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# Epigallocatechin 3-gallate applications on HT-29 and MCF-7 cell lines and evaluation of tumor suppressor gene methylation

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**Abstract:** Epigallocatechin 3-gallate (EGCG) is an antitumor molecule and shows this activity by binding to the active center of a methyltransferase enzyme (DNMT1). The methylation of DNA sequences of tumor suppressor and DNA repair genes is observed in different stages of carcinogenesis. In this study, we analyzed the effect of EGCG on the methylation status of 25 tumor suppressor genes in cancer cell lines HT-29 and MCF-7. HT-29 and MCF-7 cells were incubated with 10 μM, 20 μM, and 50 μM and 1 μM, 5 μM, and 10 μM EGCG for 48 h, respectively. We found promoter hypermethylation of (1) *CDH13, GATA5*, and *RAR*β genes in MCF-7 cell line and (2) *RARβ*, *ESR1*, *PAX6*, *WT1*, *CADM1*, *CHFR, CDH13*, and *GATA5* genes in HT-29 cell line. However, (3) after EGCG application, no changes in methylation status were detected in our samples. Our results suggest that methylation status of tumor suppressor genes did not change with different EGCG doses.

**Key words:** Epigallocatechin 3-gallate, epigenetics, methylation, breast cancer, colorectal cancer

## 1. Introduction

EGCG is the major component of catechins found in green tea and exists in four active forms: (1)-epigallocatechin-3-gallate (EGCG), (2)-epigallocatechin (EGC), (3)-epicatechin-3-gallate (ECG), and (4)-epicatechin (EC) (Zeng et al., 2014). Catechins, which are polyphenols, constitute nearly 30%–40% of the dry weight of green tea (Lecumberri et al., 2013; Zeng et al., 2014), which is one of the most popular beverages in the world, after water. EGCG is an important compound, as it has antitumor activity. It exerts this activity by binding to the active center of DNA methyltransferase-1 (DNMT1) enzyme, as previously demonstrated by in vitro activity assays (Lee et al., 2005; Pandey et al., 2010). Thus, it is involved in the changes in gene expression by an epigenetic mechanism decreasing DNA hypermethylation and is a potential anticancer drug for cancer prevention or treatment (Fang et al., 2003; Chen et al., 2011).

Cancer development and progression is a multistep process evolving through both genetic and epigenetic mechanisms. The loss of function of tumor suppressor genes is a fundamental step that is quite well known in cancer development. Aberrant promoter methylation, especially hypermethylation of tumor suppressor genes, is an important mechanism of epigenetic silencing (Baylin and Herman, 2000; Brooks, 2009; Nystrom and Mutanen, 2009; Arends, 2013). Nearly half of all known tumor suppressor genes might be inactivated by hypermethylation (Arai et al., 2006).

Breast and colorectal cancers are common cancers and constitute a major public health problem; thus, they are important targets for research and the implementation of treatment (Cho et al., 2010; Colussi et al., 2013). It has been reported that CpG island methylation of tumor-related genes is an important event in both the pre-invasive and progression stages of breast cancer (Park et al., 2011). Colorectal cancer is another common cause of morbidity and mortality, and dietary components play an important role in the pathogenesis and, therefore, prevention of this cancer (Singh and Fraser, 1998). In recent years, specific nutritional components, such as catechins, have been reported to be bioactive molecules acting on cancer cells (Fang et al., 2003; Li and Tollefsbol, 2010; Nandakumar et al., 2011).

In this study, considering (1) EGCG is a potential anticancer drug, (2) it binds to DNA methyltransferase enzyme, (3) promoter methylation of tumor suppressor genes is an important mechanism of epigenetic silencing, and (4) epigenetic silencing is important to the carcinogenesis of adenocarcinomas, we aimed to evaluate

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the effects of EGCG on the methylation status of 25 tumor suppressor gene promoters in colorectal (HT-29) and breast cancer (MCF-7) cell lines. Methylation-specific multiplex ligation probe amplification (MS-MLPA) was used for the analysis that determines both the methylation status of genes of interest and the copy number changes. We tested the effects of different EGCG concentrations on the methylation status of tumor suppressor gene promoters in HT-29 and MCF-7 cell lines and also assayed effects of these application concentrations on cell proliferation.

## 2. Materials and methods

### 2.1. Cell lines

Human colon adenocarcinoma (designation: HT-29, ATCC number: HTB-38) and human breast adenocarcinoma (designation: MCF-7, ATCC number: HTB-22) cell lines were obtained from the American Type Tissue Culture Collection (ATCC). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 IU/mL), and streptomycin (100 µg/mL) (media and serums from Biochrom AG, Berlin, Germany). All cells were cultured at 37 °C in the humidified atmosphere of a 5% $CO_2$ incubator (Heraeus, Hanau, Germany).

### 2.2. EGCG treatment

Commercially available (−)-epigallocatechin gallate was used in the experiments (Sigma-Aldrich, St Louis, MO, USA). Powdered EGCG was dissolved in water to prepare 5 mg/mL stock solution and stored at –20 °C. For each cell line (MCF-7 and HT-29), 2 million cells were transferred to 9 different 25 $cm^2$ tissue culture flasks. Three of these flasks were assigned as the control group, and EGCG was not applied to these flasks. The other 6 flasks were used in duplicate for EGCG application. HT-29 and MCF-7 cells were incubated with 10 µM, 20 µM, and 50 µM (Zhang, 2012) and 1 µM, 5 µM, and 10 µM (Lee et al., 2005; Hsu et al., 2011) EGCG, respectively for 48 h, as defined by previous studies. At the end of 48 h for all groups, DNA samples were extracted from both untreated control cells and EGCG-treated cells by using QIAamp DNA mini kit (Qiagen, Carlsbad, Germany), according to the manufacturer's instructions.

### 2.3. MS-MLPA analysis

SALSA MLPA kit ME002-B1 Tumor Suppressor 2 kit (MRC Holland, Amsterdam, the Netherlands) was utilized to analyze the methylation status of promoter regions of 25 different tumor suppressor genes (Table). MLPA reactions were performed according to the manufacturer's instructions. Briefly, 250 ng of genomic DNA extracted from control and EGCG applied cell lines in 5 µL of TE buffer (10 mM Tris-HCl, pH 8.2; 0.1 mM EDTA) was denatured. Oligonucleotide probes were hybridized to the genomic DNA for 16 h. Following the hybridization reaction, hybridized probes were ligated via ligation reaction. All reaction tubes were divided into two tubes, one for copy number analyses and the second for methylation analyses. To determine the methylation status of the promoter regions, a restriction endonuclease reaction was performed by using *HhaI* enzyme. As a methylation-sensitive restriction endonuclease, *HhaI* can only digest an unmethylated DNA target. Following *HhaI* digestion, PCR reaction was performed in all tubes. PCR products were denatured and loaded onto an ABI 3500 capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA). Band analysis was performed by GeneMapper software (Applied Biosystems), and Coffalyser Net program (MRC Holland) was used to determine copy number analysis and methylation status of the EGCG-treated and untreated samples. The assay uses 14 reference probes (Figures 1 and 2; last 14 data points). The undigested PCR runs were analyzed to obtain the copy number ratio in samples. The ratios were calculated by dividing the peak area obtained from each PCR reaction by the sum of the area of reference probes (Figures 1A and 2A). Fragment analysis of digested samples was performed to determine unmethylated regions. Since reference probes do not have *HhaI* restriction sites, the total of peak area values obtained from reference probes served as controls to methylated regions that were not digested by the enzyme. The methylation ratio was obtained by dividing each digested probe's peak area by the sum of the area of reference probe peaks (Figures 1B and 2B). Differences in methylation status between EGCG-treated and untreated cells were compared using independent samples t-test. SPSS version 20.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis, and $P < 0.05$ was considered significant.

### 2.4. Assay for cell proliferation

The effects of EGCG application on the proliferation of MCF-7 and HT-29 cells were colorimetrically tested by biochemical reduction of MTT (3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma-Aldrich). Briefly, EGCG was serially diluted in half from 400 to 1 µM in 96-well microtiter plate rows, except for the untreated cells and medium control wells. Plates were incubated for 48 h. The inhibition of cell proliferation was determined after 4 h of incubation with 20 µL of MTT (5 mg/mL). The optical density of the sodium dodecyl sulfate (10%) (Sigma-Aldrich) solubilized tetrazolium salt was measured at 540 nm with an ELISA reader (Biotek Instrument ELx800, Winooski, VT, USA). Inhibition of cell proliferation and inhibitory concentration 50 ($IC_{50}$) values (EGCG concentrations at which 50% of cells are viable) were calculated from the logarithmic trend lines of the proliferation versus EGCG concentration graphs. Data of the triplicate experiments are expressed as mean ±

**Table.** List of the tumor suppressor genes in which the methylation status of promoter regions was analyzed. Genes are listed according to their chromosomal locations and nominated using the Universal Protein Resource (UniProt) catalog of information on proteins (http://www.uniprot.org).

| UniProt number | Gene alias | Gene name | Chromosomal location |
|---|---|---|---|
| Q15350 | *TP73* | Tumor protein p73 | 1p36.32 |
| P52701 | *MSH6* | DNA mismatch repair protein | 2p16.3 |
| P10826 | *RARB* | Retinoic acid receptor beta | 3p24.2 |
| P40337 | *VHL* | Von Hippel–Lindau disease tumor suppressor | 3p25.3 |
| P03372 | *ESR1* | Estrogen receptor | 6q25.1 |
| Q02548 | *PAX5* | Paired box protein | 9p13.2 |
| P42771 | *CDKN2A* | Cyclin-dependent kinase inhibitor 2A | 9p21.3 |
| B2CW77 | *KLLN* | Killin | 10q23.31 |
| P16455 | *MGMT* | Methylated-DNA--protein-cysteine methyltransferase | 10q26.3 |
| P16070 | *CD44* | CD44 antigen | 11p13 |
| P26367 | *PAX6* | Paired box protein | 11p13 |
| P19544 | *WT1* | Wilms tumor protein | 11p13 |
| P09211 | *GSTP1* | Glutathione S-transferase P | 11q13.2 |
| Q13315 | *ATM* | Serine-protein kinase | 11q22.3 |
| Q9BY67 | *CADM1* | Cell adhesion molecule 1 | 11q23.3 |
| Q96EP1 | *CHFR* | E3 ubiquitin-protein ligase | 12q24.33 |
| P51587 | *BRCA2* | Breast cancer type 2 susceptibility protein | 13q13.1 |
| P06400 | *RB1* | Retinoblastoma-associated protein | 13q14.2 |
| P07996 | *THBS1* | Thrombospondin-1 | 15q14 |
| Q9ULZ3 | *PYCARD* | Apoptosis-associated speck-like protein containing a CARD | 16p11.2 |
| R55290 | *CDH13* | Cadherin-13 | 16q23.3 |
| P38398 | *BRCA1* | Breast cancer type 1 susceptibility protein | 17q21.31 |
| P04637 | *TP53* | Cellular tumor antigen p53 | 17p13.1 |
| Q15831 | *STK11* | Serine/threonine-protein kinase | 19p13.3 |
| Q9BWX5 | *GATA5* | Transcription factor | 20q13.33 |

standard error of means (SE). Differences in $IC_{50}$ between cell lines were statistically evaluated by independent samples t-test at the 0.05 level using SPSS version 20.0 (IBM Corp., USA).

## 3. Results

### 3.1. Effects of EGCG on the methylation status of tumor suppressor genes in MCF-7 and HT-29

Copy number analysis of the undigested (Figures 1A and 1B) MCF-7 and HT-29 samples demonstrated that 25 tumor suppressor genes (Table) had neither duplications nor deletions in their promoter regions. Similarly, there were no changes in the copy numbers of the EGCG-treated samples. Methylation status of the promoter regions of 25 tumor suppressor genes was detected by fragment analysis of *HhaI*-digested samples. Each digested probe's peak area was divided by the combined peak areas of the control probes lacking a *HhaI* restriction site, and methylation ratios were obtained (Figures 1B and 2B). Accordingly, promoter regions of *CDH13, GATA5,* and *RAR*β genes were found to be hypermethylated in all samples from the MCF-7 cell line, either EGCG-treated or untreated (Figure

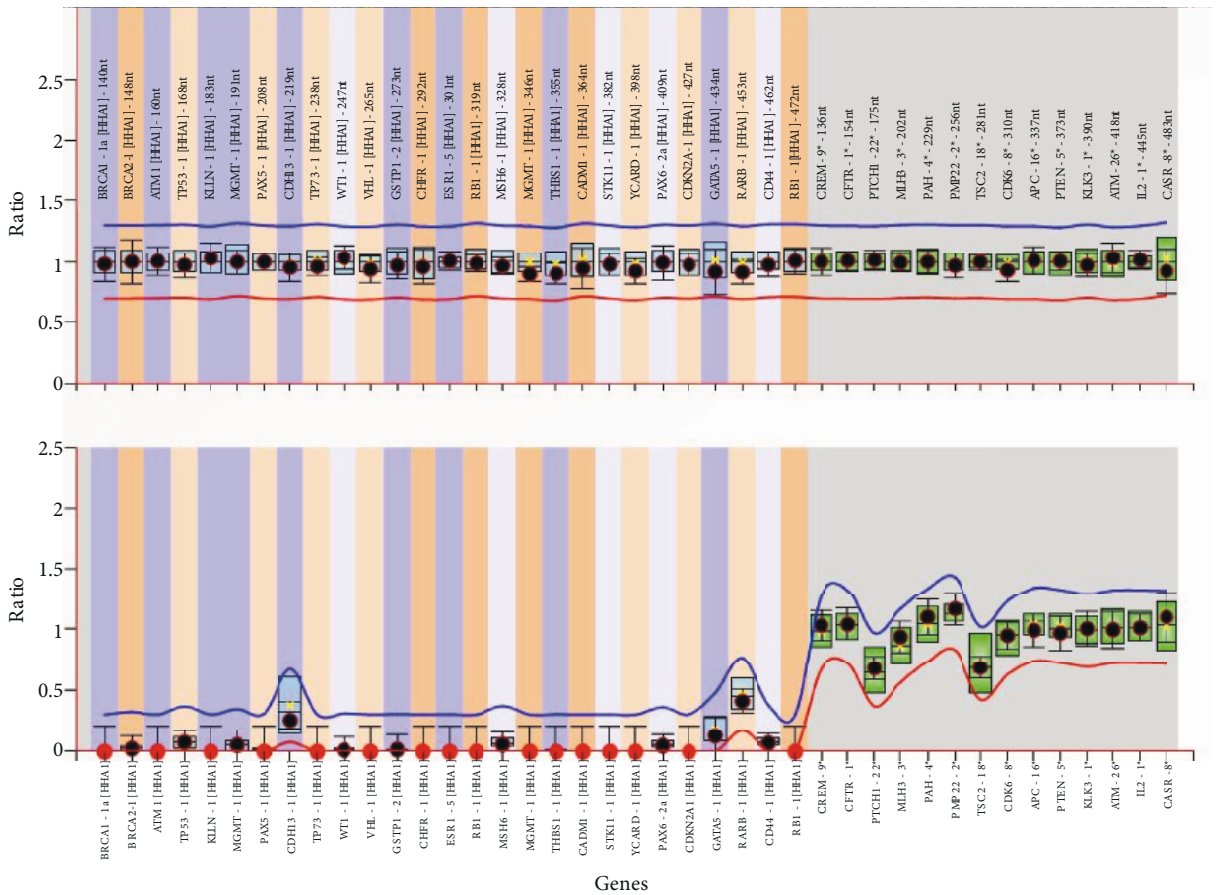


**Figure 1**. Methylation-specific MLPA analysis of MCF-7 cells. X-axis shows tumor suppressor genes (1–25) and reference probes (26–40). Y-axis represents (A) copy number analysis of the tumor suppressor genes and (B) methylation profile of the tumor suppressor genes with respect to reference probes. The red line and blue line in panel A represent minimum normalized ratio with deletions and maximum normalized ratio with duplications in the regions, respectively. The red line and blue line in panel B represent minimum normalized ratio of unmethylated promoter regions and maximum normalized ratio of methylated regions, respectively. Promoter regions of *CDH13*, *GATA5*, and *RARβ* genes were hypermethylated in MCF-7 cell line.

1B). However, we did not detect a statistically significant difference between treated and untreated groups. *RAR*β, *ESR1*, *PAX6*, *WT1*, *CADM1*, *CHFR*, *CDH13*, and *GATA5* genes were hypermethylated in the HT-29 cell line in addition to these three genes (Figure 2B). However, we did not detect changes in methylation status in our samples in HT-29 after EGCG application.

### 3.2. Antiproliferative effects of EGCG on MCF-7 and HT-29 cells

MTT analysis revealed a concentration-dependent growth inhibition by EGCG in both cell lines (Figure 3). $IC_{50}$ was 11.2 ± 1.4 µM for MCF-7 cells, whereas it was 136.3 ± 2.1 µM for HT-29 cells. MTT results demonstrated that EGCG caused significantly ($P < 0.05$) higher cytotoxicity in MCF-7 cells compared to HT-29 cells. Next, we calculated the effect of application concentrations on cell viability. Viability of MCF-7 cells was 91.5 ± 1%, 65.3 ± 3.4%, and 52.0 ± 3.0% at 1, 5, and 10 µM EGCG concentrations, respectively. Similarly, viability of HT-29 cells was 93.2 ± 1%, 88.7 ± 0.9%, and 77.7 ± 1.9% at 10, 20, and 50 µM EGCG concentrations, respectively.

## 4. Discussion

Dietary components may play key roles in influencing DNA methylation status, resulting in regulation of gene expression. As cancer is a multistep process, gene activation and silencing throughout epigenetic mechanisms, as well as gene mutations, play important roles in carcinogenesis. In recent years, dietary components have been widely studied, and their potential use in cancer treatment and prevention strategies has been discussed (Li and Tollefsboy, 2010; Hardy and Tollefsbol, 2011).

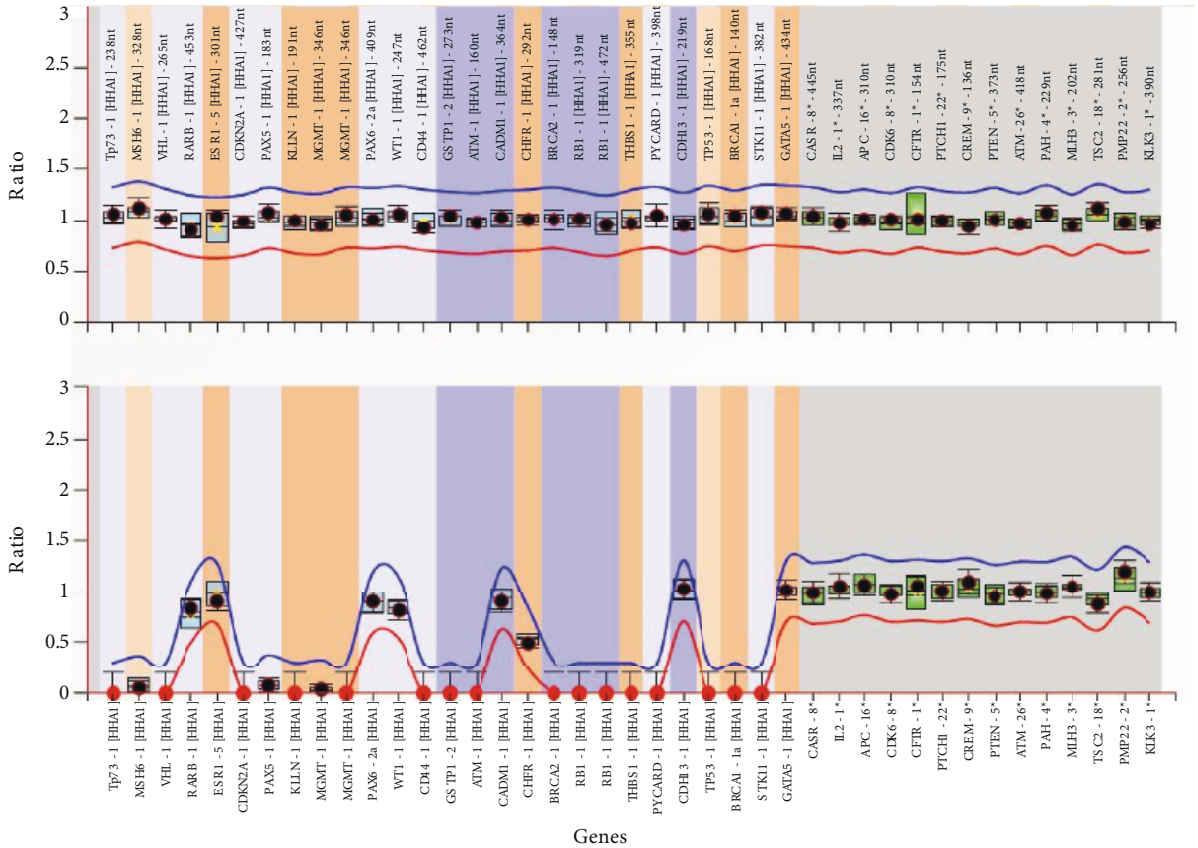


**Figure 2**. Methylation-specific MLPA analysis of HT-29 cells. X-axis shows tumor suppressor genes (1–25) and reference probes (26–40). Y-axis shows (A) copy number analysis of the tumor suppressor genes and (B) methylation profile of the tumor suppressor genes with respect to reference probes. The red line and blue line in panel A represent minimum normalized ratio with deletions and maximum normalized ratio with duplications in the regions, respectively. The red line and blue line in panel B represent minimum normalized ratio of unmethylated promoter regions and maximum normalized ratio of methylated regions, respectively. Promoter regions of *RARβ*, *ESR1*, *PAX6*, *WT1*, *CADM1*, *CHFR*, *CDH13*, and *GATA5* genes were hypermethylated in HT-29 cell line.

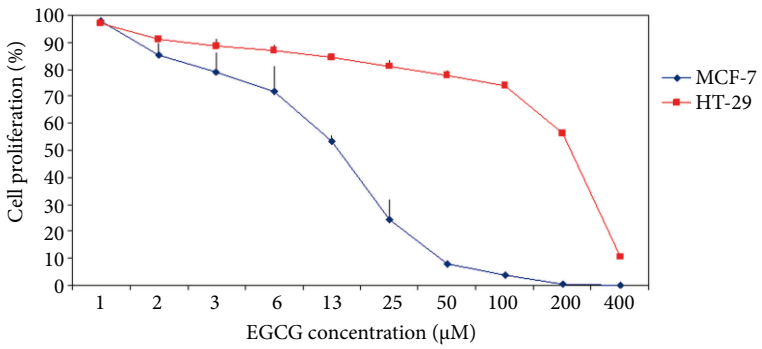


**Figure 3**. Effect of EGCG on the proliferation of MCF-7 and HT-29 cells after 48 h. Cell proliferation data are expressed as the percentage of untreated cell controls. EGCG was more cytotoxic to MCF-7 cells (blue line) than HT-29 cells (red line). Error bars represent standard error of means obtained from independent triplicates.

Promoter methylation is an important epigenetic change in cancer progression (Nystrom and Mutanen, 2009). We found that *CDH13, GATA5*, and *RARβ* genes are methylated in the MCF-7 cell line: in addition to these genes, *ESR1*, *PAX6*, *WT1*, *CADM1*, and *CHFR* were also hypermethylated in HT-29 cell line. EGCG is known to be an inhibitor of DNMT1 enzyme activity. It has also been reported to be a chromatin remodeling agent (Li and Tollefsboy, 2010). Whenever EGCG inhibits DNMT1 enzyme activity or affects chromatin remodeling in the promoter region of a gene, changes in gene expression occur, resulting in reactivation, as in the case of tumor suppressor genes. In this study, we investigated the demethylation effect of EGCG on the promoters of tumor suppressor genes in MCF-7 and HT-29 cell lines.

Different EGCG concentrations did not change the methylation status of the promoter regions, suggesting that it has no demethylating effect on the cell lines studied. Various factors including target availability, intracellular bioavailability dependent on cellular uptake, biotransformation or efflux, and EGCG application concentration might contribute to the present results obtained from cells of different histological origin. Meanwhile, the presence or abundance of other DNA methyltransferases (DNMT3a and DNMT3b) might influence the demethylating effect of EGCG on these cell lines via DNMT1. It should be noted that DNMT1 activity assays using nuclear extracts and analysis of gene expression levels are needed for broader conclusions. Results of the present study demonstrated that EGCG was more cytotoxic to MCF-7 cells than HT-29, i.e. $IC_{50}$ was approximately 10-fold higher in MCF-7 cells. Differences in intracellular signaling pathways between HT-29 and MCF-7 cells may affect the cytotoxic potency of EGCG since it binds to EGFR and PDGFR (Liang et al., 1997; Sachinidis et al., 2000) and inhibits protein kinases (Chung et al., 1999, 2001). When studying cells of different histological origin, cytotoxic variations in response to test materials should be considered. We investigated the effect of EGCG on cell viability at different doses and found that the range of cell viability after all EGCG concentrations tested was 50%–92% and 78%–93% in MCF-7 and HT-29 cell lines, respectively. However, we did not observe any changes in the DNA methylation status in the two cell lines after application of specified doses of EGCG. We conclude that the possible reasons are incubation doses that are below the DNMT1 inhibitory concentration or cell lines that are resistant to epigenetic effects of EGCG. In fact, according to previously reported data, nontoxic concentrations (<15% cell inhibition) of EGCG and catechin partially inhibited the methylation status of the promoter regions of the *RARb* gene (Lee et al., 2005) in breast cancer cells. In order to assess whether sublethal EGCG treatment may have caused the lack of demethylating effect on the cell lines tested, we also performed MS-MLPA analysis of MCF-7 cells treated with 50 and 100 μM EGCG, at which 8% and 4% cell viability was attained, respectively. Similar to the results demonstrated, we did not observe any significant changes in the DNA methylation status of the treated cells at these concentrations (data not shown).

EGCG is transported into the cell by passive diffusion and converted to the methylated metabolites and glucuronides. However, EGCG is pumped out by multidrug resistance-associated proteins (MRPs), as evidenced by increased EGCG accumulation in HT-29 cells in the presence of the MRP inhibitors indomethacin and probenecid (Hong et al., 2002). Both HT-29 and MCF-7 cells were found to express MRP1 (Alvarez et al., 1998; Kars et al., 2006).

In conclusion, although our study did not support the hypothesis that EGCG intake in early stages of colorectal and breast cancers could be preventive, especially when the importance of consuming dietary products in these tumors is taken into consideration, further studies could be designed on this basis with early-stage and numerous cancer cell lines.

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