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ORIGINAL ARTICLE

Autophagy inhibition contributes to epigallocatechin-3-gallate-mediated apoptosis in papillary thyroid cancer cells

Ling Bu[1,2] · Tingting Zheng[1] · Chaoming Mao[1] · Fei Wu[3] · Xiao Mou[1] · Chengcheng Xu[1] · Xuan Luo[1] · Qingyan Lu[1] · Liyang Dong[1] · Xuefeng Wang[1]

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Abstract

Background Epigallocatechin-3-gallate is a natural polyphenolic compound that induces apoptosis in papillary thyroid cancer cells. However, its underlying molecular mechanism was not completely clarified.

Objectives The present study demonstrated the role of apoptosis and autophagy in EGCG-treated papillary thyroid cancer cells and the relationship between these processes.

Results EGCG significantly suppressed the viability of TPC-1 papillary thyroid cancer cells at an IC50 of 17.2 μM. EGCG induced TPC-1 cell apoptosis and cell cycle arrest at S phase and downregulated the protein expression of cyclin A and cyclin-dependent kinase-2. EGCG decreased reactive oxygen species levels, upregulated Bax expression, downregulated Bcl-2 expression and increased cytochrome C levels in the cytosol. Treatment with EGCG also increased the levels of cleaved caspase 3, cleaved caspase 9 and cleaved poly(ADP-ribose) polymerase. EGCG induced an autophagic response via the upregulation of the autophagy-related protein LC3-II and suppression of the AKT/mTOR signalling pathway. Autophagy inhibition further enhanced EGCG-induced cell apoptosis and ROS suppression, which indicated that autophagy played a cytoprotective role in EGCG-treated TPC-1 cells.

Conclusion Taken together, these results demonstrated that autophagy inhibition was beneficial to EGCG–mediated apoptosis in papillary thyroid cancer cells.

Keywords Epigallocatechin-3-gallate · Apoptosis · Autophagy · ROS · Papillary thyroid cancer

Introduction

Thyroid cancer is the most frequent malignancy of the endocrine system, and the incidence of thyroid cancer has increased rapidly (Wang and Sosa 2018). The increased incidence is almost entirely attributable to papillary thyroid cancer. Patients with papillary thyroid cancer are traditionally treated with ^{131}I ablation after total thyroidectomy with thyroid stimulating hormone (TSH) suppression, and most patients have a good prognosis (American Thyroid Association, et al. 2009; Sawka et al. 2004). However, approximately 10% of advanced thyroid cancer patients have a poor prognosis (Tumino et al. 2017). Therefore, the development of potential alternative therapies for the treatment of papillary thyroid cancer is necessary.

Green tea is the most popular beverage consumed worldwide, and higher tea consumption may reduce the risk of thyroid cancer (Ma et al. 2015). Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea and it inhibits the proliferation of different kinds of thyroid cancer cells (Allegri et al. 2018; Wu et al. 2019). EGCG treatment suppressed proliferation and induced apoptosis of ARO anaplastic thyroid carcinoma cells via suppression of the EGFR/ERK pathway and cyclin B1/ cyclin-dependent kinase 1 (CDK1) complex (Lim and Cha 2011). EGCG also

✉ Chaoming Mao
jq1001@ujs.edu.cn

[1] Department of Nuclear Medicine, The Affiliated Hospital of Jiangsu University, 438 Jiefang Road, Zhenjiang 212001, China

[2] Department of Pharmacy, Jiangsu Health Vocational College, 69 Huangshanling Road, Nanjing 211800, China

[3] Department of Nuclear Medicine, The First People's Hospital of Yancheng, 166 Yulong west Road, Yancheng 224005, China

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inhibited the growth of human thyroid papillary (FB-2) and follicular (WRO) carcinoma cells in a dose-dependent manner (Amicis et al. 2013). However, the mechanisms underlying the effects of EGCG in papillary thyroid cancer cells are not completely understood. Previous studies indicated the involvement of EGCG in apoptotic or autophagy-induced cell death (Mukhtar et al. 2012; Wang et al. 2014). Whether EGCG induced apoptosis and autophagy in papillary thyroid cancer cells and the relationship between these processes remain unknown.

The present study examined the potential effects and mechanisms of EGCG on papillary thyroid cancer cells and provides new insight for the prevention and adjuvant therapy of papillary thyroid cancer.

Results

EGCG inhibits the proliferation of papillary thyroid cancer cells but not human thyroid follicular epithelial cells

To ascertain the ability of EGCG to inhibit papillary thyroid cancer cell growth, the effects of EGCG treatment were compared in the papillary thyroid cancer cell line TPC-1 and the human thyroid follicular epithelial cell line Nthy-ori 3-1 (Fig. 1a, b), The results revealed that 24 and 48 h of EGCG treatment with selectively inhibited the growth of TPC-1 cells in a time- and dose-dependent manner but did not affect Nthy-ori 3-1 cells. The anti-proliferative efficacy of EGCG in TPC-1 cells was more effective at 48 h post-treatment than at 24 h. Based on these results, the IC50 concentration (17.2 μM) and a processing time of 48 h were selected for subsequent experiments.

EGCG promotes apoptosis in papillary thyroid cancer cells

To elucidate whether EGCG-induced cell growth inhibition was mediated via alterations in cell apoptosis, the effect of EGCG on cell apoptosis was evaluated using flow cytometric studies. The results showed that EGCG (17.2 μM) markedly promoted apoptosis of TPC-1 cells compared to the control group (Fig. 2a, b). Nuclear fragmentation and chromatin condensation were observed in DAPI-stained cells after treatment with EGCG (17.2 μM) for 48 h (Fig. 2c), Cell cycle arrest is an important cause of cell apoptosis, and cell cycle phase distribution was evaluated using flow cytometric analysis. After treatment with EGCG (17.2 μM) for 48 h, an appreciable arrest of cells at S phase, no accumulation of cells in G2/M phase and a decrease in G0/G1 cells were observed during apoptosis (Fig. 2d, e). To further confirm the EGCG-mediated cell cycle arrest in TPC-1 cells, S phase cycle-related proteins, including cyclin A and cyclin-dependent kinase-2 (CDK2), were examined. As shown

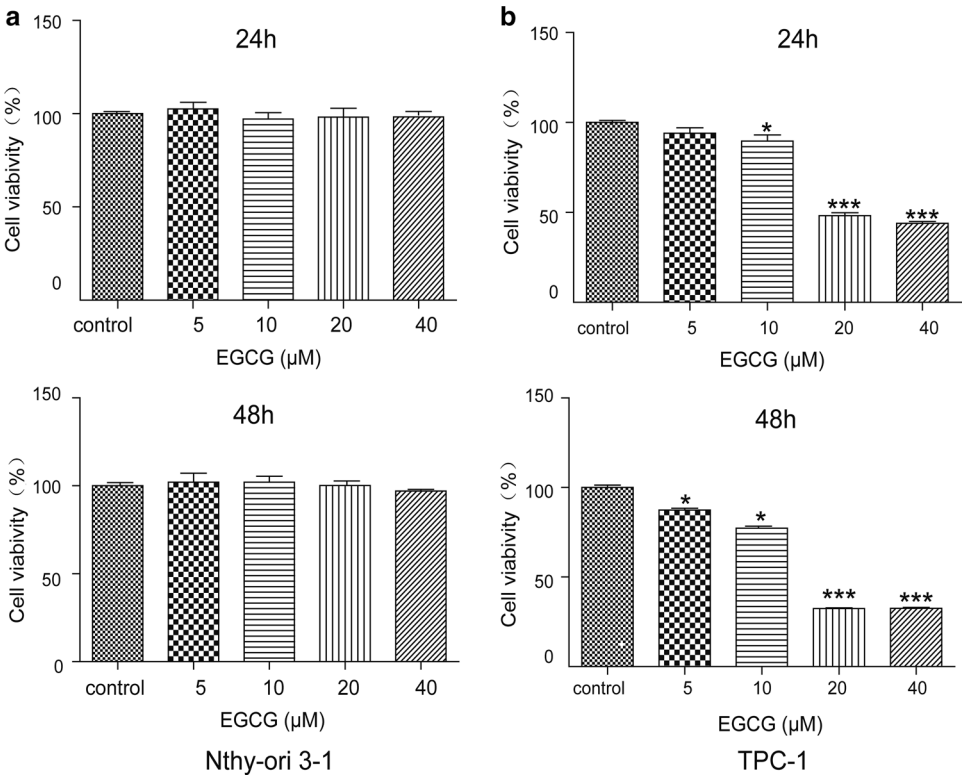

Fig. 1 Effect of EGCG on the proliferation of papillary thyroid cancer cells. **a** Nthy-ori 3–1 cells and **b** TPC-1 cells were treated with the indicated concentrations of EGCG for 24 h and 48 h, and the percentage of cell viability was estimated using the MTT assay. Data are expressed as the mean ± SEM of three independent experiments. Significant differences and P values were calculated by one-way ANOVA. $*p < 0.05$, $***p < 0.001$ *vs.* control

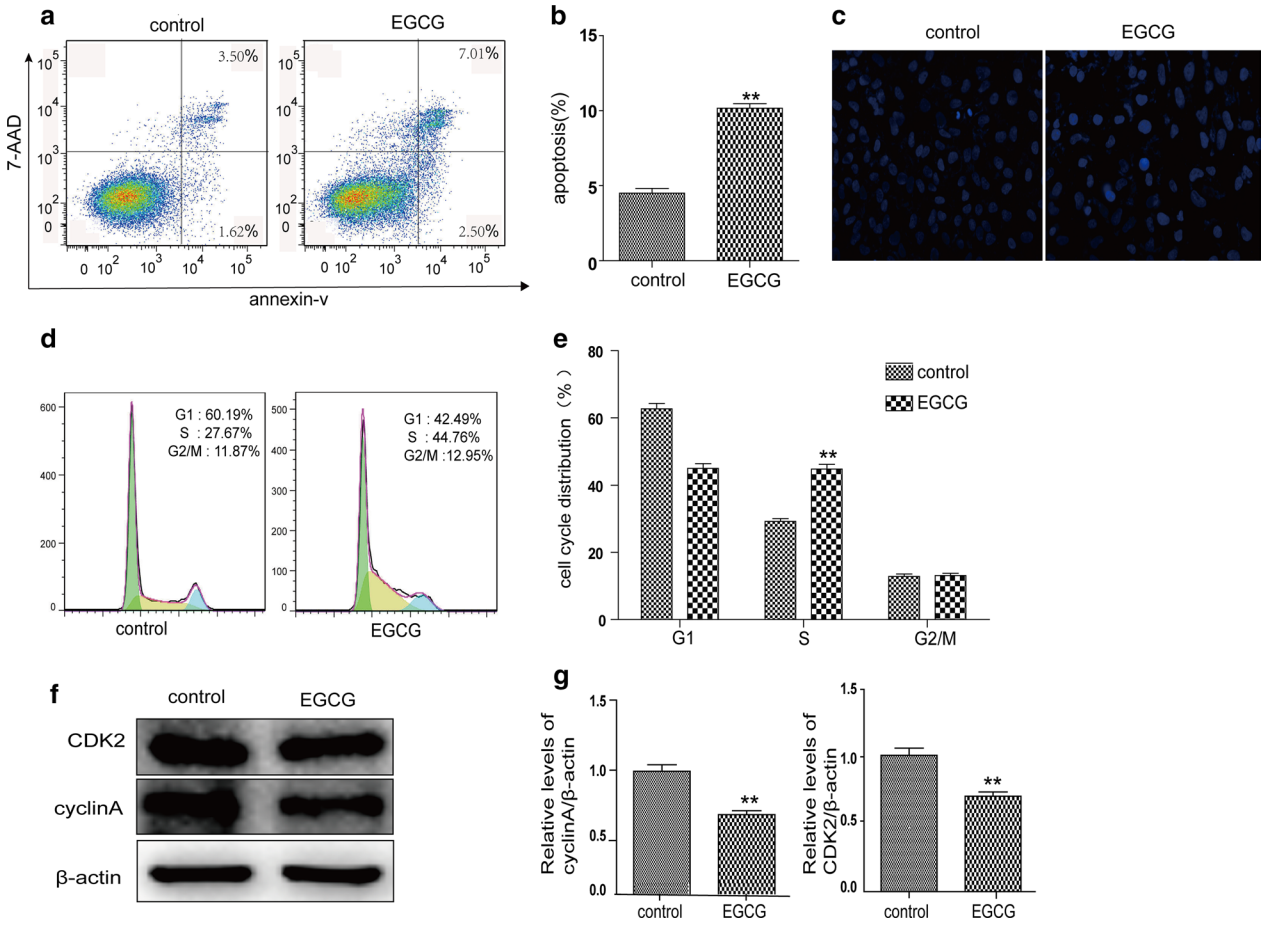

Fig. 2 Analysis of EGCG-induced apoptosis in papillary thyroid cancer cells. TPC-1 cells were treated with EGCG (17.2 μM) for 48 h, **a** stained with annexin V/7-AAD and analysed by flow cytometry. **b** The percentages of apoptotic TPC-1 cells are presented in bar charts. **c** The morphological changes in TPC-1 cells treated with EGCG (17.2 μM) were captured using fluorescence microscopy with Hoechst 33342 staining. **d** The cell cycle distribution was analysed by flow cytometry, and **e** quantification of cells in different phases is presented in bar graphs. **f** The protein levels of cyclin A and CDK2 were analysed by Western blot analysis, (**g**) and quantification of the protein levels of cyclin A and CDK2 was normalized to the control and presented in bar graphs. Data are expressed as the mean ± SEM of three independent experiments. Significant differences and *p* values were calculated with Mann–Whitney *U* tests. $^{**}p < 0.01$ *vs.* control

in Fig. 2f, g, EGCG significantly decreased the expression of cyclin A and CDK2 proteins.

The mitochondria-dependent pathway is involved in EGCG-induced apoptosis

Previous studies demonstrated increased ROS levels in EGCG-treated cancer cells (Zhang 2012). To determine whether EGCG treatment induced oxidative stress in TPC-1 cells, intracellular ROS levels were measured using a fluorescence probe (H2DCFDA). The results showed that EGCG decreased ROS levels in TPC-1 cells (Fig. 3a, b). To further determine the mechanism of EGCG-induced apoptosis, the expression of apoptosis-associated proteins was examined using Western blotting. Our results revealed that EGCG downregulated the expression of Bcl-2, upregulated the expression of Bax, and stimulated the release of cytochrome C (Cyt c) (Fig. 3c, d). EGCG treatment increased the expression of cleaved caspase 9, cleaved caspase 3, and cleaved poly(ADP-ribose) polymerase (PARP) (Fig. 3e, f).

EGCG induces autophagy in papillary thyroid cancer cells

The relationship between autophagy and apoptosis is complex, and autophagy is involved in the modulation

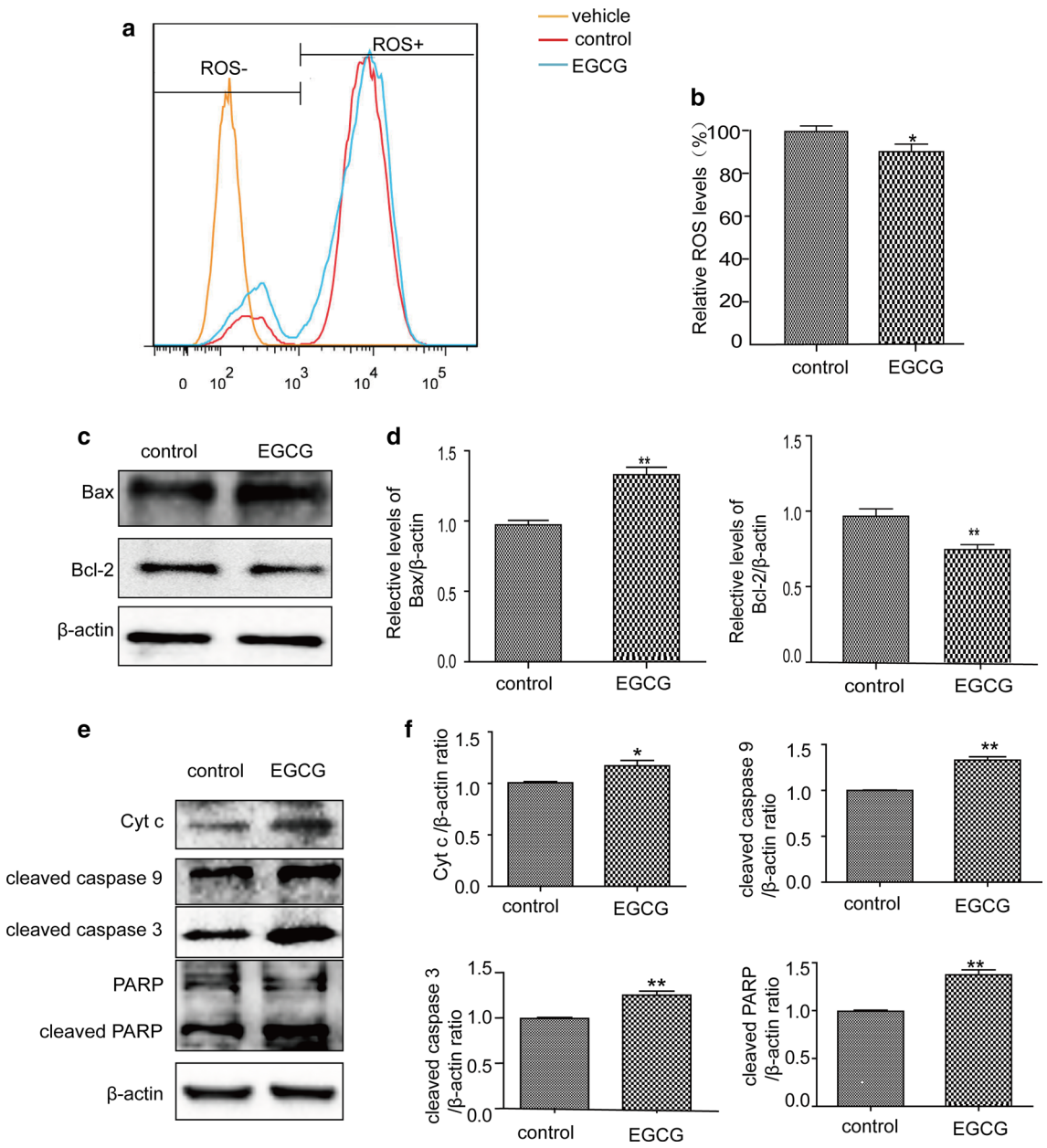

Fig. 3 Effects of EGCG on the levels of ROS and apoptosis-associated proteins in papillary thyroid cancer cells. TPC-1 cells were treated with EGCG (17.2 μM) for 48 h, (**a**) and ROS levels were measured by flow cytometry. **b** Relative quantitative data are presented in bar graphs. **c** Western blot analysis was performed to assess the levels of Bax and Bcl-2, and (**d**) relative quantitative data for Bax and Bcl-2 are presented in bar graphs. (**e**) The expression levels of Cyt c, cleaved caspase 3, cleaved caspase 9 and cleaved PARP in TPC-1 cells were estimated by Western blotting, and (**f**) relative quantitative data for Cyt c, cleaved caspase 3, cleaved caspase 9 and cleaved PARP are presented in bar graphs. Data are expressed as the mean ± SEM of three independent experiments. Significant differences and *p* values were calculated with Mann–Whitney *U* tests. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* control

of specific mechanisms of cell death (Kasprowska-Liśkiewicz 2017). To evaluate the autophagy status in EGCG-treated TPC-1 cells, the expression of autophagy-related proteins LC3B-II, P62, p-AKT and p-mTOR was detected using immunoblot analysis. The levels of LC3B-II protein in the EGCG-treated group were significantly increased compared to the control group (Fig. 4a, b). The levels of P62, p-AKT and p-mTOR proteins were decreased compared to those of the control (Fig. 4c, d).

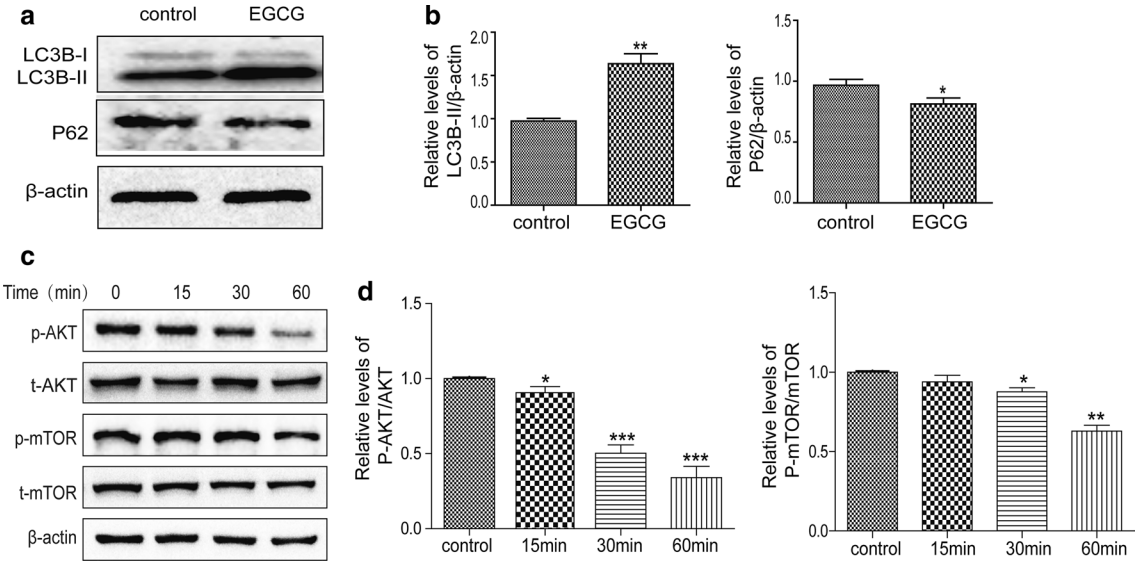

Fig. 4 Analysis of EGCG-induced autophagy in papillary thyroid cancer cells. **a** TPC-1 cells were treated with EGCG (17.2 μM) for 48 h, and the levels of LC3I/II and P62 were measured by Western blotting. **b** Relative quantification of the LC3I/II and P62 proteins is presented in bar graphs. **c** TPC-1 cells were treated with EGCG (17.2 μM) for 0, 15, 30 and 60 min, and Western blotting was performed to analyse the protein levels of p-AKT, t-AKT, p-mTOR, and t-mTOR. **d** Relative quantitative data are presented in bar graphs. Data are expressed as the mean ± SEM of three independent experiments. Significant differences and *p* values were calculated via one-way ANOVA and Mann–Whitney *U* tests. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ *vs.* control

Inhibition of autophagy contributes to EGCG-induced apoptosis in papillary thyroid cancer cells

To determine the relationship between EGCG-induced autophagy and apoptosis, TPC-1 cells were treated with the autophagy inhibitor 3-MA for 2 h before a 48 h EGCG incubation. Pre-treatment with 3-MA decreased the expression of LC3-II in EGCG-treated TPC-1 cells (Fig. 5a, b). Notably, EGCG in combination with 3-MA enhanced EGCG-induced growth inhibition (Fig. 5c) and induced a higher percentage of apoptotic cells (shown in Fig. 5d, e) than EGCG treatment alone. ROS levels were further decreased in the EGCG with 3-MA group compared to EGCG-treated TPC-1 cells (Fig. 5F, G). Taken together, these results suggest that autophagy plays a protective role in EGCG-induced cell death in TPC-1 cells.

Discussion

EGCG is the most potent compound in green tea extract, and the cancer-preventive effects of EGCG have provoked great interest (Khan and Mukhtar 2018; Link et al. 2010). Our results revealed that EGCG obviously inhibited the growth and induced apoptosis of papillary thyroid cancer cells but not normal human thyroid follicular epithelial cells. To further demonstrate the effect of EGCG on papillary thyroid cancer cells, the affection of cell cycle and its regulation were examined. Cell cycle studies showed that EGCG increased the proportion of TPC-1 cells in S phase, which is prolonged during apoptosis. The expression of CDK2 and cyclin A proteins, which are cell factors associated with the S phase of cells, was also detected. These results demonstrated that the reduction in cyclin A and CDK2 levels arrested TPC-1 cells in S phase.

ROS are produced in mitochondria and act as critical signalling molecules. Elevated rates of ROS production are detected in most cancers (Liou and Storz 2010), and excess generation of ROS contributes to cancer cell death (Feng et al. 2017). Food phytochemicals, such as capsaicin in chili pepper, acacetin in chrysanthemum, and curcumin in turmeric, induce apoptosis via the generation of ROS and blockade of cellular signal transduction in tumour cells (Pan et al. 2008). Resveratrol in a variety of plant species increased ROS generation and oxidative-related cellular lesions in resveratrol-sensitive THJ-16T anaplastic thyroid

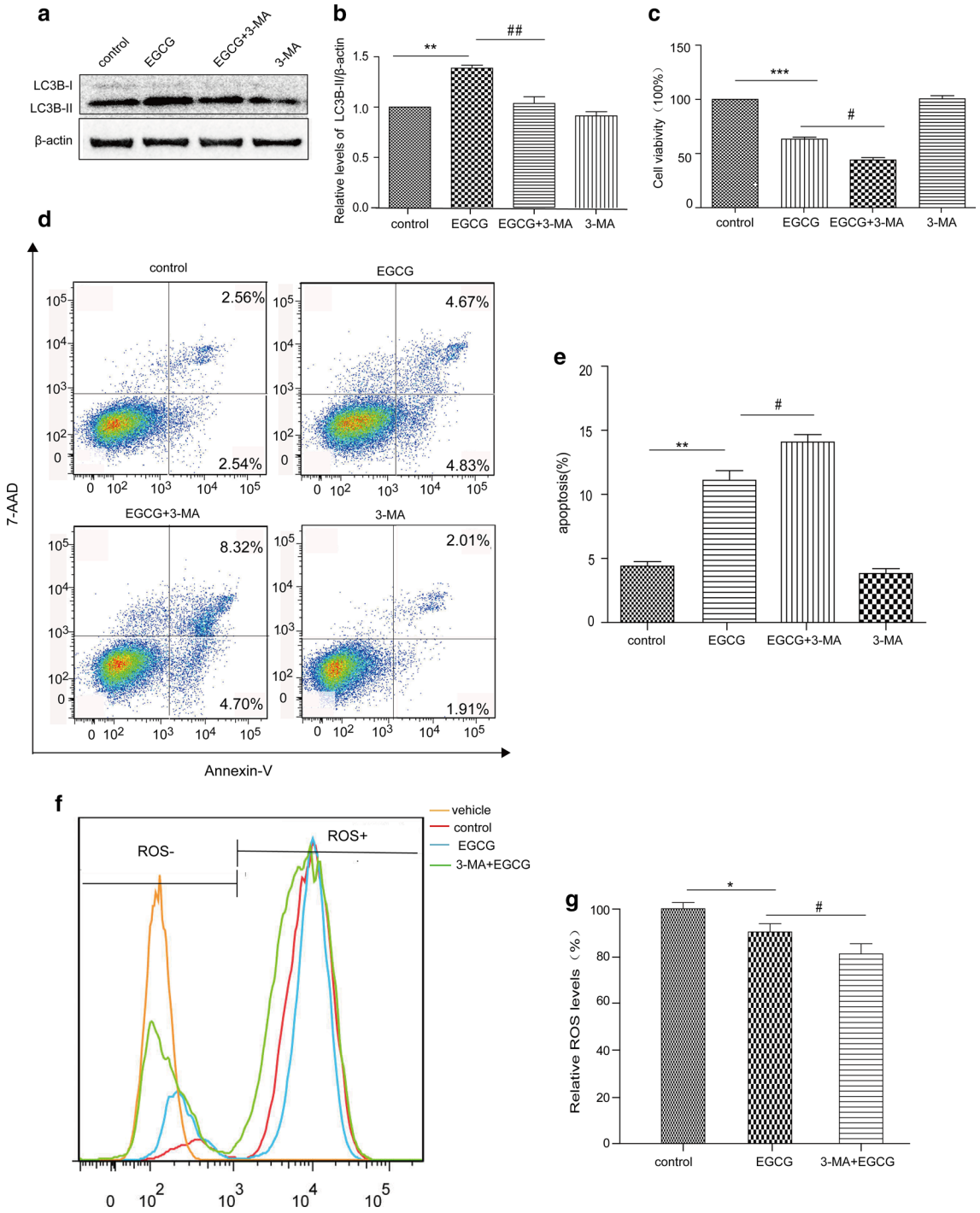

Fig. 5 Effect of autophagy inhibition on EGCG-induced TPC-1 cell death. TPC-1 cells were treated with EGCG (17.2 μM) in the presence or absence of 3-MA (0.3 mM) for 48 h. **a** The autophagy-associated protein LC3-I/II was detected by Western blot analysis, and (**b**) relative quantitative data are presented in bar graphs. **c** Cell viability was analysed by MTT assay. **d**, **e** Apoptosis was analysed by flow cytometry. **f**, **g** ROS were analysed by flow cytometry. Data are expressed as the mean ± SEM of three independent experiments. Significant differences and *p* values were calculated by one-way ANOVA. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ *vs.* control; $^{\#}p<0.05$. $^{\#\#}p<0.01$ *vs.* EGCG group

cancer cells, presumably via activation of the ROS-mitochondrial signalling pathway (Zheng 2018). Therefore, the present study investigated the effects of EGCG, as a type of bioactive dietary compound, on ROS production in papillary thyroid cancer cells. Notably, our results indicated that ROS levels were significantly decreased in EGCG-treated TPC-1

cells. Previous studies suggested that cancer cells adapted to increased levels of intracellular ROS and depended on changes in metabolic pathways, which may keep cancer cells in a stable redox state and allow them to proliferate and survive normally (Trachootham et al. 2009). Therefore, the EGCG-induced decreases in ROS in TPC-1 cells in the current study may disturb the redox balance and result in apoptosis and growth inhibition. As a previous study confirmed that oxidative stress levels played a role in mitochondria-mediated apoptosis (i.e., the intrinsic pathway) (Zhao et al. 2017), it was suspected that the decrease in ROS in TPC-1 cells affected the mechanisms associated with mitochondria-mediated apoptosis. First, several proteins associated with the intrinsic apoptosis pathway were examined, including Bcl-2, Bax, Cyt C, cleaved caspase 9, cleaved caspase 3, and cleaved PARP. The results demonstrated that EGCG induced a significant increase in the pro-apoptotic protein Bax and a decrease in the anti-apoptotic protein Bcl-2, and promoted the release of Cyt C in TPC-1 cells. EGCG activated caspase 9 and caspase 3, which further induced the proteolytic cleavage of PARP. In conclusion, these results indicated that the intrinsic apoptosis pathway was involved in the mechanism of EGCG-mediated apoptosis.

Autophagy has an important homeostatic function to eliminate pathogens and metabolize damaged cytoplasmic organelles and long-lived proteins (Yi et al. 2014). Autophagy is also involved in tumorigenesis, cancer development and treatment outcome (Choi et al. 2013). Treatment with EGCG increased the levels of LC3-II, which is a marker for autophagosome formation, and decreased P62 levels in the current study. The P62 protein is an autophagy substrate that is efficiently degraded during autophagy (Mizushima et al. 2010). The AKT/mTOR signalling pathway is a major regulatory pathway of autophagy in eukaryotic cells, and activation of mTOR signalling inhibits autophagy (Jung et al. 2010). The present study observed a downregulation of p-AKT and p-mTOR, which supports EGCG-induced autophagy activation.

The mechanism that regulates survival-supporting or death-promoting roles for autophagy is controversial (Fulda and Kögel 2015; Kenific et al. 2010). To elucidate whether autophagy played a role in the mechanism underlying the cytotoxic effects of EGCG or its potential cytoprotective effect, the influence of EGCG on cell viability via autophagy suppression was evaluated using 3-MA in TPC-1 cells. The results demonstrated significant increases in growth inhibition and cell death with combined EGCG and 3-MA treatment compared to EGCG treatment alone, which indicates that EGCG-induced autophagy may play a cytoprotective role in TPC-1 cells. Notably, ROS levels were further decreased with combined EGCG and 3-MA treatment compared to EGCG treatment alone. These results suggest that the decrease in ROS levels promotes mitochondrial dysfunction and triggers mitochondria-mediated apoptosis.

In summary, the current study provides evidence that EGCG may be a more potent and suitable compound for the inhibition of papillary thyroid cancer cells. Autophagy inhibition in combination with EGCG exacerbated the anticancer effects. EGCG significantly inhibited the growth of papillary thyroid cancer cells by decreasing the levels of ROS, inducing the ROS-related pathway of apoptosis and inducing cell cycle arrest at S phase. Notably, EGCG induced autophagy in TPC-1 cells, which partially counteracted EGCG-induced apoptosis of papillary thyroid cancer cells (Fig. 6). The gene expression of EGCG in human

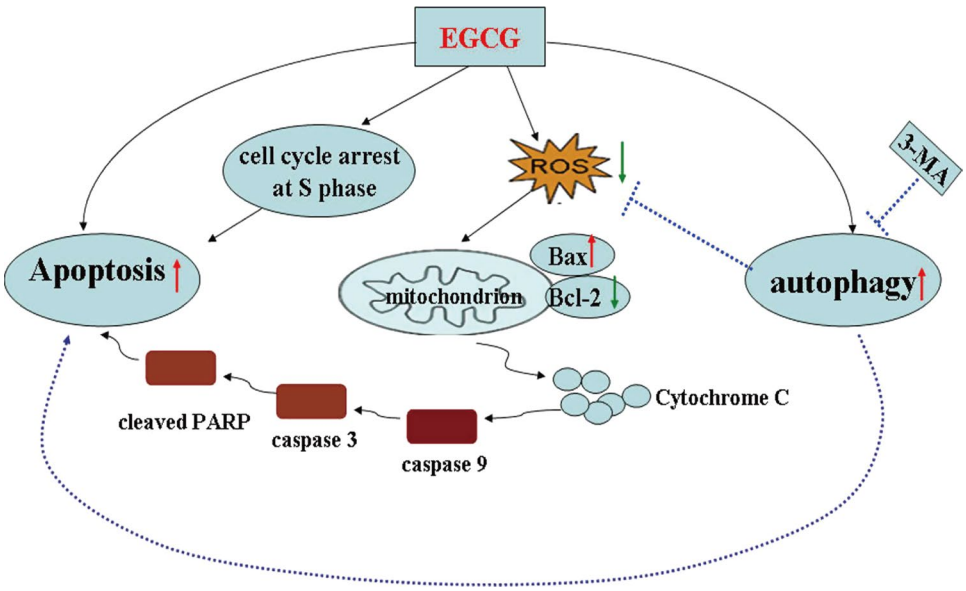

Fig. 6 Proposed model of EGCG on papillary thyroid cancer cells. EGCG induces TPC-1 cell apoptosis by decreasing the levels of ROS, inducing the ROS-related pathway of apoptosis and inducing cell cycle arrest at S phase. EGCG induces an autophagic response. Notably, autophagy inhibition further enhanced EGCG-induced cell apoptosis and ROS suppression, which triggered a higher percentage of apoptosis. The results indicate that autophagy plays a cytoprotective role in EGCG-treated TPC-1 cells

cervical cancer cells and LNCaP cancer cells was detected using cDNA microarray, which suggests a role of EGCG gene regulation (Ahn et al. 2003; Wang and Mukhtar 2002). However, more detailed molecular mechanisms of the genomic responses underlying EGCG-induced apoptosis in papillary thyroid cancer cells remain to be elucidated. Further investigation is needed to determine the clinical efficacy of EGCG in human subjects with papillary thyroid cancer. Our current observation that EGCG co-treatment with an autophagy inhibitor may be a novel strategy for EGCG-mediated adjuvant therapy for papillary thyroid cancer.

Methods

Cell lines

The human papillary thyroid cancer cell line TPC-1 and the human thyroid follicular epithelial cell line Nthy-ori 3-1 were maintained in Dulbecco's modified Eagle's and RPMI 1640 medium, respectively, supplemented with 10% heat-inactivated foetal bovine serum and 100 units/ml penicillin–streptomycin at 37 °C in a humidified incubator in a 5% CO_2 atmosphere. Cells in the logarithmic growth stage were seeded on plates at different densities for subsequent studies.

Drugs and reagents

EGCG was dissolved in phosphate-buffered saline (PBS) to prepare a 5 mg/ml stock solution, which was stored at − 20 °C. MTT was dissolved in PBS to prepare a 5 mg/ml stock solution, which was stored at − 20 °C. Rabbit anti-human primary antibodies targeting cleaved caspase 9, cleaved caspase 3, cleaved PARP, Cyt c, Bax, Bcl-2, cyclin A, CDK2, LC3B-II, P62, p-mTOR, p-AKT, t-mTOR, t-AKT and β-actin were obtained from Cell Signaling Technology. Goat anti-rabbit IgG-HRP was used as a secondary antibody.

Cell proliferation assay

Cells were plated at a density of 2500 cells per well in a 96-well plate in 10% FBS medium and treated with the indicated concentrations 12 h after plating. MTT (20 µl of 5 mg/ml) was added to each well for an additional 4 h. The blue MTT formazan was dissolved in 100 µl of dimethyl sulfoxide (DMSO) per well after removal of the culture medium. The cell proliferation inhibition ratio was calculated according to the absorbance at 490 nm (A value) in each well using an ELISA analyser. The cell proliferation inhibition ratio (%) = (A value of control group–blank group value)/(a value of treated group- blank group value) × 100%.

Hoechst 33342 staining

Cells were plated at a density of 5×10^4 cells per well in a 24-well plate in 2% FBS medium with or without the addition of EGCG for 48 h. The cells were fixed in 4% formaldehyde in PBS for 10 min and stained with 100 µl 1X Hoechst 33342 for 10 min at room temperature in the dark. The cells were washed three times with distilled water. Morphological changes in the nuclei were observed under a fluorescence microscope.

Apoptosis assay

Cells were plated at a density of 5×10^4 cells per well in a 24-well plate in 2% FBS medium with or without the addition of EGCG for 48 h. The cells were trypsinized and incubated for 30 min with 7-AAD and annexin V. The cells were analysed using flow cytometry. Each experiment was repeated three times.

Western blot analysis

Proteins were extracted from TPC-1 cell lines using a protein extraction kit supplemented with a protease inhibitor cocktail. Protein concentrations were measured using a BCA protein assay kit, and equal amounts of protein lysates were loaded on 8%, 10% and 12% SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% dry skimmed milk and incubated with specific primary antibodies against proteins and β-actin (standard control). Blots were incubated with the appropriate HRP-conjugated secondary antibody, and signals were detected using the Pierce ECL Plus substrate and scanned with a Fluor Chem FC3 camera system. Images were analysed using AlphaView software (AIC, Santa Clara, CA, USA).

Cell cycle assay

TPC-1 cells (2×10^5 cells) were plated on 6-well plates in 2% FBS medium with or without EGCG for 48 h. The collected cells were fixed with 70% ice-cold ethanol at − 20 °C overnight. The cells were centrifuged, washed twice with cold 1X buffer solution, stained with 0.25 mg/ml RNase and 5 µl propidium iodide (PI) (Beyotime Biotechnology, Shanghai, China) for 30 min in the dark at room temperature and analysed using a flow cytometer (FACS Calibur; BD Biosciences, USA).

ROS analysis

TPC-1 cells (5×10^4 cells) were plated in 24-well plates in 2% FBS medium with or without EGCG for 48 h. The cells

were incubated with fresh FBS-free medium containing 5 μM dichlorofluorescein diacetate (DCFH-DA) at 37 °C for 30 min in the dark. After three washes, the cells were resuspended in FBS-free medium. Intracellular ROS generation was detected by measuring fluorescence using a flow cytometer.

Statistical analysis

Differences were evaluated using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). The descriptive data are presented as the mean ± SEM, and numerical data were compared between two groups using a homogeneity of variance test or the Mann–Whitney test. Differences in the mean values of multiple groups were analysed using one-way ANOVA with the Tukey–Kramer multiple comparison test, as appropriate. A p value < 0.05 was considered statistically significant.

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Author contributions Chaoming Mao, Ling Bu and Tingting Zheng were responsible for the conception and design of the study, for analysis and interpretation of data and for drafting and revising the manuscript. Fei Wu, Xiao Mou, Chengcheng Xu, Xuan Luo and Qingyan Lu were responsible for the conception and design of the study, data acquisition, interpretation of data and review of the manuscript. Liyang Dong and Xuefeng Wang were responsible for the conception and design of the study, for data acquisition and for reviewing the manuscript. All authors have read and approved the manuscript.

Declarations

Conflict of interest Ling Bu declares she has no conflict of interest. Tingting Zheng declares she has no conflict of interest. Chaoming Mao declares he has no conflict of interest. Fei Wu declares she has no conflict of interest. Xiao Mou declares she has no conflict of interest. Chengcheng Xu declares she has no conflict of interest. Xuan Luo declares she has no conflict of interest. Qingyan Lu declares she has no conflict of interest. Liyang Dong declares he has no conflict of interest. Xuefeng Wang declares she has no conflict of interest.

Ethics statement The study includes only laboratory studies on a stable established cell line. No ethical or institutional approval was required.

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