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Original Article

Epigallocatechin gallate enhances 5-fluorouracil antitumor activity in MCF7 cells by regulating the expression of Bcl-xL

Sijing Sun[1*], Yizhuang Dai[2*], Zhongzhi Lu[1], Min Li[1], Zhen Zhai[1], Xiaolong Ren[1], Dongsheng Li[1]

[1]Third Department of General Surgery, The First Hospital of Zibo City, Zibo, Shandong, China; [2]Department of General Surgery, People's Hospital of Zoucheng City, Zoucheng, Shandong, China. [*]Equal contributors.

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Abstract: Objective: The aim of our study was to investigate whether EGCG could enhance 5-Fluorouracil (5-FU) antitumor activity in MCF7 cells and to reveal its potential mechanisms. Methods: MCF7 cells were treated with different concentrations of EGCG and/or 5-FU for 48 h. Cell viability, apoptosis, colony formation ability, migration and invasive ability were assessed at different concentrations of 5-FU. In addition, the protein expression levels of apoptosis-related protein were determined. The expression of B-cell lymphoma (Bcl)-xL was silenced by small interfering RNA (siRNA). After transfection, the cell viability, apoptosis, and colony formation ability were determined again. Results: EGCG showed no cytotoxicity on MCF7 cells. The cell viability, colony formation ability, migration and invasive ability were significantly decreased while the percentages of apoptosis were statistically increased when combined of EGCG and 5-FU compared to independent application of 5-FU (all $P < 0.05$) with the increasing of concentrations of 5-FU. Besides, 5-FU alone or combination of EGCG and 5-FU induced cell apoptosis by increasing the expression of Bax, cleaved Caspase-3, cleaved Caspase-9, and cleaved poly (ADP-ribose) polymerase (PARP) but decreasing Bcl-2 expression. However, the expression of Bcl-xL was significantly increased by 5-FU but was decreased by additional application of EGCG. Moreover, no significant differences were found in cell viability, apoptosis, and colony formation ability after silencing the expression of Bcl-xL. Conclusion: Our results indicate that EGCG sensitizes MCF7 cells to 5-FU antitumor activity by regulating the expression of Bcl-xL. Combination of EGCG and 5-FU exhibits synergism in breast cancer.

Keywords: Epigallocatechin gallate, 5-fluorouracil, breast cancer, sensitivity, Bcl-xL

Introduction

Breast cancer is the most prevalent diagnosed malignancy among women worldwide, and has become the second leading cause of cancer-related deaths in women, accounting for approximately 12% of all new cancer cases [1]. It has been reported that China has accounted for 12.2% of total cases in the world and 9.6% of related deaths by 2008 with an increasing health burden [2, 3]. Current treatments for breast cancer include surgery, chemotherapy (CT), radiation therapy (RT), endocrine therapy (ET), and targeted therapy. Among all the treatments, CT plays a significant role in management of breast cancer. However, drug resistance limits this efficacy, particularly the multiple drug resistance (MDR). 5-Fluorouracil (5-FU) is a first-line anticancer agent in the treatment of breast cancer due to its strong anticancer activity. 5-FU exerts antitumor effects through inhibition of thymidylate synthase (TS) and incorporation of its metabolites into RNA and DNA [4]. Although the conventional modalities of treating breast cancer, there is no standard regimen after failure of 5-FU in patients with advanced or recurrent breast cancer, and the prognosis still remains unsatisfactory. Previous studies have shown that 5-FU is resistant to breast cancer cells with a 2.5% low sensitivity and a 20% medium sensitivity [5]. However, resistance mechanisms poorly understood. Therefore, overcoming drug resistance and understanding their molecular mechanisms is essential to develop novel therapeutic strategies for breast cancer.

Recently, epigallocatechin gallate (EGCG), a well-known cancer chemopreventive agent derived from green tea, has been gained much attention in medical research. It has been well demonstrated that the effect of conventional cancer therapies could be enhanced by EGCG through its additive or synergistic effects along with amelioration of deleterious side effects [6]. For example, it has been reported that EGCG markedly enhanced the growth-inhibitory effects of 5-FU in hepatocellular carcinoma cells and human head and neck squamous cell carcinoma cell lines [7, 8]. Besides, Zhang *et al.* has suggested that EGCG enhances RT in breast cancer [9]. The reported effects include the modulations of the specific genes expression, induction of apoptosis, and the regulation of multiple cellular signaling pathways [10-14]. However, the exact mechanisms underlying the potential anticancer effects of EGCG on breast cancer cells are unclear.

Therefore, the purpose of our study is to investigate the effects of the combination of EGCG and 5-FU on MCF7 cells and to reveal the underlying mechanisms. Our study might provide new insight into novel therapeutic targets for breast cancer.

Material and methods

Cell culture and treatment

Human mammary gland adenocarcinoma cell line MCF7 cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA). All these cells were grown at Dulbecco Modified Eagle Medium (DMEM) (Gibco; Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 1% L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies), and 100 μg/mL streptomycin (Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 24 h, these cells were seeded in six-well cell culture plates at 1×10^5 cells/well and treated with or without different concentrations of EGCG (Sigma, St. Louis, MO, USA) (0 μM, 1 μM, 5 μM, 10 μM, or 15 μM) or 5-FU (Sigma) (0 μg/ml, 0.1 μg/ml, 1 μg/ml, 10 μg/ml, or 100 μg/ml) for 48 h.

Transfection

The small interfering RNA (siRNA) expressing plasmids specifically targeting B-cell lymphoma (Bcl)-xL and control siRNA (no silencing) (si-control) were synthesized and provided by GenePharma Co (Shanghai, China). Cells were transiently transfected using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Briefly, these cells were seeded in 6-well plates at a density of 5×10^5 cells/well. After 24 h of incubation, the cells were transfected with si-Bcl-xL or si-control and incubated for an additional 16 h. At 48 h after the transfection, cell lysates were collected from each dish and the expression of Bcl-xL was determined by Western blotting. Thereafter, cells were subjected to cell viability, apoptosis, and colony assays.

Cell viability

Cell viability was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl -2-H-tetrazolium bromide (MTT). After 48 h of culture with EGCG, 5-FU, or transfection, 20 μl MTT (5 mg/ml) was added to each well and incubated for 4 h at 37°C. Thereafter, stop solution was added to terminate the reaction after incubation overnight at room temperature. Absorbance at 490 nm was measured using a Synergy plate reader (Biotek, Winooski, VT).

Annexin-V binding assay

Apoptosis is characterized by the translocation of phospholipid phosphatidylserine from the inner leaflet of the plasma membrane to the cell surface, and can be assessed using an Annexin V-fluorescein isothiocyanate (FITC)-labeled annexin V (annexin V-FITC) antibody. Briefly, MCF7 cells transfected with or without si-control or si-Bcl-xL were seeded at the density of 1×10^5 cells/well in 6-well plates, incubated overnight, treated with or without the indicated concentrations of 5-FU (1 μg/ml or 10 μg/ml) and/or EGCG (10 μM), and further incubated for 48 h. To determine the apoptosis of MCF7 cells, these cells were washed twice with ice-cold phosphate buffer saline (PBS) and incubated with Annexin V-FITC for 10 min in the dark at room temperature, followed by addition of propidium iodide (PI) for additional 5 min. Cells were then examined under a fluorescent microscope (Olympus, Tokyo, Japan).

APOPercentage apoptosis assay

Apoptosis was also detected using APOPercentage apoptosis assay (Biocolor Ltd., New-

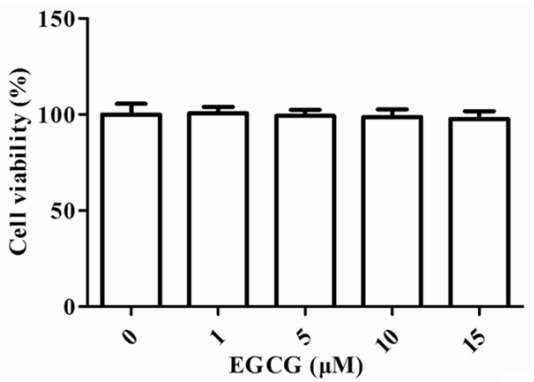

Figure 1. EGCG shows no cytotoxicity on MCF7 cells. EGCG, epigallocatechin gallate.

ton Abbey, UK) according to the manufacturer's instructions. This method is based on the transfer and exposure of phosphatidylserine to the outer surface of the membrane. After treatment with 5-FU and/or EGCG, the cells were harvested, washed with PBS, and stained with APOPercentage dye for 30 min, the number of purple-red cells identified as apoptotic cells were counted and multiple representative pictures were taken using a light microscopy (Olympus, Tokyo, Japan).

Colony formation assay

MCF7 cells were seeded onto six well-plates at 1×10^3 per well in growth medium. Then, the cells treated with or without 5-FU or EGCG, or transfected with si-Bcl-xL were allowed to grow for 14 days at 37°C under 5% CO_2 until visible clones appeared. Thereafter, the cells were collected, washed with PBS, stained with Giemsa for 10 min, washed with ddH_2O, and subjected to photograph. The number of colonies was determined under fluorescence microscopy (Olympus, Tokyo, Japan).

Cell migration and invasion assay

Cell migration and invasion were detected by Transwell migration chambers (8 μm pore size; Becton, Dickinson Labware, Franklin Lakes, NJ). For invasion assay but not migration assay, the membranes were coated with 1 ml of diluted ECM solution (Sigma-Aldrich, Shanghai, China). Briefly, cells (1×10^5 cells/well) were collected and suspended in serum-free DMEM on 24-Transwell membranes. The upper portion of a chamber was filled with 70 μL Matrigel, and the lower chamber was coated with 10% FBS. After 24 h of incubation at 37°C, non-invaded cells were removed, and the invaded cells were fixed in 1% glutaraldehyde, stained with 0.5% crystal violet reagent, and then counted in eight random microscopic fields per membrane using light microscopy.

Western blotting

Following treatment, the cells were collected and washed with PBS. Protein was extracted and the concentration was quantified using Bio-Rad protein assay (Bio-Rad Laboratories, Germany). Protein samples (30 μg per lane) were separated on a 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Thereafter, the protein was blotted onto polyvinylidene difluoride membranes, blocked in Tris buffer solution (TBS) supplemented with 5% nonfat dry-milk, 0.2% fetal calf serum (FCS) and 0.1% Tween-20 for 2 h, and probed with primary antibodies (1:1,000 for Bax, Bcl-2, Bcl-xL, Caspase-3, cleaved Caspase-3, Caspase-9, cleaved Caspase-3, poly (ADP-ribose) polymerase (PARP), cleaved PARP, Tubulin or 1:5,000 for GAPDH at 4°C overnight). All antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). The membranes were then washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature. Proteins were visualized by enhanced chemiluminescence.

Statistical analysis

All experiments were carried out three times and the results of multiple experiments are expressed as the means ± standard deviation (SD). Statistical analyses were performed using Statistical Package for the Social Science (SPSS) statistical software (version 18.0; SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) used to analyze differences among groups. Statistical significance was set at $P < 0.05$.

Results

EGCG shows no cytotoxicity on MCF7 cells

We first determined the effects of EGCG on breast cell cytotoxicity. At 48 h of incubation with different concentrations of EGCG (0 μM, 1 μM, 5 μM, 10 μM, or 15 μM), cell viability was determined using MTT. As shown in **Figure 1**, we found that EGCG showed no cytotoxicity at different concentrations on cell viability. The

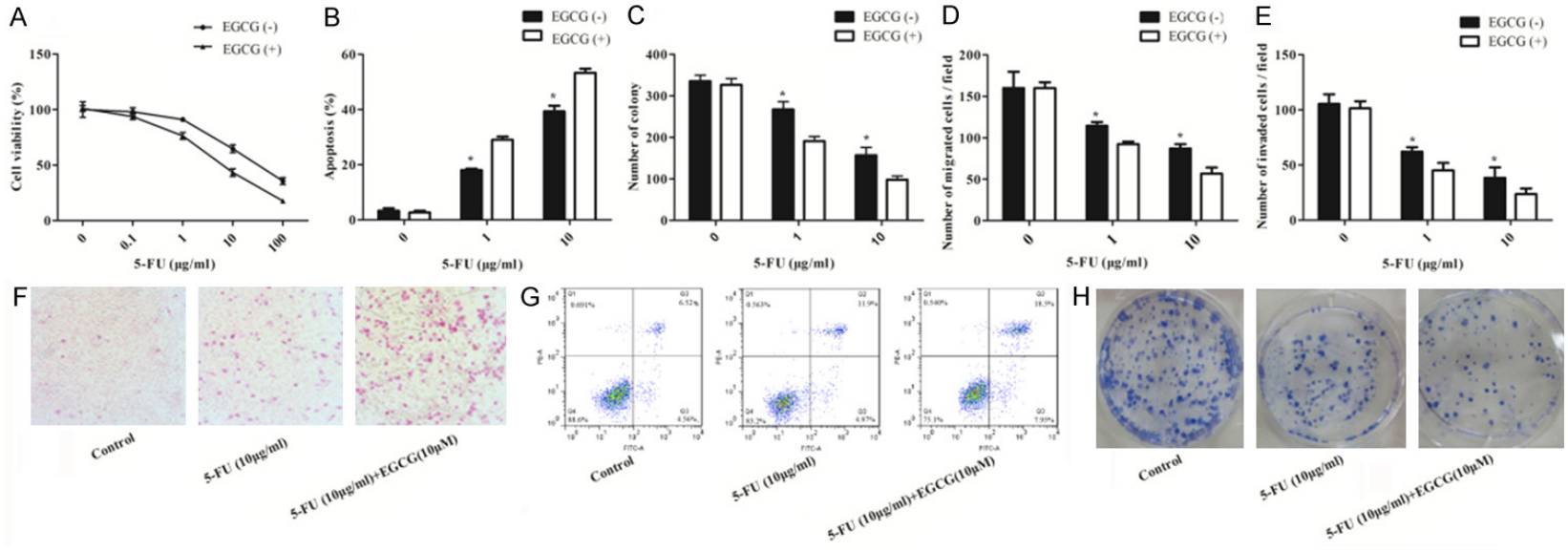

Figure 2. EGCG enhances 5-FU on antitumor activity in MCF7 cells. A. Cell viability is significantly decreased when combined of EGCG and 5-FU; B. The percentage of apoptosis is significantly increased when combined of EGCG and 5-FU; C. The colony formation ability is significantly decreased when combined of EGCG and 5-FU; D. The number of migrated cells is significantly decreased when combined of EGCG and 5-FU; E. The number of invaded cells is significantly decreased when combined of EGCG and 5-FU; F. Representative pictures of apoptosis (10 μg/ml 5-FU + 10 μM EGCG); G. Representative pictures of flow cytometry (10 μg/ml 5-FU + 10 μM EGCG); H. Representative pictures of colony formation ability (10 μg/ml 5-FU + 10 μM EGCG). EGCG, epigallocatechin gallate; 5-FU, 5-fluorouracil. $^{*}P < 0.05$ compared with 5-FU + EGCG.

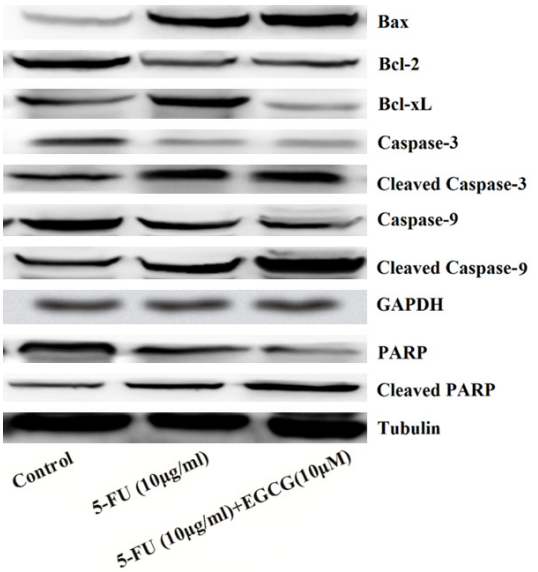

Figure 3. EGCG enhances 5-FU on antitumor activity in MCF7 cells by regulation of Bcl-xL expression. Bcl, B-cell lymphoma; PARP, poly (ADP-ribose) polymerase.

concentration of EGCG was 10 μM in our subsequent experiments.

EGCG enhances 5-FU antitumor activity in MCF7 cells

Next, we determined the effects of combination of EGCG and 5-FU on breast cancer. The cell viability, apoptosis, colony formation ability, migration and invasive ability were assessed at different concentrations of 5-FU. The MTT results showed that the cell viability was significantly lower when combined of EGCG and 5-FU than independent application of 5-FU ($P < 0.05$). Besides, the cell viability was decreased with the increasing of concentrations of 5-FU (**Figure 2A**). The percentages of apoptosis were statistically increased by combination of EGCG and 5-FU compared to only application of 5-FU with the increasing of concentrations of 5-FU (both $P < 0.05$). Additionally, the number of colony, migrated and invaded cells were all significantly reduced by combination of EGCG and 5-FU with the increasing of concentrations of 5-FU (all $P < 0.05$) (**Figure 2B-H**).

EGCG enhances 5-FU antitumor activity in MCF7 cells by regulation of Bcl-xL expression

To further explore the possible underlying mechanism for enhancement of sensitivity to 5-FU induced by EGCG, the protein expression levels of apoptosis-related protein (Bax, Bcl-2, Bcl-xL, Caspase-3, cleaved Caspase-3, Caspase-9, cleaved Caspase-9, PARP, and cleaved PARP) were determined. The results showed that 5-FU or combination of EGCG and 5-FU induced MCF7 cell apoptosis by increasing the expression of Bax, cleaved Caspase-3, cleaved Caspase-9, and cleaved PARP but decreasing the expression of Bcl-2. However, it was noteworthy that the expression of Bcl-xL was significantly increased by 5-FU but was significantly decreased by additional application of EGCG, indicating that EGCG might enhance the 5-FU antitumor activity by regulating the expression of Bcl-xL. The combination of EGCG and 5-FU significantly increased the expression of Bax, cleaved Caspase-9, and cleaved PARP, which further promoted the apoptosis of breast cancer cells (**Figure 3**).

EGCG shows no increased sensitivity to 5-FU in MCF7 cells after transfection with si-Bcl-xL

To investigate the effects of silence of Bcl-xL on the sensitivity induced by EGCG on 5-FU, the cell viability was determined after silencing the expression of Bcl-xL. As shown in **Figure 4A**, the results showed that the expression of Bcl-xL was significantly suppressed by transfection with si-Bcl-xL. Besides, the MTT results demonstrated that no significant differences were found in cell viability between 5-FU + si-Bcl-xL and 5-FU + si-Bcl-xL + EGCG (**Figure 4B**). We further measured the percentages of apoptosis and colony formation ability. Although the percentages of apoptosis were significantly increased by application of 5-FU + si-Bcl-xL and 5-FU + si-Bcl-xL + EGCG compared to 5-FU + si-control group, there were no significant differences between 5-FU + si-Bcl-xL and 5-FU + si-Bcl-xL + EGCG. Additionally, no significant differences were found in colony formation ability between the two groups (**Figure 4C-G**). These results indicated that EGCG showed no increased sensitivity to 5-FU in MCF7 cells after silencing the expression of Bcl-xL.

Discussion

In the present study, we found that EGCG could significantly enhance 5-FU antitumor activity in MCF7 cells by increasing apoptosis and decreasing the cell viability, colony formation ability, migration and invasive ability. Additionally, EGCG could statistically reduce the expression of Bcl-xL that was increased by 5-FU,

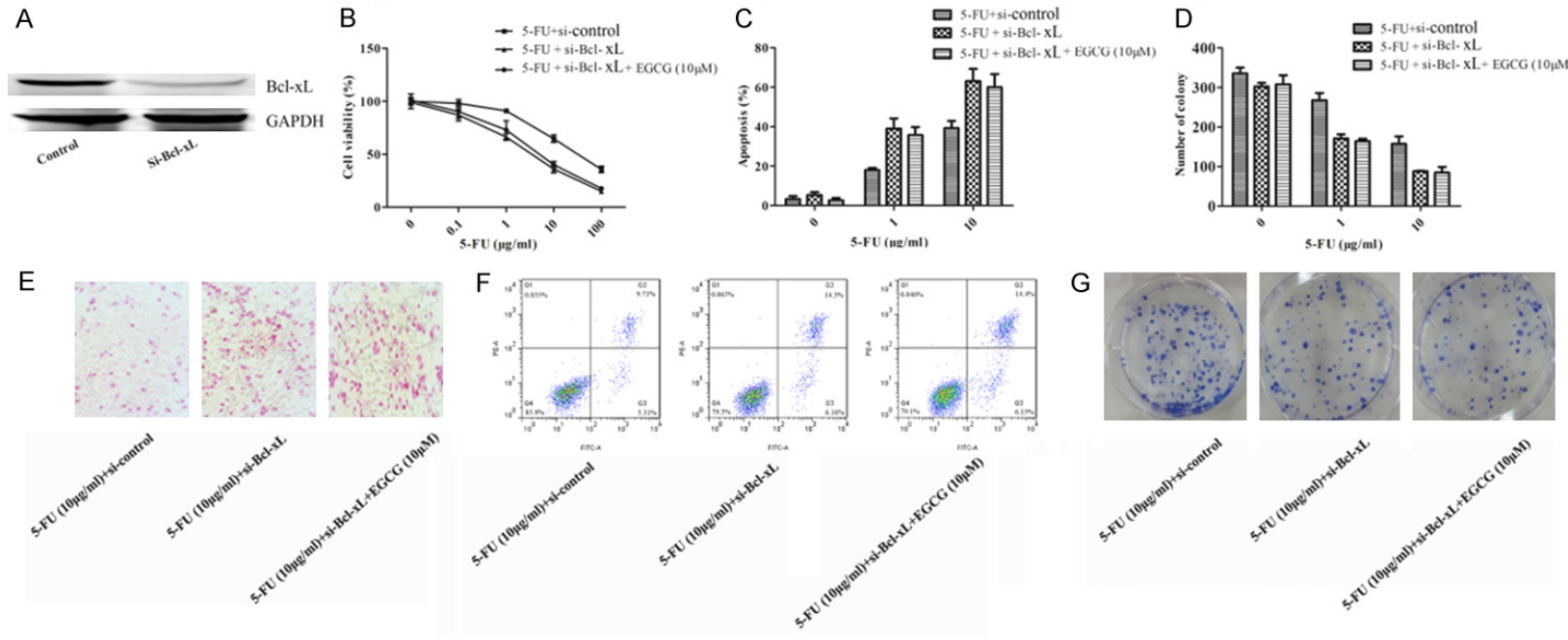

Figure 4. EGCG shows no increased sensitivity to 5-FU in MCF7 cells after transfection with si-Bcl-xL. A. Representative pictures of Western blotting after transfection with si-Bcl-xL; B. EGCG shows no increased cell viability to 5-FU after transfection with si-Bcl-xL; C. EGCG shows no increased apoptosis to 5-FU after transfection with si-Bcl-xL; D. EGCG shows no increased colony formation ability to 5-FU after transfection with si-Bcl-xL; E. Representative pictures of apoptosis (10 μg/ml 5-FU + 10 μM EGCG); F. Representative pictures of flow cytometry (10 μg/ml 5-FU + 10 μM EGCG); G. Representative pictures of colony formation ability (10 μg/ml 5-FU + 10 μM EGCG). EGCG, epigallocatechin gallate; 5-FU, 5-fluorouracil; siRNAs, small interfering RNA.

leading to increased apoptosis of breast cancer cells. Therefore, combination of EGCG and 5-FU greatly improved the resistance efficacy, which might be through regulating the expression of Bcl-xL.

CT is one of the most effective therapeutic methods to treat various tumors including breast cancer, and has also been confirmed to be effective against in the vast majority of advanced triple negative breast cancer (TNBC) and human epidermal growth factor receptor-2 (HER2)-positive breast cancers [15]. Among all the chemotherapeutic agents, anti-cancer drug 5-FU causes cell death, which has been considered as a mainstay of treatment for breast cancer. However, CT has encountered huge challenges because of the development of drug resistance and severe side-effects [16, 17]. Resistance to 5-FU is a multifactorial event in which a great many factors have been reported to be involved including metabolism mechanisms, molecular mechanisms, transport mechanisms, and protection from apoptosis [18]. Unfortunately, there is so far no effective treatment strategy for the treatment of resistant breast cancer. Consequently, it is essential to explore the underlying resistance mechanism and to look for a new agent that could enhance the effect of 5-FU on anticancer therapy.

Consumption of tea is a commonly practiced dietary habit among the population. Green tea, the second most consumed beverage worldwide, contains high amounts of the potent antioxidants polyphenols and has showed significant beneficial effects on human health [19, 20]. EGCG is the most abundant polyphenol in green tea and has potent antioxidant and chemopreventive effects [21]. EGCG has been shown to inhibit different processes of cancers including the initiation, progression and metastasis. Besides, EGCG has been proposed as a chemo/radiosensitizer of cancer cells showing the additive or synergistic effects [22]. In addition to additive or synergistic effects, a decreased risk of side effects caused by anticancer agents is also observed in chemosensitization by reduction the doses of drugs. Several different mechanisms have been demonstrated or postulated to elucidate the enhancement of chemotherapy by EGCG. For example, increased intracellular drug concentration [23-25], increased cell cycle arrest and apoptosis (high expression of some Bcl-2 family proteins, Caspase-3, and cleaved PARP), modulation of pro-angiogenic and pro-invasive molecular pathways [26-28], regulation of chemoresistance-related proteins [29], interaction with hormone receptor [30-32], and antioxidant/pro-oxidant activity [33] are proposed events related to the improvement of anticancer. Combination of EGCG and 5-FU has been reported in varies of cancers [8, 22]. However, the antitumor effects of combination of EGCG and 5-FU on breast cancer have been poorly documented until now.

Therefore, we investigated the antitumor effects of combination of EGCG and 5-FU on breast cancer and explored the underlying mechanism. We found that EGCG enhanced antitumor effect on MCF7 cell growth induced by 5-FU, and a combination of EGCG and 5-FU showed a synergic effect. Combination of EGCG and 5-FU caused a significant decrease in cell viability, colony formation ability, migration and invasive ability but induced a significant increase in apoptosis compared to only application of 5-FU. Furthermore, we studied the apoptosis by determining the apoptosis related proteins. The results showed that 5-FU or combination of EGCG and 5-FU elevated the expression of Bax, and activated Caspase-3, Caspase-9, and PARP, leading to apoptosis. However, the expression of Bcl-xL was also increased by 5-FU. This might be an important reason by which 5-FU causes drug-resistance on breast cells. Interestingly, the expression of Bcl-xL was significantly decreased by combination of EGCG and 5-FU, indicating that combination of EGCG and 5-FU increased the anticancer activity might be through an increase the expression of Bcl-xL. To confirm the results, we silenced the expression of Bcl-xL and observed the effects on the cell viability, apoptosis, and colony formation ability. The results showed that after transfection with si-Bcl-xL, no significant differences were found in cell viability, apoptosis, and colony formation ability between combination of EGCG and 5-FU and only application of 5-FU, indicating that EGCG showed no increased sensitivity to 5-FU in MCF7 cells after transfection with si-Bcl-xL. Therefore, the expression of Bcl-xL plays an important role in chemosensitization induced by EGCG to 5-FU.

In conclusion, our results suggest that EGCG sensitizes MCF7 cells to 5-FU antitumor effect by modulating the expression of Bcl-xL. The synergic effect coming from the combination

of EGCG and 5-FU might provide the basis to overcome drug resistance in breast cancer treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Dongsheng Li, The Third Department of General Surgery, The First Hospital of Zibo City, 4 Emeishan East Road, Boshan District, Zibo 255200, Shandong, China. Tel: +86-533-4251990; E-mail: lidongsheng4502@126.com

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