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Epigallocatechin-3-gallate induces autophagy-related apoptosis associated with LC3B II and Beclin expression of bladder cancer cells

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Abstract

The incidence of bladder cancer in traditional green tea-consuming countries was dramatically lower than low green tea-consuming countries. Epigallocatechin-3-gallate (EGCG), an active ingredient extracted from green tea, showed effective inhibition of formation and progression of many tumors. However, whether autophagy involved in this tumor-suppression mechanism of EGCG on bladder cancer was still unclear. In this study, we demonstrated low concentration of EGCG-induced proliferation inhibition and increased apoptosis in bladder cancer cell lines (5,637 and T24 cells) indicated by the increased expression of apoptosis-related protein (caspase9, caspase3 and BAX). In addition, low dose of EGCG also regulated autophagy pathway associated protein (LC3B II and Beclin) expression and this autophagy pathway was blocked by PI3K/AKT inhibitor; moreover, knockdown of ATG5 reversed EGCG-induced apoptosis in 5,637 cells, indicating that EGCG might inhibit the bladder cancer through autophagy pathway. Our findings indicated that EGCG should be considered as a novel therapy for bladder cancer treatment by regulating autophagy pathway.

Practical applications

Our research proved EGCG from green tea could be used as an effective anti-tumor ingredient by revealing another mechanism that epigallocatechin-3-Gallate inhibited bladder cancer cells via inducing autophagy-related apoptosis. And green tea could be considered as a kind of tumor-preventing beverage.

KEYWORDS

autophagy, apoptosis, bladder cancer, epigallocatechin-3-gallate

1 | INTRODUCTION

The incidence of bladder cancer in traditional green tea-consuming countries was dramatically lower than low green tea-consuming countries (Bray et al., 2018). Epigallocatechin-3-gallate (EGCG), an active ingredient extracted from green tea, as a proverbial tumor suppressant, had shown multi-mechanisms anti-tumor effects in many cancers (Bimonte & Cascella, 2020; Zhou et al., 2020), including inhibit initiation and progression of tumors by increasing autophagy-related apoptosis (Fang et al., 2015; Hu et al., 2015). Reports indicated that excessive activation of autophagy would lead to cell death, which was

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called autophagy-dependent cell death (Lorenzo et al., 2018). Although apoptosis and autophagy had their own signal pathways, they had common stimulators and regulatory proteins, and there was a correlation between the two pathways (Subhadip et al., 2014), and the Bcl-2 protein family was the key factor among them (Atan & Katz, 2017).

In bladder cancer, EGCG was also reported that promote apoptosis via modulation of the PI3K/Akt pathway (Qin et al., 2007) and inhibit invasion via suppression of NF-κB-regulated matrix metalloproteinase-9 (MMP-9) expression of bladder cancer cells of human (Qin et al., 2012). Furthermore, a kind of EGCG loaded gold nanoparticles had been used to treatment bladder cancer in a mouse model (Hsieh et al., 2011). However, whether autophagy was involved in its anti-cancer effect remains undiscovered. In this study, we aim to verify whether the anti-tumor properties of EGCG was associated with autophagy pathway in bladder cancer. We exposed T24 and 5,637 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 5,637 cells. Furthermore, PI3K/AKT inhibitor blocked the EGCG-mediated upregulation of Beclin1 and LC3BII in 5,637 cells, and knockdown of autophagy-related gene 5 (ATG5) reversed EGCG-induced apoptosis in 5,637 cells, suggesting the anti-tumor effect of EGCG might be through autophagy pathway.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human bladder transitional cell carcinoma cell lines (T24 and 5,637) were bought from American Type Culture Collection (ATCC, Manassas, VA, USA). T24 cells were grown in McCoy's (Modified) 5A Media (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Invitrogen Life Technologies). 5,637 cells were cultured in RPMI1640 (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies). All cells were kept under a humidified 5% CO_2 incubator at 37°C.

2.2 | shRNA transfection

For iRNA of ATG5, we transfected 50 nM ATG5 shRNA into 5,637 cells with Lipofectamine 3,000 (Life technologies, Grand Island, NY) to knock down the expression of ATG5. The cells were used for further analysis after 48 hr transfection. shRNA scramble was applied as negative control. The sequences were used as follows: ATG5-sh: 5′ TCACGTTGTCTGATATATTCTAAAGdTdT3′ (cat no: Q000009474-1-B, RiboBio Co., Ltd, Guangzhou, China).

2.3 | MTT assay

To test the impact of EGCG to T24 and 5,637 cells growth, the cells were planted into 96-well plates with 8×10^3 cells/well. After cultured for 4 hr, 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 hr. Then, 5.0 mg/ml (final concentration) of MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and incubated for another 4 hr at 37°C. The formazan dissolved in dimethyl sulfoxide (Sigma-Aldrich) was generated in each well. Absorbance of each well at 570 nm was measured with a microplate reader (Synergy™ Mx; BioTek, Winooski, VT, USA). The experiments were repeated for 3 times.

2.4 | Sulforhodamine B (SRB) assay

Cells in the logarithmic growth phase were adjusted to 5×10^4 cells/ml of the density, and planted in a 96-well plate, 100 μL per well. After culturing for 4 hr, the old culture medium was discarded. The wells were washed with PBS. New culture medium was added in wells of the blank group, and the experiment groups were treated with different concentrations of EGCG as 2.3. After 24, 48 and 72 hr, OD values (wavelength was 550 nm) were measured with a microplate reader. The experiments were repeated for three times.

2.5 | Flow cytometric analysis of the cell apoptosis

The cells after treatment as above-mentioned were trypsinized and re-suspended with ice-cold PBS, then incubated for 15 min in dark using Annexin V/PI detection kit (Molecular Probes Inc., Eugene, OR) according to the manufacturer's instructions. Cell apoptosis was analyzed by flow cytometry (Beckman Coulter, USA). The experiments were repeated for three times.

2.6 | Electron microscopy analysis

After fixed in 3% glutaraldehyde diluted with 0.1 M phosphate buffer (pH 7.4), the cells treated 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 using a LVEM5 transmission electron microscope (TEM) (Quantum Design, Beijing, China).

2.7 | Western blot

Protein in the cells was extract with RIPA lysis buffer (Boster, Wuhan, China), and protein concentration was measured with BCA Protein Assay Kit (Thermo Scientific, USA). Total protein was separated on

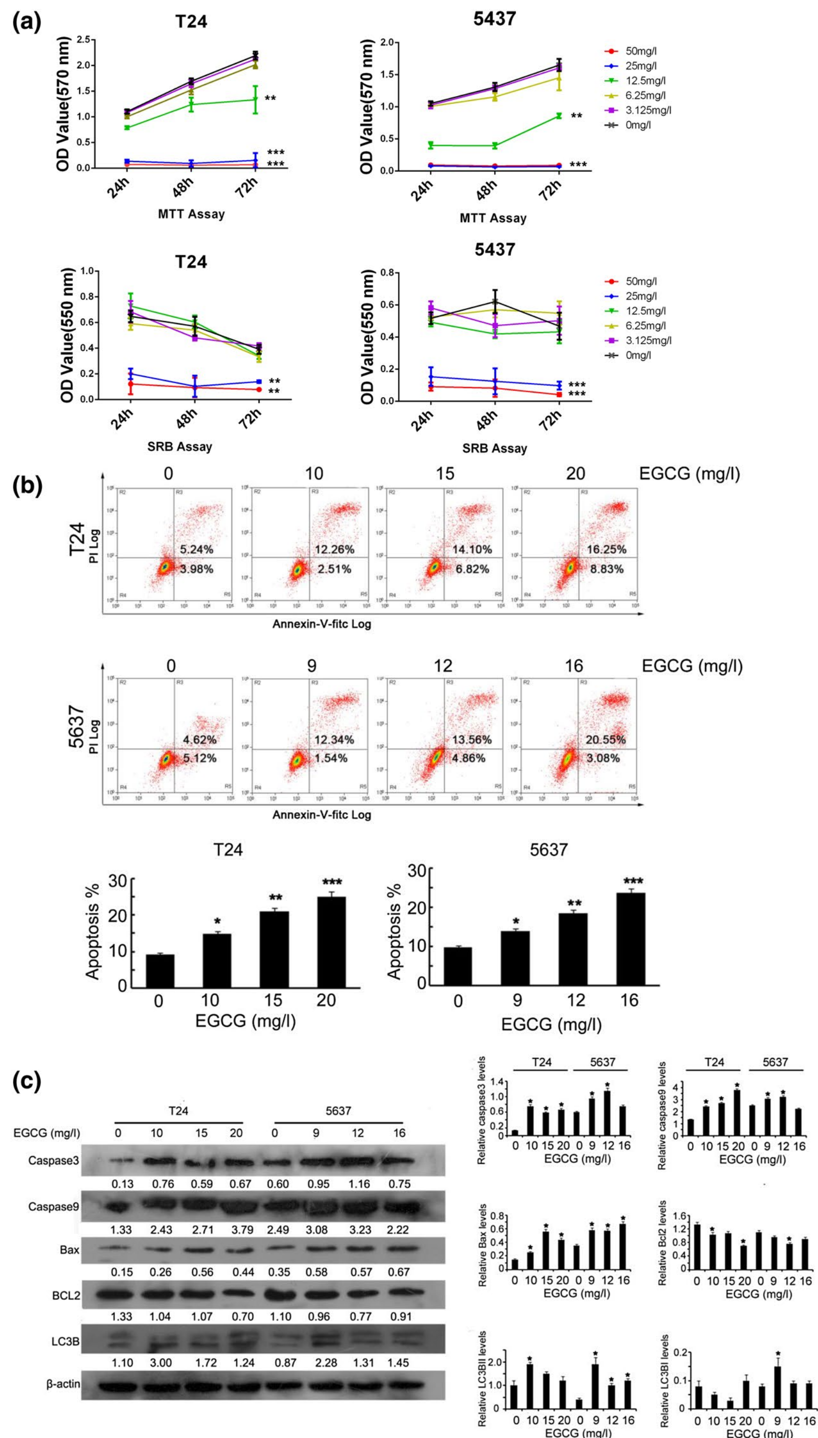

FIGURE 1 (a): EGCG induces proliferation inhibition in a dose-dependent manner in bladder cancer cells. MTT and SRB assays were used to measure the cell proliferation after T24 cells (right) and 5,637 cells (left) exposed to a range of concentrations of EGCG (0, 3.125, 6.25, 12.5, 25 and 50 mg/L) for 24 hr. Data were presented by means ± *SD*, **p* < .05, ***p* < .01, ****p* < .001 versus 0 mg/L group. (b): 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 5,637 cells (lower) exposed to a range of concentrations of EGCG (10, 15, 20mg/L for T24 cells and 9,12,16 mg/ L for 5,637 cells) for 24 hr. The histogram: quantification of cell apoptosis in T24 cells (left) and 5,637 cells (right). Data were presented by means ± *SD*, **p* <.05, ***p* <.01, ****p* <.001 versus. 0 mg/L group. **1C:** EGCG regulates apoptosis and autophagy related protein expression. Western Blot was used to measure apoptosis and autophagy related protein expression after T24 cells and 5,637 cells exposed to a range of concentrations of EGCG (10, 15, 20mg/L for T24 cells and 9,12,16 mg/ L for 5,637 cells) for 24 hr. Data were presented by means ± *SD*, **p* < .05 versus 0 mg/L group

10% SDS-PAGE and blotted onto nitro cellulose membranes (Boster, Wuhan, China). The membranes were blocked for 2 hr with 5% skim dry milk diluted with tris-buffered saline (TBS) and incubated with primary antibodies (rabbit polyclonal anti-caspase 3, anti-caspase 9, anti-Bax, anti-BCL-2, anti-LC3B and anti-β-actin, and anti-Beclin-1 (Abcam, UK); anti-mTOR, anti-p-S6K(Thr389), and anti-p-mTOR, anti-4E-BP1, anti-p-4E-BP1(ser65), anti-S6K (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 the secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1h under 37°C. Chemiluminescence enhancer (Wuhan Boster) was used to detect the signal on the membrane with a densitometry using Image-Pro plus software 6.0 (Media Cybernetics, Rockville, MD, USA) and normalized to the expression of the internal control (β-actin).

2.8 | Statistical analysis

Statistical analyses were performed with software package GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data were 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. $p < .05$ was considered to indicate a statistically significant difference.

3 | RESULTS

3.1 | EGCG inhibits proliferation and induces apoptosis in a dose dependent manner in T24 and 5,637 cells

To investigate the role of EGCG on bladder cancer cell growth, we exposed T24 and 5,637 cells to serial concentrations (0, 3.125, 6.25, 12.5, 25 and 50 mg/L) of EGCG. As shown in Figure 1a, 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 5,637 cells to serial concentrations (10, 15, 20 mg/L for T24 cells, and 9, 12, 16 mg/L for 5,637 cells, respectively according to the proper gradient concentrations of EGCG to different cell lines) of EGCG. We found that EGCG effectively induced T24 and 5,637 cells apoptosis with increasing dose (Figure 1b). These results indicated that EGCG inhibited bladder cancer cell proliferation, and induced cell apoptosis in a dose-dependent manner.

3.2 | EGCG regulates apoptosis and autophagy related protein expression

To investigate the correlation between EGCG and apoptosis and autophagy, we exposed T24 cells and 5,637 cells to EGCG with a range of concentrations (10, 15, 20 mg/L for T24 cells and 9, 12, 16 mg/L for 5,637 cells). We found that EGCG treatment enhanced expression of Caspase3, 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 9 and 20 mg/L in T24 cells, while significantly reduced the expression of BCL2 at 12 mg/L in 5,637 cells. EGCG treatment led to significantly increased expression of LC3BII at 9, 12 and 16 mg/L in the 5,637 cell, whereas only low dose (9 mg/L) of EGCG lead to increased expression of LC3BII in T24 cell. EGCG treatment could inhibit the expression of LC3BI in the T24 cell in a dose dependent manner, but not reach significance. And only low dose of EGCG treatment led to significantly increased expression of LC3BI in 5,637 cell, which was not found in other dose (Figure 1c).

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 5,637 cells, especially at low dose (Figure 2a).

3.3 | EGCG enhances the expression of autophagy pathway-related proteins

In order to explore the pathway involved in autophagy affected by EGCG, we detected the key molecules expression of autophagy pathway. The results indicated that no significant difference in the expression of mTOR and p-mTOR was found in the T24 cell after EGCG treatment 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. In the 5,637 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 5,637 cells. In addition, EGCG could lead to significantly increased expression of S6K and p-S6K (Figure 2b).

3.4 | 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 5,637 cells. Thus, we chose 5,637 cells to investigate whether EGCG regulated bladder cancer cell proliferation and apoptosis through PI3K/mTOR signaling. We exposed 5,637 cells to a range of concentrations of EGCG. We found that both PI3K/AKT inhibitor (LY294002) and mTOR inhibitor (RAPA) could inhibit the proliferation of 5,637 cell. And the half maximal inhibitory concentration (IC50) was 0.7767 μM (rounded to 0.78 μM) for LY294002, and 0.2392 μg/ml (rounded to 0.24 μg/ml) for RAPA. These two concentrations were selected for further investigation (Figure 2c).

Both EGCG and LY294002 could lead to significantly increased apoptosis in 5,637 cell, and LY294002 was more effective than EGCG. When combined EGCG with LY294002 to treat the 5,637 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 3a).

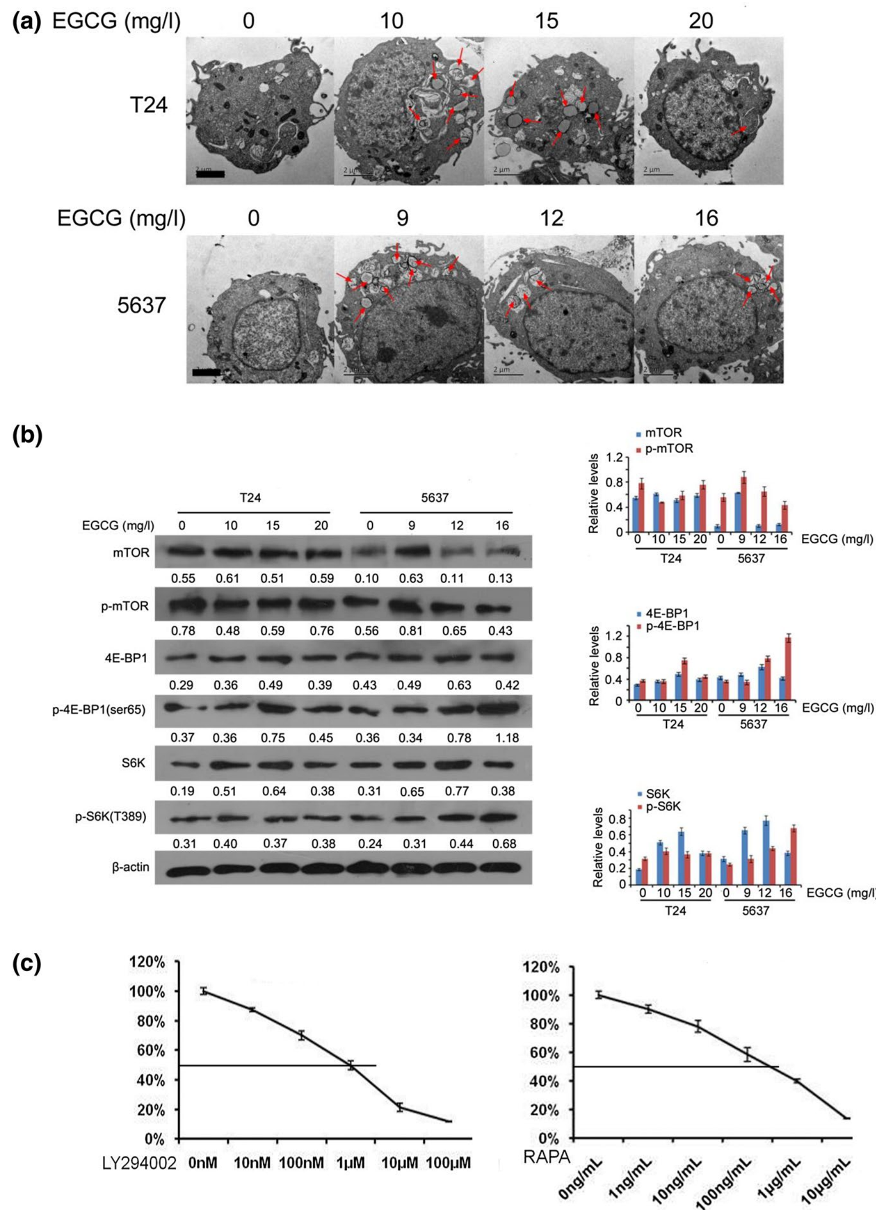

FIGURE 2 (a): EGCG lead to the formation of autophagosome formation. Transmission electron microscope was used to observer autophagosome formation (indicated by red arrow) after T24 cells and 5,637 cells exposed to a range of concentrations of EGCG (10, 15, 20 mg/L for T24 cells and 9,12,16 mg/ L for 5,637 cells) for 24 hr. (b): 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 5,637 cells exposed to a range of concentrations of EGCG (10, 15, 20 mg/L for T24 cells and 9,12,16 mg/L for 5,637 cells) for 24 hr. Data were presented by means ± *SD*, **p* < .05, ***p* < .01 versus 0 mg/L group. (c): Effects of PI3K and mTOR inhibitor on EGCG-mediated cell apoptosis. MTT was used to measure the cell proliferation after 5,637 cells exposed to a range of concentrations of LY294002 or RAPA (10 nM, 100 nM, 1 Mm, 10 μM, 100 μM for LY294002 and 1 ng, 10 ng, 100 ng, 1 μg, 10 μg, 100 μg for RAPA) for 24 hr. Data were presented by means ± *SD*. The half maximal inhibitory concentration (IC50) was 0.7767 μM (rounded to 0.78 μM) for LY294002, and 0.2392 μg/ml (rounded to 0.24 μg/ml) for RAPA

3.5 | 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 with PI3K inhibitor, but not mTOR inhibitor. Thus, we exposed 5,637 cells to PI3K/AKT inhibitor (LY294002) or mTOR inhibitor (RAPA) for 24 hr, and then detected the expression of the key molecules of autophagy pathway. As shown in Figure 3b, 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 5,637 cells. No difference in the expression of S6K was observed after EGCG, LY29400, EGCG+LY294002, RAPA or EGCG+RAPA treatment to the 5,637 cell. EGCG, RAPA

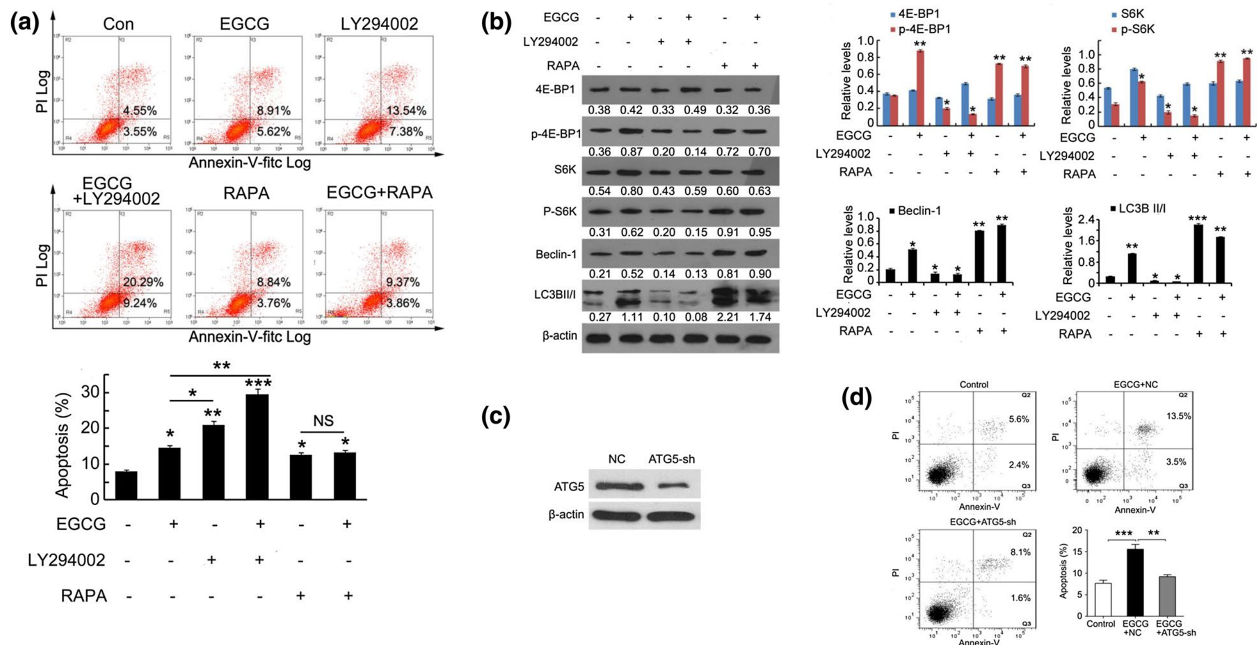

FIGURE 3 (a): Flow cytometry analysis was used to measure the cell apoptosis after 5,637 cells exposed to a low concentration of EGCG (9 mg/L), LY294002 (0.78 μM), EGCG combined with LY294002(EGCG + LY294002), RAPA (0.24 μg/ml), RAPA combined with EGCG (EGCG + RAPA) for 24 hr. Data were presented by means ± *SD*, **p* < .05, ***p* < .01, ****p* < .001. (b): PI3K inhibitor blocks EGCG-promoted autophagy in bladder cancer cells. Western blot was used to measure autophagy pathway-related protein after 5,637 cells exposed to a low concentration of EGCG (9 mg/L), LY294002 (0.78 μM), EGCG combined with LY294002 (EGCG + LY294002), RAPA (0.24 μg/ml), RAPA combined with EGCG (EGCG + RAPA) for 24 hr. Data were presented by means ± *SD*, **p* < .05, ***p* < .01, ****p* < .001 versus control group. (c): Knockdown of ATG5 reverses EGCG-induced apoptosis in 5,637 cells. A: ATG5 expression was measured by western blot after shRNA transfection. (d): Flow cytometry analysis was used to measure the cell apoptosis after indicated treatment. Data were presented by means ± *SD*, ***p* < .01, ****p* < .001

or EGCG+RAPA treatment had no effect on the expression of S6K. However, LY294002 and EGCG+LY294002 treatment lead to significantly decreased expression of p-S6K. EGCG, RAPA or EGCG+RAP treatment induced a remarkably enhanced expression of Beclin. EGCG induced enhancement of Beclin expression, 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 5637cells (Figure 3c,d). These results suggest that the anti-tumor effect of EGCG might be through autophagy pathway.

4 | DISCUSSION

EGCG was a well-known anti-tumor ingredient which was extracted from green tea (Hwang et al., 2020). Besides, the mechanisms of the anti-tumor effects (Wei et al., 2020; Zhang et al., 2020), induction of autophagy-related apoptosis for example (Chen et al., 2020), were well-studied in many cancers (Kumazoe et al., 2020; Naujokat & McKee, 2020), including bladder cancer (Dettlaff et al., 2017). However, whether autophagy was involved in its anti-cancer effect remains undiscovered. In this study, we found that EGCG induced proliferation inhibition and apoptosis in bladder cancer cell through regulating autophagy pathway. In addition, our results suggested that low dose of EGCG enhanced autophagy activation and formation in bladder cancer indicated by upregulation of LC3BII and Beclin. These effects were reversed by PI3K/AKT inhibitor.

There was crosstalk between autophagy and apoptosis (Amaravadi et al., 2007; Gordy and He, 2013; Gordy & He, 2013; Wu et al., 2015). EGCG was able to inhibit the formation and development of tumors by inducing cancer cell apoptosis (Philips et al., 2009). By MTT assay, SRB 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 (caspase3, 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 (Levine et al., 2008; Zhou et al., 2000). Furthermore, we analyzed the typical autophagy-related protein LC3B II/I (Klionsky et al., 2007; Lee et al., 2014). Interestingly, low dose of EGCG treatment significantly increased the expression of LC3BII in the 5,637 and T24 cell. By the electron microscope observation, we found that EGCG caused autophagosome formation, a typical characteristic of autophagy (Yang & Klionsky, 2010). These results indicate that low dose of EGCG

lead to bladder cancer cell death through autophagy-mediated apoptosis.

mTOR was a major modulator of autophagy, and its phosphorylation (p-mTOR) can inhibit autophagy (Cuyas et al., 2014; Nazio et al., 2013). In the 5,637 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 was the downstream molecules of mTOR targeted protein (Corradetti & Guan, 2006). 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 5,637 cell. As EGCG only caused altered expression of mTOR in 5,637 cell, thus, we mainly focused on the role of EGCG in this cell line. PI3K/AKT/mTOR pathway was involved in the activation of autophagy (Heras-Sandoval et al., 2014; Yang & Klionsky, 2010), and mTOR was a downstream target of PI3K/AKT pathway (Hay, 2005; Manning & Cantley, 2007). In line with previous reports (Cai et al., 2012; Wang et al., 2015), we observed that both PI3K/AKT and p-mTOR inhibitor could induce apoptosis in 5,637 cell. Interestingly, when 5,637 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 LC3B||), which were selectively reversed to even under baseline level when bladder cells were co-treated with LY294002. Thus, it was 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 was context dependent. Activation of autophagy pathways could reduce cell death (Eisenberg-Lerner et al., 2009), but in some cases, autophagy may act as a pro-apoptotic role (He et al., 2012; Heitz et al., 2009). In addition, Autophagy was able to collaborate with apoptosis to sensitize glioma cells to death stimuli (Thorburn et al., 2014). 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 was 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.

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CONFLICTS OF INTEREST

The authors declared that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Writing-original draft; Writing-review & editing: Zhaofa Yin. *Conceptualization; Data curation; Formal analysis; Methodology; Project administration; Resources; Software*: Juan Li. *Project administration; Validation*: Le Kang. *Methodology*: Jianguo Luo. *Methodology*: Xiangyang Liu. *Methodology*: Ling Zhang. *Methodology*: Yuting Li. *Investigation; Methodology*: Jiarong Cai.

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