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Green tea polyphenols and its constituent epigallocatechin gallate inhibits proliferation of human breast cancer cells in vitro and in vivo

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Abstract

Tea [*Camellia sinensis* (Theaceae)] intake is second only to water in terms of worldwide popularity as a beverage. The Green tea polyphenols have been shown to have a protective effect in prostate cancer in various pre-clinical animal models and has been reported to be effective in several other cancer types as well. An inverse association between the risk of breast cancer and the intake of green tea has also been reported in Asian Americans. Several epidemiological studies have shown that breast cancer progression is delayed in the Asian population that consumes green tea on regular basis. In this study, we report the effectiveness of green tea polyphenols (GTP) and its constituent Epigallocatechin Gallate (EGCG) in tumor regression using both in-vitro cell culture models and in vivo athymic nude mice models of breast cancer. The anti-proliferative effect of GTP and EGCG on the growth of human breast cancer MDA-MB-231 cell was studied using a tetrazolium dye-based (MTT) assay. Both GTP and EGCG treatment had the ability to arrest the cell cycle at G1 phase as assessed by flow cytometry. The expression of Cyclin D, Cyclin E, CDK 4, CDK 1 and PCNA were down regulated over the time in GTP and EGCG treated experimental group, compared to the untreated control group as evaluated by western blot analysis for cell cycle proteins, which corroborated the G1 block. Nude mice inoculated with human breast cancer MDA-MB-231 cells and treated with GTP and EGCG were effective in delaying the tumor incidence as well as reducing the tumor burden when compared to the water fed and similarly handled control. GTP and EGCG treatment were also found to induce apoptosis and inhibit the proliferation when the tumor tissue sections were examined by immunohistochemistry. Our results suggest that GTP and EGCG treatment inhibits proliferation and induce apoptosis of MDA-MB-231 cells in-vitro and in-vivo. All together, these data sustain our contention that GTP and EGCG have anti-tumor properties.
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Keywords: EGCG; Green tea; MDA-MB-231; Tunel; Xenograft

1. Introduction

Tea [*Camellia sinesis* (Theacacea)] is considered second only to water as the most popular beverage consumed worldwide. Consumption of tea has been associated with many health benefits and their role and mechanism in cancer chemoprevention has been extensively reviewed [1–3]. Green, black and oolong tea are the three major commercial types of tea [4]. They differ in the way they are produced and also in their chemical composition. Only 20% of the tea manufactured is green tea and it is processed to prevent the oxidation of green leaf polyphenols. Majority of the polyphenols are oxidized during black tea production and oolong tea consists of a partially oxidized product

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[4]. The biological activity of green tea is due to different catechin and epigallocatechin gallate is identified as the principal antioxidant contributing approximately 30% of the total antioxidant capacity of green tea [5].

Green tea's beneficial effect in prostate cancer has been extensively studied using preclinical transgenic models [6–10] and nude mice xenograft models [11]. Breast cancer is the most common cancer in women and makes up one tenth of all new cancer diagnoses worldwide [12]. Epidemiological studies suggest that increased consumption of green tea is also related to improved prognosis of human breast cancer [13]. An inverse association between the risk of breast cancer and the intake of green tea has also been reported in Asian-Americans [14,15]. The medicinal properties of these phytochemicals are often attributed to their antioxidative and/or anti-inflammatory activities [16]. Recent studies have shown that multiple mechanisms are involved in their beneficial effects with respect to tumor initiation, promotion and progression [17–20]. However, it is still not clear whether these actions occur in animals or humans because of the limited bioavailability of EGCG following oral administration [21]. Studying the cancer chemopreventive effect of polyphenols and the pathways affected by them has been useful in understanding the molecular mechanism(s) involved in cancer promotion as well as to understand the properties of cancer cells. Although green tea and its constituents have been shown to inhibit breast cancer [22–24], the mechanism(s) of the inhibition is not completely known. In this study, we evaluated the anti-proliferative activity of GTP and its key constituent EGCG both in-vitro using MDA-MB-231 breast carcinoma cell line and in-vivo using nude mice xenograft model. Both GTP and its constituent EGCG decreased the proliferation of the tumor cell line by arresting the progression of the cell through G1 phase of the cell cycle. Furthermore, we have also observed that GTP and EGCG were capable of delaying the tumor incidence as well as reducing the tumor burden in-vivo using MDA-MB-231 human breast carcinoma xenograft in athymic nude mice. These results suggest that Green tea constituents may affect breast cancer cell on a cellular level and further studies are important to elucidate the precise mechanism(s) of inhibition of breast cancer by green tea.

2. Materials and methods

2.1. Materials

EGCG and GTP were obtained from LKT laboratories (St Paul, Minnesota, MN). The estrogen receptor-negative MDA-MB-231 breast cancer cell line was obtained from ATCC (Manassas, VA). The cells were maintained in monolayer in Eagle's minimum essential medium supplemented with 5% fetal bovine serum, nonessential amino acids, 2× vitamin solution, penicillin and streptomycin. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and were maintained at 70–90% confluence in T-150 flasks. Medium was changed daily. Cells were dislodged for both passaging and harvesting by a brief incubation in 0.25% trypsin and 0.02% EDTA. Cell were stained with trypan blue and counted using a hematocytometer. For injection, the cells were re-suspended in the medium at a concentration of 5×10^6 cells/ml.

2.2. Cell proliferation assay

MDA-MB-231 cell proliferation in the presence of various concentrations of EGCG and GTP was determined using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) cell proliferation kit (Boehringer Manheim, Indianapolis, IN) as per manufacturer's protocol. Briefly, cells were plated in 96-well tissue culture plates in a range of 3000 cells/well in a final volume of 100 ul of medium and were allowed to attach overnight. The cells were then treated once with varying doses of EGCG and GTP and observed after 24, 48, 72 and 96 h. After completion of the treatment, the cells were incubated with MTT for 3–4 h at 37 °C. Cells were lysed, and the reduced intracellular formazan product was dissolved in the buffer provided in the kit. MTT is reduced to a colored water insoluble formazan salt by only metabolically active cells, which is quantitated in a conventional ELISA plate reader at 570 nm.

2.3. Cell cycle analysis

The effect of EGCG and GTP on cell cycle distribution was determined by flow cytometry after staining the cells with propidium iodide. Briefly, 5×10^5 cells were seeded and allowed to attach overnight. The medium was replaced with fresh complete medium containing the desired concentration of EGCG and GTP. Both the floating and adherent cells were collected, washed with phosphate-buffered saline (PBS), and fixed with 70% ethanol at −80 °C. The fixed cells were then centrifuged and washed with PBS. The cells were then treated with 1 unit of DNase-free RNase to the cell suspension (10^6 cells in 1 ml), and incubated for 30 min at 37 °C. 50 μl of 1 mg/ml propidium iodide was added directly to the cell suspension and were passed in a Coulter Epics XL Flow Cytometer. The data was analyzed using modfit software.

2.4. Western blotting

Cells were harvested, pelleted and homogenized with ice-cold homogenization buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton ×100, 1% sodium deoxycholate, 0.1% SDS to which 1mM DTT, 1 mM PMSF,

1 μg/ml pepstatin A, 10 μg/ml Leupeptin, 10 μg/ml aprotinin, 25 mM NaF, and 100 μM Na_3VO_4. The homogenate was passed 10 times through a 25-guage needle and centrifuged at 14,000×*g* for 20 min at 4 °C. The supernatant protein extract was transferred to fresh tubes, aliquoted and stored at −80 °C. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). 50 μg of protein extracts in 13 μl of RIPA were thawed on ice and mixed with 2 μl of 10× sample reducing agent (Invitrogen, Carlsbad, CA) and 5 μl of 4× sample buffer (Invitrogen, Carlsbad, CA). Samples were heated at 100 °C for 5 min and loaded on 4–12% Bis-Tris gels for fractionation at 120 V. Predetermined molecular weight standards (Invitrogen) were used as markers. Protein on the gel was blotted onto nitrocellulose membranes at 225 mA for 120 min at 4 °C. After transfer, the membranes were incubated with blocking buffer (5% skim milk in wash buffer [1× TBS and 0.1% Tween 20]) for 1 h at room temperature. Protein expression for various cell cycle regulatory proteins was detected by incubating with appropriate primary antibodies (BD biosciences, San Jose, CA) in blocking buffer. The membranes were washed three times with wash buffer and were then incubated with the appropriate secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at dilutions of 1:2000 blocking buffer. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) according to the specifications of the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were scanned and optical densities of the band were quantitated.

2.5. Animals

Female athymic nude mice (NCr-nu/nu), 5 weeks old were purchased from NCI (Fredrick, MD) and were maintained in cages with housing in a specifically designed pathogen-free isolation facility. All experiments were done in accordance with the United States Public Health Service policy and the University laboratory animal review board.

2.6. Tumor cell inoculation

The mice ($n=45$) were weighed and were anesthetized followed by an inoculation of 5×10^6 cells in 100 μl of culture medium in the mammary fat pad. Mice were allowed to recover and were randomized into three groups of 10 animals each. 15 animals, which were not manipulated, were divided into 3 groups of 5 each and were used as control. The mice were weighed and the inoculation site was palpated at weekly intervals. The growth rates were determined by weekly measurement of two diameters of the tumor with a vernier caliper. Experiment was terminated 10 weeks after tumor cell inoculation. At the time of animal sacrifice, macroscopic examination of metastases was noted in lung, liver and kidney. Part of lung and tumor tissue was immediately frozen and the rest were fixed in 10% neutral-buffered formalin and were embedded in paraffin.

2.7. Feeding regimen

One group of animals received 1% GTP (made in autoclaved distilled water) as a sole source of drinking water and the other group received a dose of 1 mg/animal of EGCG in 100 μl of autoclaved distilled water. The control group was similarly handled and received 100 μl of autoclaved distilled water. The treatment was started the day after cell inoculation. Throughout the experiment, the animals had access to laboratory chow ad libitum.

2.8. Immunohistochemical detection of PCNA

PCNA expression in tissue sections was analyzed by immunostaining using a monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Deparaffinized sections were rehydrated and treated with 10 mM citrate buffer (pH 6.0) at 95 °C for 20 min. Endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide in methanol followed by two washes with PBS. The sections were then treated with normal horse serum (Vector Laboratories, Burlingame, CA) followed by a wash in PBS. The sections were incubated with mouse monoclonal anti-PCNA antibody overnight at 4 °C in humidified chamber. Slides were incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 10 min at room temperature with pre and post washes with PBS. Sections were incubated with conjugated horseradish peroxidase streptavidin complex for 5 min followed by another incubation with DAB substrate for peroxidase (Vector Laboratories, Burlingame, CA) for 7 min and a wash with PBS. The sections were counterstained with Hematoxylin.

2.9. In situ apoptosis detection by TUNEL staining

Apoptotic cell death in deparaffinized tissue sections was determined by Terminal deoxynucleotidyl transferase (TdT) mediated D-Uridine Tri Phosphate nick end labeling (TUNEL) technique using apoptag peroxidase In situ apoptosis detection kit (Chemicon International, Inc., Temecula, CA) as per manufacturers information. This method is based on the specific binding of TdT to 3′-OH end of DNA and ensuring synthesis of a polydeoxynucleotide polymer. Briefly sections were digested using proteinase K and the endogenous peroxidase activity was blocked using 3% hydrogen peroxide in PBS. The sections were then placed in equilibration buffer and incubated with working strength of TdT enzyme in a humidifying chamber at 37 °C for 1 h. The reaction was terminated with stop/wash buffer, provided with the kit. The apoptotic nuclei were stained by direct immunoperoxidase detection of digioxigenin-labeled DNA in test sections.

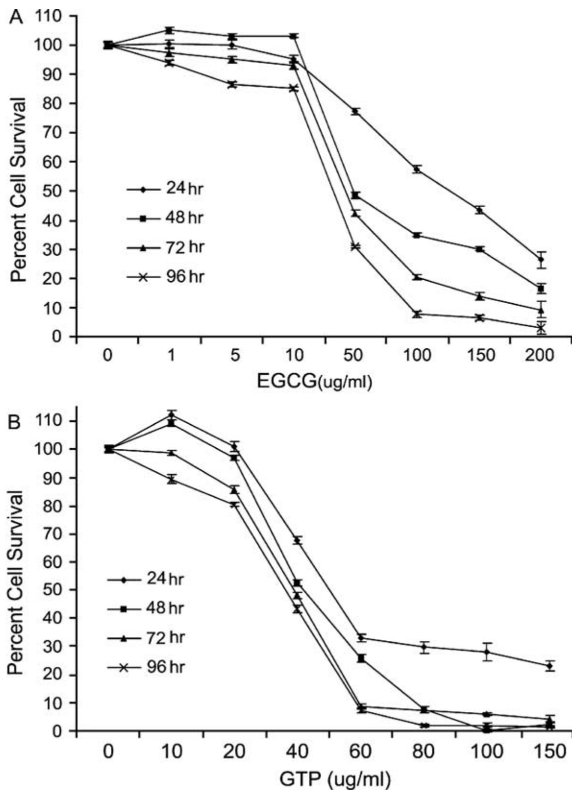

Fig. 1. EGCG and GTP inhibits the proliferation of MDA-MB-231. (A) Effect of different concentrations of EGCG on the proliferation of MDA-MB-231 incubated for 24,48,72,96 h. (B) Effect of different concentrations of GTP on the proliferation of MDA-MB-231 incubated for 24,48,72,96 h. The data are presented as the mean plus/minus SE($n=10$).

2.10. Quantitative analysis of proliferation and apoptosis

We examined 10 fields (PCNA immunostaining or TUNEL) that were blinded and randomly selected for each slide. Each field then was photographed at a magnification of 20×. For each photomicrograph we manually counted the numbers of total cells and positively stained nuclei for PCNA or TUNEL. The percentage of positive cells of the total number of cells was calculated for each image and a mean value was obtained for treated and untreated groups.

2.11. Statistical analysis

Unless otherwise indicated, data are presented as means ± SEM of 3–5 different observations. Data were analyzed by using one-way analysis of variance (ANOVA). Significance was defined as a *P*-value of less than 0.05

3. Results

3.1. EGCG and GTP treatment inhibits growth of MDA-MB-231 in-vitro

We examined the effect of increasing concentration of EGCG and GTP on the growth of the estrogen receptor-negative MDA-MB-231 human breast cancer cells for 24, 48, 72 and 96 h using MTT assay. Treatment with EGCG (1–200 μg/ml) and GTP (10– 150 μg/ml) inhibited the growth of MDA-MB-231 breast cancer cells in a concentration dependent manner with an IC_{50} of 50 μg/ml and 40 μg/ml for EGCG and GTP, respectively, at 48 h (Fig. 1). Extensive inhibition of cell growth was observed in polyphenol treatment groups receiving high concentrations (>50 μg/ml) of either EGCG or GTP.

3.2. EGCG and GTP treated cells are arrested in G1 phase of the cell cycle

Cell cycle distribution analysis of MDA-MB-231 cells treated with varying concentration of EGCG (50 and 80 μg/ml) and GTP (40 and 60 μg/ml) and harvested at 12 and 24 h showed a G1 arrest (Table 1). Both EGCG and GTP treatments increased the percentage of cells in G1 at 12 h (56% control vs. ~76% treatment) and at 24 h (43% control vs. ~71% treatment). The percentage of cells in S-phase was significantly reduced by both EGCG and GTP at 12 h followed by a reduction of cell in G2 in 24 h. In order to examine the molecular mechanism(s), underlying changes in cell cycle pattern, we investigated the effect of EGCG and

Table 1
Flow cytometric analysis of the effect of EGCG and GTP on MDA-MB-231

	12 h			24 h		
	G1	S	G2	G1	S	G2
Control	56.21	42.54	1.25	43.23	31.15	25.62
EGCG 50	76.04	19.32	4.64	71.12	20.69	8.19
EGCG 80	74.87	19.86	5.27	71.75	19.51	8.74
GTP 40	74.8	20.12	5.08	70.05	23.45	6.5
GTP 60	77.19	18.7	4.11	72.46	20.36	7.18

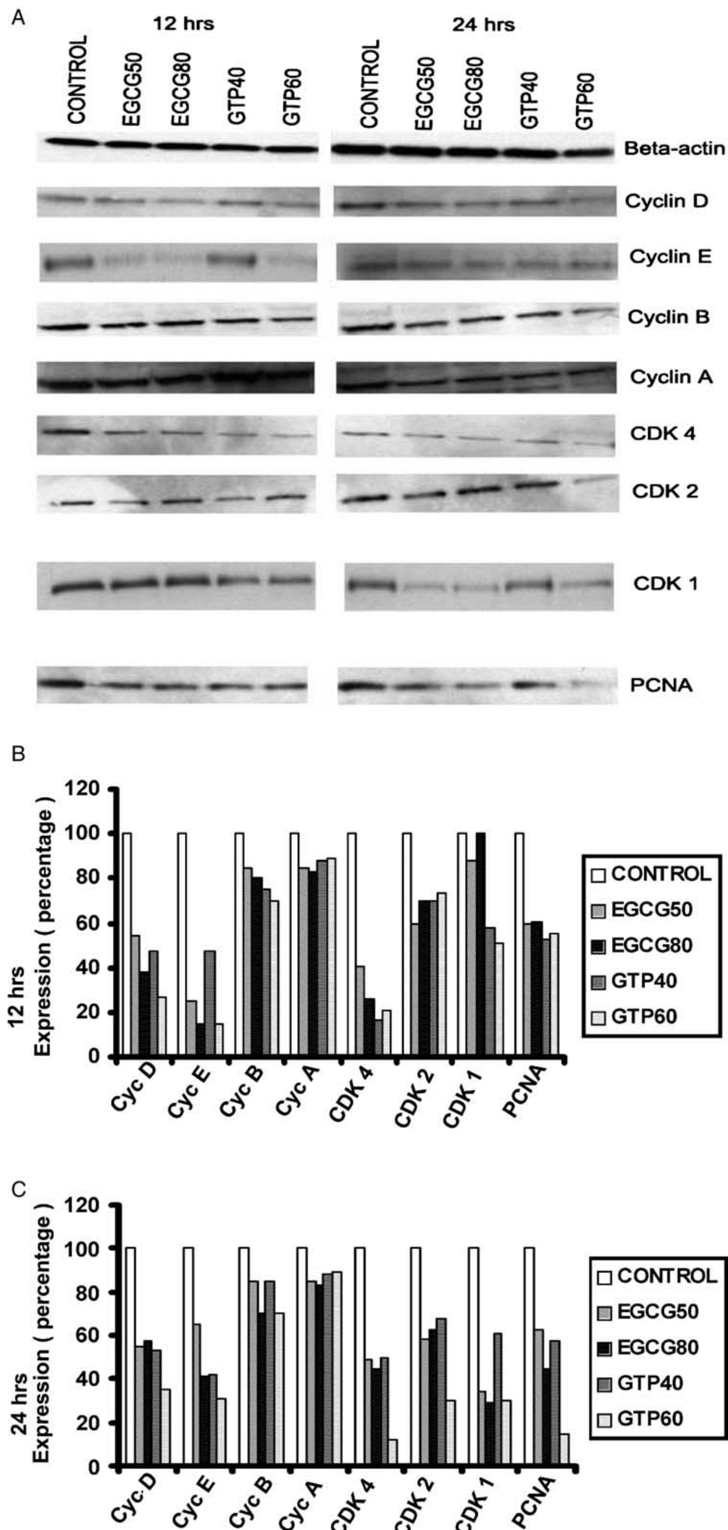

Fig. 2. Effect of EGCG and GTP on Cyclins and CDK. (A) Representative picture of Westernblot of cell cycle proteins of MDA-MB-231 treated with 50 and 80 ug/ml of EGCG and 40 and 60 ug/ml GTP for 12 and 24 h. (B) Quantificaton of 12 h expression of cell cycle proteins. (C) Quantificaton of 24 h expression of cell cycle proteins. Data are representatives of two individual experiment.

GTP on various cyclins and CDKs involved in cell cycle control. We approached this study by treating MDA-MB-231 with two different concentrations of EGCG (50 and 80 μg/ml) and GTP (40 and 60 μg/ml) on MDA-MB-231 by treating them for a period 12 and 24 h (Fig. 2A). We observed more than 50% reduction in the expression of Cyclin E, Cyclin D and CDK 4 in treatment involving both concentrations EGCG and GTP for 12 h (Fig. 2B). A similar pattern was observed in 24 h treatment (Fig. 2C). Reduction in the expressions of PCNA and CDK 1 were also observed for both EGCG and GTP treatments. A moderate reduction in other Cyclins and CDKs were also seen.

3.3. Effect of the oral administration of EGCG and GTP on the growth of human tumor xenograft in nude mice

EGCG solution (1 mg/0.1 ml/mouse) was administerd by oral gavage and 1% GTP was administered as the sole source of water to nude mice bearing tumors established from MDA-MB-231. All of the animals appeared healthy with no loss of body weight (Fig. 3 A). The average GTP consumption was constant throughout the cages and was estimated at around 3 mg/mouse/day. The tumor incidence was decreased (25 and 55%) in EGCG and GTP treated animals as compared to the untreated control after 2 weeks of treatment. At the end of 10 weeks, 10 and 20% of mice in EGCG and GTP treatment, respectively, did not develop tumors whereas all the animals in the untreated control group developed tumors. Although the difference in the tumor development was not significant after 10 weeks, there was a delay in the average time to onset (Fig. 3B). At the end of 10 weeks, the tumor volume was also reduced by 45 and 61% in the EGCG treated and GTP treated, respectively, as compared to the untreated controls and was found to be statistically significant ($P<0.05$) (Fig. 3 C).

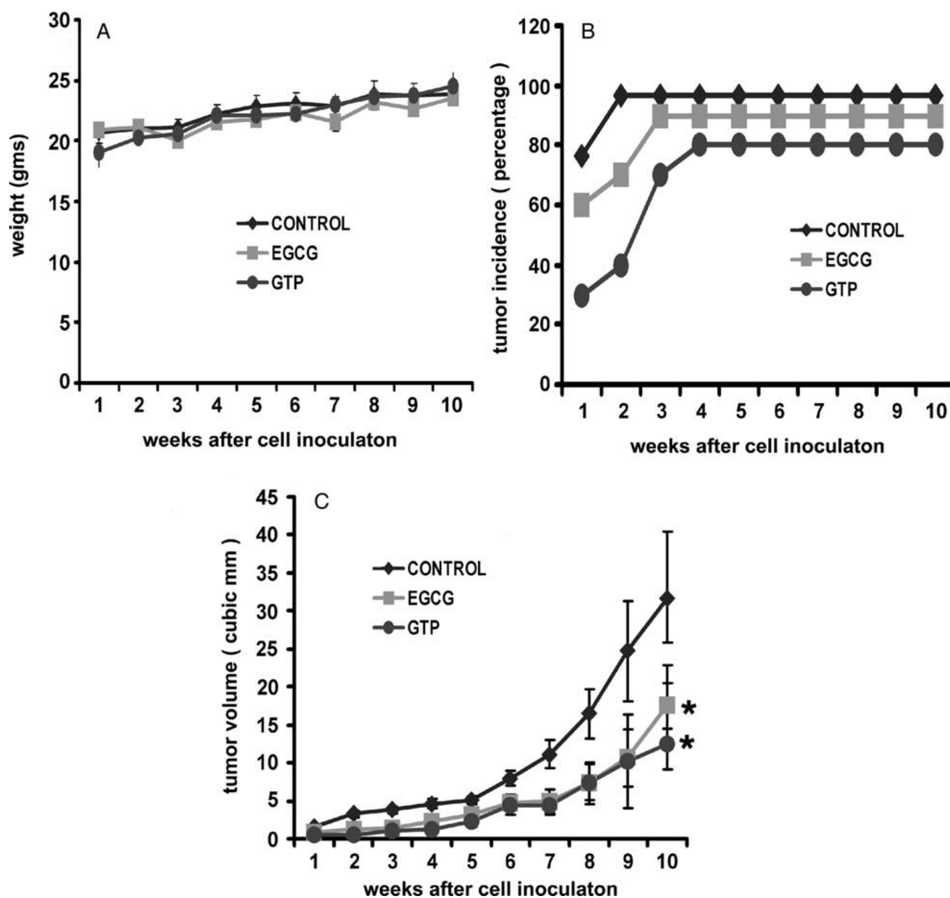

Fig. 3. Effect of EGCG and GTP In-vivo. (A) Effect of EGCG and GTP on body weight of nude mice. (B) Effect of EGCG and GTP on tumor incidence of nude mice. (C) Effect of EGCG and GTP. Data for weight and tumor volume are mean plus/minus SE ($n=10$). *Significantly different compared with controls, $P<0.05$.

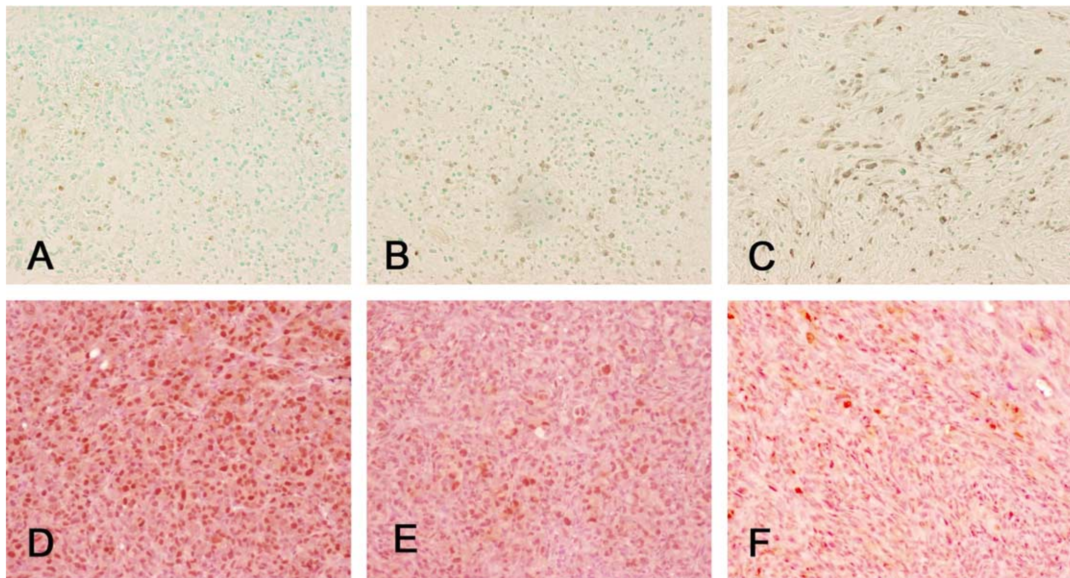

Fig. 4. Immunohistochemical staining for TUNEL and PCNA in MDA-MB-231 xenograft tumor masses. Top panels: TUNEL staining in tumor masses from (A) control, (B) EGCG treated, and (C) GTP treated animals. The TUNEL staining was pronounced in the center of the tumor mass. A greater number of TUNEL positive cells were observed in tumors from polyphenol treated animals. Nuclei were counterstained with methyl green. Bottom panels: PCNA staining from tumor masses in (D) control, (E) EGCG treated, and (F) GTP treated animals. Polyphenol treatment decreases the number of proliferating cells in tumor masses. Counterstained with hematoxylin. All images 200×.

3.4. EGCG and GTP decrease proliferation and increases apoptosis of human tumor xenograft in nude mice

As there was a significant reduction in the tumor volume by the polyphenol treatment, we examined the effect of EGCG and GTP on the tumor growth in xenograft by performing TUNEL assay. The tissues were also stained with PCNA, a proliferation biomarker.

EGCG and GTP fed animals showed increased apoptosis (Fig. 4 A–C) and decreased proliferation (Fig. 4 D–F) when compared with the untreated controls. GTP treatment showed a higher tumor cell death in-vivo as around 80±10 SEM cells/field were apoptotic, whereas, it was around 60±8 SEM cells/field in EGCG as compared to 23±5 SEM cells/field in control (Fig. 5). The PCNA-positive cells in control mice xenograft was 87±12 SEM cells/field. A decrease in the PCNA positive cells in EGCG (24±5.0 SEM cells/field) and GTP (33±4.3 SEM cells/field) indicated the anti-proliferative effect of these polyphenols (Fig. 5).

4. Discussion

Despite significant advances in the treatment of breast cancer, this disease not only remains the second most frequent cause of cancer death but also one of the most commonly diagnosed cancers among women in 2005 [25]. Epidemiological data from more than 250 case control and cohort studies shows an inverse relationship between the risk of certain types of cancer

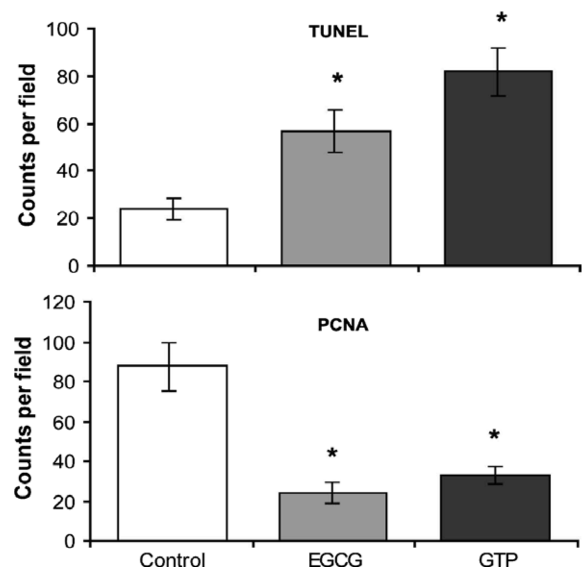

Fig. 5. The number of TUNEL and PCNA positive cells in microscopic (20×) fields from 6 samples were averaged. Error bars indicate SEM. *Significantly different compared with controls, $P<0.05$.

and consumption of dietary phytochemicals and fibers [26]. Previous studies in mice supported the anti-metastatic potential of green tea [27,28], which is rich in polyphenols. Green tea has also shown to significantly reduce the risk of breast and ovarian cancer in Asian women [15,29]. One of the key issues in chemoprevention with phytochemicals is to find out whether the activity and molecular mechanism(s) of the single active compound isolated and the extract are similar. Once they are found that they are similar, the extract may preferentially replace single active compound, as the cost incurred to identify and to develop these isolated compounds are very expensive. The present research documents a parallel study showing the effect of GTP and EGCG treatment in-vivo in a human tumor xenograft in nude mice as well as in in-vitro cell culture models involving estrogen receptor negative MDA-MB-231. The data presented herein suggest that GTP and its principal constituent EGCG are effective in suppressing the proliferation of MDA-MB-231, a highly invasive estrogen receptor- negative breast cancer cell line as shown by growth inhibition and apoptosis induction both in-vivo and in-vitro. The study also explains the anti-proliferative effect of these compounds as a function of their G1 blocking capacity.

Many of the molecular alterations that accompany carcinogenesis lead to uncontrolled proliferation and growth and the ability of the transformed cells to evade apoptosis. Both EGCG and GTP had a significant anti-proliferative effect. It has been shown that the anticancer effect of some potential phytochemicals like resveratrol and sulphoraphane take place through differential regulation of the cell cycle and subsequent events leading to cell death [30,31]. Flow cytometry analysis showed that both EGCG and GTP treatments had a profound effect on the cell cycle control as cells accumulated in the G1 phase of the cell cycle. Cyclin E, one of the key regulators of cell cycle has been found to be over-expressed in primary breast carcinoma tissue. Cyclin E has the potential to be used independently to predict the risk of visceral breast cancer relapse after surgery [32]. Cyclin E expression was generally high in estrogen receptor-negative tumors, suggesting a potential role for cyclin E in mechanisms responsible for estrogen-independent tumor growth. [33]. Over-expression of cyclin D1 in the mammary gland of transgenic mice induces mammary carcinoma [34]. Cyclin D expression is deregulated frequently in human neoplasias and agents that can downregulate cyclin D1 expression could be helpful in the prevention as well as treatment of human neoplasias [35]. Subsequent analysis of the cell cycle protein expression after EGCG and GTP treatment showed downregulation of cyclin D and Cyclin E, key cell cycle proteins, involved in G1/S progression. This indicates GTP and EGCG may be potential chemopreventive agents against breast tumors. EGCG was found to induce G1 phase ckis and thereby causing an arrest in G1 phase of the cell cycle in prostate carcinoma cells [36]. Furthermore, we also observed a tumor inhibitory activity of both EGCG and GTP in nude mice xenograft model. Both incidence and mean tumor volume were significantly reduced by GTP and EGCG treatment. Immunohistochemical staining showed a decrease in the proliferating cell nuclear antigen and an increase in apoptosis in tumors from animal treated with EGCG and GTP. Earlier reports have indicated that induction of apoptosis by green tea catechin treatment may be due to a decrease in Bcl-2 and an increase in Bax level [37]. Failure of normal apoptotic machinery in neoplastic cells underpin both tumorigenesis and drug resistance. Neoplastic cells undergo changes that diminish their susceptibility to apoptosis [38–40]. Thus for, the phytochemical agent to be accepted and developed as a potential anti-cancer drug, it has to demonstrate a direct toxic activity on these resilient neoplastic cells. Our data indicate that both EGCG and GTP effectively suppresses the proliferation as well as induces apoptosis in highly invasive estrogen negative MDA-MB-231 both in-vitro and in-vivo. Moreover, GTP as a mixture was needed in less concentration when compared to EGCG to induce similar cytotoxic effects in this cancer cell line. Though epidemiological studies and preclinical experimental studies, including this one, suggest the cancer chemopreventive and chemotherapeutic nature of these phytochemicals, we acknowledge that human intervention trials will be the ultimate proof of the aforementioned beneficial nature of these phytochemicals.

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