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Epigallocatechin-3-gallate promotes apoptosis in human breast cancer T47D cells through down-regulation of PI3K/AKT and Telomerase

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ABSTRACT

Background: Green tea has antioxidant, anti-tumor and anti-bacterial properties. Epigallocatechin-3-gallate (EGCG) in green tea is highly active as a cancer chemopreventive agent. In this study, we designed a series of experiments to examine the effects of EGCG on proliferation and apoptosis of estrogen receptor α-positive breast cancer (T47D) cells.
Methods: Cells were treated with EGCG (0–80 μM) and tamoxifen (0–20 μM), as the positive control, up to 72 h. Cell viability was determined by MTT assay. Apoptosis investigated by real time PCR of apoptosis and survival (Bax, Bcl-2, p21, p53, PTEN, PI3 K, AKT, caspase3 and caspase9 and hTERT) genes and by western blot of Bax/Bcl-2 proteins expressions.
Results: The results showed that EGCG decreased cell viability as concentration- and time-dependently. IC_{50} values were 14.17 μM for T47D and 193.10 μM for HFF cells, as compared with 3.39 μM and 32.75 μM for tamoxifen after 72 h treatment, respectively. Also, EGCG (80 μM) significantly increased the genes of PTEN, CASP3, CASP9 and decreased AKT approximately equal to tamoxifen. In gene expression, EGCG (80 μM) significantly increased Bax/Bcl-2 ratio to 8-fold vise 15-fold in tamoxifen (20 μM)–treated T47D cells during 72 h. In protein expression of Bax/Bcl-2, EGCG significantly increased 6-fold while this ratio augmented 10-fold in tamoxifen group. EGCG significantly decreased 0.8, 0.4 and 0.3 gene expression of hTERT in 24, 48 and 72 h, respectively.
Conclusions: This study suggests that EGCG may be a useful adjuvant therapeutic agent for the treatment of breast cancer.
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Introduction

The most common cancer among women is breast cancer (17.1 per 100,000 person-year), which has mainly affected Iranian women about a decade earlier than western countries [1,2]. Presently, common treatments for breast cancer are chemotherapy, radiotherapy and surgery. Current systemic therapies for breast cancer are often limited by major organ damage, short-term efficacy due to the emergence of drug resistance and poor prognosis [3]. So, the search for new antitumor agent's development with improved efficacy and side-effect profile has been continued. Researchers believe that dietary phytochemical agents may influence chemotherapy treatment and help cure patients with cancer. Different natural compounds can improve the efficiency of chemotherapeutic agents, decrease resistance of chemotherapeutic drugs, and lower as well as alleviate adverse side-effects of chemotherapy [4]. As a result, researchers attempt to employ different herbs and their effective agents both *in vitro* and *in vivo* for cancer therapy. Most herbs contain antioxidant agents that could be consumed to prevent cancer or potentiate chemotherapy. Experimentally, several medicinal plants and herbal ingredients have been reported to have anticancer effects [5]. Also, a number of phytochemicals isolated from medicinal plants have been shown to decrease cell proliferation, induce apoptosis, retard metastasis and inhibit angiogenesis [6]. Currently, some of these plant-derived compounds are widely used for the chemotherapy of patients with cancer. For example, taxol analogues, vinca alkaloids (vincristine, vinblastine), and podophyllotoxin analogues have played an important role in the treatment of such patients [7]. Green tea is a popular beverage in

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Asia. Epidemiological studies have suggested that drinking green tea is effective in the treatment of different diseases. Based on many *in vivo* and *in vitro* studies, the biological activity of green tea is mediated by its major polyphenolic constituent, epigallocatechin gallate (EGCG), which is a potent antioxidant [8]. The beneficial effects of EGCG are reported in the treatment of cancer, cardiovascular diseases, diabetes, neurodegenerative diseases, and liver diseases. It reduces the risk of cancer developing in the prostate, bladder, stomach, oesophagus and lung [9–13]. In the pioneer study confirmed that EGCG affected the ERα-positive cells more than ERα-negative breast cancer cells [14]. Therefore, we chose T47D cells (as estrogen receptor α-positive breast cancer cell model) to evaluate the effects of EGCG on proliferation and apoptosis compared with tamoxifen (as positive control). It was also aimed to determine whether antitumor effects of EGCG are associated with altering of PI3K/AKT and telomerase genes expressions.

Materials and methods

Cell lines and reagents

The estrogen receptor positive human breast cancer cell line T47D and normal Human Foreskin Fibroblast cell line HFF were obtained from Pasteur Institute of Iran. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, trypsin, penicillin and streptomycin were obtained from Gibco BRL Life Technologies (USA). The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) 4-, OH-tamoxifen and EGCG (>95%) were purchased from Sigma-Aldrich company (USA). Tripure was purchased from Invitrogen (USA). Real-time PCR Master Mix and cDNA synthesis Kit were obtained from Roche Diagnostic (Switzerland) and Fermentas (Lithuania), respectively. The enhanced chemiluminescence (ECL) detection kit and polyvinylidene difluoride (PVDF) membranes were purchased from GE Healthcare (UK) and Millipore (USA), respectively. Primary antibodies to Bcl-2, Bax, β-actin, and secondary antibody were obtained from Cell Signaling Technology (USA).

Cell culture

T47D and HFF cells were maintained at 37 °C in a humidified atmosphere (90%) containing 5% CO2 and DMEM with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were seeded overnight and then, incubated with various concentrations of EGCG (10–80 μM) and tamoxifen (2.5–20 μM) for 24, 48 and 72 h. For each concentration and time course study, there was a control sample which remained untreated and received the equal volume of medium. All the different treatments were carried out in triplicate.

Cell viability

The cell viability was determined using a modified MTT assay. Briefly, the cells were seeded (5000 cells/well) on to the flat bottomed 96-well culture plates and allowed to growth for 24 h and then, treated with EGCG (0, 10, 20, 40 and 80 μM) and tamoxifen (0, 2.5, 5,10 and 20 μM) for 24, 48 and 72 h. After removing the medium, the cells were labeled with MTT solution (5 mg/ml in PBS) for 2 h and the resulting formazan dye was solubilized with DMSO (100 μl). The absorption was measured at 570 nm (620 nm as a reference) in an ELISA plate reader [15].

Real-time PCR

Total RNA was extracted from EGCG (10–80 μM)- and tamoxifen (20 μM)-treated T47D cells using Tripure according to the manufacturer's instruction. Then, 1 μg of RNA was applied to cDNA synthesis using reverse transcription kit. Quality of the cDNA samples was confirmed by PCR reaction detecting glyceraldehyde phosphate dehydrogenase (GAPDH) expression. Quantitative PCR reaction was carried out on step one real time PCR system using SYBR Green PCR Master Mix. The reaction mixture consisted of 1X Q-PCR master mix and 5 μM of the primers for Bax, Bcl-2, p21, p53, PTEN, PI3K, AKT, caspase3, caspase9, hTERT and GAPDH (Table 1). The primer sequences were designed by Allel ID software. Thermal cycling condition was programmed as: an initial denaturation step for 10 min at 95 °C followed by 40 cycles including a denaturation step for 15 s at 95 °C and annealing step for 1 min at 60 °C. Fluorescent data were acquired in the extension step. Finally, for each reaction, a melting curve analysis was performed from 55 to 95 °C to confirm the specifity of each reaction. Each sample was analyzed in triplicate and GAPDH was applied as the normalizer gene. Fold changes in gene expression were calculated by delta–delta CT method [16].

Western blot analysis

Bax and Bcl-2 protein were evaluated in treated-T47D cells with EGCG (80 μM) and tamoxifen (20 μM) during 72 h. Extracted proteins were separated by SDS-PAGE and then, performed immunoblotting. Briefly, after treatment T47D cells were homogenized with lysis buffer (10 mM Tris–HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM Na3VO4, 1 mM PMSF, and pH 7.5) and the lysates centrifuged at 18000 × *g*, 1 h, at 4 °C to precipitate the particulates. Then, equal amounts of total

Table 1
Primers used for real-time PCR.

Gene	Forward primer	Reverse primer	Genbak Code	Produt size (bp)
BAX	GCCTCCTCTCCTACTTTG	CTCAGCCCATCTTCTTCC	NM_001291428.1	102
Bcl**2.**	CCAAGAAAGCAGGAAACC	GGATAGCAGCACAGGATT	NM_000633.2	170
P21	AACGGCGGCAGACCAGCAT	GAGACTAAGGCAGAAGATGTAGAGCG	NM_000389.4	150
P53	GGAACTCAAGGATGCCCAG	CAAGAAGTGGAGAATGTCAGTC	NM_001126113.2	155
AKT	GCACCTTCATTGGCTACA	CCGCTCCGTCTTCATCAG	NM_001014431.1	104
PI_3**K**	TGCGGAAACTGACGGACGATGA	CGGAGCGGAGGTGCCAGAA	NM_005026.3	162
CASP**3**	AGAACTGGACTGTGGCATT	GCTTGTCGGCATACTGTTT	NM_004346.3	191
CASP**9**	CTTTGTGTCCTACTCTACTTTCC	AACAGCATTAGCGACCCTA	NM_032996.3	151
PTE**N**	AGTAGAGGAGCCGTCAAATC	ATCAGAGTCAGTGGTGTCAG	NM_000314.4	109
hTER**T**	TGTACTTTGTCAAGGTGGATGTGA	GCTGGAGGTCTGTCAAGGTAGAG	NM_001193376.1	195
GAPD**H**	GAAGTCAGGTGGAGCGAGG	TGGGTGGAATCATATTGGAACAT	NM_001256799.2	200

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