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*Original Article*

# Inhibitory effect of epigallocatechin-3-gallate on bladder cancer cells via autophagy pathway

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**Abstract:** Despite advances in the treatment of genitourinary malignancies, no novel therapies have been approved by the US Food and Drug Administration for urothelial carcinoma, including bladder cancer, in the last 20 years. Epigallocatechin-3-gallate (EGCG), an active ingredient in green tea, is known to effectively inhibit formation and development of tumors. However, the effects and mechanisms of EGCG on bladder cancer are still unclear. In the present study, we demonstrated that low concentration of EGCG induced retarded proliferation and increased apoptosis in bladder cancer cell lines (T24 and 5637 cells) indicated by the increased expression of apoptosis related protein (caspase 9, caspase 3 and BAX). In addition, low dose of EGCG also regulated autophagy pathway associated protein (LC3B II and Beclin), which was blocked by PI3K/AKT inhibitor; moreover, knockdown of ATG5 reversed EGCG-induced apoptosis in 5637 cells, indicating that EGCG might inhibit the bladder cancer through autophagy pathway. Our findings suggest that EGCG may be a novel therapy for bladder cancer treatment by regulating autophagy pathway.

**Keywords:** Epigallocatechin-3-gallate, bladder cancer, autophagy, apoptosis

## Introduction

Epigallocatechin-3-gallate (EGCG) is the mainly catechin extracted from green tea, accounting for 50-80% in a brewed cup [1]. EGCG is known to effectively inhibit formation and development of tumors by increasing autophagy-related apoptosis [2, 3]. Autophagy is an important cellular degradation process. During this process, damaged macromolecules are removed from cell to maintain cell integrity function [4]. Autophagy initially forms an autophagosome to engulf damaged cytosolic proteins or organelles, and then the autophagosome fuses with lysosome to produce an autolysosome. Autophagosome maturation is regulated by a series of kinases, such as mammalian target of rapamycin (mTOR) [5], and the initiators Beclin1 and LC3BII/I, which is involved in autophagy induction [6]. PI3K/Akt and mTOR pathway links apoptosis and autophagy by shifting Beclin1's action from autophagy to apoptosis [7]. Genetic defects of Beclin1 can cause an increasing of cancer incidence, including bladder cancer [8]. However, the role of EGCG associated with autophagy in bladder cancer cell is still unclear.

In the present study, we aim to investigate whether the anti-tumor properties of EGCG is associated with autophagy pathway in bladder cancer. We exposed T24 and 5637 cells to different concentration of EGCG, and then assessed cell proliferation, apoptosis and alterations of autophagy pathway. In addition, we also examined whether EGCG caused autophagosome formation by using electron Microscopy analysis. Our data demonstrated that EGCG could repress cell proliferation and enhance apoptosis in bladder cancer cells in a dose-dependent manner. Autophagosome was increased by EGCG treatment in T24 and 5637 cells. Furthermore, PI3K/AKT inhibitor blocked the EGCG-mediated upregulation of Beclin1 and LC3BII in 5637 cells, and knockdown of ATG5 reversed EGCG-induced apoptosis in 5637

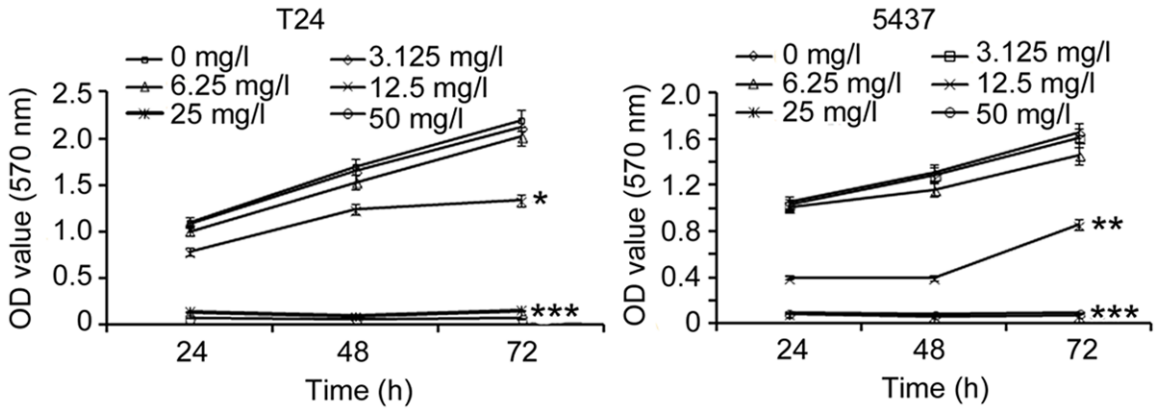



**Figure 1.** EGCG induces proliferation inhibition in a dose dependent manner in bladder cancer cells. MTT was used to measure the cell proliferation after T24 cells (right) and 5637 cells (left) exposed to a range of concentrations of EGCG for 24 h. Data are presented by means ± SD, **P*<0.05, ***P*<0.01, ****P*<0.001 vs. 0 mg/L group.

cells, suggesting that the anti-tumor effect of EGCG might be through autophagy pathway.

## Materials and methods

### *Cell culture*

The human bladder transitional cell carcinoma cell lines (T24 and 5637) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Human T24 cells were cultured in McCoy's (Modified) 5A Media (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen Life Technologies). 5637 cells were grown in RPMI1640 (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies). All cells were cultured in a humidified 5% $CO_2$ incubator at 37°C.

### *shRNA transfection*

In order to knock down the expression of ATG5, we transfected 50 nM ATG5 shRNA into 5637 cells by using Lipofectamine 3000 (Life technologies, Grand Island, NY). After 48 h transfection, the cells were used for further analysis. And siRNA scramble was used as negative control. The sequences were used as followings: ATG5-sh: 5' TCACGTTGTCTGATATATTCTAAAGdTdT3' (cat no: Q000009474-1-B, RiboBio Co., Ltd, Guangzhou, China).

### *MTT assay*

To test the effect of EGCG on T24 and 5637 cells growth, the cells were seeded into 96-well plates at $1\times10^3$ cells/well. After cultured for 4 h, the cells were treated with various doses of EGCG (0, 3.125, 6.25, 12.5, 25 and 50 mg/L, Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Then, MTT (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 5.0 mg/ml were added to each well, and incubated for additional 4 h at 37°C. The formazan generated in each well was dissolved in 150 ml of dimethyl sulfoxide (Sigma-Aldrich). Absorbance of each well at 570 nm was measured using a microplate reader (Synergy™ Mx; BioTek, Winooski, VT, USA).

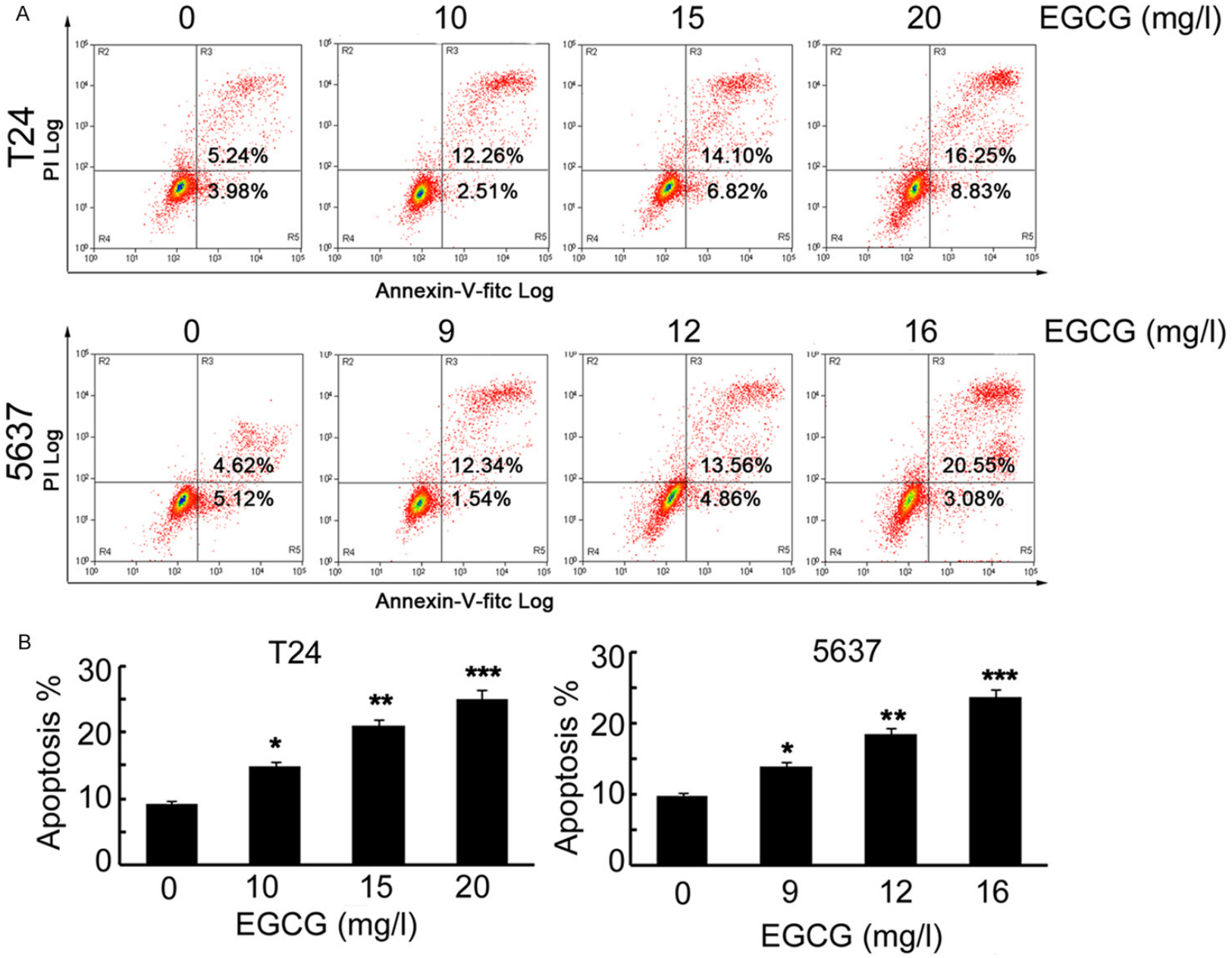
To test the effect of PI3K/AKT inhibitor (LY294002) and mTOR inhibitor (RAPA) on 5637 cells growth, the cells were seeded in 96-well plates ($5\times10^3$ cells/well). After cultured for 4 h, the cells were treated with EGCG (9 mg/L), LY294002 (1 μM) or RAPA (100 ng/ml) alone, or co-treated with EGCG (9 mg/L) and LY294002 (1 μM) or RAPA (100 ng/ml) for 24 h. Then, MTT assay was performed as mentioned above.

### *Flow cytometric analysis of the cell apoptosis*

Following indicated treatment, the cells were trypsinized and washed with ice-cold PBS. The cell suspensions were incubated for 15 min in dark by using Annexin V/PI detection kit (Molecular Probes Inc., Eugene, OR) according to the manufacturer's instructions. And then, the cell apoptosis was analyzed by flow cytometry (Beckman Coulter, USA). The experiments were performed in triplicate.

### *Electron microscopy analysis*

After fixed in 3% glutaraldehyde diluted with 0.1 M phosphate buffer (pH 7.4), the cells treated


A
0
10
15
20
EGCG (mg/l)
T24
PI Log
5.24%
3.98%
12.26%
2.51%
14.10%
6.82%
16.25%
8.83%
Annexin-V-fitc Log
0
9
12
16
EGCG (mg/l)
5637
PI Log
4.62%
5.12%
12.34%
1.54%
13.56%
4.86%
20.55%
3.08%
Annexin-V-fitc Log
B
T24
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30
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5637
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30
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EGCG (mg/l)

**Figure 2.** EGCG promotes apoptosis in bladder cancer cells in a dose dependent manner. A: Flow cytometry analysis was used to measure the cell apoptosis after T24 cells (upper) and 5637 cells (lower) exposed to a range of concentrations of EGCG for 24 h. B: Quantification of cell apoptosis in T24 cells (left) and 5637 cells (right). Data are presented by means ± SD, **P*<0.05, ***P*<0.01, ****P*<0.001 vs. 0 mg/L group.

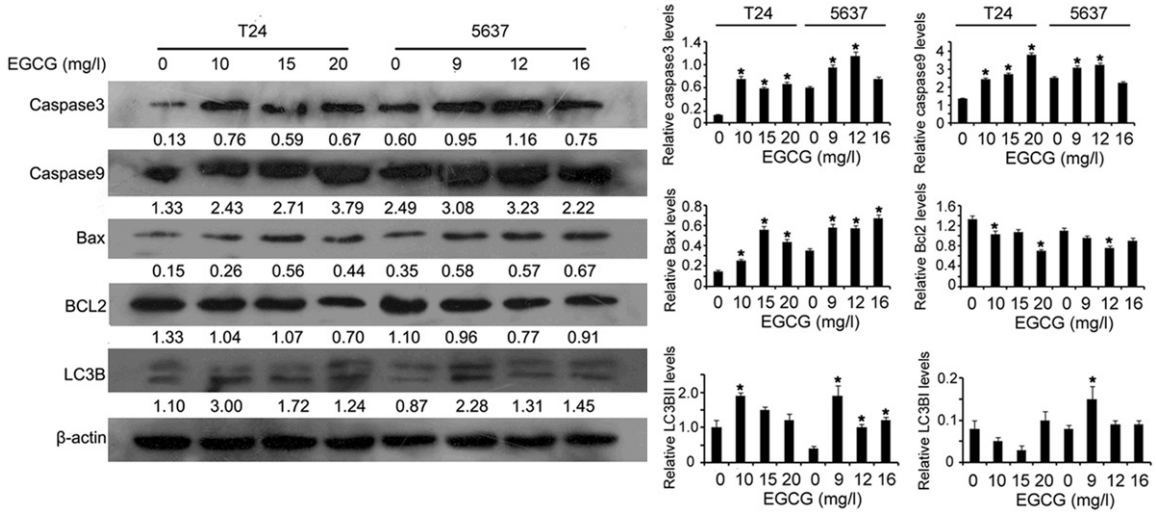


**Figure 3.** EGCG regulates apoptosis and autophagy related protein expression. Western Blot was used to measure apoptosis and autophagy related protein expression after T24 cells and 5637 cells exposed to a range of concentrations of EGCG for 24 h. Data are presented by means ± SD, **P*<0.05 vs. 0 mg/L group.

with EGCG were post-fixed in 1% osmium tetroxide (Sigma-Aldrich) in Sorensen's phosphate buffer. After dehydration in gradient ethyl alcohol, the cells were embedded in epon (Sigma–Aldrich). Samples were placed on copper grids and analyzed by using a LVEM5 transmission electron microscope (TEM) (Quantum Design, Beijing, China).

*Western blot*

RIPA lysis buffer (Boster, Wuhan, China) was used to extract protein from indicated cells. BCA Protein Assay Kit (Thermo Scientific, USA) was used to measure the protein concentration. Total 60 μg protein were separated on 10% SDS-PAGE and blotted onto 0.22 μm nitro cellulose membranes (Boster, Wuhan, China). The membranes were blocked for 2 h with 5% non fat dry milk diluted with tris-buffered saline (TBS) and incubated with primary antibodies (rabbit polyclonal anti-caspase 3 (1:1000), anti-caspase 9 (1:1000), anti-Bax (1:2000), anti-BCL-2 (1:2000), anti-LC3B (1:500) and anti-β-actin (1:3000), and rabbit monoclonal anti-Beclin-1 (1:3000), Abcam, UK; mouse monoclonal anti-mTOR (1:1000) and anti-p-S6K (Thr389) (1:1000), and rabbit monoclonal anti-p-mTOR (1:1000), anti-4E-BP1 (1:1000), anti-p-4E-BP1 (ser65) (1:1000), anti-S6K (1:1000), Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 40°C. The membranes were washed with tris-buffered saline containing Tween (TBST), and then incubated with appropriate horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, 1:3000; goat anti-mouse, 1:2000; Jackson Immuno Research, West Grove, PA, USA) for 1 h at 37°C. Enhanced chemiluminescence reagent (Wuhan Boster) was used to detect the signal on the membrane. The data were analyzed via densitometry using Image-Pro plus software 6.0 (Media Cybernetics, Rockville, MD, USA) and normalized to the expression of the internal control (β-actin).

*Statistical analysis*

Statistical analyses were performed using GraphPad Prism 5 software (Graphpad Software, Inc., La Jolla, CA, USA) and the data are presented as the mean ± standard deviation. An unpaired two-tailed Student's *t*-test or one way analysis of variance (ANOVA) with Bonferroni *t* post-test was used to analyze the data depending on conditions. *P*<0.05 was consid-

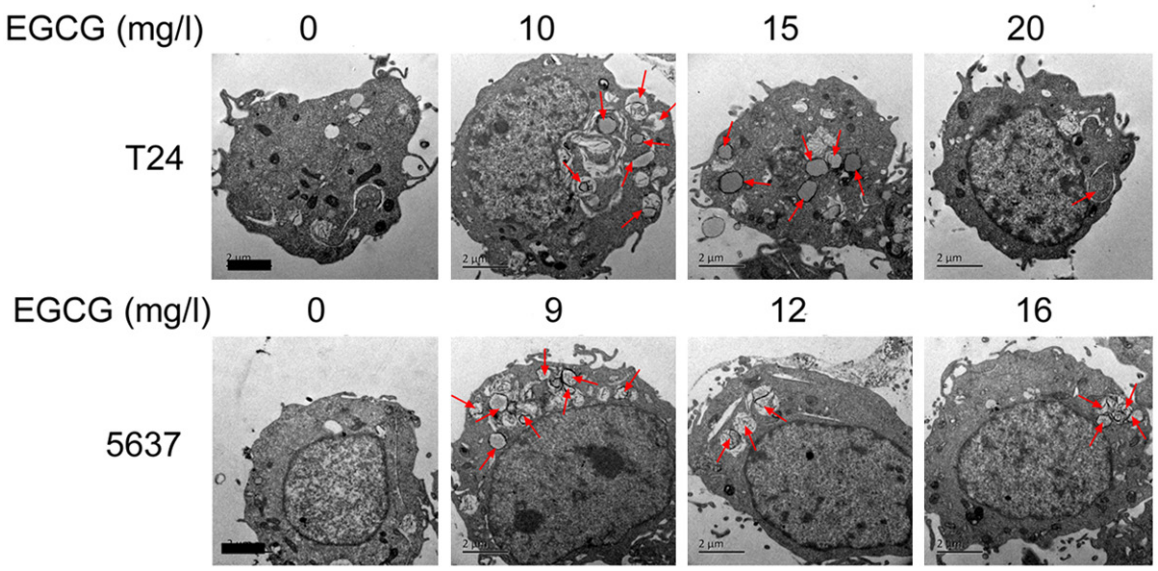


**Figure 4.** EGCG lead to the formation of autophagosome formation. Transmission electron microscope was used to observer autophagosome formation (indicated by red arrow) after T24 cells and 5637 cells exposed to a range of concentrations of EGCG for 24 h.

ered to indicate a statistically significant difference.

## Results

*EGCG inhibits cell proliferation, and induces apoptosis in a dose dependent manner in T24 and 5637 cells*

To investigate the role of EGCG on bladder cancer cell growth, we exposed T24 and 5637 cells to serial concentrations (rang 0 to 50 mg/L) of EGCG. As shown in **Figure 1**, the growth of two cell lines was significantly inhibited by EGCG with increasing dose. In addition, to investigate the role of EGCG on bladder cancer cell apoptosis, we subjected T24 and 5637 cells to serial concentrations (rang 0 to 20 mg/L for T24 cells, and rang 0 to 16 mg/L for 5637 cells, respectively) of EGCG. We found that EGCG effectively induced T24 and 5637 cells apoptosis with increasing dose (**Figure 2**). These results indicated that EGCG inhibited bladder cancer cell proliferation, and promoted cell apoptosis in a dose dependent manner.

*EGCG regulates apoptosis and autophagy related protein expression*

To investigate the correlation between EGCG and apoptosis and autophagy, we exposed T24 cells and 5637 cells to EGCG with a range of concentrations. We found that EGCG treatment enhanced expression of Caspase 3, Caspase 9 and Bax in a dose dependent manner, but not the case in the expression of BCL2 and LC3B. EGCG led to a remarkably decreased expression of BCL2 at 10 mg/L and 20 mg/L in T24 cells, while significantly reduced the expression of BCL2 at 12 mg/L in 5637 cells. EGCG treatment lead to significantly increased expression of LC3B II at 9 mg/L, 12 mg/L and 16 mg/L in the 5637 cell, whereas only low dose (10 mg/L) of EGCG lead to increased expression of LC3B II in T24 cell. EGCG treatment could inhibit the expression of LC3B I in the T24 cell in a dose dependent manner, but not reach significance. And only low dose of EGCG treatment lead to significantly increased expression of LC3B I in 5637 cell, which was not found in other dose (**Figure 3**).

Furthermore, we also want to know if EGCG affects autophagosome formation involved in apoptosis. By TEM, we found that EGCG treatment significantly increased autophagosome formation in the T24 and 5637 cells, especially at low dose (**Figure 4**).

*Effect of EGCG on the expression of autophagy pathway related protein*

In order to explore the pathway involved in autophagy affected by EGCG, we detected the key molecules expression of autophagy pathway. The results showed that no significant difference in the expression of mTOR and p-mTOR was found in the T24 cell after EGCG treatment

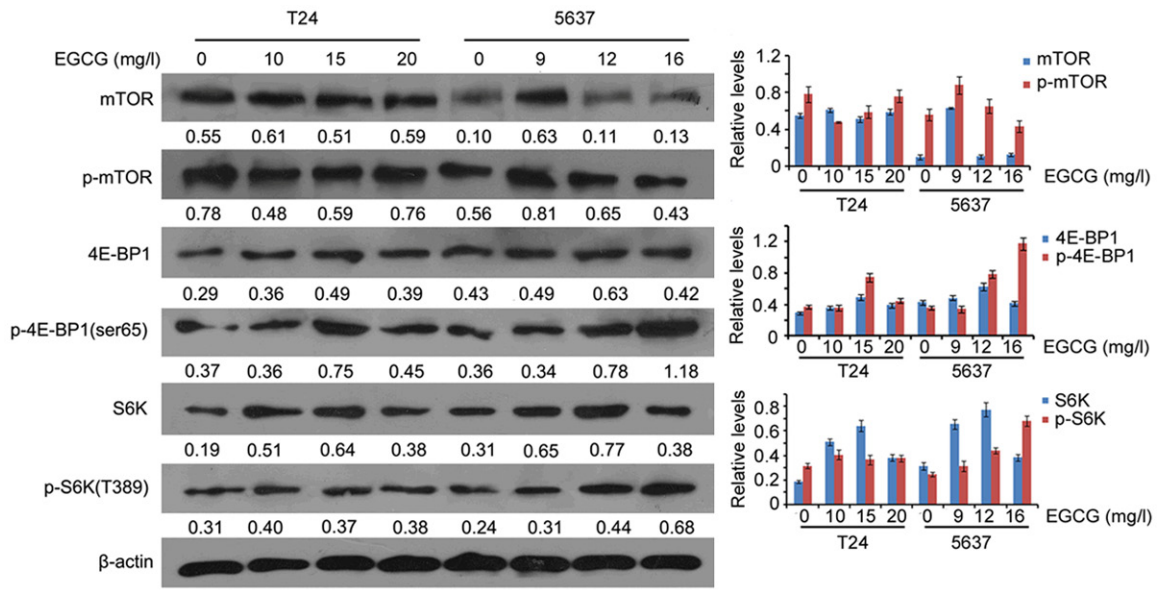


**Figure 5.** Effects of EGCG on the expression of autophagy pathway related protein. Western Blot was used to measure autophagy pathway related protein after T24 cells and 5637 cells exposed to a range of concentrations of EGCG for 24 h. Data are presented by means ± SD, **P*<0.05, ***P*<0.01 vs. 0 mg/L group.

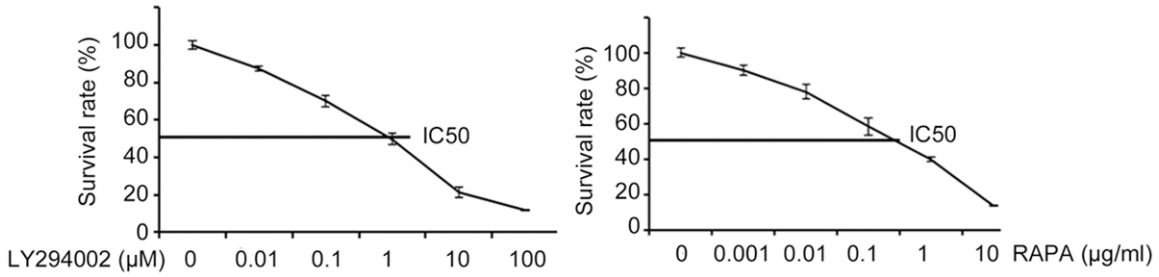


**Figure 6.** Effects of PI3K and mTOR inhibitor on EGCG-mediated cell apoptosis. MTT was used to measure the cell proliferation after 5637 cells exposed to a range of concentrations of LY294002 or RAPA for 24 h. Data are presented by means ± SD.

even at high dose, while EGCG significantly increased the expression of p4E-BP1 at 15 mg/L, and increased the expression of S6K at 10 mg/L and 15 mg/L. On the other hand, in the 5637 cell, low dose of EGCG led to significantly increased expression of mTOR and p-mTOR, while high dose of EGCG significantly increased the expression of p4E-BP1 in the 5637 cells. In addition, EGCG could lead to significantly increased expression of S6K and p-S6K (**Figure 5**).

*Effects of PI3K or mTOR inhibitor on EGCG-mediated cell apoptosis*

As mentioned above, we have known that EGCG affected the PI3K/mTOR signaling in 5637 cells. Thus, we chose 5637 cells to investigate whether EGCG regulated bladder cancer cell proliferation and apoptosis through PI3K/mTOR signaling. We exposed 5637 cells to a range of concentrations of EGCG. We found that both PI3K/AKT inhibitor (LY294002) and mTOR inhibitor (RAPA) could reduce proliferation of 5637 cells. And the half maximal inhibitory concentration (IC50) is 1 μM for LY294002, and 100 ng/ml for RAPA. These two concentrations were selected for further investigation (**Figure 6**).

Both EGCG and LY294002 could lead to significantly increased apoptosis in 5637 cell, and LY294002 was more effective than EGCG. When combined EGCG with LY294002 to treat

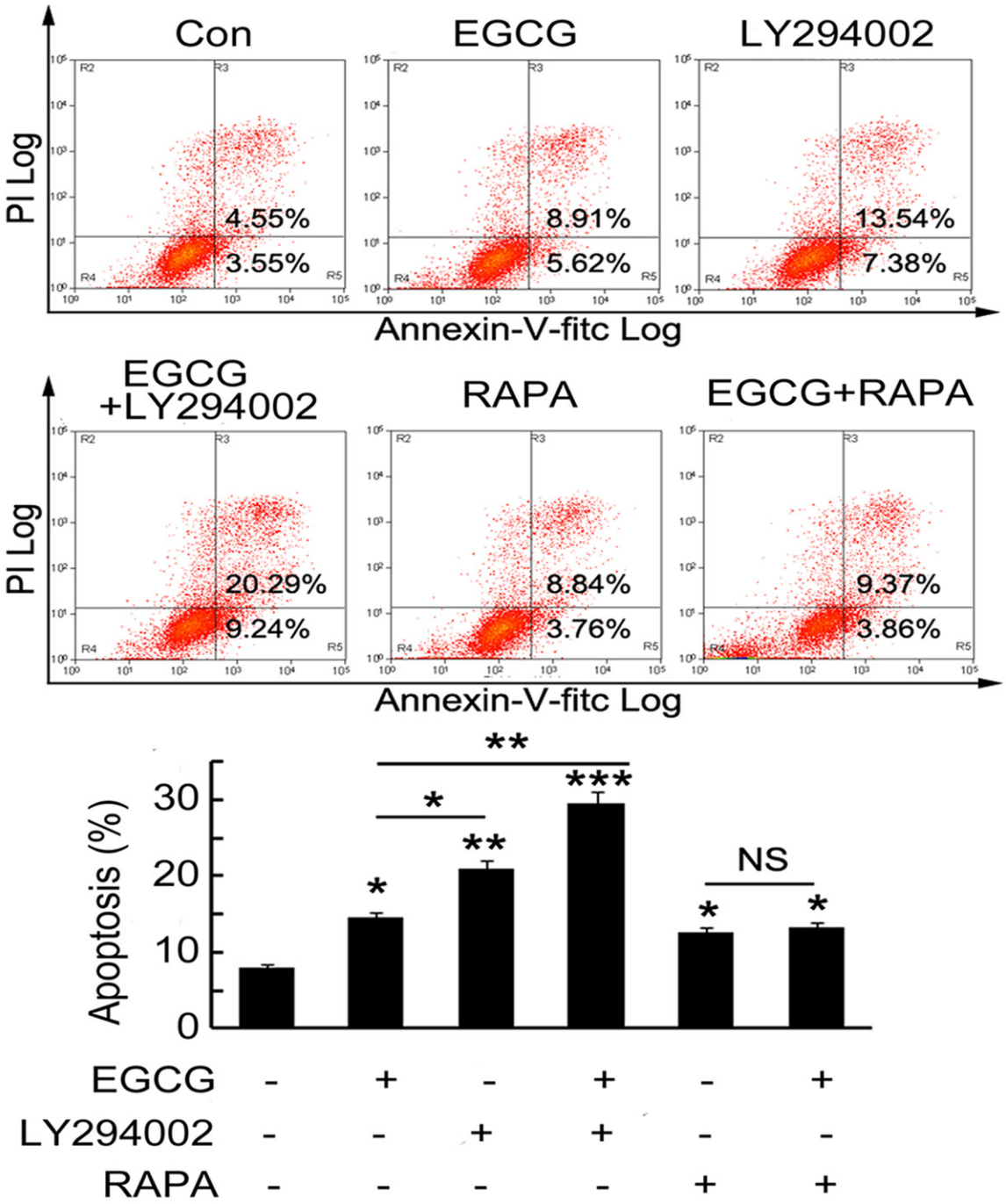


**Figure 7.** EGCG promotes apoptosis in bladder cancer cells in a dose dependent manner. Flow cytometry analysis was used to measure the cell apoptosis after 5637 cells exposed to a low concentrations of EGCG, LY294002, EGCG combined with LY294002 (EGCG + LY294002), RAPA, RAPA combined with EGCG (EGCG + RAPA) for 24 h. Data are presented by means ± SD, $*P<0.05$, $**P<0.01$, $***P<0.001$.

the 5637 cell, a synergistic effect was found, as this treatment showed the most efficiency in cell apoptosis. Although RAPA was found effective in cell apoptosis inducement, no synergistic effect was found when combined RAPA with EGCG (**Figure 7**).

*PI3K inhibitor blocks EGCG-promoted autophagy in bladder cancer cells*

We have shown that EGCG enhanced autophagy formation and activated autophagy signaling at low dose, and EGCG had a synergistic effect

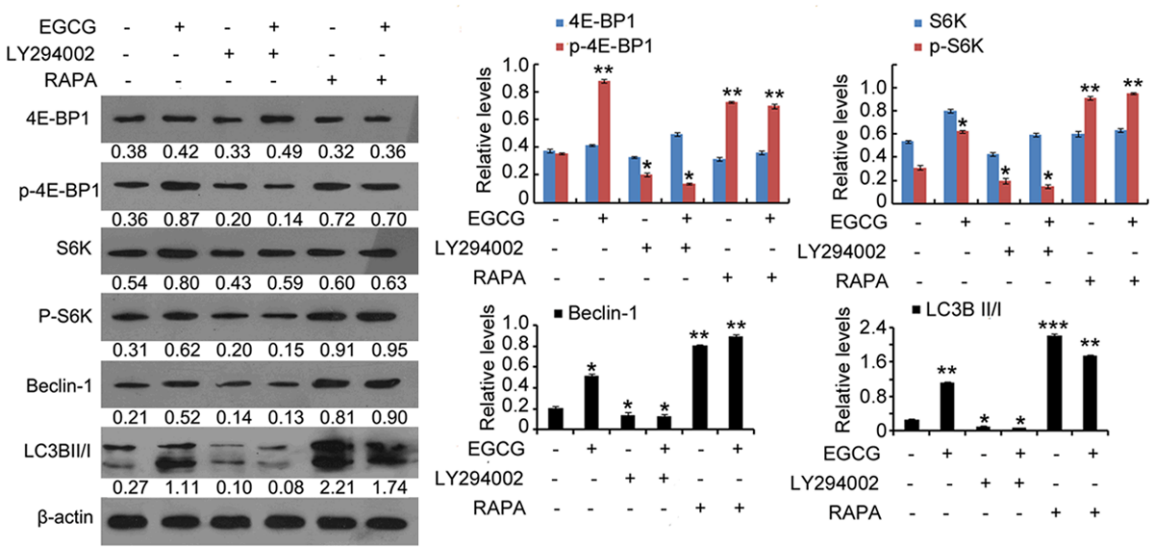


**Figure 8.** PI3K inhibitor blocks EGCG-promoted autophagy in bladder cancer cells. Western Blot was used to measure autophagy pathway related protein after 5637 cells exposed to a low concentrations of EGCG, LY294002, EGCG combined with LY294002 (EGCG + LY294002), RAPA, RAPA combined with EGCG (EGCG + RAPA) for 24 h. Data are presented by means ± SD, **P*<0.05, ***P*<0.01, ****P*<0.001 vs. control group.

with PI3K inhibitor, but not mTOR inhibitor. Thus, we exposed 5637 cells to PI3K/AKT inhibitor (LY294002) or mTOR inhibitor (RAPA) for 24 h, and then detected the expression of the key molecules of autophagy pathway. As shown in **Figure 8**, EGCG treatment induced a significantly enhanced expression of p-4E-BP1, which was reversed to even under baseline after combined with LY294002 treatment. Both RAPA and EGCG + RAPA treatment lead to increased expression of p-4E-BP1 with no significant difference between two groups in 5637 cells. No difference in the expression of S6K was observed after EGCG, LY29400, EGCG + LY294002, RAPA or EGCG + RAPA treatment to the 5637 cell. EGCG, RAPA or EGCG + RAPA treatment had no effect on the expression of S6K. However, LY294002 and EGCG + LY29-4002 treatment lead to significantly decreased expression of p-S6K. EGCG, RAPA or EGCG + RAPA treatment induced a remarkably enhanced expression of Beclin. EGCG induced enhancement of Beclin, which was blocked by LY294002 treatment. EGCG treatment induced an enhanced expression of LC3B II/I, which was reversed by co-treated with LY294002. Both RAPA and EGCG + RAPA treatment could lead to increased expression of LC3B II/I. We also showed that knockdown of ATG5 reversed EGCG-induced apoptosis in 5637 cells (**Figure 9**). These results suggest that the anti-tumor effect of EGCG might be through autophagy pathway.

## Discussion

In the present study, we reported that EGCG exhibited an inhibitory effect on bladder cancer through regulating autophagy pathway. We found that EGCG induced proliferation inhabitation and apoptosis in bladder cancer cell. In addition, our results suggested that low dose of EGCG enhanced autophagy activation and formation in bladder cancer indicated by upregulation of LC3B II and Beclin. These effects were reversed by PI3K/AKT inhibitor.

There is crosstalk between autophagy and apoptosis [9-11]. EGCG is able to inhibit the formation and development of tumors by inducing cancer cell apoptosis [12]. By MTT assay and flow cytometry analysis, we found that EGCG inhibited bladder cancer cells proliferation and increased apoptosis in a dose dependent manner. In line with the result of flow cytometry analysis, we found that EGCG could enhance expression of apoptosis related protein (caspase 3, caspase 9 and Bax), and decrease expression of anti-apoptosis factor BCL2. BCL2 and Caspase 9 were previously suggested to be involved in the initiation of autophagy [13, 14]. Furthermore, we analyzed the typical autophagy related protein LC3B II/I [15, 16]. Interestingly, low dose of EGCG treatment led to significantly increased expression of LC3BII in the 5637 and T24 cell. By the electron microscope observation, we found that EGCG caused

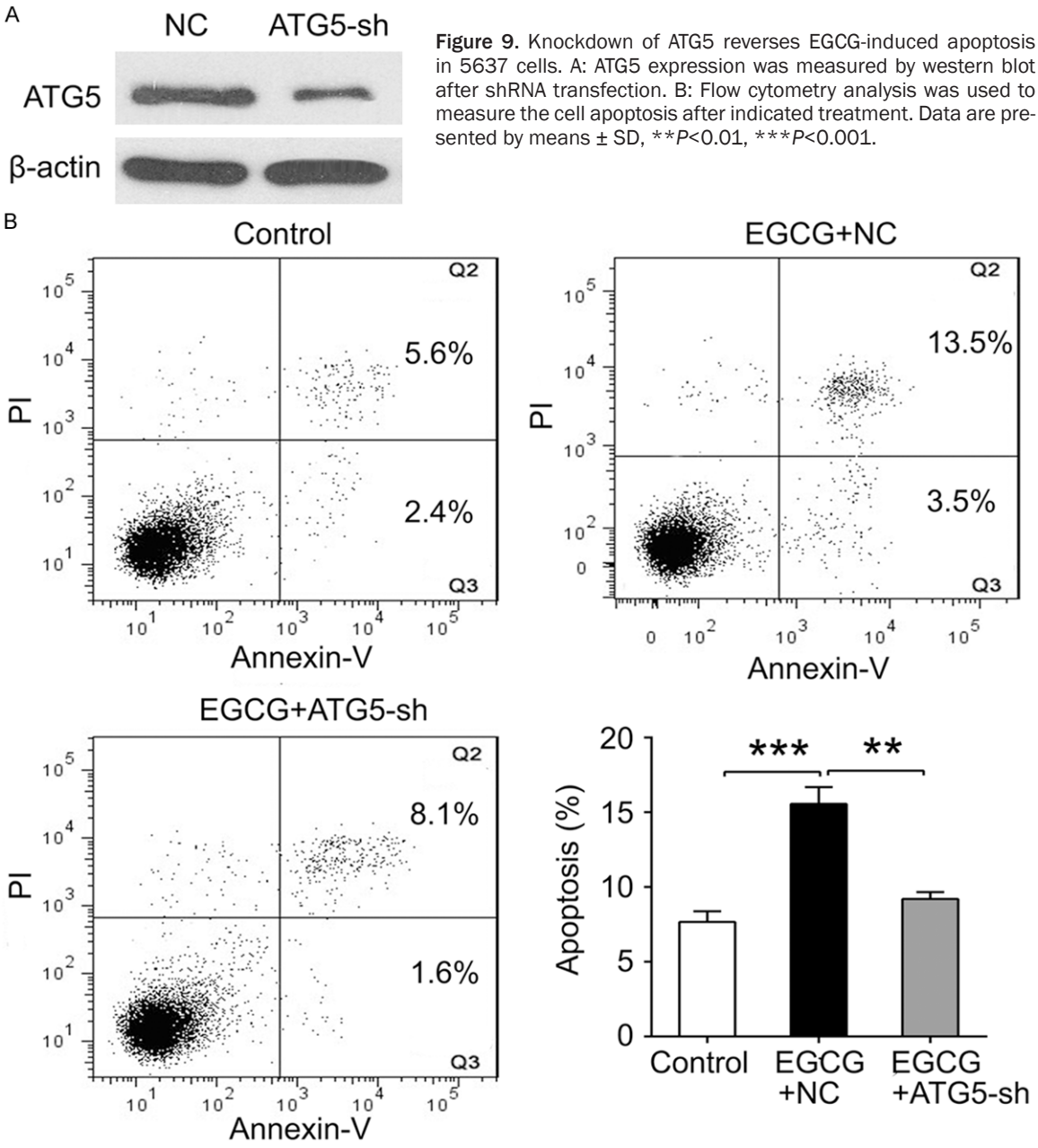


**Figure 9.** Knockdown of ATG5 reverses EGCG-induced apoptosis in 5637 cells. A: ATG5 expression was measured by western blot after shRNA transfection. B: Flow cytometry analysis was used to measure the cell apoptosis after indicated treatment. Data are presented by means ± SD, $^{**}P<0.01$, $^{***}P<0.001$.

autophagosome formation, a typical characteristic of autophagy [17]. These results indicate that low dose of EGCG lead to bladder cancer cell death through autophagy-mediated apoptosis.

mTOR is a major modulator of autophagy, and its phosphorylation (p-mTOR) can inhibit autophagy [18, 19]. In the 5637 cell, low dose of EGCG led to significantly increased expression of mTOR, resulting in a decreased ratio of p-mTOR to mTOR, thus activating autophagy pathway. 4E-BP1 and S6K is the downstream molecules of mTOR targeted protein [20]. We found that low dose EGCG treatment decreased the ratio of p-S6K to S6K, but could not alter the ratio of p-4E-BP1 to 4E-BP1 in 5637 cell. As EGCG only caused altered expression of mTOR in 5637 cell, thus, we mainly focused on the role of EGCG in this cell line. PI3K/AKT/mTOR pathway is involved in the activation of autophagy [17, 21], and mTOR is a downstream target of PI3K/AKT pathway [22, 23]. In line with previous reports [24, 25], we observed that both PI3K/AKT and p-mTOR inhibitor could induce apoptosis in 5637 cell. Interestingly, when

5637 cells were co-treated with EGCG and PI3K/AKT inhibitor, but not RAPA, a synergistic effect was found in enhancement of apoptosis. We found that EGCG treatment led to a remarkably enhanced expression of autophagy related proteins (Beclin and LC3BII), which were selectively reversed to even under baseline level when bladder cells were co-treated with LY-294002. Thus it is reasonable to infer that the synergistic effect of EGCG with PI3K/AKT inhibitor to induce apoptosis might be via activation of autophagy. Autophagy contributed to apoptosis is context dependent. Activation of autophagy pathways can reduce cell death [26], but in some cases, autophagy may act as a pro-apoptotic role [27, 28]. In addition, Autophagy is able to collaborate with apoptosis to sensitize glioma cells to death stimuli [29]. In the present study we found that inhibition of autophagosome formation by knockdown of ATG5 was able to rescue EGCG-induced apoptosis in bladder cancer cells. Thus it is reasonable to infer that low dose of EGCG lead to the inactivation of PI3K/AKT/mTOR pathway, resulting in growth inhibition of bladder cancer cell by facilitating crosstalk between apoptosis and autophagy.

In conclusion, our data demonstrate that EGCG induces bladder cell apoptosis by activating autophagy pathway. Thus, drinking green tea containing EGCG might be a promising strategy in bladder cancer prevention.

## Disclosure of conflict of interest

None.

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