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PRECLINICAL STUDY

Potentiation of growth inhibition and epigenetic modulation by combination of green tea polyphenol and 5-aza-2′-deoxycytidine in human breast cancer cells

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Abstract Epigenetic therapy by DNA demethylating agent 5-aza-2′-deoxycytidine (5-aza 2′dC) is clinically effective in acute myeloid leukemia; however, it has shown limited results in treatment of breast cancer and has significant toxicity to normal cells. Green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) has anti-cancer and DNA demethylating properties with no significant toxicity toward normal cells. Therefore, the objective of this study was to evaluate the therapeutic efficacy of a combination of non-toxic, low dose of 5-aza 2′ dC with EGCG, on growth inhibition of breast cancer cells. Human breast cancer cell lines (MCF-7, MDA-MB 231) and non-tumorigenic MCF-10A breast epithelial cells were treated with either 5-aza 2′ dC, EGCG, or their combination for 7 days. Cell growth inhibition was determined by cell count, cell viability, cell cycle, and soft agar assay, whereas genes expression changes were determined by quantitative real-time PCR and/or Western blot analysis. Histone modifications and global DNA methylation changes were determined by Western blot and RAPD-PCR, respectively. The results revealed significantly greater inhibition of growth of breast cancer cells by co-treatment with 5-aza 2′ dC and EGCG compared to individual treatments, whereas it has no significant toxicity to MCF-10A cells. This was further confirmed by gene expression analysis. Changes in DNA methylation and histone modifications were also greater in cells with combination treatment. Findings of this study suggest that potentiation of growth inhibition of breast cancer cells by 5-aza 2′ dC and EGCG combination treatment, at least in part, is mediated by epigenetic mechanism.

Keywords Breast cancer · Epigenetic therapy · EGCG · 5-Aza-2′-deoxycytidine

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Introduction

Breast cancer is the second most commonly diagnosed cancer among women in the United States [1]. Although tremendous progress has been made in breast cancer treatment, it is often hindered by de novo or acquired drug resistance [2, 3]. Current chemotherapeutic drugs used in breast cancer mainly encompass anthracyclines, taxanes, and platinum-based drugs. However, in addition to cancerous cells, these drugs can also kill normal cells and have adverse side effects like cardio-toxicity, neutropenia, neurotoxicity, muscle fatigue, and abnormal blood clotting [4–6]. The inadequate clinical response and toxicity of anti-cancer drugs to normal cells [7] suggest a need for alternate targets and treatment strategies for breast cancer.

Accumulating evidence suggests that in addition to the genetic alterations, the epigenetic changes play an important role in breast cancer development. Global DNA methylation, concurrent with loci-specific hypermethylation is a common hallmark of breast cancer [8]. Global hypomethylation is linked to chromosomal instability and activation of oncogenes, whereas loci-specific hypermethylation leads to transcriptional silencing of tumor suppressor genes. For example, genes involved in pathways including cell cycle regulation (*cyclin D2*, *p16INK4a*, *14-3-3σ*), DNA repair (*MGMT*, *hMLH1*, *BRCA1*), growth inhibitory signaling (*RARβ*, *RASSF1A*, *HIN1*, *SFRP1*), cell adhesion (*CDH1*), angiogenesis (*maspin*), carcinogen

detoxification (*GSTP1*), hormone regulation (*ERα*, *PR*), and apoptosis (*TWIST*, *HOXA5*, *DAPK1*) are silenced by promoter hypermethylation in breast cancer [9, 10]. In contrast to genetic changes like mutations that are non-reversible, epigenetic changes can be reversed and therefore epigenetically silenced genes in cancer can be reactivated. It is because of this reversible nature of epigenetic alterations in target molecules, a new strategy known as "epigenetic therapy" has recently emerged for cancer therapy [11]. Epigenetic alterations can also serve as biomarkers of cancer risk. A recent study has associated methylation of ATM gene in white blood cells with breast cancer risk [12].

Decitabine or 5-aza-2′-deoxycytidine (5-aza 2′ dC) is a nucleoside analog and well-established DNA demethylating agent. Evidence suggests that 5-aza 2′dC demethylates DNA by various mechanisms, such as inhibiting the activity of DNA methyltransferase enzyme [13] and degradation of DNA methyltransferase 1 (DNMT1) by ubiquitination-dependent proteasomal pathway [14]. Pharmacological action of 5-aza 2′ dC is mediated by its incorporation into DNA, leading to formation of irresolvable DNA-5-aza 2′ dC complex. Interestingly, 5-aza 2′ dC acts as demethylating agent only at low doses, and higher doses of this drug cause cell death due to cytotoxicity [15]. Doses of 5-aza 2′ dC that are currently used in the clinic have shown limited results in solid tumors when given as a single agent, and higher doses cause cytotoxicity to normal cells [16]. To overcome this limitation of 5aza-2′dC for its clinical use as a chemotherapeutic agent, it has been suggested that combination of epigenetic modifiers may be more successful in DNA demethylation-mediated re-expression of silenced genes in breast cancer [15].

(−)-epigallocatechin-3-gallate (EGCG) is the major catechin among the polyphenols in green tea (*Camellia sinensis*) [17]. Evidence suggests that EGCG can inhibit the growth of breast cancer cells in vitro and breast tumor in vivo [18]. It has been reported that EGCG can induce cancer cell-specific apoptosis and cell cycle arrest and has no or minimal toxicity toward normal cells [19]. Mechanistically, EGCG inhibits breast cancer growth by interfering with multiple signaling pathways including NF-κB, MAPK, and EGFR [20]. Additionally, EGCG is an epigenetic modifier, which can re-express epigenetically silenced tumor suppressor genes such as *RARβ*, *hMLH1*, *GSTP1*, and $p16^{INK4a}$ by downregulation of DNA methyltransferase and histone deacetylase activity in cancer cells [21–24]. Treatment of human prostate cancer LNCaP cells with green tea polyphenols causes time-dependent inhibition of HDACs 1, 2, and 3 expressions and upregulation of histone acetylation [25]. As EGCG selectively kills cancer cells and has shown great promise in growth inhibition of cancer cells, it presents a high potential for development as an effective and minimally cytotoxic cancer therapeutic. However, the concentrations of EGCG used in vitro studies for growth inhibition are unachievable through daily intake of tea in patients [26], thus limiting its potential as a monotherapeutic agent in cancer.

While chemotherapeutic or epigenetic drugs have been used in combination with EGCG or 5-aza 2′ dC so as to synergize tumor suppression or sensitize cancer cells, the potential of combination treatment of 5-aza 2′ dC and EGCG on growth inhibition of breast cancer cells is not known. Both compounds are established DNA methyltransferase inhibitors, therefore their combination may have greater effect on re-expression of genes that are silenced by promoter hypermethylation in breast cancer. In addition to the tumor growth suppression by restoration of epigenetically silenced tumor suppressor genes, EGCG-mediated cell cycle arrest and apoptosis through targeting cell signaling pathways can further add to inhibition of breast cancer growth. This suggests that extended co-treatment with non-toxic, low doses of 5-aza 2′ dC and EGCG may potentiate growth inhibition of breast cancer through synergistically reactivating tumor suppressors as well as drug-specific mechanisms, while minimizing cytotoxicity to normal cells. Therefore, the objective of this study was to evaluate the efficacy of 5-aza 2′ dC and EGCG co-treatment, at low concentrations as compared to their individual treatment on growth inhibition of breast cancer cells in vitro.

Materials and methods

Chemicals and reagents

5-Aza-2′-deoxycytidine (5-aza 2′ dC), (−)-epigallocatechin-3-gallate (EGCG), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). TRizol for RNA isolation was purchased from Invitrogen, Inc. (Carlsbad, CA).

Cell culture and treatment

Human breast cancer cell lines MCF-7 [ER positive] and MDA-MB 231 [ER negative] as well as the human normal breast epithelial cell line MCF 10A were obtained from American Type Culture Collection (ATCC). MCF 10A is a non-tumorigenic human breast epithelial cell line and has been a widely accepted model for normal breast epithelium [27, 28]. MCF 10A cells were maintained in MEBM mammary epithelial cell growth media. MCF-7 and MDA-MB 231 cells were propagated in Dulbecco's modified eagle minimal medium (DMEM F12) supplemented with 5 % fetal bovine serum (FBS) and 1 % antibiotic–

antimycotic solution. Cell culture of all the three cell lines were maintained at 37 °C in a 5 % CO_2-containing humidified incubator. Cells were treated with either 5-aza 2′ dC (5 μM), EGCG (50 μM), combination of EGCG and 5-aza 2′ dC or DMSO (0.001 %) as vehicle control for 7 days. Stock solutions for treatment were made in DMSO and diluted prior to use. Treatments were given in cell culture media specific for each cell line. Media were changed, and fresh treatment was given after every 48 h. All the three cell lines were used to evaluate the effect of treatments on cell growth. Since the observed effects of treatment on growth were greater in MCF-7 cells than MDA-MB231, only MCF-7 cells were used for subsequent molecular and mechanistic analysis.

Cell count analysis

Cell count analysis was performed to assess the effects of 5-aza 2′ dC, EGCG, and their combination on the growth inhibition of MCF10A, MCF-7, and MDA-MB 231 cells. Briefly, 100,000 cells were seeded in 6-well plates. After 24 h of seeding, cells were treated with either 5-aza 2′ dC (5 μM), EGCG (50 μM), combination of EGCG and 5-aza 2′ dC or DMSO (vehicle control) for 7 days. Treatments were given in cell culture media specific for each cell lines. Media were changed, and fresh treatment was given after every 48 h. At day 7, cells were detached by trypsinization and suspended in cell culture media, and viable cells were counted with a Nexcelom Cellometer cell counter. Each treatment was run in triplicates and the experiment was repeated twice.

MTT assay for cell viability

Methyl thiazolyl tetrazolium (MTT) assay was performed to determine the effects of EGCG and 5-aza2′dC treatments on cell viability. Cells were seeded in triplicates in 96-well plates at a density of 2,500 cells per well. After 24 h of seeding, cells were treated with 5-aza2′dC, EGCG, and their combination as mentioned above. Media were changed, and fresh treatment was given after every 48 h. MTT assay was performed at day 7 of the treatment. Briefly, media were removed, cells were washed with 1X PBS, fresh culture media with MTT (1 mg/mL final concentration in media) were added to each well, and the plate was incubated at 37 °C for 4 h. Media were removed, and formazan was solubilized by adding 200 μL of DMSO in each well. The optical density (OD) was measured at 570 and 650 nm (as background). Each treatment was run in triplicates and the experiment was repeated twice. Cell viability was converted into percentage with respect to control as 100 %.

Cell cycle analysis

Cells were seeded in 25-cm^2 flasks and treated as mentioned earlier. The optimum cell seeding density was chosen so as to maintain sub-confluency at the time of cell fixation. After 7 days of treatment, cells were fixed in 70 % cold ethanol and stored at 4 °C for 24 h. Fixed cells were stained with Guava® Cell Cycle Reagent (Millipore, CA, USA), and cell cycle analysis was performed using Guava EasyCyte™ flow cytometer (Millipore, CA,USA) as per manufacturer's instructions. A total of 5,000 gated events were counted and data were acquired for each sample by Guava Incyte software (Millipore, CA). Each treatment was run in triplicates and the experiment was repeated twice.

Soft agar assay

Anchorage-independent growth of MCF-7 cells was determined by soft agar assay. Briefly, a base layer of agar (0.5 %) in cell culture media was plated in six-well plates. MCF-7 cells were treated as mentioned earlier for 72 h, mixed in top layer agar solution (0.35 %) and then plated at a density of 5,000 cells per well. Each treatment group was plated in triplicates. Plates were incubated at 37 °C in a 5 % CO_2-containing humidified incubator. After plating, cells were treated with different drug regimes as mentioned previously after every 48 h. After 7 days of colony growth, cells were stained with 0.001 % crystal violet dye, and colonies were visualized and counted under a microscope.

RNA extraction and real-time PCR

Total RNA was isolated from control and treated MCF-7 cells by TRIzol method and one-step real-time PCR kit with SYBR green dye (Bio-Rad Laboratories, Hercules, CA) was used for amplification of total RNA (50 ng) as per manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) amplifications were performed in 96-well reaction plates using MyiQ2 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) with the following PCR amplification conditions: Reverse transcription at 50 °C for 10 min, RT enzyme inactivation at 95 °C for 5 min, followed by 40 cycles each of 10 s denaturation at 95 °C and 30 s annealing and extension at 60 °C. Specificity of the PCR products was verified by melt curve analysis. Threshold cycle number (Ct value) was determined using iCycler IQ2 optical system software (Bio-Rad version 3.0a). The Ct value of each gene of interest was normalized to Ct value of GAPDH, and fold change in the gene expression was calculated using the delta–delta Ct method [29]. Amplification reaction for each treatment was performed in triplicates, and the experiment was repeated

twice. A non-template control was included in each experiment. Primer sequences used for qRT-PCR are given in Table 1.

DNA extraction and methylation-sensitive random amplification of polymorphic DNA (MS-RAPD)

Methylation-sensitive random amplification of polymorphic DNA was carried out to study the effect of treatment on DNA methylation patterns [30]. MS-RAPD is based on digestion of DNA by isoschizomeric restriction enzymes, methylation sensitive (MspI) and methylation insensitive (HpaII). Both the enzymes recognize CCGG; however, Hpa II is unable to cut when internal cytosine residue is methylated, whereas MspI can cleave with internal cytosine methylated, and therefore can discriminate between methylated and unmethylated cytosine.

Total DNA was extracted from treated and control MCF-7 cells by SDS/proteinase K digestion and phenol–chloroform extraction method as described before [31]. DNA was quantified spectrophotometrically, and quality was verified by agarose gel electrophoresis. 2.5 μg of DNA was used for single digestion with HpaII and MspI at 37 °C overnight. RAPD-PCR was performed using digested and undigested DNA with random 10-mer primers. Reaction was carried out in a 25-μL reaction mixture containing 100 μM dNTPs (Applied Biosystems, Foster City, CA), 100 nM primer, 0.5 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), 2.5 μL enzyme assay buffer, 2.5 mM $MgCl_2$, and 75 ng DNA. The PCR amplifications were performed in a DNA thermal cycler (GeneAmp PCR System 2700) programmed for 45 cycles as follows: one cycle of 3.5 min at 92 °C, 1 min at 34 °C, and 2 min at 72 °C followed by 44 cycles of 1 min at 92 °C, 1 min at 34 °C and 2 min at 72 °C, followed by a final extension cycle of 15 min at 72 °C. PCR products were resolved on 1.5 % agarose gel and visualized by ethidium bromide staining.

Western blot analysis

Protein lysates were prepared from 7-day treated and control MCF-7 cells and resolved by SDS-PAGE gel electrophoresis. After electrophoretic separation, the protein bands were transferred to a nitrocellulose membrane. The membrane was blocked with 5 % non-fat milk solution (non-fat dried milk in 1X TBS) for an hour at room temperature and then incubated with primary antibody overnight at 4 °C. Dilutions of antibodies used are as follows: *GAPDH* 1:200 (Santa Cruz, Cat# sc-25778), *DNMT1* 1:100 (Santa Cruz, Cat# 10219), *H3K27me3* 1:200 (Millipore, Cat#17-622), *MBD4* 1:100 (Santa Cruz, Cat# sc-10754), *HDAC1* 1:100 (Santa Cruz, Cat# sc-6298), *p-Ac-H3 (Ser11/K15)* 1:200 (Santa Cruz, Cat#33361). After incubation, membranes were given three washes, each of 10 min with 1X TBST washing buffer (1X TBS containing 0.05 % tween 20) and then incubated with appropriate horseradish peroxidase conjugated secondary antibodies at 1:1,000 (anti-rabbit), 1:500 (anti-goat and anti-mouse) dilutions for an hour at room temperature. Membranes were washed three times, each for 10 min with 1X TBST buffer. Protein bands were visualized with chemiluminescence detection system (Amersham, NJ). Band intensity was quantified with ImageJ software. Intensity value for protein of interest was normalized to GAPDH and plotted as histogram.

Table 1 Sequences of forward and reverse primers used in analysis of gene expression by real-time PCR

Gene name	Forward primer sequence (5′→3′)	Reverse primer sequence (5′→3′)
GAPDH	GGTGGTCTCCTCTGAGTTCAACA	GTTGCTGTAGCCAAATTCGTTGT
Bax	TTTGCTTCAGGGTTTCATCCC	CAGTTGAAGTTGCCGTCAGA
Bcl2	GGATGCCTTTGTGGAACTGT	AGCCTGCAGCTTTGTTTCAT
Survivin	AGCCAGATGACGACCCCATT	GCAACCGGCCGAATGCTTTT
CCND1	AACTACCTGGACCGCTTCCT	CCACTTGAGCTTGTTCACCA
CCNA1	ACATGGATGAACTAGAGCAGGG	GAGTGTGCCGGTGTCTACTT
CCNG1	TCTGATATCGTGGGGTGCGGT	ACCACAGACGTTTGGCTGAC
CCNG2	GGTCCAACTTCTCGGGTTGT	CCGGGGTAGCCTCAATCAAA
CCNE2	TGTCAAGACGAAGTAGCCGTT	ACAGGTGGCCAACAATTCCTA
Wnt1	CCTCCACGAACCTGCTTACA	TCCCCGGATTTTGGCGTATC
RASSF1A	AAGTTCACCTGCCACTACCG	AAGGTCAGGTGTCTCCCACT
DNMT1	GTGGGGGACTGTGTCTCTGT	GAAAGCTGCATGTCCTCACA
DNMT3a	CCTGAAGCCTCAAGAGCAGT	AGCCAAGTCCCTGACTCTCA
DNMT3b	ACCAGTGGTTAATAAGTCGAAGG	CTCGGCTCTGATCTTCATCCC
HDAC1	TGGAAATCTATCGCCCTCAC	TCTCTGCATCTGCTTGCTGT
MBD4	CAGGCAAAATGGCAATACCT	GTTTTTGCCCGAAGCTCGTA

Statistical analysis

Student's t test (independent-two sample, unequal variance) was performed to determine the significant difference between treatments. Values with $p < 0.05$ were considered to be statistically significant.

Results

Effect of 5-aza 2′ dC, EGCG, and their combination on cell growth and cell viability

To determine the effect of 5-aza 2′ dC, EGCG, and their co-treatment on breast cancer cell growth in vitro, cell count analysis and MTT assay were performed after treating cells for 7 days. The results of cell count analysis and MTT assay are given in Fig. 1. Treatment with 5-aza 2′ dC alone resulted in statistically significant ($p < 0.05$) growth inhibition of both MCF-7 and MDA-MB231 cells by 22.7 and 23.4 percent, respectively, as compared to vehicle-treated control cells (Fig. 1a). Treatment with EGCG alone significantly inhibited the growth of MCF-7 cells by 23.5 percent, whereas growth inhibition of MDA-MB 231 cells by 18.5 % was statistically not significant. The combination of 5-aza 2′ dC and EGCG, however, significantly inhibited the growth of both MCF-7 and MDA-MB 231 cells by 46.5 and 39.5 percent, respectively, thereby suggesting a synergistic effect of co-treatment on growth inhibition of these breast cancer cells. There was no significant growth inhibition of MCF-10A cells after treatment with 5-aza 2′ dC and EGCG both alone and in combination.

To further validate the result of cell count, MTT assay for cell viability was also performed. The result of MTT assay revealed that treatment of MCF-7 cells with 5-aza 2′ dC and EGCG alone for 7 days caused significant decrease in their viability by 23.57 and 27.08 %, respectively, as compared to the vehicle-treated control cells (Fig. 1b). The combination of 5-aza 2′ dC and EGCG, however, resulted in greater decrease in MCF-7 cell viability by 45.12 % as compared to cells treated with either 5-aza 2′ dC or EGCG alone. Similarly, treatment with 5-aza 2′ dC and EGCG both alone resulted in 42.01 and 37.26 % decrease in viability of MDA-MB 231 cells, whereas their combination resulted in even greater decrease by 51.4 % in this cell line (Fig. 1b). Decreases in viability of MCF-7 and MDA-MB231 cells treated with 5-aza 2′ dC and EGCG combination were statistically significant as compared to both vehicle-treated control cells and cells treated individually with either of these two compounds. There was no significant change in the viability of MCF-10A cells treated with either 5-aza 2′ dC or EGCG alone as well as their combination. Therefore, the MTT data further confirmed the potentiation of growth inhibition in these two breast cancer cell lines by combination of 5-aza 2′ dC and EGCG as compared to cells treated alone with either of these two compounds.

Changes in cell cycle by treatment with 5-aza 2′ dC and EGCG

Cell cycle analysis was performed to determine whether EGCG and 5-aza 2′ dC decrease the growth of MCF-7 and MDA-MB 231 cells by induction of apoptosis and/or

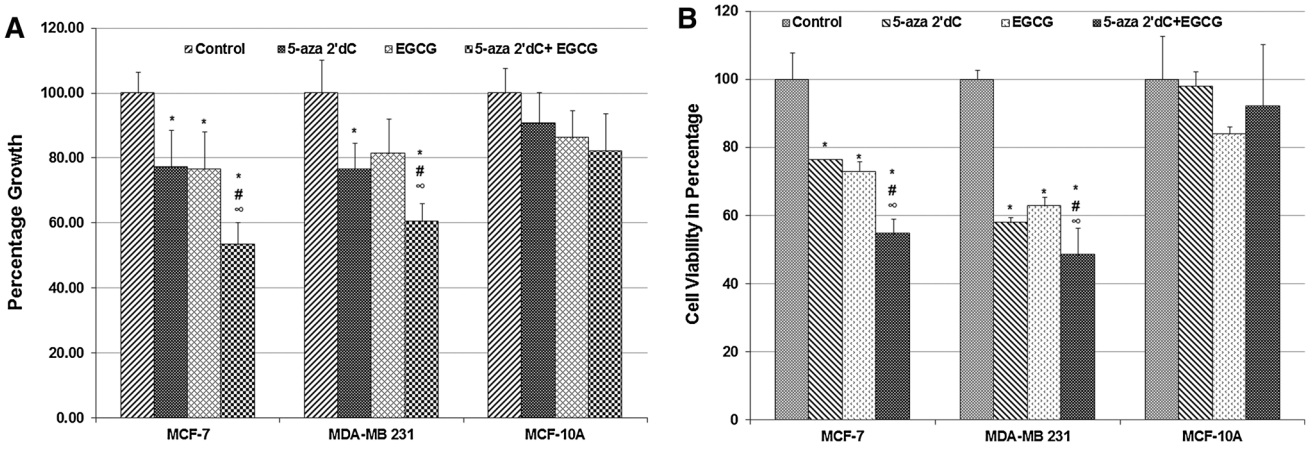


Fig. 1 *Bar graph* representation of the effect of 5-aza 2′ dC (5 μM), EGCG (50 μM), and their combination treatments on growth inhibition of MCF-10A, MCF-7, and MDA-MB 231 cells as measured by cell count analysis (Fig. 1a) and cell viability by MTT assay (Fig. 1b). Cells were treated with 5-aza 2′ dC (5 μM), EGCG (50 μM), 5-aza 2′ dC, and EGCG combination or vehicle (DMSO) control for 7 days, and cell count analysis and MTT assay were performed as described in Materials and Methods. Average value of cell counts and MTT assay from each treatment group was converted into percentage with vehicle control as 100 % and bar graph was plotted. An *asterisk* indicates statistically significant difference as compared to vehicle control (DMSO). Symbol *hash* indicates significant difference as compared to corresponding concentration of 5-aza 2′ dC. Symbol *infinity* indicates significant difference as compared to corresponding concentration of EGCG

delayed cell cycle progression. The flow cytometry histograms are given in Fig. 2a, and the percentage of cells in pre-G1 is given as bar graph in Fig. 2b. As compared to vehicle control, an increase in pre-G1 sub-population (apoptotic cells) was observed in all the treated groups of both MCF-7 and MDA-MB 231 cells (Fig. 2a, b). Interestingly, the percentage of pre-G1 cell population was significantly higher in cells treated with the combination of EGCG and 5-aza 2′ dC than cells treated individually with either EGCG or 5-aza 2′ dC. For example, treatment of MCF-7 cells with 5-aza 2′ dC and EGCG alone resulted in 15.8 and 21.2 percent cells, respectively, in pre-G1, whereas cells co-treated with 5-aza 2′ dC and EGCG had 30.5 percent cells in pre-G1. Similarly, MDA-MB 231 cells treated with 5-aza 2′ dC and EGCG alone had 7.3 and 16.9 percent cells, respectively, in pre-G1, whereas their combination resulted in 24.2 percent cells in pre-G1. As compared to MCF-7 and MDA-MB 231 cells, the percentage of pre-G1 cells in MCF-10A was extremely low in both 5-aza 2′ dC and EGCG alone and their combination treatment groups. Treatment of MCF-10A cells with 5-aza 2′ dC and EGCG alone resulted in 0.1 and 0.6 percent cells, respectively, in pre-G1, whereas cells co-treated with 5-aza 2′ dC and EGCG had 1.5 percent cells in pre-G1. Therefore, the cell cycle data suggest that apoptosis may be one of the mechanisms through which co-treatment of 5-aza 2′ dC and EGCG potentiates the growth inhibition of breast cancer cells but had no adverse effect on the growth of normal breast epithelial cells.

Co-treatment of 5-aza 2′ dC and EGCG significantly reduces tumorigenicity of MCF-7 cells in vitro

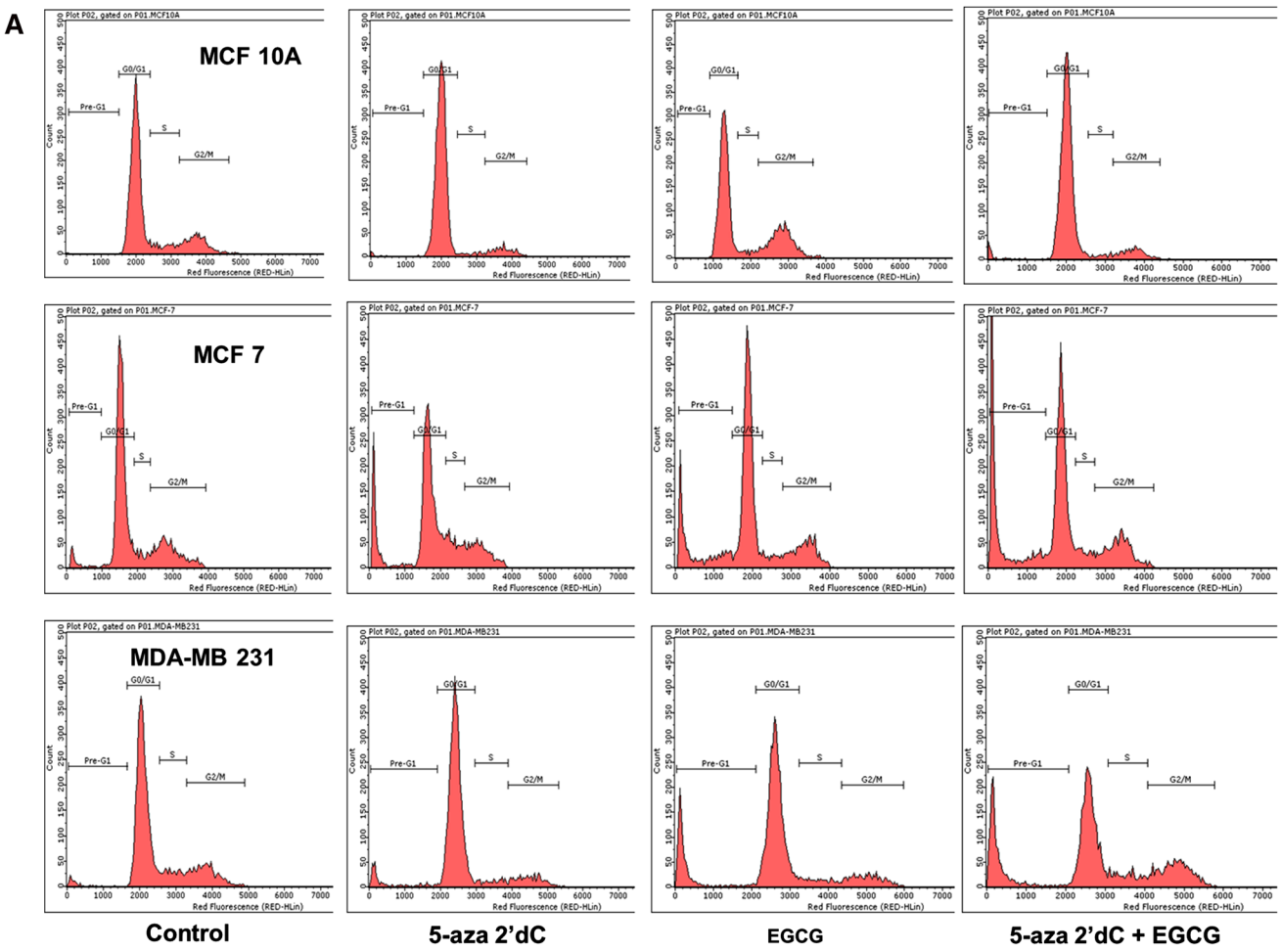
Soft agar colony formation assay was performed in order to determine the effect of 5-aza 2′ dC and EGCG on the colony formation of MCF-7 cells. The result of soft agar assay showed that both, 5-aza 2′ dC and EGCG, as well as their combination significantly reduced the anchorage-independent growth of MCF-7 cells (Fig. 3). As compared to control, there was a significant reduction in the number of soft agar grown colonies by 47.5, 58.4, and 90.5 % in 5-aza 2′ dC, EGCG, and combination treatment, respectively. In addition to the decreased number of colonies, decrease in the size of colonies was also observed in all the treatment groups as compared to vehicle control. The combination treatment resulted in the maximum reduction in number as well as in size of colonies. Thus, the data of soft agar assay suggest that co-treatment resulted not only in greater reduction of breast cancer cell growth but also anchorage-independent growth, an indicator of cell tumorigenic potential, of MCF-7 cells as compared to control and individual treatments.

Effect of 5-aza 2′ dC and EGCG treatment on gene expression

To further understand the growth inhibitory effect of 5-aza 2′ dC, EGCG, and their co-treatment at molecular level, the expression of genes involved in cell cycle, apoptosis, tumor suppression, and proliferation of breast cancer cells was analyzed at transcript level by quantitative real-time PCR. The changes in the expression of each category of genes are as follows:

Changes in expression of genes involved in cell cycle, survival, growth, and tumor suppression

The representative genes for cell cycle (*CCND1*, *CCNE2*, *CCNA1*, *CCNG1*, *CCNG2*), cell survival (*Bcl2*, *Bax* and *survivin*), and growth signaling (*Wnt1*, *RASSF1A*) were analyzed (Fig. 4). Among the cell cycle-related genes, the expression of *CCNE2* and *CCNA1* was significantly downregulated in all the treatment groups as compared to control. Although there was no significant change in the G1 cyclin *CCND1* in 5-aza 2′ dC-treated cells, its expression was significantly downregulated in EGCG-treated cells. Co-treatment of 5-aza 2′ dC and EGCG leads to significantly greater downregulation in *CCND1* expression (0.108-fold change) as compared to individual treatments. Similarly, co-treatment leads to significantly greater downregulation of another G1 cyclin, *CCNE2* (0.09-fold change) as compared to the treatment with 5-aza 2′ dC (0.279-fold change) and EGCG (0.52-fold change) alone. Decreased expression of *CCNA1* was also observed in all the treated groups, however, co-treatment did not lead to greater decrease as compared to individual treatments. The expression of *CCNG1* was significantly downregulated in 5-aza 2′ dC-treated cells (0.689-fold change) as well as cells co-treated with 5-aza 2′ dC and EGCG (0.71-fold change), but not in those treated with EGCG (1.27-fold change) alone. The expression of another negative cell cycle regulatory gene, cyclin *CCNG2* was significantly upregulated in EGCG-treated (2.99-fold change) and co-treated cells (1.65-fold change). However, no significant difference was observed in the expression of *CCNG2* in 5-aza 2′ dC-treated cells (1.19-fold change) as compared to control. The expression of pro-apoptotic gene, *Bax* was significantly increased in all the treated cells as compared to control, however, the expression of *Bax* in co-treated cells (2.69-fold change) was significantly increased as compared to 5-aza 2′ dC (2.12-fold change) but not EGCG (2.59-fold change). The expression of anti-apoptotic gene, *Bcl2* was significantly downregulated in 5-aza 2′ dC and EGCG-treated cells. Co-treated cells had significantly more downregulation in *Bcl2* (0.179-fold change) as compared to cells treated with either 5-aza 2′ dC (0.488-fold change) or



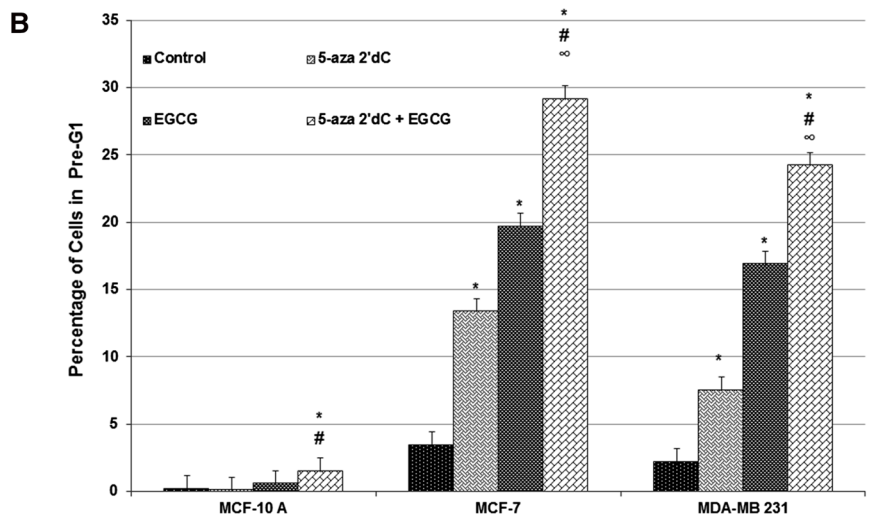


Fig. 2 **a** Representative flow cytometry histogram of percentage of cells in pre-G1 phase of cell cycle from MCF-10A, MCF-7, and MDA-MB 231 cells treated with 5-aza 2′ dC (5 μM), EGCG (50 μM), EGCG and 5-aza 2′ dC combination, or vehicle (DMSO) control for 7 days. Cells were treated, fixed, stained, and analyzed by flow cytometry as described in Materials and Methods. **b** *Bar graph* of percentage of cells in pre-G1 phase of cell cycle from MCF-10A, MCF-7, and MDA-MB 231 cells treated with 5-aza 2′ dC (5 μM), EGCG (50 μM), EGCG and 5-aza 2′ dC combination, or vehicle (DMSO) control for 7 days. An *asterisk* indicates statistically significant difference as compared to vehicle control (DMSO). Symbol *hash* indicates significant difference as compared to corresponding concentration of 5-aza 2′ dC. Symbol *infinity* indicates significant difference as compared to corresponding concentration of EGCG

EGCG (0.248-fold change). Maximum alteration of *Bax:Bcl2* ratio, in favor of apoptosis (*Bax:Bcl2* > 1) was observed in co-treated cells (*Bax:Bcl2* = 15.027) as compared to that in cells treated with either 5-aza 2′ dC(*Bax:Bcl2* = 4.34) and EGCG (*Bax:Bcl2* = 10.44). Similar trend of downregulation of pro-survival genes, *Survivin* and *Wnt1*, was also observed. Significantly greater downregulation of *Wnt1* was observed in co-treated cells (0.372-fold change) as compared to cells treated with either 5-aza 2′ dC (0.85-fold change) or EGCG (0.546-fold change). Expression of *RASSF1A* gene, known to have tumor suppressor properties was significantly upregulated in all treatments as compared to control. As compared to individually treated cells, significantly greater upregulation of *RASSF1A*, was observed in co-treated cells.

Expression of epigenetic regulatory genes at transcripts level

To evaluate the role of epigenetic mechanism on the growth inhibitory effect of 5-aza 2′ dC and EGCG, the expression of genes involved in DNA methylation (*DNMT1*, *DNMT3a*, and *DNMT3b*), histone deacetylation (*HDAC1*), and methylated DNA recognition (*MBD4*) was analyzed by real-time PCR (Fig. 5). The expression of the three DNMTs (*DNMT1*, *DNMT3a*, and *DNMT3b*) analyzed in this study was decreased in all treatments. Cells co-treated with EGCG and 5-aza 2′ dC had significantly greater downregulation in *DNMT1* and *DNMT3b* transcripts expression as compared to that in cells treated with either 5-aza 2′ dC or EGCG. A similar trend of maximum decrease in co-treated cells was not observed for *DNMT3a*. Expression of methyl-binding domain protein *MBD4* was significantly downregulated by both EGCG and co-treatment, though no significant difference was seen between the two. No significant change in *MBD4* was observed in 5-aza 2′ dC-treated cells. Although there was a decrease in expression of *HDAC1* in co-treated and EGCG-treated cells, it was statistically not significant.

Expression of epigenetic regulatory proteins and histone modifications

Western blot analysis was performed to further confirm the changes in the expression of some of the epigenetic regulatory genes (DNMT1, HDAC1, and MBD4) at protein level.

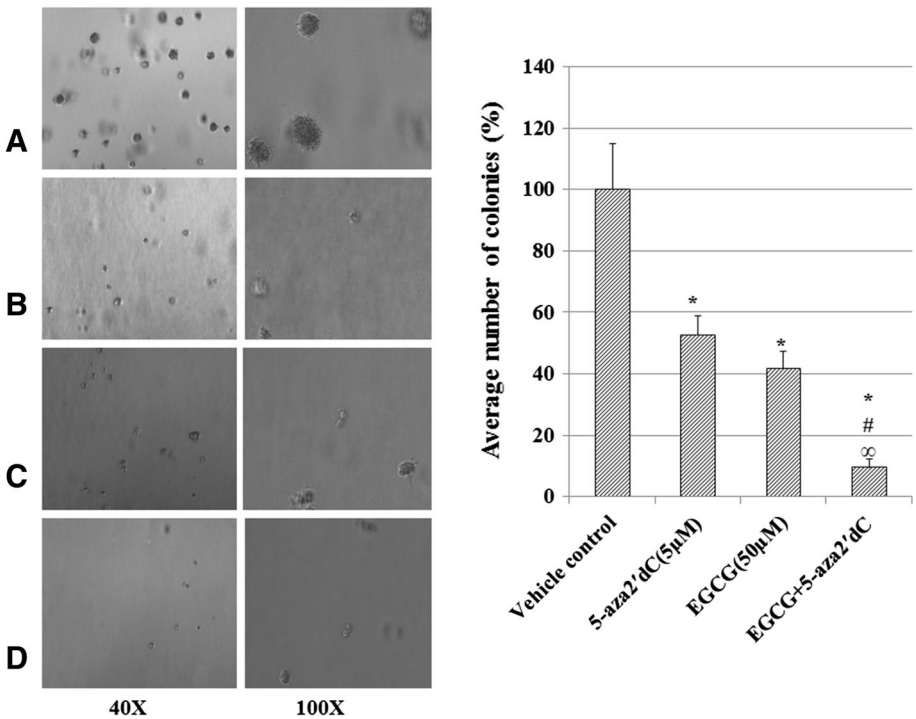


Fig. 3 Representative photographs of soft agar grown colonies of MCF-7 cells treated with DMSO as vehicle control (**a**), 5-aza 2′ dC (**b**), EGCG (**c**), and combination of EGCG and 5-aza 2′ dC (**d**). Cells were treated with these chemicals for 72 h and then grown in soft agar for 7 days. Details of cell treatments and seeding on soft agar are discussed in materials and methods section. The representative images for the number and size of cell colonies formed in each treatment as observed under ×40 and ×100 magnifications are given (*left panel*). The histogram represents the average number of colonies (%) in each treatment group (*right panel*). Data are represented as mean ± SEM. An *asterisk* indicates statistically significant difference as compared to vehicle control (DMSO). Symbol *hash* indicates significant difference as compared to corresponding concentration of 5-aza 2′ dC. Symbol *infinity* indicates significant difference as compared to corresponding concentration of EGCG

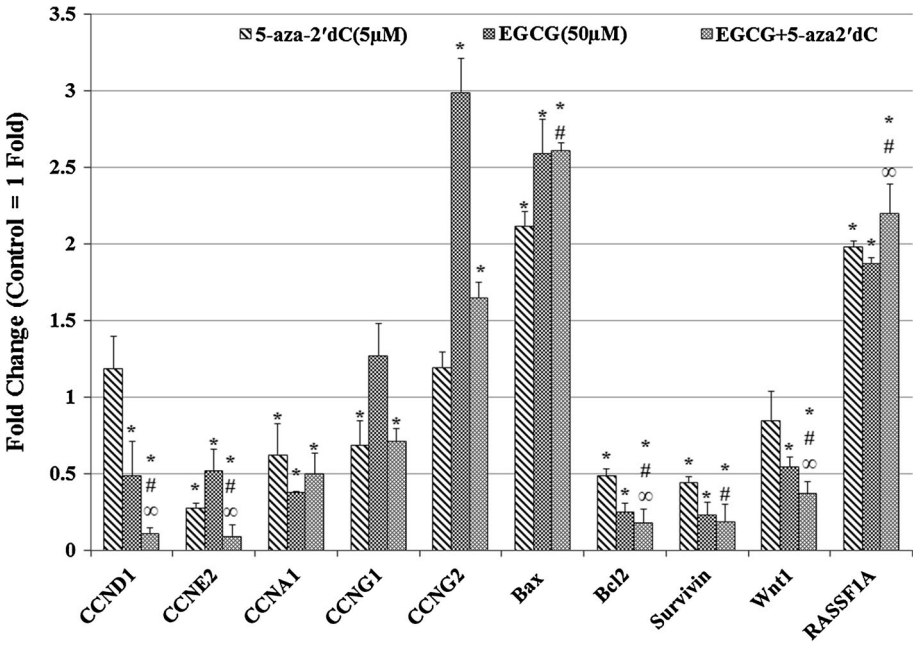


Fig. 4 Quantitative real-time PCR analysis of gene expression in MCF-7 cells treated with 5-aza 2′ dC (5 μM), EGCG (50 μM), EGCG and 5-aza 2′ dC combination and DMSO as vehicle control for 7 days. One-step RT-PCR with total cellular RNA was performed as described in Materials and Methods. Fold change in gene expression was determined by delta–delta Ct method and plotted as mean ± SEM. An *asterisk* indicates statistically significant difference as compared to vehicle control (DMSO). Symbol *hash* indicates significant difference as compared to corresponding concentration of 5-aza 2′ dC. Symbol *infinity* indicates significant difference as compared to corresponding concentration of EGCG

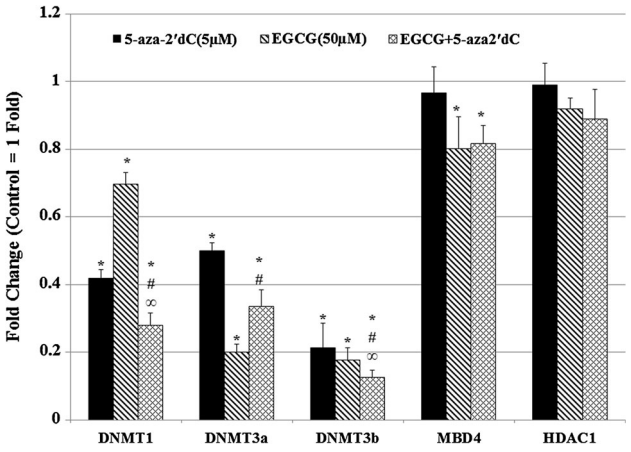


Fig. 5 Quantitative real-time PCR analysis of gene expression in MCF-7 cells treated with 5-aza 2′ dC (5 μM), EGCG (50 μM), EGCG, and 5-aza 2′ dC combination and DMSO for 7 days. One-step RT-PCR with total cellular RNA was performed as described in Materials and Methods. Fold change in gene expression was determined by Ct method (GAPDH-normalization gene) and plotted as mean ± SEM. An *asterisk* indicates statistically significant difference as compared to vehicle control (DMSO). Symbol *hash* indicates significant difference as compared to corresponding concentration of 5-aza 2′ dC. A symbol *infinity* indicates significant difference as compared to corresponding concentration of EGCG

The level of histone H3 modifications (H3K27me3 and p-Ac-H3 (S11/K15)) was also measured at protein level. The result (Fig. 6) revealed that the expression of HDAC1, DNMT1, and MBD4 was significantly downregulated in cells treated with either 5-aza 2′ dC, EGCG, or their co-treatment. As compared to cells treated with either 5-aza 2′ dC or EGCG, co-treated cells had significantly greater downregulation of HDAC1 and MBD4. Interestingly, the expression of DNMT1 was undetectable in 5-aza 2′ dC-treated cells, and co-treatment of 5-aza 2′ dC and EGCG leads to significantly greater downregulation of DNMT1 as compared to EGCG alone. Co-treated cells had significantly greater decrease in methylated H3 (H3K27me3) when compared to 5-aza 2′ dC and vehicle control-treated cells, but not in EGCG-treated cells. The level of histone H3 acetylation (p-Ac-H3 (S11/K15)) was significantly decreased in co-treated cells as compared to vehicle control and cells treated with either 5-aza 2′ dC or EGCG.

Detection of methylation changes by MS-RAPD-PCR

The effect of 5-aza 2′ dC and EGCG treatments on genome-wide methylation pattern was evaluated by methylation-sensitive RAPD-PCR. As compared to control, there was no change in the RAPD fingerprint generated from undigested DNA (Fig. 7). This indicates the absence of mutational changes due to 5-aza 2′ dC and EGCG treatment. As compared to undigested DNA, the methylation-sensitive (MS) RAPD-PCR from restriction-digested DNA revealed several

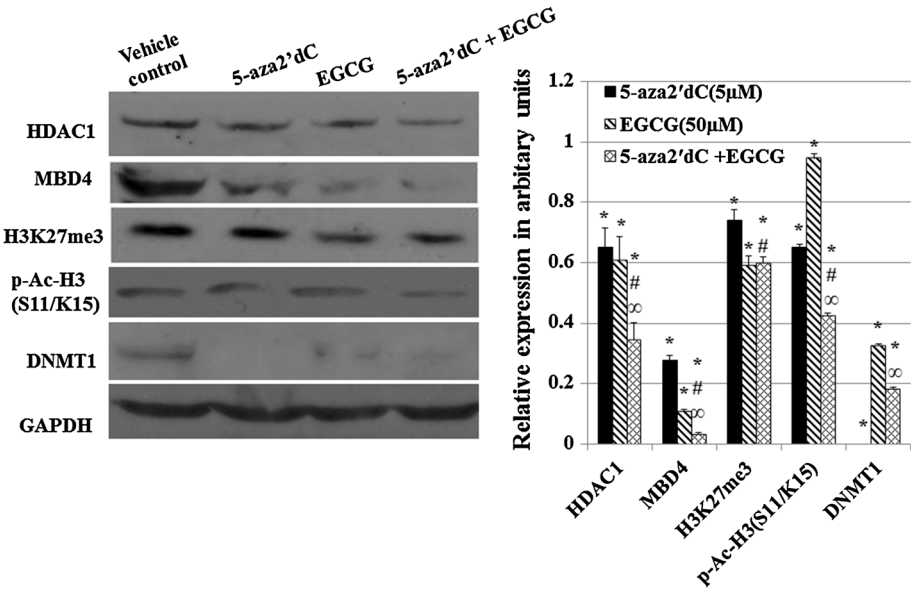


Fig. 6 Representative Western blot images (*left panel*) and *histograms* representing their relative band intensity (*right panel*). Western blot analysis with total cellular protein lysates was performed as described in Materials and Methods. Relative amount of respective epigenetic regulatory proteins or histone modifications was determined by densitometry analysis. Band intensity of each protein was normalized by GAPDH intensity from the same sample and then plotted as mean ± SEM. An *asterisk* indicates statistically significant difference as compared to vehicle control (DMSO). Symbol *hash* indicates significant difference as compared to corresponding concentration of 5-aza 2′ dC. A symbol *infinity* indicates significant difference as compared to corresponding concentration of EGCG

changes in RAPD fingerprint. Comparison of DNA fingerprint generated from MspI-digested DNA revealed the presence of 500 and 300 bp bands in treated cells, whereas the intensity of these two bands was faint/hardly detectable in vehicle control, thereby suggesting that hypomethylation was induced at these loci by 5-aza 2′ dC and EGCG. This was further confirmed by the presence of these bands in control and their loss in intensity in the treated group of cells from Hpa II-digested RAPD fingerprint. Similar pattern of 5-aza 2′ dC- and EGCG-induced hypomethylation was observed in RAPD fingerprint generated from additional set of primers (data not shown).

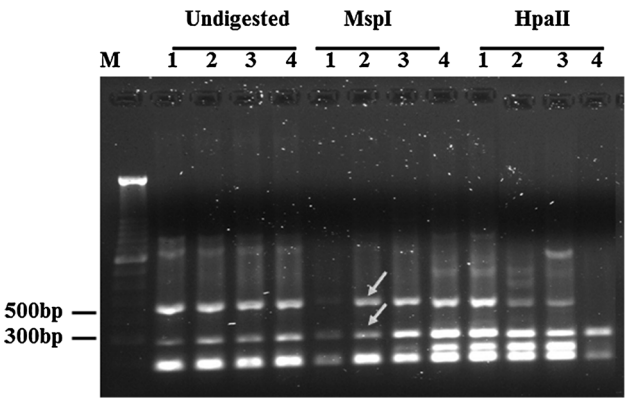


Fig. 7 Representative MS-RAPD fingerprint generated using the total DNA from cells treated with 5-aza 2′ dC (5 μM), EGCG (50 μM), EGCG, and 5-aza 2′ dC combination and DMSO as vehicle control for 7 days. Total DNA was digested with MspI and Hpa II restriction enzymes, and MS-RAPD-PCR was performed as described in Materials and Methods. Random primer sequence used to generate the RAPD fingerprint is 5′-AAAGCTGCGG- 3′. Treatments are represented as follows: *M* Marker; *1* DMSO; *2* 5-aza 2′ dC (5 μM); *3* EGCG (50 μM); *4* EGCG (50 μM) and 5-aza 2′ dC (5 μM). Changes in methylation pattern are indicated by *arrows*

Discussion

The major finding of this study is that co-treatment with non-toxic, low concentrations of 5-aza 2′ dC and EGCG is more efficient in inhibiting growth of breast cancer cells as compared to individual treatments. Additionally, for the first time our data revealed that this potentiation of growth inhibition by co-treatment is, at least in part, mediated by their synergistic action on epigenetic modulators in breast cancer cells.

Both 5-aza 2′ dC and EGCG inhibit growth of cancer cells by several mechanisms including apoptosis and cell cycle arrest. 5-aza 2′ dC induces G2/M cell cycle arrest and apoptosis by altering expression of cyclins and aberrantly methylated apoptotic genes [32–37]. Similarly, EGCG can regulate the expression of cell cycle regulatory and apoptotic genes to decrease cancer cell growth and proliferation by inducing cell cycle arrest and apoptosis [38–41]. EGCG has been shown to inhibit the growth of cancer cells by

regulating the cell surface growth factor receptors, such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), insulin-like growth factor receptor (IGFR) involved in receptor tyrosine kinase (RTK)-dependent signaling pathways for cell proliferation, survival, and angiogenesis [42–44]. Another mechanism for EGCG—mediated growth inhibition of cancer cells involve inhibition of various transcription factors that are frequently upregulated in cancer cells, such as STAT1, STAT3, AP-1, NF-kB, Sp1, and FOXO1 [45–48].

These reports suggest that both EGCG and 5-aza′dC have potential to inhibit growth of cancer cells by these two agent-specific mechanisms as described above. However, the efficacy of 5-aza′dC and EGCG is limited in solid tumors when given as single agent [26, 49]. It has been suggested that these compounds can be combined with other chemotherapeutic drugs or HDAC inhibitors to augment their effects [15, 50]. Therefore, in this study, we further investigated the mechanisms through which co-treatment of 5-aza 2′ dC and EGCG resulted in greater growth inhibition of breast cancer cells. Significantly, greater downregulation of *cyclin D1*, *cyclin E2*, and *Bcl2* mRNA in MCF-7 cells co-treated with EGCG and 5-aza 2′ dC as compared to those treated with either compound alone suggests that these genes are the common target for these two compounds. Additionally, we also observed drug-specific changes in the expression of certain genes, such as *cyclin G1*, *cyclin G2*, and *cyclin A1*, thereby suggesting that these genes are uniquely targeted by either 5-aza′dC or EGCG. Therefore, the observed potentiation in growth inhibition by co-treatment may be due to alteration of common as well as drug-specific targets, which otherwise may not be possible to achieve by single-agent treatment.

In addition to these above-mentioned genetic targets, studies have shown that both 5-aza 2′ dC and EGCG are epigenetic modifiers and inhibit DNMTs [13, 22]. Additionally, EGCG has HDAC inhibitory activity [24]. Elevated levels of class 1 HDAC enzymes in breast cancer tissue suggest their role in breast cancer development [51]. EGCG reduces the expression of HDAC 3 not only at transcript level but also by targeting this protein for degradation in HCT 116 cancer cells [52]. These reports suggest that both EGCG and 5-aza 2′ dC can synergistically act through epigenetic mechanism. Consistent with this theory, many genes including *RASSF1A*, *Wnt1, CCND1, CCNA1, Bax*, and *survivin*, that were synergistically either up or downregulated by co-treatment in our study, are known to be regulated by epigenetic mechanisms [34, 53, 54]. Therefore, the epigenetic effect of EGCG and 5-aza 2′ dC was also examined in this study. Our data indicate that co-treatment of EGCG and 5-aza 2′ dC can synergistically target several epigenetic regulatory genes. For example, significantly greater downregulation of *DNMT1*, *DNMT 3b* transcripts, and HDAC1 and MBD4 proteins was observed in co-treated cells as compared to individually treated cells. Besides the change in expression of epigenetic regulatory genes, co-treatment also resulted in greater effects on DNA methylation and histone modifications as compared to the individual treatments. For example, methylation-sensitive RAPD results revealed increase in DNA hypomethylation in co-treated MCF-7 cells. Similarly, co-treatment resulted in decrease in histone H3K27 trimethylation and histone H3 acetylation (p-Ac-H3 (S11/K15)). The antibody used in this study for histone acetylation detects both phosphorylation of serine 11 and acetylation at lysine 15. Phosphorylation of serine 11 facilitates acetylation of H3 and is associated with mitogen-activated c-fos oncogene expression in a MAP kinase-dependent manner [55, 56]. The observed decrease in p-Ac-H3 (S11/K15) by co-treatment of EGCG and 5-aza 2′ dC suggests that these two compounds mediate growth inhibition by p-Ac-H3 (S11/K15)-dependent inactivation of oncogenes in MCF-7 breast cancer cells. Given the role of these epigenetic regulatory proteins and histone modifications in repression of critical genes such as tumor suppressors and DNA repair genes in cancer [57–61], the data of this study suggest that the observed potentiation of growth inhibition by co-treatment is, at least in part, mediated by epigenetic mechanisms, such as epigenetic reactivation of tumor suppressors and apoptotic pathways in breast cancer cells.

An important aspect of this study was the dose of 5-aza 2′ dC and EGCG selected for treatment. 5-aza 2′ dC has a dual dose-dependent mechanism of action. High dose of 5-aza 2′ dC leads to formation of cytotoxic DNA adducts, whereas low dose induces gene expression changes that favor reduced proliferation and apoptosis [15]. EGCG has been reported to induce apoptosis in several cancer cell lines without affecting the normal cells [62–64]. The result of this study from cell count, MTT assay for cell viability, and cell cycle analysis further confirmed that low dose 5-aza 2′ dC and EGCG alone or their combination has no cytotoxic effect on MCF-10A normal breast epithelial cells, whereas these two compounds induce significant cytotoxicity to MCF-7 and MDA-MB 231 breast cancer cells. While the exact mechanism for this differential action is not known, pro-oxidative effect of EGCG has been attributed for its cancer cell-specific apoptosis. Studies have shown that, in general, normal cells have a better antioxidant system as compared to cancer cells, where the antioxidant system integrity is compromised [65, 66], therefore normal cells are better suited to scavenge H_2O_2 produced by EGCG than cancer cells [19, 67–69]. This may be a potential reason for the cancer cell specificity of EGCG for inducing apoptosis. It is interesting to note that EGCG can induce apoptosis or necrosis based on its dose. Lower concentrations (10–50) μM of EGCG have been reported

to favor apoptosis, whereas higher concentrations (100–400 μM) are associated with necrosis in MCF-7 cells. This dose-dependent effect has been attributed to apoptosis favoring changes in intracellular ROS, mitochondrial membrane potential, and ATP levels, which are induced by lower dose of EGCG [70]. These previous reports and the result of this study suggests that cancer cell-specific cytotoxicity can be achieved by a combination of these two compounds at concentrations low enough to be safe for normal cells.

Recent evidence suggests that combination of chemotherapeutic or naturally occurring compounds has advantages over single-agent treatments. For example, combination of EGCG with chemotherapeutic drugs such as tamoxifen or dietary phytochemicals like resveratrol can synergistically inhibit proliferation of breast cancer cells [71, 72]. Similarly, 5-aza 2′ dC can be combined with HDAC inhibitors to synergistically decrease cancer cell proliferation and growth [73, 74]. Due to the reversible nature of epigenetic targets and the minimal toxicity of bioactive dietary compounds toward normal cells unlike conventional chemotherapeutic drugs, combination of EGCG and 5-aza 2′ dC has great promise for clinical applications in breast cancer treatment. Therefore, these previous reports and the findings of this study further highlight the importance of combination treatment for efficient inhibition of cancer growth.

In summary, our data suggest that combination of 5-aza′dC and EGCG is more effective in terms of breast cancer cell growth inhibition as compared to individual treatments. Our study indicates that the combination of 5-aza 2′ dC with green tea polyphenol EGCG can target cancer growth and proliferation by multiple mechanisms including modification of epigenetic machinery.

Conflict of interest The authors hereby disclose that there are no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work, and therefore there is no conflict of interest.

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