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ORIGINAL ARTICLE

Epigallocatechin gallate from green tea exhibits potent anticancer effects in A-549 non-small lung cancer cells by inducing apoptosis, cell cycle arrest and inhibition of cell migration

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Summary

Purpose: *Green tea (Camellia sinensis) is considered as a rich source of epigallocatechin gallate (EGCG) which has been shown to exert impressive pharmacological properties. The anticancer properties of EGCG have been extensively studied however, its anticancer activity has not been explored in lung cancer. The present study was therefore designed to evaluate the anticancer effects of EGCG against non-small cell lung cancer (NSCLC) cell line A-549 and normal human fibroblast FR-2 cells.*

Methods: *Cell viability was assessed by CCK8 assay, apoptosis by DAPI, annexin V/propidium iodide (PI) and flowcytometery and cell cycle analysis by flow cytometry. Cell migration capacity was investigated by wound-healing assay and protein expression was examined by Western blotting.*

Results: *The results revealed that EGCC could inhibit the proliferation of A-549 cells in a concentration-dependent manner and exhibited an IC_{50} of 25 μM against the IC_{50} of 100 μM against the normal human fibroblasts. Further evaluation revealed that EGCG exerts its anticancer effects via induction of apoptosis, modulation of Bax/blc-2 ratio and by triggering G2/M cell cycle arrest. Furthermore, EGCG could also inhibit the migration of A5-49 cells in a concentration-dependent manner.*

Conclusion: *In conclusion, based on our results, we believe that EGCG could prove to be an important lead molecule for the treatment of lung cancer.*

Key words: *apoptosis, cell cycle arrest, epigallocatechin gallate, green tea, lung cancer*

Introduction

Green tea, botanically known as *Camellia sinensis*, is an important medicinal plant. It is a rich source of secondary metabolites such as flavonoids and phenolics [1]. *C.sinensis* has been reported to exhibit impressive pharmacological properties and to exert anticancer and neuroprotective effects [2]. Although a number of secondary metabolites have been isolated from green tea, EGCG (Figure 1) is one of its principal components [3]. The role of EGCG in cancer inhibition has been studied extensively. A recent study has reported the correlation between regular consumption of green tea and the suppression of tumor progression [4]. Moreover, EGCG has several advantages as compared to the conventional drugs because it is commonly available, inexpensive and can be easily isolated from green tea. More importantly, it can be taken p.o. and is non-toxic [5]. In the current study, we evaluated the antiproliferative effect of EGCG against the NSCLC cell line A-549.

Lung cancer is the most prevalent and commonly detected cancer across the globe. It is con-

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sidered as one of the most lethal types of malignancies affecting the health of the populations around the world [6]. Although several drugs are used clinically for the treatment of lung cancer, these drugs create side effects and drug resistance over a period of time. Thefore. there is urgent need to explore for drugs with high efficacy, low toxicity, inexpensive and easily available [7,8].

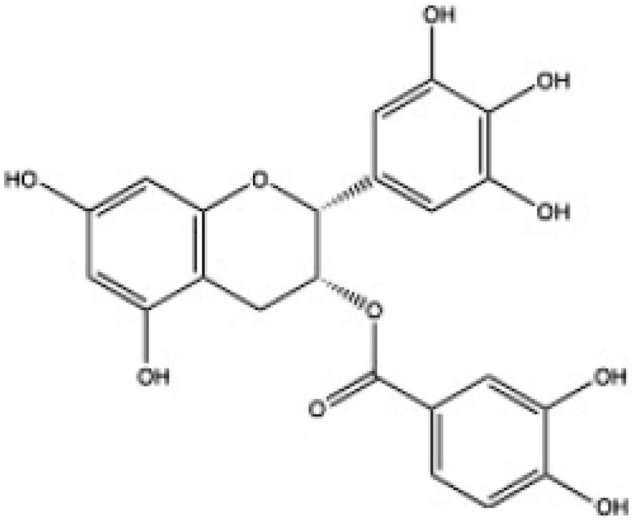

Figure 1. Chemical structure of epigallocatechin gallate (EGCG).

Methods

Chemical reagents and cell culture

DAPI, RNase A triton X-100 and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co. (St. Louis, USA). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine, antibiotics were obtained from Invitrogen Life Technologies. (Grand Island, NY, USA). NSCLC cell line A-549 and normal human fibroblast cell line FR-2 were procured from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin and maintained in a humidified atmosphere containing 5% CO_2.

Cell viability assay

The viability of lung cancer cell line A549 and normal FR-2 cells was examined by employing CCK8 assay. For this, 5×10^3 cells were cultured in a 96-well plate and incubated overnight at 37°C in a humidified, 5% CO_2 atmosphere. After incubation the cells were exposed to different concentrations of EGCG (0-500 µM) for 24 hrs. Afterwards, 10 µL of CCK8 was added into each well for a new 1-hr incubation at 37°C. Finally, absorbance at optical density $(OD)_{450}$ nm was checked by microplate spectrophotometer (BioRad, Segrate, Italy).

Apoptosis assay

A549 cells were seeded at a density of $2x10^5$ cells/well in 96-well plates and treated with varied concentrations of EGCG after 24-h incubation. DAPI staining was carried out and the cells were then washed with PBS, fixed in formaldehyde (10%) and washed again with PBS. The DAPI-stained cells were then examined by fluorescence microscope. For annexinV/PI a similar procedure as that of DAPI was followed except for the cells stained with annexinV/PI investigation was performed using flow cytometry.

Cell cycle analysis

To estimate the dissemination of A549 cells in different phases of the cell cycle, the EGCG-administered cells (0, 12.5 25 and 50 µM EGCG) were collected and washed twice with PBS. Thereafter, the cells were fixed with ethanol (70%) for 1 hr and then washed again with PBS. The cells were finally resuspended in solution of PI (50µl /ml) and RNase1 (250 µg/ml). This was followed by incubation for 30 min at room temperature. The cell distribution was finally determined by fluorescence-activated cell sorting cater-plus cytometer.

Cell migration assay

A549 cells were seeded at a 5×10^4 cell density in 96-well plates and kept overnight to adhere. When the cells reached 80% confluence, a wound was scratched across each well by wound Maker device. Afterwards the cells were washed with PBS to remove the detached cells.

Western blotting

Total protein from cancer and normal cells was isolated in RIPA lysis buffer. Equal protein extracts from each group were run on SDS PAGE and then transferred to a polyvinylidene fluoride membrane. This was followed by blocking with 5% non-fat milk and incubation at room temperature for 1 h. Thereafter the membranes were incubated with a specific primary antibody at 4°C overnight. This was followed by washing in washing buffer and incubation for 1 h with the suitable secondary antibody. The protein bands of interest were visualised by an ECL Advanced Western Blot Detection Kit.

Statistics

Experiments were carried out in triplicate. Data is presented as mean±SD and the values were considered significant at *p<0.01, **p, 0.001, ***p<0.0001. One way ANOVA followed by Tukey's test were used for statistical analysis using Graph Pad 7 software.

Results

EGCG inhibits A-549 cancer cell growth

To evaluate the anticancer potential of EGCG, the lung cancer A-549 cells and normal human fibroblasts FR-2 were treated with varied concentrations of EGCG (0-500 µM) (Figure 2). The

results indicated that EGCC exhibited significant anticancer activity on EGCG cells by reducing cell viability in a concentration-dependent manner. The IC_{50} of EGCG against lung A-549 cells was 25 μM. Importantly, the antiproliferative effects of EGCG on normal FR-2 cells were less pronounced as evidenced from the IC_{50} of 100 μM.

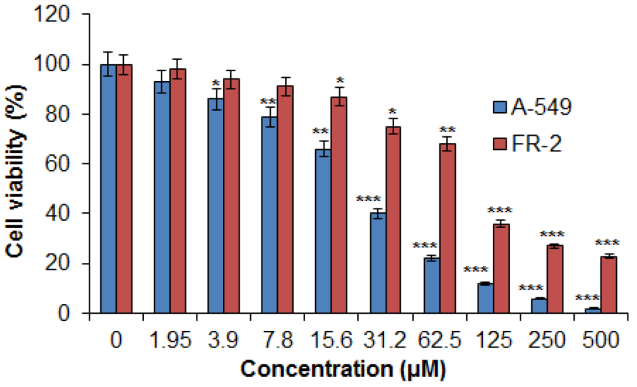

Figure 2. Effect of indicated doses of EGCG on viability of A-549 and FR-2 cells determined by CCK8 assay. Experiments were carried out in triplicate. Data are presented as mean±SD and the values were considered significant at *p<0.01, **p, 0.001, and ***p<0.0001.

EGCG induces apoptosis in A549 cells

We investigated if EGCG exerts its anticancer effects via induction of apoptosis in A549 cells. The A549 cells were administered 0, 12.5, 25 and 50 μM EGCG (Figure 3) and the results indicated that EGCG induced apoptosis in a concentration-dependent manner as evidenced from DAPI staining. Furthermore, the apoptotic cell populations increased from 0.70% in the control to 36.75% at the concentration of 50 μM (Figure 4). To explore if the apoptosis followed the mitochondrial pathway, we determined the expression of Bax and Bcl-2. The results indicated that EGCG caused significant and concentration-dependent reduction in the expression of Bax with concomitant upregulation of Bcl-2 expression (Figure 5).

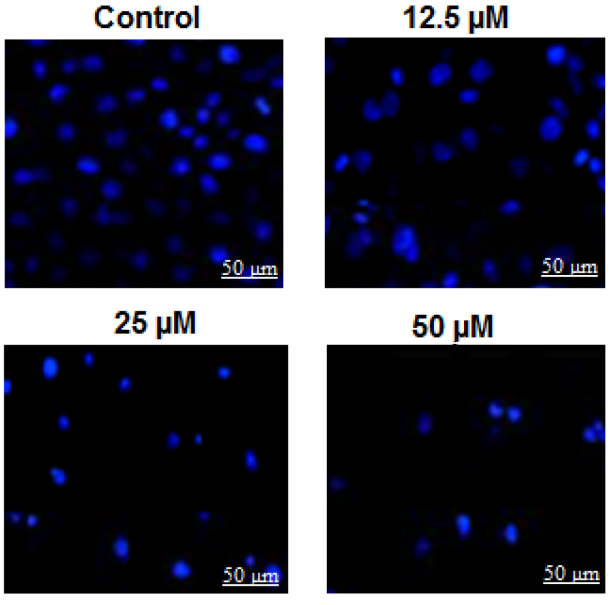

Figure 3. DAPI staining of A549 cells at indicated concentrations reveals induction of apoptosis. Experiments were carried out in triplicate. As depicted in the Figure, EGCG exerted apoptosis in A431 cells in a concentration-dependent manner.

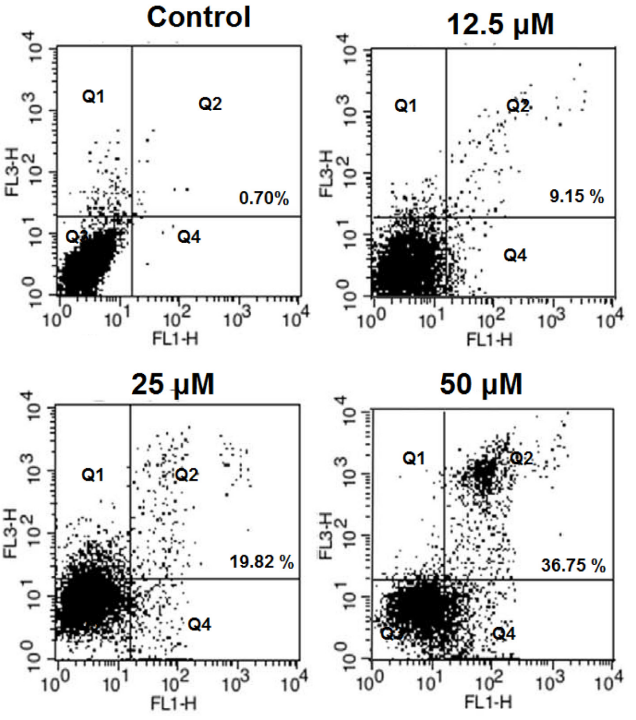

Figure 4. Estimation of apoptotic cell population by annexin V/PI staining followed by flow cytometry at indicated concentrations of EGCG. Experiments were carried out in triplicate. As depicted in the Figure, the apoptotic cell population increased in a concentration-dependent manner.

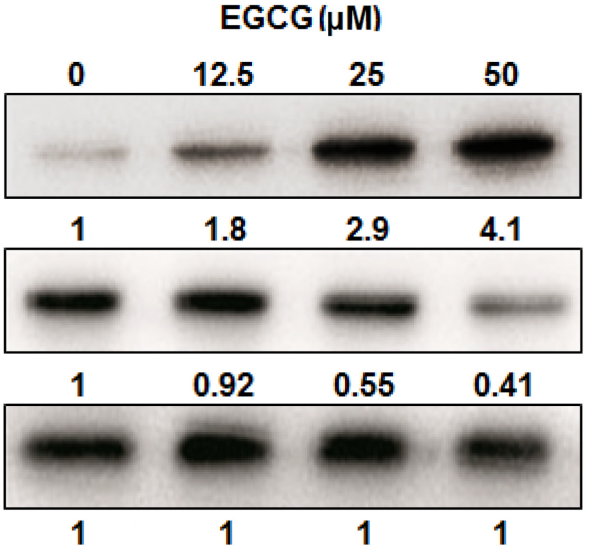

Figure 5. Effect of EGCG at indicated concentrations showing the expression of Bax and Bcl-2 in A549 cells as determined by Western blotting. Experiments were carried out in triplicate. The results showed that EGCG caused upregulation of Bax and downregulation of Bcl-2 in a concentration-dependent manner.

EGCG triggers cell cycle arrest in A549 cells

To examine the effect of EGCG on the cell cycle phase distribution of A549 cells, the cells were treated with 0, 12.5, 25 and 50 μM of EGCG for 24 hrs. The results revealed that the number of cells at G2 phase increased in a dose-dependent manner causing cell cycle arrest (Figure 6). At 50 μM there was a marked increase in the number of cells at G2 phase.

EGCG inhibits cell migration

We then investigated if EGCG could suppress the migration of A-549 cancer cells at the 0, 12.5 , 25 and 50 μM concentration using the wound healing assay. The results of this assay showed that EGCG reduced the migratory capability of A-549 cells (Figure 7).

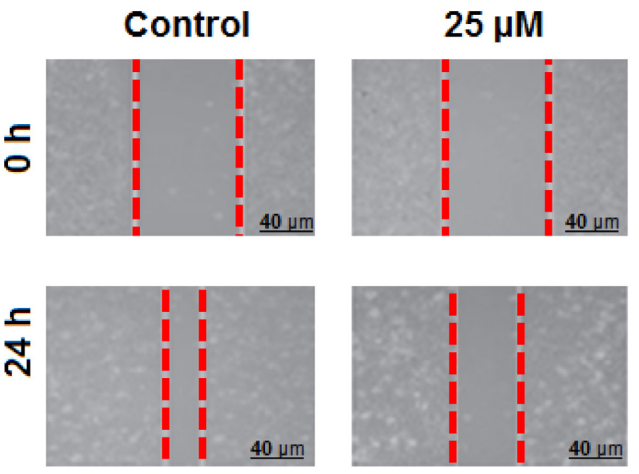

Figure 7. Wound healing assay showing the effect of EGCG at indicated concentrations on cell migration of A549 cells. Experiments were carried out in triplicate. It was observed that EGCG inhibited A431 cell migration.

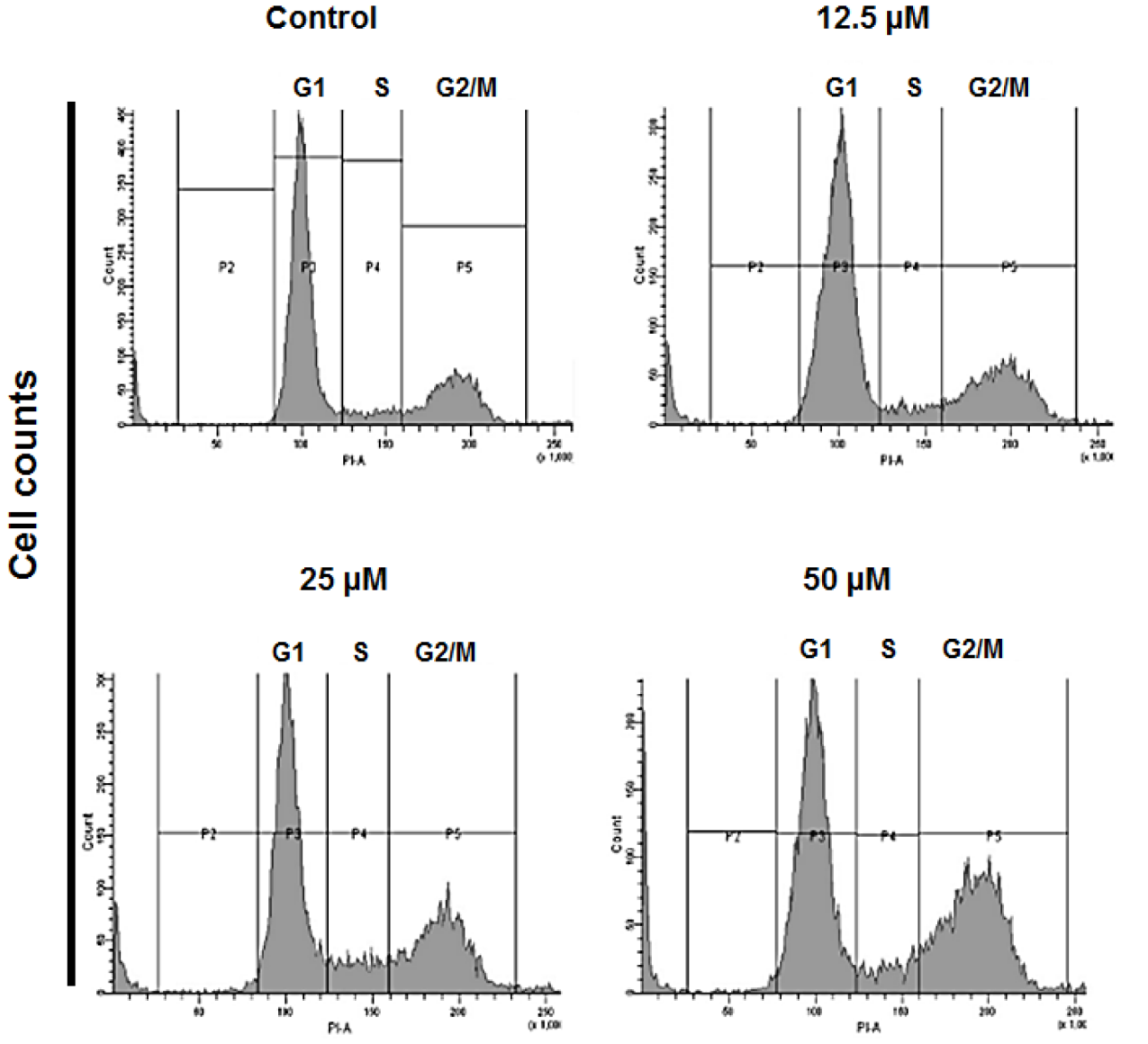

Figure 6. Effect of EGCG at indicated concentrations on cell cycle distribution of A-549 cells as determined by flow cytometry. Experiments were carried out in triplicate. It was observed that EGCG caused G2/M cell cycle arrest in a concentration-dependent manner.

Discussion

Lung cancer is included among the most aggressive and prevalent, common types of cancers across the globe. In 2008, lung cancer was reported as the leading cause of cancer-related deaths in men and second leading cause in women [9]. Currently, there are limited treatment options available for this disease. The problem is worsened by the fact that most of the lung cancer cases are detected at advanced stages and the treatment options at these stages are very limited [6]. Therefore, it is not strange that lung cancer causes tremendous morbidity and mortality across the world. It is very obvious that there is an urgent need to explore and develop more effective therapies for the treatment of this malignancy. Natural products such as medicinal plants have long been considered as good sources of anticancer compounds and they continue to play a principal role in drug discovery [10]. In the current study we observed that EGCG could inhibit the growth of NSCLC cell line A-549 and exhibit a minimum inhibitory concentration (MIC) of 25 μM. Interestingly, EGCG showed very high MIC (100 μM) against normal human fibroblasts. These results are promising since it is considered essential that the potential anticancer lead molecules show less toxicity against normal cells. We further investigated as to how EGCG exerts its anticancer effects and our results indicated that EGCG prompts apoptosis in A-549 cells in a concentration-dependent manner as evidenced from both DAPI and annexin V/PI staining. The resuts of this study are in concordance with studies where several natural products have been shown to induce apoptosis in cancer cells. It has been reported that apoptosis is an essential defense mechanism to prevent the growth and spread of cancer cells and most of the chemotherapeutic agents exploit this mechanism to destroy cancer cells [11,12]. Several previous studies have reported that EGCG suppresses the protein expression levels of the anti-apoptotic Bcl-2 and upregulates the protein expression levels of apoptotic proteins such as Bax. This causes concomitant activation of caspases and ultimately apoptotic cell death [13,14]. We therefore investigated the effect of EGCG on the expression of Bax and Bcl-2 and the results indicated that EGCG could upregulated the expression of Bax which was associated with simultaneous downregulation of Bcl-2. Moreover, anticancer agents that could block cell cycle of cancer cells are also considered essential anticancer agents. In the present study we observed that EGCG induced G2/M cell cycle arrest in lung cancer A-549 cells. These results are in agreement with previous studies [13,14] where EGCC has been reported to block cell cycle progression. It has also been reported that EGCG-encapsulated chitosan nanoparticles induce cell cycle arrest in cancer cells [15]. Moreover, EGCG has been shown to trigger cell cycle arrest in pancreatic cells [13]. Taken together these results indicate that EGCG exerts anticancer effects via induction of apoptosis and cell cycle arrest.

Another aspect of drug development paradigms is to identify drugs that could inhibit metastasis of cancer cells [16]. After invasion, cancer cells move through the extracellular matrix, enter the bloodstream and disseminate to other parts of the body, making it very difficult to treat cancer at this stage [17]. In our study we observed that EGCG could significantly inhibit the migration of cancer cells. These results are in concordance with previous studies where EGCG has been reported to inhibit cancer cell metastasis, by suppressing the expression of matrix metalloproteinases [18].

Conclusion

We conclude that EGCG exerts its anticancer effects via induction of apoptosis and cell cycle arrest. Importantly, it shows less cytoxicity against normal human fibroblasts and inhibits cell migration and invasion and therefore we believe that EGCG could prove a lead molecule for the treatment of lung cancer. However, more studies involving evaluation of EGCG against many cancer cells cell lines and *in vivo* models are urgently required.

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Conflict of interests

The authors declare no conflict of interests.

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