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Epigallocatechin-3-gallate Induces Growth Inhibition and Apoptosis of Human Anaplastic Thyroid Carcinoma Cells Through Suppression of EGFR/ERK Pathway and Cyclin B1/CDK1 Complex

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Background: Anaplastic thyroid carcinoma (ATC) is one of the most lethal cancers because of its aggressiveness and the lack of efficacious therapy. Epigallocatechin-3-gallate (EGCG), a major catechin in green tea, was shown to possess remarkable therapeutic potential against various types of human cancer cells in in vitro and in vivo models. The aim of this study was to investigate the effect of EGCG on the proliferation and apoptosis of ARO cells—human ATC cells.

Study design: Experimental study.

Methods: Human ATC cell line, ARO, was treated with EGCG. Cell viability was assessed by MTT assay. Inhibition of EGFR/MAPK pathway and cell cycle-related proteins by EGCG were measured by Western blot analysis. In addition, cell cycle analysis was measured by flow cytometry.

Results: EGCG treatment inhibited the growth of ARO cells in a dose-dependent manner. Furthermore, EGCG suppressed phosphorylation of EGFR, ERK1/2, JNK, and p38. These changes were associated with increased p21 and reduced cyclin B1/CDK1 expression. In addition, EGCG treatment increased the accumulation of sub-G1 cell, activated caspase-3 and cleaved PARP.

Conclusions: Taken together, EGCG inhibits cell proliferation and induces apoptosis via suppression of the EGFR/ERK pathway and cyclin B1/CDK1 complex in ATC cells.

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KEY WORDS: anaplastic carcinoma; thyroid gland; EGCG; apoptosis

INTRODUCTION

Anaplastic thyroid carcinoma (ATC) is a relatively uncommon cancer arising from the thyroid gland, but remains one of the most lethal human malignancies, in contrast to the excellent prognosis of other thyroid cancers, such as follicular or papillary thyroid carcinoma [1]. Patient survival from presentation is extremely low, with a median survival of less than 6 months. It is because current treatments for ATC, including surgery, radiotherapy, or chemotherapy, are largely ineffective [2]. Development of novel therapeutic or preventive strategies is therefore desirable.

The epidermal growth factor receptor (EGFR) is a transmembrane receptor consisted of an extracellular domain for ligand binding, a transmembrane domain, and an intracellular domain with tyrosine kinase activity [3]. EGFR and its downstream effectors play a key role in the pathogenesis, migration, and invasiveness of many types of cancer, including head and neck, breast, and lung cancers [4–6]. In ATC, several studies have demonstrated that high levels of EGFR protein, and EGFR over-expression were common findings [7]. Moreover, in patients whose thyroid tumors bound more EGF, the prognosis was reported to be poor [8,9]. This observation suggests there is potential for targeted molecular therapy in the treatment of ATC. Some human epidemiologic studies showed that naturally occurring dietary substances are capable of slowing or blocking the carcinogenic process without adverse effects [10]. Of these substances, green tea is the most studied for its chemopreventive effect on various human cancers [11]. Of the four known catechins found in green tea, including (–)-epigallocatechin-3-gallate (EGCG), (–)-epicatechin-3-gallate (ECG), (–)-epigallocatechin, and (–)-epicatechin, EGCG has been identified as the major catechin and was found in 50–80% of green tea [11]. Some studies reported that EGCG inhibited cancer cell proliferation, and induced apoptosis via EGFR signaling blockade in some cancer cells [12]. Therefore, we hypothesized that EGCG may exert anti-cancer effects on ATC cells, and investigated the ability of EGCG to inhibit ATC cell growth by blocking epidermal growth factor (EGF)-dependent EGF receptor signaling and downstream ERK activation.

MATERIALS AND METHODS

Cell Lines and Reagents

Human ATC cell lines comprised of ARO cells were used in our study, and were maintained in RPMI 1640 medium (Gibco, Auckland, NZ) supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 U/ml and streptomycin 100 U/ml). The cells were cultured at 37°C with 5% CO_2 in humidified condition. EGCG, ECG, Genistein, Resveratrol, and Capsaicin were purchased from Sigma, St. Louis, MO. All the primary antibodies for the following proteins: EGFR from Upstate Biotechnology (Waltham, MA), phospho-EGFR, phospho-ERK1/2, ERK1/2, phospho-JNK,

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Conflict of Interest: None.

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JNK, phospho-p38, p38, p53, caspase-3, and PARP were purchased from Cell Signaling Technology (Beverly, MA). p16, p21, p27, CDK1, CDK2, CDK6, cyclin A, cyclin B1, cyclin D1, cyclin E, and cyclin I from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies, anti-rabbit Ig G or anti-mouse Ig G, were purchased from Jackson ImmunoResearch (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Cell Proliferation Assay

The inhibitory effects of various polyphenols, such as EGCG, ECG, Genistein, Resveratrol, and Capsaicin, on ARO cells were assessed by MTT assay. Briefly, ARO cells (7×10^3 cells/ml) were incubated in a 96-well plate and grown up to at 70% confluency of cell. The cells were then treated with the indicated concentration of each polyphenol, and cultured at 37°C under humidified atmosphere with 5% CO_2 for 24 hr. Subsequently, 20 μl MTT solution (5 mg/ml in PBS) was added to each well and placed at room temperature for 3 hr. After 3 hr incubation, the absorbance was measured on a SpectraMax 190 (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm.

Cell Cycle Analysis

The ARO cells were plated in a 6-well plate at 70% confluency, and treated with EGCG at various concentrations (0, 50, 100, and 200 μM). As control, 0.1% DMSO was used. After incubating in a humidified incubator (5% CO_2 in air at 37°C) for 24 hr, cells were collected, washed with PBS, and fixed in 70% ethanol at −20°C overnight. Subsequently, the cells were washed once with PBS, resuspended in PBS containing 200 μg/ml RNase A, incubated at 37°C for 1 hr, and stained with 50 μg/ml propidium iodide (Sigma). The distribution of cells in each cycle phase was analyzed using FACS caliber instrument (Becton Dickinson, Franklin Lakes, NJ). All experiments were performed in triplicate.

Western Blot Analysis

The ARO cells treated with different EGCG concentrations or 0.1% DMSO were lysed with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, mixture of protease inhibitors, and a mixture of phosphatase inhibitors) on ice for 30 min. The lysates were centrifuged at 14,000 rpm at 4°C for 20 min, and the protein concentration of clear lysates was determined with the Coomassie protein assay kit (Pierce, Rockford, IL). Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was used to separate the proteins, and they were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) that contained 0.1% Tween-20 at room temperature for 1 hr, and then incubated with primary antibodies overnight in a cold room at 4°C. The membrane was washed the next day with TBST solution and incubated with corresponding horseradish peroxidase-conjugated secondary antibody for 1 hr. Finally, the immunoreactive bands were visualized by enhanced chemiluminescence detection.

RESULTS

EGCG Inhibits Proliferation of ARO Cells

First, we examined the anti-proliferative effect of various polyphenols, including EGCG, ECG, Genistein, Reveratrol, and Capsaicin, on ARO cells. We performed MTT assay of each polyphenols at a dose of 50 μM, a maximal dose of dose-dependent studies. As shown in Figure 1a, EGCG exerted the strongest inhibition among the polyphenols, and therefore, EGCG was selected for further experiments. Next, we examined whether EGCG inhibited the proliferation of ARO cells using the MTT assay. EGCG treatment inhibited the growth of ARO cells in a dose-dependent manner (Fig. 1b). Interestingly, EGCG treatment at 100 μM resulted in 80% inhibition of ARO cells after 24 hr of treatment.

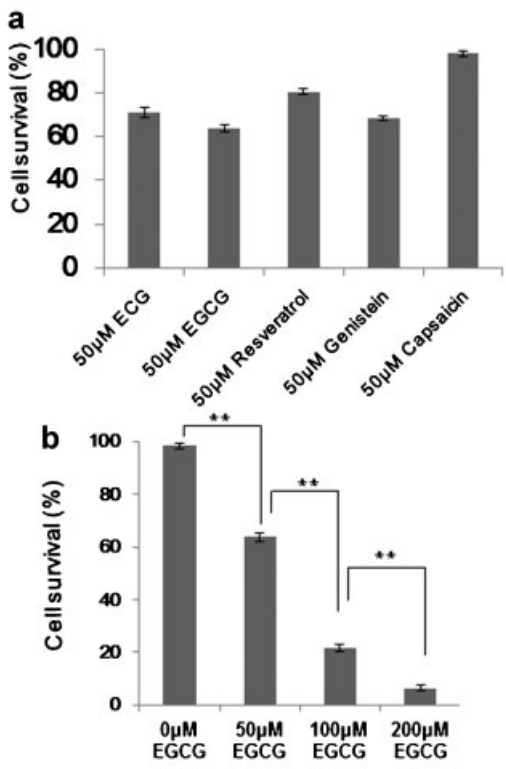

Fig. 1. Effects of polyphenol on the proliferation of ARO cells. **a**: EGCG showed the most powerful anti-proliferative activity against ARO cells among various polyphenols, such as EGCG, ECG, Genistein, Resveratrol, Capsaicin. ARO cells were treated with 50 μM of each polyphenols for 24 hr, and growth inhibition of ARO cells were assessed by MTT assay. 50 μM is a maximal dose of dose-dependent studies of different polyphenols (data not shown). **b**: EGCG inhibited growth of ARO cells in a dose-dependent manner. $^{**}P < 0.01$.

EGCG Inhibits the Proliferation of ARO Cells by Suppressing EGFR Phosphorylation and Its Downstream ERK

Next, we examined whether EGCG could inhibit the EGFR signaling pathway. As shown in Figure 2, ARO cells treated with EGCG decreased EGF-induced, EGFR phosphorylation in a dose-dependent manner. Furthermore, we assessed for potential involvement of the mitogen activated protein kinase (MAPK) pathway as a downstream pathway of EGFR in ARO cells treated with EGCG. As shown in Figure 2, EGCG significantly inhibited ERK1/2 activation in a dose-dependent manner, and EGCG treatment at 100 μM resulted in near complete inhibition of ERK1/2 phosphorylation. In addition, EGCG exerted slight inhibitory effect on EGF-induced JNK and p38 phosphorylation (Fig. 2).

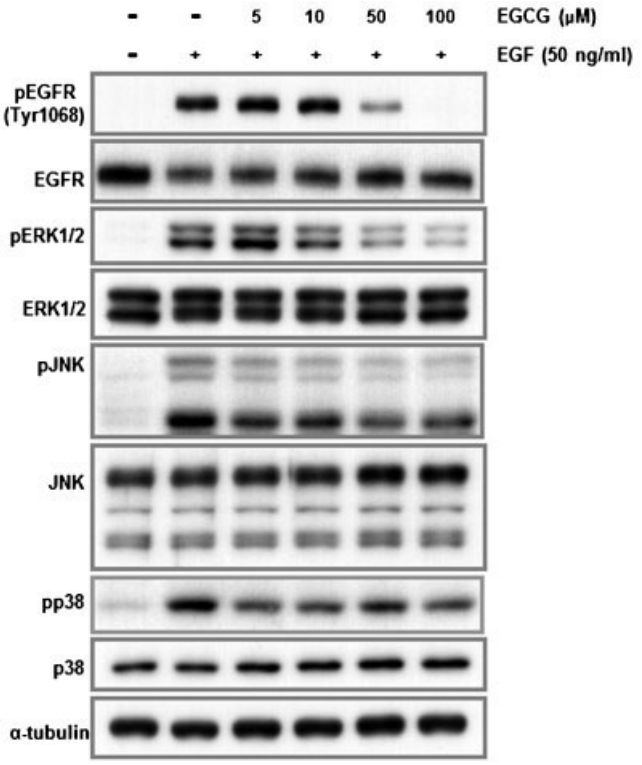

Fig. 2. Effects of EGCG on EGFR-dependent downstream kinases. After ARO cells were plated in serum-free medium for 24 hr and treated for 30 min at various concentrations (5, 10, 50, 100 μM) of EGCG prior to stimulation with 50 ng/ml EGF. After 10 min, whole cell lysates were prepared and immunoblotting was performed.

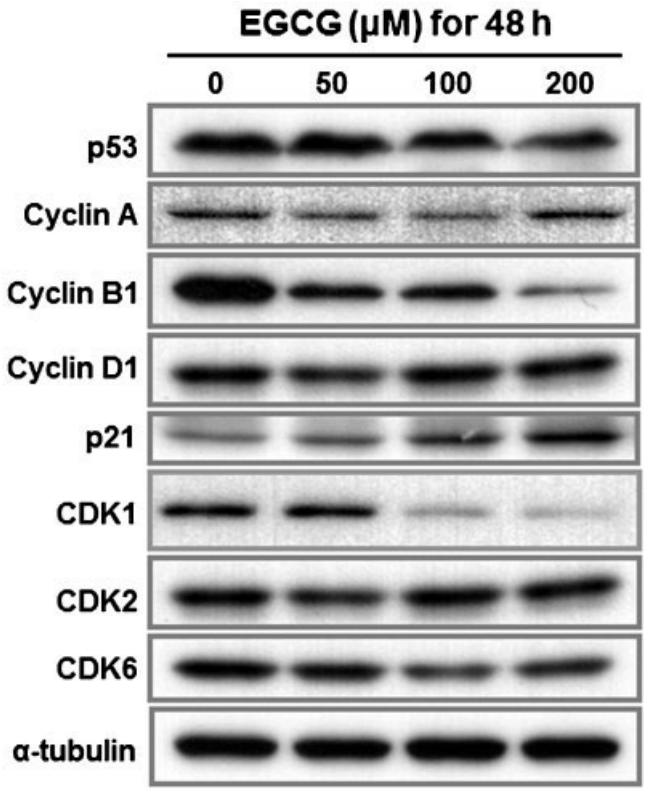

Fig. 3. Effects of EGCG on cell cycle associated proteins. ARO cells were grown in standard RPMI medium for 24 hr, and treated with the indicated EGCG concentration (0, 50, 100, 200 μM) for 48 hr. The cells were lysed and prepared for immunoblotting.

EGCG Suppresses Cell Cycle Related Proteins in ARO Cells

After treatment of ARO cells with EGCG, cell cycle-related proteins, such as cyclin B1 and CDK1, were down-regulated, whereas p21 was up-regulated in a dose-dependent manner. This phenomenon occurred in a p53-independent manner. In contrast, the activity of other cyclins, including cyclins A, D1, E and I, and CDK2, CDK6 remained unchanged (Fig. 3).

EGCG Induces Apoptosis of ARO Cells

Next, we performed flow cytometric analysis to check the appearance of sub-G1 peak, a specific fraction for apoptosis in cell cycle distribution. In the present study, we observed that EGCG treatment increased the accumulation of sub-G1 cells in a dose-dependent manner (Fig. 4a). Furthermore, clear cleaved caspase-3 and the cleavage of PARP into active forms were seen in the treatment with EGCG at 100 μM for 48 hr. These results suggest that EGCG stimulated apoptosis in ARO cells (Fig. 4b).

DISCUSSION

Failure of current therapeutic modalities for ATC, including surgery, radiotherapy, chemotherapy, or combined therapy, motivate researcher to improve the understanding of the mechanism that underlies ATC carcinogenesis. Some studies indicated that the EGFR pathway might play an important role in ATC carcinogenesis. Nobuhara et al. [13] observed that EGFR was almost universally expressed in ATC cell lines. Ensinger et al. [14] also demonstrated that all ATC with poorly differentiated thyroid carcinoma parts exhibited EGFR over-expression on immunohistochemistry. In addition, over-expression of EGFR in papillary thyroid carcinoma tends to be associated with high frequency of lymph node metastasis, increased tumor size and advanced clinicopathologic stage [15]. All these previous observations suggest that EGFR may be associated with ATC carcinogenesis, and interrupting EGFR function by targeted therapy in ATC presents potential for therapeutic development.

Point mutations of BRAF are found in approximately 45% of thyroid papillary carcinoma [16] and many ATC with papillary components are derived from BRAF-mutated PTC [17]. Therefore, BRAF mutations appear to play a role in the development of a subset of ATC [18]. Constitutive activation of MAPK signaling pathway is mainly involved in cell survival and proliferation and is a frequent event in many human cancer types, especially in thyroid cancer. This occurs through activating mutations or overexpression of upstream activators of MAPK, including receptor tyrosine kinase (RTK)s, RAS, and BRAF, suggesting that RTK–RAS–BRAF–MAPK cascade signaling play a role in the oncogenic potential of ATC and targeting this pathway may be a alternative treatment approach for ATC [19]. In addition, inhibitors of the MAPK pathway may increase efficacy of RAI therapy in cancers with BRAF mutations [20].

Recently, many studies have shown strong chemotherapeutic effect of EGCG against numerous cancer, including that of lung, breast, colon, and head and neck [21–24].

One anti-tumor mechanism exerted by EGCG is inhibition of EGFR-mediated signaling pathway, including the MAPK pathway [11]. There are three major types of MAPKs in mammalian cells:

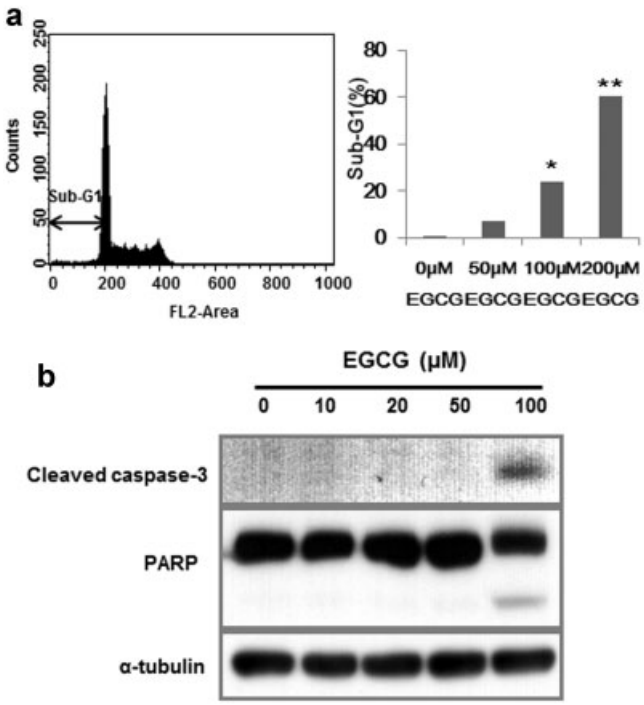

Fig. 4. The effects of EGCG on cell cycle and apoptosis-related proteins. ARO cells were plated in a 6-well plate for 24 hr, and were treated at various EGCG concentrations (0, 50, 100, 200 μM). After 24 hr, cell cycle was examined by flow cytometry. In addition, ARO cell lysates were extracted, and immunoblotting for caspase-3 and PARP was performed. **a**: EGCG treatment increased sub-G1 population of ARO cells in a dose-dependent manner. $^{*}P < 0.05$, $^{**}P < 0.01$. **b**: EGCG treatment activated caspase-3 and cleaved the PARP in ARO cells.

extracellular signal-regulated protein kinases (ERK), p38 MAPKs, and the c-Jun NH2-terminal kinases (JNK). EGCG has been shown to inhibit the MAPK pathway [11]. Our studies also showed that MAPK activity is reduced by EGCG in a dose-dependent manner. Specifically, EGCG exerted almost complete inhibition of EGF-induced ERK1/2 phosphorylation, as well as slight inhibition of EGF-induced JNK and p38 phosporylation.

Dysregulation of cyclin-dependent kinase (CDK) activity was frequently observed in human cancer, and tumor cells have specific requirements for each CDKs [25]. Some studies have shown that EGCG affected a number of factors associated with cell cycle progression, and direct inhibition of CDKs is considered as the primary event [11]. Particularly, dysregulation of CDK1 activity may be responsible for tumor development in malignances lacking p21 expression [25]. Our study demonstrated that EGCG reduced the levels of CDK1/cyclin B1 complexes, and also induced the expression of p21 without change in p53 expression in ATC cells.

Apoptosis is considered to be a protective mechanism against cancerous change in an organism, by eliminating genetically damaged cells [26]. A defect in the apoptosis pathway is therefore a common event in many types of cancer. As a result, enhancement of apoptosis in cancer cells may provide therapeutic benefits against certain human cancer. Various studies have shown induction of apoptosis by EGCG in many types of cancer [11]. Our study demonstrated EGCG induces apoptosis of ATC cells, increases sub-G1 cell numbers, caspase-3 and PARP cleavage.

CONCLUSION

Our study demonstrated that EGCG inhibits the proliferation of ATC cells by suppressing the EGFR/ERK signaling pathway. In addition, EGCG suppressed the expression of CDK1/cyclin B1 complex while increased p21 expression. Furthermore, EGCG causes apoptotic cell death in ATC cells. Taken together, these results suggest that EGCG may be an alternative agent for the treatment of anaplastic thyroid cancer.

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