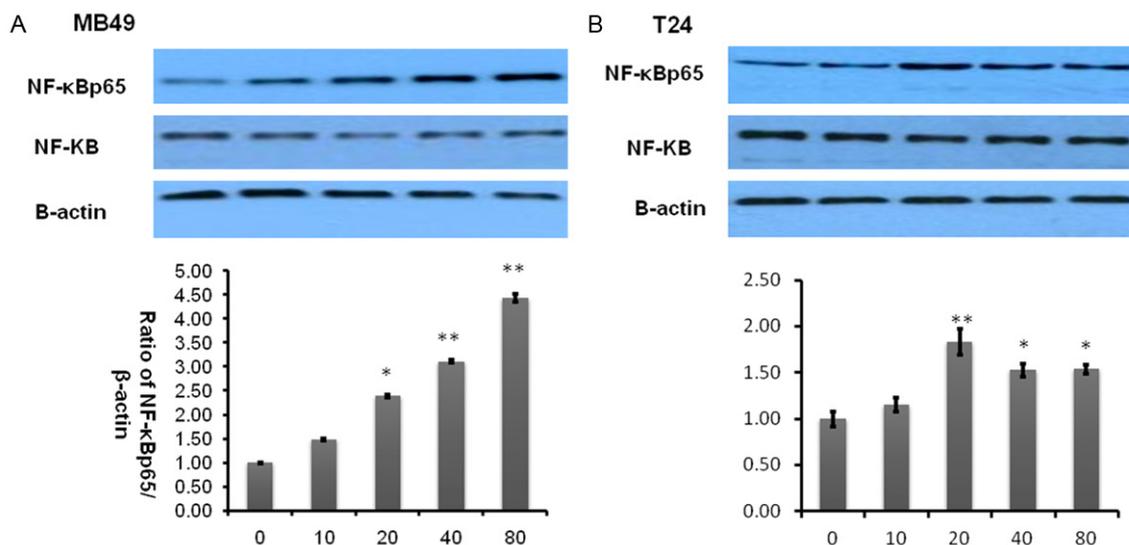
Figure 5. Quercetin activates NF-κBp65. A, B. Represent western blottings of NF-κBp65 and NF-κB after MB49 and T24 bladder cancer cell lines treated by quercetin for 2 h. β-actin was included as a loading control. The ratio of different proteins to β-actin was calculated by the band density of Western blots of each cell line using Image J software (*P<0.05, **P<0.01 compared with control).

1.2×10^4 cells onto 96-well culture plates and after 24 hours treated cells with different concentrations of quercetin (40 µM, 80 µM, 160 µM). 24 hours later, 100 µl binding buffer, 1 µL of Annexin V-FITC and 1 µL of propidium iodide were added to cells at room temperature in the dark for 15 minutes, kept in a low temperature and then observed by fluorescence microscopy.

Measurement of cell migration

A total of 5×10^3 cells were seeded onto a six-well plate and allowed to reach full confluence. The monolayer was wounded using a cocktail stick. Cells were incubated with serum-free DMEM medium, for the time period as stated. Digital images were taken at times 0, 6 h, 12 h and 24 h. The mean area was calculated using Image J software. The experiments were repeated three times.

Protein characterization

Western blot assessment was performed using regular procedure. Primary antibody was added in BSA and allowed to incubate overnight at

4°C, washed with TBS/0.05% Tween-20 for 5 times (10 min per time) before the secondary antibody was added and then incubated for an additional hour at room temperature. The membrane was again washed 3 times before adding Pierce Super Signal chemiluminescent substrate (Rockford, IL, USA) and then immediately imaged on Chemi Doc (Bio-Rad, Hercules, CA, USA). The films were scanned using EPSON PERFECTION V500 PHOTO and quantified by Image J (NIH, Bethesda, MD, USA).

Statistical analysis

All data are presented as mean±SEM. Statistical analyses were carried out using ANOVA analysis and statistical significance was assumed at a value of *p*<0.05.

Results

Quercetin inhibits bladder cancer cell lines proliferation

In order to determine whether quercetin affected the proliferation of the bladder cancer cell lines in vitro, we analyzed the effects of the
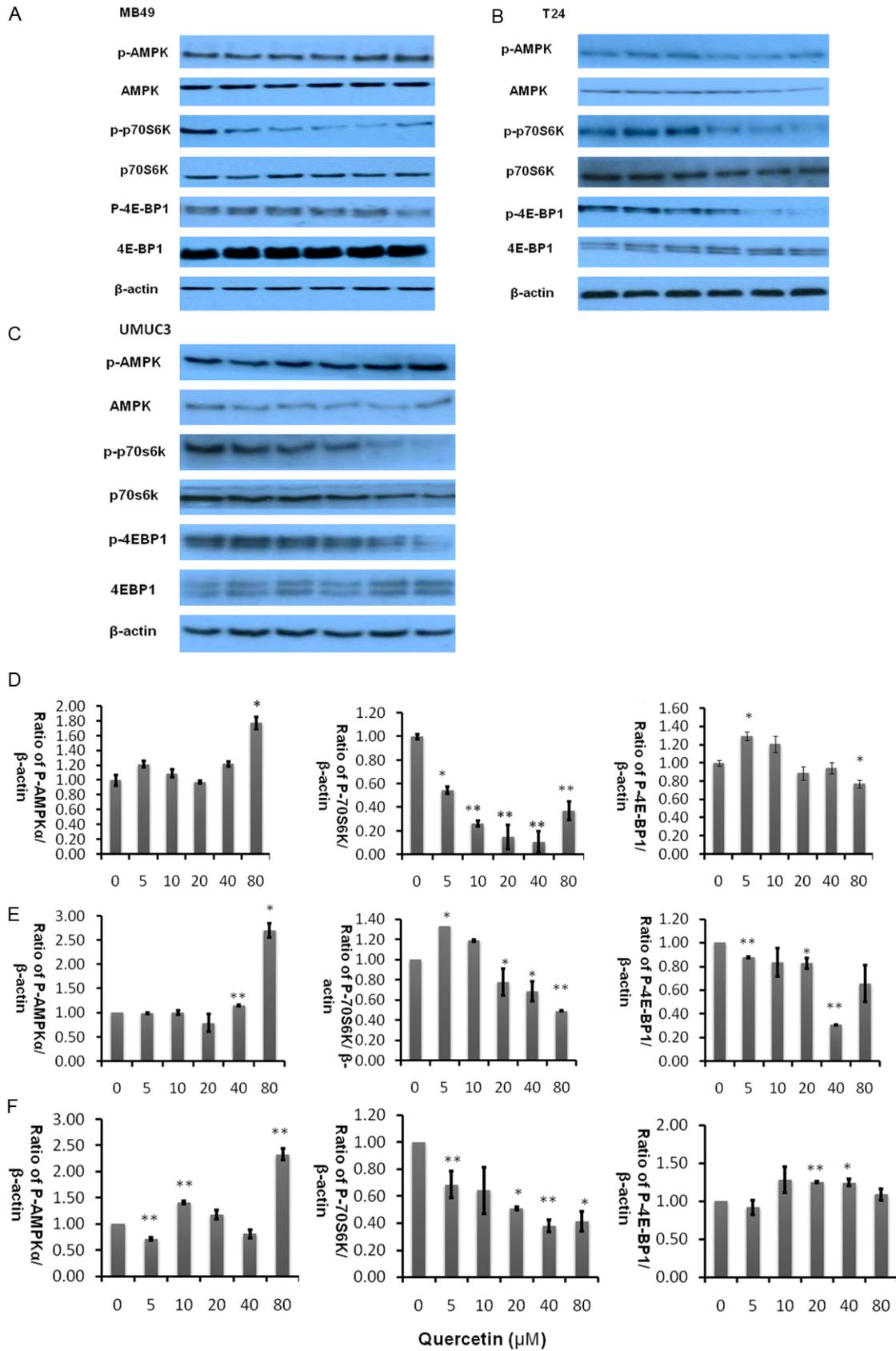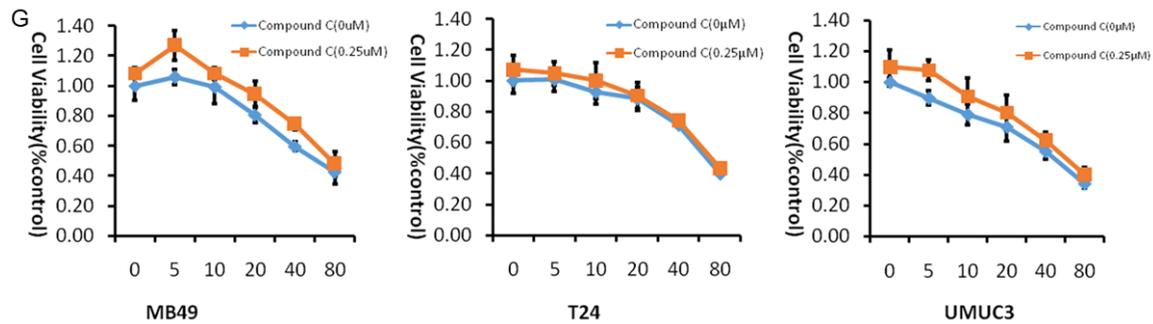
Figure 6. Quercetin activates AMPK intracellular signaling pathways at various concentrations. A-C. Represent western blot of p-AMPK, p-p70S6K, p-4EBP1, t-AMPK, t-p70S6K and t-4EBP1 of three bladder cancer cell lines MB49, T24, and UMUC3 respectively treated with quercetin (0-80 μM) for 2 hours. β-actin was included as a loading control. D. Represents the ratio of proteins of interests to β-actin was calculated by the band density of western blots of MB49 cell line using Image J software (*P<0.05, **P<0.01 compared with control). E. Represent the ratio of proteins of interests to β-actin was calculated by the band density of western blots of T24 cell line using Image J software. *P<0.05, **P<0.01 compared with control. F. Represent the ratio of proteins of interests to β-actin was calculated by the band density of western blots of MB49 cell line using Image J software. (*P<0.05, **P<0.01 compared with control). G. Cell viability was assessed after treatment of quercetin alone or quercetin combined with AMPK antagonist compound C for 48 hours. Data were presented as cell proliferation curves.

quercetin on the bladder cancer cell lines using MTT approach. The cell line was grown in 10% FBS and was exposed to quercetin (0 μM, 5 μM, 10 μM, 20 μM, 40 μM, 80 μM, 160 μM; **Figure 1**) and cell viability determined by tetrazolium-based assay after 48 h. In the experiment, we found that quercetin led to a dose-dependent inhibition on cell proliferation, and IC$_{50}$ was shown in **Table 1**, the most sensitive cell line is UMUC3 with IC$_{50}$ 42.29 μM.

Quercetin suppresses colony formation

The ability of cell lines to form colonies in the presence of quercetin, was studied and we discovered quercetin led to dose-dependent inhibition of colony formation as shown in **Figure 2**. At a concentration of 80 μM, colony numbers were significantly decreased at three bladder cancer cells, and the most sensitive cell line was UMUC3 which is consistent with the results of MTT experimental.

Assessment of apoptosis

As shown in **Figure 3**, cellular apoptosis was considerably increased in the quercetin-treated groups compared to the control group using FITC as an apoptosis marker in a dose dependent fashion. At 160 μM, quercetin induced significance apoptotic cells positively marked with both FITC and PI. There is little difference in the three cell lines. This demonstrated that quercetin is able to induce apoptosis in bladder cancer cells.

Scratch wound healing assay

Cell scratch assay was used to analyze whether quercetin affects the migration of bladder cancer cells. As shown in **Figure 4**, cell free area of the quercetin-treated group was significantly wider than that of control in three cell lines at 24 hours (P<0.05), demonstrating that quercetin inhibits bladder cancer cell migration.

Western blot

Quercetin activates rather than suppress NF-kappaB: Transcription factor NF-κB is a very well-known pro-inflammatory biomarker. Previous reports have demonstrated that quercetin is able to suppress the activation of NF-κB [26] and then inhibit cancer cell growth [27]. To validate whether this pathway was involved in inhibitory effect of quercetin on bladder cancer cells, we performed a similar examination. However, surprisingly as shown in **Figure 5**, NF-κB was significantly activated in a dose dependent manner whether in human bladder cancer T24 or murine bladder cancer MB49 can activate NF-κB. This implies that quercetin does not suppress NF-κB associated inflammatory pathway.

Quercetin activates AMPK signaling pathway: AMPK, a central energy sensor, is known as a crucial factor in the interaction between metabolism and cancer [28]. Quercetin is known to activate AMPK in some cancer cell lines, we

therefore sought to examine whether pathways known to be influenced by AMPK activity might be affected in bladder cancer cell lines by quercetin treatment. As activation of AMPK correlates tightly with phosphorylation at Thr-172 (p-AMPK), we assessed activation of AMPK by determining p-AMPK and its primary downstream targeting enzyme including p-p70s6k and p-4EBP1. Treatment of bladder cancer cells with quercetin for 2 hours induced a significant increase in the level of phosphorylation of p-AMPK while p-p70s6k, p-4EBP1 decreased as shown in **Figure 6A-C**.

Next, we added AMPK specific inhibitor compound C to determine whether activating AMPK signaling pathway is essential for quercetin to exert anti-tumor effect. As shown in **Figure 6G**, in three bladder cancer cell lines, quercetin and Compound C have shown varying degrees of antagonism, the ability of quercetin to inhibit cancer cell proliferation was reduced after adding compound C, implying that inhibition of AMPK accelerates proliferation of cancer cells.

Discussion

Bladder cancer remains one of the main malignancies that affect the genitourinary tract [29-31]. In fact, the main problem of this disease is that even if it is superficial at diagnostic moment, the current treatment, which is transurethral resection followed by BCG intravesical administration, is not fully effective in preventing tumor recurrence and progression. Therefore, fairly amount of research groups has studied several natural products, produced by secondary metabolism of plants, in an attempt to find new and better treatments to bladder tumors. Quercetin, which exists in nearly all plants, has a long history as a normal part of the human diet [32]. An increasing number of studies have revealed the biological and pharmacological activities of quercetin, which are beneficial to human health including its selective antiproliferative effects and cell death, predominantly through an apoptotic mechanism in cancer cell lines but not in normal cells [33]. Recent data from literature indicate the potential role of quercetin for pharmacologic treatment, including its activity against bladder cancer [28, 34]. However, the underlying molecular mechanisms remain poorly understood. In the present study, we found that quercetin inhibited cell proliferation and induced cell death via activating AMPK signaling pathway.

In this work, proliferation data obtained from MTT assays revealed that quercetin treatment significantly suppressed three bladder cancer cells proliferation. Furthermore, colony information and migration data obtained from clonogenic assay and scratch wound assay confirmed the suppression effect of quercetin on bladder cancer cell proliferation or motility. The mechanism of quercetin in bladder cancer cells was analysized by Western blots and the results showed that the expression of p-AMPK was increased while p-p70s6k was strongly decreased after treated with quercetin. These data collectively demonstrate that quercetin exerts anticancer effect on bladder cancer via AMPK signaling pathway. We also found out that quercetin led to a dose-dependent cell apoptosis through FITC apoptosis assay. Wound healing assay also reveals that quercetin suppresses migration of bladder cancer cells.

In conclusion, the results of this study show the anti-proliferative potential of quercetin on tumor cells by activating AMPK signaling pathway and provides solid foundation on the clinical application of quercetin in bladder cancer treatment.

Disclosure of conflict of interest

None.

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