EPIGENETIC DISEASE & ACCESSIBLE TREATMENT

January 3, 2016

BY: ANONYMOUS

Epigenetics is the study, in the field of genetics, of cellular and physiological phenotypic trait variations that are caused by external or environmental factors that switch genes on and off and affect how cells read genes instead of being caused by changes in the DNA sequence.

-**Epigenetic diseases** include, but are not limited to; all forms of Cancer, Diabetes 1 & 2, Psychological Disorders, and Auto-Immune Diseases.

-A great way to understand Epigenetics is to watch the video **"Nova - Ghost in Your Genes"** https://d.tube/#!/v/sophisto007/QmRTSuzRpaAzuHKMnyXvchCze5oE6Ut8GPzadaxF3EBdTq

-Many, if not most, of **Epigenetic markers are made of Methane.**

-**If you eliminate the Epigenetic methyl markers, you eliminate the Disease.**

-**Decitabine** is a synthetic drug used, at Temple University, to remove the methyl markers from human DNA, resetting the Epigenome.

-Here is a natural, readily available, homeopathic version of **Decitabine**:

(800mgEGCG + 10mg Bioperine) x 3/day = DNA Demethylation agent

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-**800mg Green Tea polyphenol EGCG** or higher ***(Without Bioperine it will not be effective)***

-**10mg Bioperine** (95% piperine or higher)***(This is to make the EGCG more bioavailable)***

-If you take this regimen there is a good chance you will stop cancer and other dreaded Epigenetic Diseases.

-When cells replicate, and EGCG is present, the EGCG will absorb the methane from your epigenome and it will exit the body. That means you need a continuous supply of EGCG in your system to be ready for cellular replication. After 1 year it is estimated that a human body replaces 98% of all the cells in the body, via replication. So to be 98% methane free, take this regimen 3 times a day for 1 year, resetting your Epigentics to when you were born.

-**To support my research the following documents are on file at the United States National Library of Medicine National Institutes of Health website:**

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Epigenetic effects of green tea polyphenols in cancer

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Abstract

Epigenetics describes heritable alterations of gene expression and chromatin organization without changes in DNA sequence. Both hypermethylation and hypomethylation of DNA can affect gene expression and the multistep process of carcinogenesis. Epigenetic changes are reversible and may be targeted by dietary interventions. Bioactive compounds from green tea (GT) such as (–)-epigallocatechin gallate have been shown to alter DNA methyltransferase activity in studies of esophageal, oral, skin, Tregs, lung, breast and prostate cancer cells, which may contribute to the chemopreventive effect of GT. Three out of four mouse model studies have confirmed the inhibitory effect of (–)-epigallocatechin gallate on DNA methylation. A human study demonstrated that decreased methylation of CDX2 and BMP-2 in gastric carcinoma was associated with higher GT consumption. It is the goal of this review to summarize our current knowledge of the potential of GT to alter epigenetic processes, which may be useful in chemoprevention.

Keywords

cancer; COMT; DNA methylation; DNMT; (–)-epigallocatechin gallate; epigenomics; green tea; one-carbon metabolism; polyphenols

Cancer is a disease of genetic susceptibility as well as epigenetic abnormalities [1]. Epigenetics generally refers to changes in gene expression and chromatin organization that are independent of alterations in the DNA sequence [2]. Epigenetic phenomena are modifiable by dietary and environmental factors. Changes in DNA methylation can be passed on to the next generation [3]. Epigenetic changes have been identified as promising targets for the prevention and treatment of cancer [4].

DNA methylation is the most widely studied epigenetic modification in mammals. DNA methylation results in the addition of a methyl group to the carbon-5 position in the pyrimidine ring of cytosine in the CpG dinucleotide of genomic DNA. The distribution of CpG dinucleotides throughout the human genome is not uniform, and are frequently enriched in the promoter regions of genes, especially in regions of large repetitive sequences such as

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centromeric repeats, LINE-1 and ALU retrotransposon elements [5]. Short CpG-rich regions are also called 'CpG islands' and are present in more than 50% of human gene promoters [6]. Hypermethylation of CpG islands within gene promoters has been shown to lead to gene silencing, while promoters of transcriptionally active genes are typically hypomethylated [7].

In addition to DNA methylation, other epigenetic changes, such as histone modification and miRNAs, can affect gene expression. Histone modifications typically occur as post-translational alterations at the N-terminal of histones. These histone alterations include acetylation, methylation, phosphorylation, biotinylation and ubiquitination, and play a fundamental role in protein regulation throughout life [8–10]. miRNAs appear to have a fundamental role in the biology of the cell. They constitute a class of ncRNA molecules, which have now emerged as key players in regulating the activity of mRNA. miRNAs are small RNA molecules approximately 22 nucleotides in length, which affect the activity of specific mRNA by influencing their half-life through interference with the normal mRNA degradation process or mRNA translation into proteins [11]. This review will focus on the epigenetic effects of green tea (GT) mediated through changes in DNA methylation.

Nutrition can potentially affect epigenetic phenomena at multiple points in DNA methylation [12]. First, nutrients are the main source of methyl groups or act as coenzymes for the one-carbon metabolism that regulates methyl transfer and DNA synthesis. For example, B vitamins, such as folic acid, vitamin B2, B6 and B12, are involved as coenzymes with methionine, choline, betaine and serine as universal methyl donors [13]. Second, a number of phyto-chemicals found in plant foods and in dietary supplements alter the epigenetic processes by influencing enzyme activities such as 5-cytosine DNA methyltransferase (DNMT). Phyto-chemicals including polyphenols (green tea catechins, quercetin, myricetin), soy isoflavones (genistein), parthenolide, curcumin, resveratrol, isothiocyantes and butyrate, an intestinal product from fiber, affect the activities of methylation enzymes [9,12,14–16]. Third, dietary components, such as retinoic acid and vitamin D, bind to their receptors and modulate gene expression leading to competitive downregulation of methylating enzymes [17,18]. In addition, other phyto chemicals, such as garlic diallyl disulfide, sulfurophane and indol-3-carbinol, impact the epigenome through histone modulation and regulation of miRNAs [17].

DNA methylation is catalyzed by the enzyme 5-cytosine DNMT with *S*-adenosylmethionine (SAM) as the methyl donor. There are three main DNMT enzymes: DNMT1, DNMT3a and DNMT3b [19,20]. DNMT1 is a maintenance methyltransferase maintaining DNA methylation patterns in DNA replication during cell division [16], whereas both DNMT3a and DNMT3b are involved in *de novo* methyltransferase processes, providing an important function during development (differentiation) [21,22]. DNA methylation has evolved as an attractive target in cancer therapeutics. Altered DNMT gene expression and enzyme activity is seen in numerous diseases including cardiovascular diseases [23,24], Type 2 diabetes [25], obesity [26], possibly neurodegenerative diseases [27] and cancer [3,9,28]. In cancer, both DNA hypo- and hyper- methylation have been demonstrated to be associated with disease progression. Methy lation during cancer development includes hypermethylation of specific gene promoters, in addition to generalized hypomethylation. DNA hypermethylation in cancer often causes the silencing of tumor suppressors and other genes important for cellular growth, regulation and differentiation [29]. DNA hypomethylation has been shown to result in chromosomal instability and increased mutation events [30]. Changes in cellular DNA methylation in colorectal, pancreatic, prostate, bladder, breast and ovarian cancer have been reviewed by Heichman *et al.* [31]. For example, Yang *et al.* demonstrated a decrease in global cytosine hypomethylation comparing low-grade prostate epithelial neoplasia, high-grade prostate epithelial neoplasia and prostate cancer tissue, using

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immunohistochemistry in the prostate [32]. However, hypomethylation is not as commonly observed, with only a handful of specific genes being hypomethylated in prostate cancer. The majority of genes are characterized by site-specific hypermethylation [33]. The evaluation of a panel of methylation markers, such as APC, RARb2, TIG1 and GSTP1, demonstrated that utilizing the information derived from the methylation status of the gene panel, in combination with histological tissue evaluation, increased the percentage of detection of carcinoma from 64 to 97% compared with using histological tissue evaluation alone [34]. Analysis of the methylation status of 219 prostatectomy tissue samples using a panel of three genes (*APC*, *HOXD3* and *TGFβ*) demonstrated that an increase in methylation was associated with prostate cancer progression [33]. Evaluation of DNA methylation of these three genes was superior for the prediction of biochemical recurrence compared with individual genes [33]. Importantly, many of these methylation events were also found in early high-grade prostatic intraepithelial neoplasia lesions [32], suggesting that aberrant DNA methylation changes occur early during carcinogenesis. The epigenetic changes in prostate cancer have been summarized in a review by Ho *et al.* [35].

The number of genes identified with altered methylation in breast cancer is rapidly growing. Breast cancer studies indicate that epigenetic alterations, such as promoter hypermethylation leading to gene silencing are involved in processes in carcinogenesis, including DNA repair (*BRCA1*), xenobiotic detoxification (*GSTP1*), apoptosis (*HOXA5*, *RASSF1A* and *TWIST1*), tissue invasion and metastatic processes (*CDH1* and *CDH13*) [36,37]. These genes were not only hypermethylated in tumor cells, but also in normal epithelium surrounding the tumor site [37]. Methylated genes are utilized as cancer biomarkers in the clinical laboratory including not only breast but other major tumor tissues, such as colorectal, pancreatic, prostate, ovary, lung and bladder cancer [31].

Based on the reversible nature of epigenetic alterations, epigenetic therapy has strong potential for the prevention and treatment of chronic disease. Increasing interest in the potential of changing diet and lifestyle, or consuming dietary supplements to alter the epigenome, has led to a growing body of research focusing on the potential of dietary components and natural products as epigenetic agents in chemoprevention and cancer treatment. This review will summarize our current knowledge of the effect of green tea polyphenols on epigenetic processes in cell culture, animal and human epidemiological and clinical intervention studies.

Green tea polyphenol bioavailability & metabolism

The active compounds in green tea are polyphenols (GTPs), also known as flavan-3-ols, which include (–)-epigallocatechin gallate (EGCG), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC) and (–)-epicatechin (EC) **(Figure 1)** [38]. The most abundant polyphenol in green tea is EGCG and it is known to have anticancer effects through many different mechanisms [39–43]. In cell culture and animal models of lung, digestive tract, bladder, liver, prostate, breast and skin cancer, the most commonly observed anticancer mechanisms of EGCG include inhibition of proliferation, induction of apoptosis and cell-cycle arrest at G0/G1 [44]. EGCG induces apoptosis by multiple pathways including the inhibition of the PI3K/AKT/p-BAD cell survival pathway leading to downregulation of Bcl-2 and upregulation of Bax, as well as activation of the FASR/caspase-8 pathway [45]. EGCG also affects other factors implicated in proliferation and cell death, such as MAPK pathways (phosphoErk1/2) and growth factors (IGF1, IGF receptor and IGFBP-3) [46]. In addition, EGCG has been shown to affect cell cycle regulation through inhibition of class I histone deacetylase (HDAC) enzyme activity leading to increased accessibility of the promoter region and increased expression of p21/waf1 and Bax [47]. In protein-binding assays, it was demonstrated that EGCG binds several proteins (vimentin, IGF-1 receptor and

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GRP 78 kDa) with high specificity at very low concentrations. However, in cell culture experiments, due to nonspecific protein binding, higher concentrations of EGCG were required to inhibit the same targets [44]. Treatment with EGCG or GT extract also demonstrated inhibition of angiogenesis, invasion, VEGF, MMP2 and MMP9. Finally, EGCG has been demonstrated to exhibit anti-inflammatory activity via COX-2 and NFκB inhibition. The concentration of phosphorylated NFκB/p65 in HT-1080 cells was inhibited by EGCG treatment in a dose-dependent manner, while NFκB/p65 remained the same [48]. Most of these mechanisms still need to be confirmed in human intervention studies.

GTPs are mainly absorbed from the small intestine [49,50]. The absorption is regulated by several multidrug MRP transporters and monocarboxylated transporter [51]. According to their function, the transporters are either located at the basal membrane (MRP1) or at the apical membrane (MRP2). GTPs are taken up into epithelial cells and metabolized leading to enhanced excretion or reduced chemopreventive activity [52,53]. Nongallated GTPs such as EGC and EC undergo glucuronidation and sulfation, whereas gallated GTPs, EGCG and ECG, are mainly present in the free form [54]. All GTPs with at least one catechol group undergo methylation by catechol-*O*-methyl transferase (COMT). COMT catalyzes the transfer of methyl groups from SAM to one of the hydroxyl catechol groups, with the equimolar formation of *S*-adenosyl-L-homocysteine (SAH) [55]. EGCG and ECG contain two catechol ring structures. EGCG is readily methylated at 4′ and 4″ positions to form 4″-*O*-MeEGCG and 4′,4″-di-*O*-methyl-EGCG [56].

EGC and EC are the major catechins circulating in blood [49], with approximately 30% of EGC occurring in methylated form as 4′-*O*-MeEGC [57]. Although EGCG is not well absorbed in the small intestine due to its abundance in GT, it can be found in human tissue of men consuming six cups of GT daily [58,59]. In urine, EGC, 4′-*O*-MeEGC and EC are the predominant catechins, occurring in conjugated form [60]. In mouse and human prostate tissue, the major catechins present are EGCG and ECG [56,61,62]. Approximately 50% of EGCG occurs in the methylated form as 4″-*O*-MeEGCG or 4′,4″-*O*-di-*O*-methyl-EGCG in human prostate tissue obtained at prostatectomy after consumption of six cups (48 oz) of GT daily for 3–5 weeks [58,63]. A similar degree of methylation of EGCG was found after GT consumption in mouse tissues, including lung, kidney and xenograft prostate tumors [62]. However, there are strong differences in the methylation activity depending on the organ. For example, COMT activity is much higher in liver and kidney tissue compared with prostate and lung [64,65]. Methylation significantly decreases the biological activity of EGCG. Inhibition of proliferation and stimulation of apoptosis were significantly reduced in lymph node carcinoma of the prostate (LNCaP) cancer cells treated with 4″-*O*-MeEGCG compared with EGCG [58]. In human leukemia HL60 cells, the inhibition of proteasomal activity was significantly decreased using methylated EGCG compared with the nonmethylated form [66].

Enzyme kinetic analyses revealed that EGCG, in addition to being a substrate for COMT, also exhibits strong inhibition of COMT activity [67]. The most potent inhibitors among the catechins were those containing a galloyl-type D-ring (EGCG, 4″-*O*-methyl-EGCG, 4′,4″-di-*O*-methyl-EGCG and ECG), independent from their methylation status [68]. Protein aggregate formation, as well as computational molecular modeling studies, demonstrated a direct binding of EGCG to COMT [68,69].

Since GTPs are substrates for catechol methyl ation reactions, it has been suggested that COMT-mediated methylation might decrease the intracellular concentration of SAM and, at the same time, increase the concentration of SAH, a feedback inhibitor of various SAM-dependent methylation reactions. Therefore, excessive consumption of catechol-containing polyphenols may affect other methylation processes such as DNA methylation [68].

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Potential mechanisms of DNMT inhibition by EGCG and other GTPs have been extensively investigated by Lee *et al.* [70]. Kinetic analyses using EC as a model inhibitor showed that DNA methylation was competitively inhibited *in vitro* mainly by increasing the formation of SAH. By comparison, the strong inhibitory effect of EGCG on DNMT-mediated methylation was largely due to its direct inhibition of the DNMT enzyme activity, independent of its own methylation [70].

GTPs & DNA methylation

Cell culture experiments

GTPs have been shown to inhibit DNA methylation leading to hypomethylation and activation of epigenetically silenced genes [71–77]. Extensive *in vitro* experiments have been performed in a variety of cancer cell lines to evaluate the effect of GTPs on DNA methylation **(Table 1)**. Original studies on the investigation of the effect of EGCG on DNA methylation in cell culture were performed by the laboratory of CS Yang (NJ, USA) and are summarized in a review article by Fang *et al.* [55].

Esophageal cancer

Studies conducted by Fang *et al.* demonstrated that treatment of esophageal cancer cells with EGCG (5–50 μM, 1–6 days) exhibited a time- and dose-dependent inhibition of DNMT activity reversing hypermethylation in several tumor suppressor genes (*p16*, *RARβ*, *hMLH1* and *MGMT)* [73].

Skin cancer

Treatment of A431 skin cancer cells with 10 μM of EGCG for 6 days decreased global DNA methylation as determined by 5-methylcytosine content using the QIAamp® DNA mini kit (Qiagen, Limburg, The Netherlands). In addition, EGCG treatment inhibited gene and protein expression of DNMT1, DNMT3a and DNMT3b leading to increased expression of p16(INK4a) and p21/Cip1 [75]. A comparison of the demethylation activity of the main four GTPs demonstrated that EGCG exhibited very similar activity compared with ECG, but the activity of both was higher than EGC and EC (EGCG = ECG > EGC > EC = GC) [75,78].

Tregs & Jurkat T cells

Wong *et al.* reported that treatment of Tregs and Jurkat T cells with 2–10 μM (1–2 days) of EGCG reduced global DNA methylation and *DNMT* gene expression leading to increased mRNA expression of IL-10 and Foxp3, a master switch that controls the development and function of Tregs and Jurkat T cells *in vitro* [71].

Breast cancer

Studies conducted by Li *et al.* found that treatment with EGCG at 10 μM for 3 days led to reactivation of estrogen receptor (ER)-α expression in ER-α-negative MDAMB-231 breast cancer cells [77]. A combination treatment of the same cells with EGCG and a HDAC inhibitor, trichostatin A, synergistically increased the activation of ER-α expression in ER-α-negative MDAMB-231 breast cancer cells [77]. More recent work demonstrated that sulforaphane (10 μM) enhanced the DNMT1-inhibitory effect of EGCG (20 μM) in paclitaxel-resistant ovarian cancer cells (KOV3TR-ip2) and, at the same time, enhanced the inhibition of hTERT and Bcl-2 [79].

Although overwhelming *in vitro* evidence supports the anticarcinogenic properties of GT treatment, the limited chemical stability of EGCG at alkaline pH under normal physiological conditions is of concern for the translation of our findings to clinical studies. To increase the

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chemical stability of EGCG, synthetic analogs have been generated, which show stronger anti-cancer activity with more stability and efficacy [80,81]. For example, studies comparing EGCG to an EGCG analog (peracetylated EGCG [pEGCG] and EGCG octa-acetate) demonstrated increased inhibition of hTERT expression by inducing DNA hypomethylation and promoter deacetylation, mediated by inhibition of DNMTs and histone acetylases, respectively [76,82]. Both EGCG (40 μM) and pEGCG (20 μM) inhibited the proliferation of human ER-positive (MCF-7) and ER-negative (MDAMB-231) breast cancer cells in a dose- and time-dependent way, but showed no effect on normal MCF10A cells.

Lung cancer

Treatment with 10–50 μM of EGCG for 3 days led to promoter demethylation and restoration of WIF-1, an antagonist of *Wnt*proto-oncogene in non-small-cell lung cancer (H460 and A549) cells [83].

Oral carcinoma

EGCG (20–50 μM for 3 and 6 days) treatment of oral carcinoma cells (SCC9 and HSC3) partially reversed the hypermethylation status of the *RECK* tumor suppressor gene leading to a significant increase of the expression of *RECK* mRNA [72].

Prostate cancer

Treatment of LNCaP human prostate cancer cells with a GT extract (polyphenon E) was associated with a time- and dose-dependent activation of GSTP1. The hypermethylation and downregulation of GSTP1 has been associated with the development of several types of cancer, including cancer of the prostate [84]. The inhibition of DNMT1 protein expression in LNCaP prostate cancer cells treated with 10 μg/ml of polyphenon E was strongest at 14 days of treatment and was associated with decreased methylation of the promoter region of GSTP1 [84].

Despite the abovementioned studies, the ability of EGCG to inhibit DNA methylation remains controversial since there are also two published studies that did not find evidence that EGCG treatment will alter the methylation status and reverse gene silencing. Chuang *et al.* reported that purified EGCG did not inhibit DNA methylation at single copy loci or repetitive DNA elements in three different human cancer cell lines [85]. They examined the effect of EGCG compared with 5-aza-2′-deoxycytidine (5-Aza-Cd) on DNA methylation and RNA expression of six different genes/repetitive elements (*p16*, *RARβ*, *LINE-1*, *MAGE-A1*, *MAGE-B2* and *Alu*) in three separate cell lines (T24, HT29 and PC3) [85]. Similarly, Stresemann *et al.* reported that EGCG treatment did not produce a significant effect on DNA methylation in HCT116 human colorectal cancer cells [86]. Treatment with 20–30 μM of EGCG for 6 days did not alter global methylation or the methylation status of TIMP3 in TK6, Jurkat and KG-1 cancer cell lines, including 2–50 μM for 3 days of EGCG [86]. The authors suggested potential reasons for the discrepancies between their studies and previously published studies, including different methods of analysis, possible gene specificity or cell line specificity of EGCG, or that the treatment method might have been ineffective to show efficacy. Stresemann *et al.* argued that in some *in vitro* cell culture conditions, cellular effects induced by EGCG could probably be attributed to the oxidative stress induced by this compound [86]. At neutral or alkaline pH, EGCG undergoes auto-oxidation, resulting in dimerization of EGCG and EGC to form homo- and hetero-dimers in an alkaline environment with concurrent formation of H_2O_2 [87,88]. This process is ubiquitous in *in vitro* experiments and during the intestinal digestion, but the degree of auto-oxidation depends on the cell culture medium. In cell culture medium, the indirect contribution of H_2O_2 formation can be avoided by the addition of superoxide dismutase or catalase prior to adding the GTPs [89]. However, the majority of cell culture experiments

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did not address the H_2O_2 formation. In summary, *in vitro* cell culture studies provide clear evidence that GTP treatment can alter DNA methylation, leading to re-expression of silenced genes. To achieve changes in DNA methylation, concentrations of 20–50 μmol/l of EGCG for 3–6 days is needed. These concentrations are much higher than physiologically achievable in mouse or human tissue. Therefore, GT interventions may not be suitable for therapeutic purposes, but may play a role in long-term treatment effects on DNA methylation in cancer prevention.

Therapeutic use of DNA methylation inhibitors

In general, two classes of DNA methylation inhibitors have been developed. One class includes the nucleoside analogs, such as 5-azacytidine (azacytidine) and 5-Aza-Cd (decitabine), and the second class includes non-nucleoside analogs, such as EGCG, genistein, hydralazine and procainamide, which are not incorporated into the DNA [90]. Two nucleoside analogs have been approved by the US FDA in 2004 and 2006 for the treatment of hematologic conditions, such as myelodysplastic syndrome, and are under development to treat acute myeloid leukemia; azacytidine has also been approved for chronic myelomonocytic leukemia [90]. However, the treatment of solid tumors with nucleoside analogs only led to limited response [91]. Myelosupression is the main toxicity when nucleoside analogs are used in higher doses [91]. The short plasma half-life of nucleoside analogs remains a challenge due to high levels of hepatic cytidine deaminase, an enzyme that deactivates the nucleoside analog. A second-generation prodrug with improved pharmacological profile, increased stability and decreased toxicity, is in Phase II clinical trials in the treatment of myelodysplastic syndrome and acute myeloid leukemia [90]. In the effort to minimize side effects, promising results have been shown with a combination of several cycles of treatment with very low doses of azacytidine and the HDAC inhibitor etinostat in patients with recurrent metastatic non-small-cell lung cancer [92].

Direct comparison of the inhibition of DNA methylation by nucleoside to non-nucleoside analogs in prostate (PC-3), colon (HT29) and bladder (T24) cell lines demonstrated that nucleoside analogs such as 5-Aza-Cd are far more effective in inhibiting DNA methylation leading to reactivation of silenced genes [85]. A concentration of 1 μmol/l of 5-Aza-Cd reactivated *MAGE-A1*, *MAGE-B2* and *p16* gene expression, while 30 μmol/l of EGCG, 20 μmol/l hydralazine and 200 μmol/l procainamide were inactive [85]. Another comparison of 5-Aza-Cd to GT showed that treatment of LNCaP prostate cancer cells with 10 μM 5-Aza-Cd for 3 days induced global hypomethylation of a CpG island within the LINE-1 promoter determined by methyl-specific PCR analysis, while exposure of cells to 10 μM of GTP did not result in global hypomethylation, but in re-expression of GSTp1 and promoted maintenance of genomic integrity [84]. Sustained and long-term exposure to very high doses of GTPs, comparable in activity with pharmacologic agents, could theoretically lead to chronic changes of DNA methylation [18]. However, these considerations do not apply to any of the current chemoprevention studies being carried out with GT.

On the other hand, potential interference of EGCG with the one-carbon metabolism has been observed. In a purified enzyme system of dihydrofolate reductase (DHFR) extracted from *Stenotrophomonas maltophilia*, EGCG was shown to inhibit DHFR enzyme activity *in vitro* at concentrations of 0.1–1 μM [93]. In Caco2 colon cancer cells, treatment with approximately 40 μM of EGCG interfered with the production of nucleotides, thus compromising DNA and RNA synthesis and acting as an antifolate agent [94]. DHFR catalyzes the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, which in turn is required as a coenzyme for many one-carbon group transfer reactions, including the nucleotide biosynthesis [95]. This interference with DNA biosynthesis may explain why neural tube defects, such as anencephaly and spina bifida, which are usually associated with

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folic acid deficiency, were linked to high levels of GT consumption of the mother during the periconceptional period [96].

Studies by Lemos *et al.* observed that EGCG also exhibited a dose-dependent inhibitory effect on ^{3}H-folic acid uptake with an IC_{50} value of 7.7 μM (95% CI: 2.8–20.7) [97]. In addition, EGCG interfered with the uptake of methotrexate (IC_{50}: 10.1 μM), which may result in reduced chemotherapeutic efficacy in patients taking methotrexate by the oral route [97].

Despite issues with bioavailability and stability, tea catechins have emerged as promising chemopreventive agents because many of the effects observed *in vitro* can be replicated in various animal models. However, to further increase the stability and bioavailability of GTPs, Saez-Ayala *et al.* synthesized two catechin-derived compounds, 3-*O*-(3,4,5-trimethoxybenzoyl)-(–)-catechin and 3-*O*-(3,4,5-trimethoxybenzoyl)-EC in an attempt to improve the chemopreventive activity of GTPs [98]. The antiproliferative and proapoptotic activities of both compounds were analyzed with various cancer cell lines. 3-*O*-(3,4,5-trimethoxybenzoyl)-(–)-catechin was superior compared with 3-*O*-(3,4,5-trimethoxybenzoyl)-EC in inhibiting proliferation and inducing apoptosis in both melanoma and non-melanoma cell lines [98].

Evidence from *in vivo* animal studies

The most convincing evidence to demonstrate that nutrition can modulate the epigenetic status of mammals was provided by studies with mice carrying the *Avy* gene [99,100]. In this mouse model, methylation affects the expression of the *Avy* gene. In the methylated form, the *Avy* gene is expressed only in the hair follicle (wild-type allele). However, in the unmethylated form, it is expressed ubiquitously resulting in the agouti (yellow) fur color and other characteristics of the agouti syndrome. Using the *Avy* model, Wolff and colleagues showed that feeding diets supplemented with high levels of folic acid (as methyl donor) to pregnant dams modified the expression of the agouti gene in the offspring [100].

Whether EGCG can reverse DNA hypermethylation and reactivate methylation-silenced genes *in vivo* still remains to be determined. Based on the evidence from *in vitro* cell culture studies, it is of interest to investigate the effect of GTPs on epigenetic processes *in vivo* **(Table 2)**. Potential mechanisms are the inhibition of DNMT1 activity, directly or competitively, by depleting its substrate SAM or causing accumulation of the inhibitor SAH [18,70].

The delay in the development of prostate cancer by administration of the DNA methyltransferase inhibitor 5-Aza-dC to transgenic adeno-carcinoma of the mouse prostate (TRAMP) mice has been demonstrated as a ‘proof of principle’ that cancer prevention may be achieved through epigenetic modifications [101]. Analysis of untreated TRAMP prostate lesions demonstrated elevated DNMT1 mRNA and protein levels in early stages of prostate cancer development (prostatic intraepithelial neoplasia), which continued through advanced prostate cancer and metastasis. In a 5-Aza-Cd intervention study, none of the 14 TRAMP mice receiving intraperitoneal injections twice weekly on consecutive days of 300 μl 5-Aza-Cd (0.25 mg/kg) developed prostate cancer at 24 weeks of age, whereas seven out of 13 (54%) control mice, injected with phosphate buffer solution, developed poorly differentiated prostate cancer [101].

In our investigation of the effect of drinking brewed GT instead of drinking water on tumor growth and DNMT activity in male severe-combined immunodeficiency mice, we determined an inhibition of DNMT1 protein and gene expression in prostate xenograft LAPC4 tumor tissue [62]. The GT contained a concentration of 0.07% of GTPs and was

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administered for 13 weeks. Tumor volume and weight were also decreased significantly in mice drinking the GT compared with the water control [62].

Intestinal cancer

Further evidence for the epigenetic activity of GT was provided by Volate *et al.* [102]. The administration of 0.6% (w/v) solution of GT as the only source of beverage from 8–16 weeks to Apc(Min/−) mice significantly increased the protein and mRNA levels of RXRa, which was downregulated in intestinal tumors of azoxy-methane-treated Apc(Min/+) control mice. Genomic bisulfite treatment of DNA extracted from the colon of these mice showed a significant decrease in CpG methylation with GT treatment in the promoter region of the *RXRα* gene, analyzed by pyrosequencing of 24 CpG sites [102]. GT treatment also reduced the number of newly formed intestinal tumors (28%; $p < 0.05$) compared with water control.

Skin cancer

In the studies by Mittal *et al.*, global DNA hypomethylation was observed in chronically UVB-exposed SKH-1 hairless mice [103]. Topical treatment with EGCG in hydrophilic cream providing 1 mg/cm^2 skin reversed DNA hypomethylation in chronic UVB-exposed skin determined by immunohistochemical detection of 5-methylcytosine [103]. The authors speculated that the epigenetic modulation contributed to the anticarcinogenic activity of EGCG. However, it is interesting that in this mouse model, the observation was contradictory to the concept that EGCG can prevent or reverse hypermethylation of certain specific genes [103].

Fang *et al.* addressed the question of whether the administration of EGCG in mice could lead to a decrease in SAM coinciding with the accumulation of SAH, which in turn could induce competitive inhibition of DNMT activity [55]. They examined this issue in their ongoing experiments on bioavailability, toxicity and cancer-preventive activities of EGCG. The results showed that only an acute intragastric treatment with high doses of EGCG (500–2000 mg/kg), significantly elevated plasma levels of homocysteine and at the same time decreased levels of plasma methionine and lowered the concentration of hepatic and intestinal SAM, as well as the SAM:SAH ratio [55]. The administration of EGCG (or polyphenon E) through drinking fluid (0.32% EGCG or 0.5% polyphenon E) decreased intestinal SAM concentrations moderately without increasing the level of SAH. No changes of hepatic SAM or SAH levels were observed with the administration of EGCG in the drinking water [55].

No effect on DNA methylation

Morey *et al.* recently tested whether oral consumption of GTPs could affect normal or cancer-specific DNA methylation *in vivo*, using the TRAMP mouse model [104]. TRAMP mice received 0.3% GTPs in drinking water beginning at 4 weeks of age. In these studies, GTP treatment did not inhibit tumor progression in TRAMP mice, whereas investigations by several other groups had demonstrated an inhibition of tumor size and number with GT treatment [105,106]. In addition, levels of 5-methyldeoxycytidine in the B1 repetitive element and methylation of the *Mage-a8* gene were not affected. In these studies, no measurements of tissue GTP were performed to confirm the bioavailability of the GTPs.

Combinations of EGCG & other natural products

There is an increased interest in the use of combinations of natural products in order to overcome multidrug resistance, limited bioavailability or to target multiple mechanisms concurrently. For example, in our laboratory a combination of GT and quercetin increased the bioavailability and decreased EGCG methylation, leading to an increase in the

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anticarcinogenic activity in a prostate cancer xenograft mouse model [64]. In addition multiple cell culture studies demonstrated that the combination of GTPs with sulforaphane increased apoptosis and altered Nrf2- and AP-1-regulated gene expression in prostate and colon cancer cells [79,107,108]. Combining natural products that alter the epigenome will enhance the epigenetic effect, since some compounds may alter DNA methylation and other natural compounds may affect histone structure and miRNA regulation [109–111].

DNA methylation in human studies

There are only a limited number of clinical trials investigating the effects of dietary polyphenol sources on DNA methylation such as tea or diets limited in folate and one-carbon metabolites **(Table 3)**. One study by Olthof *et al.* reported an increase in plasma homocysteine concentration associated with high polyphenol consumption from either black tea, chlorogenic acid or quercetin for 7 days [112].

A retrospective study published by Yuasa *et al.* examined the methylation status of six genes in primary gastric carcinomas from 106 patients in relation to past lifestyle, including dietary habits [113,114]. In this Japanese study of gastric carcinoma, high consumption of GT (seven cups or more per day compared with six cups or less) and cruciferous vegetables, as well as physical activity, was inversely related to the methylation of *CDX2* and *BMP-2,* but not *p16 (INK4A), CACNA2D3*, *GAT-5* and *ER* in gastric carcinoma tissue, which are frequently hypermethylated in gastric cancers [113]. It was pointed out by the authors that the epithelial surfaces of the esophagus and stomach may be particularly susceptible to beneficial DNMT1 inhibitory effects of GTPs, since the luminal intestinal side is exposed to high concentrations of GTPs before the polyphenols undergo metabolism.

Since it has been shown that the intestinal administration of large concentrations of GTPs will lead to a significant change in the SAM:SAH ratio [55], we also include human studies investigating the association of the one-carbon metabolism to colon cancer. A review by Lim and Song provided an excellent summary of these studies [115]. A landmark controlled feeding study to investigate the effect of a folate-deficient diet on global DNA methylation was performed in 1998. Global DNA methylation levels decreased in lymphocytes of postmenopausal women on a folate-deficient diet (56 μg/day for 5 weeks and 111 μg/day for 4 weeks) and increased on a folate-replete diet (286–516 μg/day for 3 weeks) [116]. Two previous human studies conducted in a metabolic unit demonstrated that marginal folate deficiency can change blood genomic DNA methylation [117]. Another study investigated the effect of the consumption of a sequence of a moderately deplete folate diet (118 μg/day for 7 weeks) followed by intake of a replete diet (415 μg/day) for 7 weeks in older women aged 60–85 years. During the moderate depletion phase, leukocyte global DNA methylation declined but did not significantly improve even after 7 weeks on the repletion diet [117]. Similarly, another clinical randomized and double-blinded intervention demonstrated that leukocyte global DNA methylation was increased in participants receiving folic acid supplements (400 μg/day for 10 weeks) [118]. These findings support the hypothesis that a reduction in total folate intake may be associated with reduced global DNA methylation and can be reversed with supplementation at levels of folic acid found in common multivitamin supplements (400 μg/day). However, older age may compromise or delay the recovery [117].

Taken together, the evidence from human studies, demonstrating significant dietary effects on DNA methylation in free-living human subjects, is very limited compared with *in vitro* and animal studies. Evidence for the role of GTPs in affecting DNA methylation in cancer development is mainly based on *in vitro* cell culture experiments. However, since dietary modifications induce relatively low impact changes on DNA methylation, with lower

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toxicity compared with epigenetic therapeutic drugs, dietary strategies may play an important role in the prevention of carcinogenesis. Moreover dietary exposures are long-term and potentially repeated several times daily in heavy tea drinkers. There is a critical need for future investigations in animal and human studies to reveal the potential of different bioactive and dietary components in the epigenetic regulation of chronic disease.

Conclusion

GTPs and other natural products have the potential to alter epigenetic processes through DNA methylation, histone modification and miRNA regulation. This area of research requires more information on the relative potency of these effects both for GTPs alone and for polyphenols in combination with other drugs and natural products or dietary supplements.

Future perspective

Owing to emerging technologies and decreasing cost of evaluating changes in DNA methylation, our understanding of the effects of nutrition and botanical dietary supplements on epigenetic processes will increase dramatically during the next decade. Although the effect of dietary exposure on epigenetics is subtle, due to the nature of dietary exposures being frequent and long-term, we expect that future evidence will support an emerging role of diet in regulating DNA methylation. *In vitro* cell culture studies provide clear evidence that extended GT treatment can change DNA methylation and reactivate gene expression. Future long-term *in vivo* GT studies are needed to ascertain whether permanent changes of DNA methylation are achievable. Results from these studies will contribute to cancer prevention and treatment.

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Executive summary

DNA methylation & cancer

■ Epigenetic phenomena are heritable and potentially modifiable by dietary, environmental and therapeutic factors.

■ Epigenetic processes are promising targets for interventions with nutrients and compounds derived from dietary supplements and natural products.

■ Nutrition affects epigenetic phenomena at multiple points: being a source of methyl groups, influencing enzyme activities (DNA methyltransferase and histone deacetylase), binding to receptors and modulating methylating enzymes and/or modulating histones and regulating miRNAs.

Green tea polyphenol bioavailability & metabolism

■ (–)-Epigallocatechin gallate (EGCG) is the main tea polyphenol under investigation.

■ Low bioavailability and extensive metabolic changes to less active methylated metabolites limits its potential.

■ Future research into combination treatments and using more stable EGCG preparations is needed.

Green tea polyphenols & DNA methylation

■ Green tea (GT) polyphenols have been shown to inhibit DNMT1 activity leading to reactivation of silenced genes in cultured cells from oral cavity, esophagus, lung, breast, prostate and immune cells.

■ Several animal studies confirmed the epigenetic effect of GT.

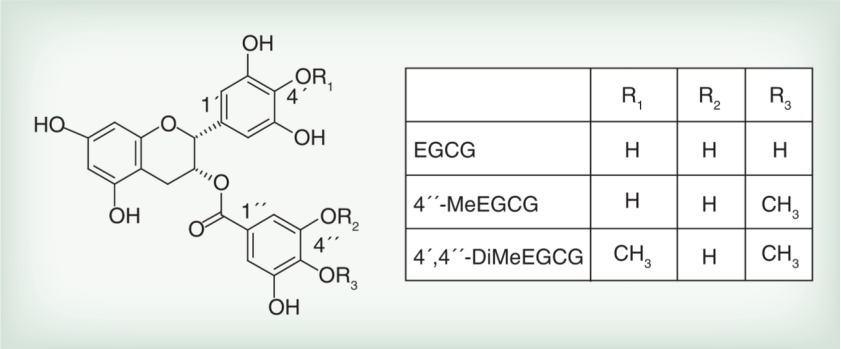
■ Additional animal and clinical studies are needed to confirm the epigenetic effect of GT.

■ Studies using combinations of different natural products such as EGCG with quercetin or sulforaphane may alter epigenetic processes more effectively.

Human studies

■ One human study demonstrated that the consumption of more than six cups of GT was inversely related to the methylation of CDX2 and BMP-2 in gastric carcinoma tissue.

■ Oral and gastric epithelial surfaces are particularly susceptible to beneficial DNMT1 inhibitory effects of tea polyphenols, since they are exposed to high concentrations of tea polyphenols before undergoing metabolism.



	R_1	R_2	R_3
EGCG	H	H	H
4´´-MeEGCG	H	H	CH_3
4´,4´´-DiMeEGCG	CH_3	H	CH_3

Figure 1. (–)-Epigallocatechin gallate and its methylated metabolites
4″-MeEGCG: 4″-*O*-methyl-epigallocatechin gallate; 4′,4″-DiMeEGCG: 4′,4″-di-*O*-methyl-(–)-epigallocatechin gallate; EGCG: (–)-epigallocatechin gallate.

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Table 1

Cell culture studies to investigate the effect of green tea or epigallocatechin gallate on DNA methylation and gene expression.

Study (year)	Tissue/cell line	Genes	GT/EGCG dose and duration of treatment (μmol/l, days)	Effect	Ref.
Fang *et al.* (2007)	Esophagus	*p16, RAR*β, *hMLH1* and *MGMT*	5–50 EGCG, 1-6	Decreased *DNMT1*, gene reactivation	[55]
Wong *et al.* (2011)	Immune T cells, Jurkat	*FOXP3*	2–10 EGCG, 1–2	Decreased DNMT1	[71]
Kato *et al.* (2008)	Oral SCC9 and HSC3	*RECK* tumor suppressor	20–50 EGCG, 3–6	Reversed hypermethylation, gene reactivation	[72]
Gao *et al.* (2009)	Lung H460 and A549	*WIF-1*	10–50 EGCG, 3	Demethylation	[83]
Li *et al.* (2010)	Breast MDAMB-231	*ER-α*	10 EGCG, 3	Reversed hypermethylation, gene reactivation	[77]
Nandakumar *et al.* (2011)	Skin A431	*p16(INK4a)* and *p21/Cip1*	10 EGCG, 6	Decreased *DNMT1*, gene reactivation	[75]
Pandey *et al.* (2010)	Prostate	*GSTP1*	10 PolyE, 14	Decreased *DNMT1*, gene reactivation	[84]
Chuang *et al.* (2005)	T24, HT29 and PC3	*p16, RARb, MAGE-A1, MAGE-B2* and *Alu*	20–30 EGCG, 6	No effect	[85]
Streseman *et al.* (2006)	TK6, Jurkat and KG-1	*TIMP3*	2–50 EGCG, 3	No effect	[86]

EGCG: Epigallocatechin gallate; GT: Green tea; PolyE: Polyphenon E.

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Table 2

Animal studies to investigate the effect of green tea or epigallocatechin gallate on DNA methylation and gene expression.

Study (year)	Tissue	Animal model	GT exposure	Effect	Ref.
Henning *et al.* (2012)	Prostate	C57BL/6J–LAPC4 xenograft	Brewed GT (0.075% GTP), 13 weeks	Decreased tumor volume, weight and decreased DNMT1	[62]
Volate *et al.* (2009)	Intestine	Apc(Min/-)	GT 0.6%	Decreased CpG methylation of RXRα	[102]
Mittal *et al.* (2003)	Skin	UVB-exposed SKH-1 hairless mice	1 mg/cm^2 skin area EGCG cream prior to UV exposure	Decreased global DNA hypomethylation	[103]
Morey *et al.* (2009)	Prostate	TRAMP mice	GTP 0.1–0.6% in drinking water, 8–20 weeks	No decreased methylation and no decrease in tumor volume	[119]

EGCG: Epigallocatechin gallate; GT: Green tea; GTP: Green tea polyphenol; Min: Multiple intestinal neoplasia; TRAMP: Transgenic adenocarcinoma of the mouse prostate.

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Table 3

Human studies to investigate the effect of green tea or epigallocatechin gallate on DNA methylation and gene expression.

Disease	Participants (n)	GT exposure	Effect	Ref.
Healthy	–	Polyphenols, 7 days	Increased plasma SAH	[112]
Gastric carcinoma	Japanese (55)	More than seven cups compared with less than six cups GT/days	Methylation status of *CDX2* was not significantly associated with low intake of tea	[113]
Gastric primary carcinoma	Japanese (106)	More than seven cups compared with <6 cups GT/days	Correlation between CDX2 and BMP-2 methylation and low tea intake	[114]

EGCG: Epigallocatechin gallate; GT: Green tea; SAH: S*-adenosyl-*L*-homocysteine.*

Piperine Enhances the Bioavailability of the Tea Polyphenol (−)-Epigallocatechin-3-gallate in Mice[1]

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ABSTRACT (−)-Epigallocatechin-3-gallate (EGCG), from green tea (*Camellia sinensis*), has demonstrated chemopreventive activity in animal models of carcinogenesis. Previously, we reported the bioavailability of EGCG in rats (1.6%) and mice (26.5%). Here, we report that cotreatment with a second dietary component, piperine (from black pepper), enhanced the bioavailability of EGCG in mice. Intragastric coadministration of 163.8 μmol/kg EGCG and 70.2 μmol/kg piperine to male CF-1 mice increased the plasma C_{max} and area under the curve (AUC) by 1.3-fold compared to mice treated with EGCG only. Piperine appeared to increase EGCG bioavailability by inhibiting glucuronidation and gastrointestinal transit. Piperine (100 μmol/L) inhibited EGCG glucuronidation in mouse small intestine (by 40%) but not in hepatic microsomes. Piperine (20 μmol/L) also inhibited production of EGCG-3″-glucuronide in human HT-29 colon adenocarcinoma cells. Small intestinal EGCG levels in CF-1 mice following treatment with EGCG alone had a C_{max} = 37.50 ± 22.50 nmol/g at 60 min that then decreased to 5.14 ± 1.65 nmol/g at 90 min; however, cotreatment with piperine resulted in a C_{max} = 31.60 ± 15.08 nmol/g at 90 min, and levels were maintained above 20 nmol/g until 180 min. This resulted in a significant increase in the small intestine EGCG AUC (4621.80 ± 1958.72 vs. 1686.50 ± 757.07 (nmol/g·min)). EGCG appearance in the colon and the feces of piperine-cotreated mice was slower than in mice treated with EGCG alone. The present study demonstrates the modulation of the EGCG bioavailablity by a second dietary component and illustrates a mechanism for interactions between dietary chemicals. J. Nutr. 134: 1948–1952, 2004.

KEY WORDS: • *epigallocatechin-3-gallate* • *piperine* • *green tea* • *bioavailability* • *mice*

Tea (*Camellia sinensis*) is a beverage with a worldwide popularity second only to that of water. Studies with animal models showed that green tea has preventive activity against cancer of the oral cavity, esophagus, stomach, intestine, colon, liver, lung, prostate, skin, and other sites (1). Epigallocatechin-3-gallate (EGCG)[3] is the major catechin component of green tea and may be a major active constituent (**Fig. 1**). Studies with human cancer cell lines have shown that EGCG possesses a number of activities related to cancer prevention, such as inhibition of activator protein 1 and nuclear factor κB transactivation and epidermal growth factor receptor signaling. It is not known, however, whether these actions occur in animals or humans because of the limited bioavailability of EGCG following oral administration (1,2).

Previously, we reported that the absolute bioavailability of EGCG in CF-1 mice and Sprague-Dawley rats is 26.5 and 1.6%, respectively (3,4). In these studies, the glucuronidated, sulfated, and aglycone forms of EGCG were quantified as total EGCG. EGCG undergoes methylation, glucuronidation, and sulfation in vivo; we showed that EGCG is largely present as the glucuronide in the plasma of treated mice (1,5,6). Modulation of the factors affecting EGCG bioavailability might increase plasma and tissue levels of this compound as well as its cancer preventive activity.

Piperine (Fig. 1), an alkaloid derived from black pepper (*Piper spp.*), has been reported to inhibit glucuronidation activity in rats and guinea pigs (7,8). Singh et al. (7) reported that piperine inhibited rat hepatocyte-mediated glucuronidation of 3-hydroxybenzo[a]pyrene with an IC_{50} of 50 μmol/L. It was reported that coadministration of piperine and curcumin to humans and rats enhanced the bioavailability of curcumin by 2000% and 154%, respectively (9). The authors suggested that this increase was due to inhibition of the glucuronidation of curcumin.

Because black pepper (world production was 47.6 million kg in 1999) and green tea are widely consumed dietary components, it is predictable that coexposure to piperine and EGCG will occur in dietary situations. We hypothesized that piperine may represent a potential dietary modulator of the bioavailability of EGCG by virtue of its ability to inhibit glucuronidation. Herein, we report the results of a study to determine whether coadministration of piperine affects the bioavailability of EGCG in mice.

[1] Supported by NIH Grant CA 88961 (to C.S.Y.).
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E-mail: joshua_lambert@hotmail.com.
[3] Abbreviations used: AUC, area under the curve; CEAS, coulochem electrode array system; ECD, electrochemical detection; EGCG, epigallocatechin-3-gallate; GI, gastrointestinal tract; MRP, mutidrug resistance related protein; PGP, *p*-glycoprotein.

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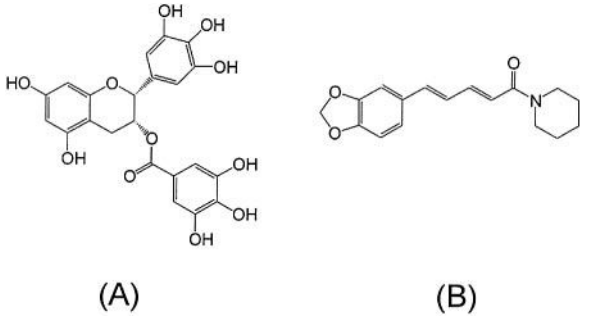

FIGURE 1 Structure of (*A*) epigallocatechin-3-gallate and (*B*) piperine.

MATERIALS AND METHODS

Chemicals. EGCG (100% pure) was provided by Tokyo Food Techno. β-D-Glucuronidase (G-7896, EC 3.2.1.31, from *Escherichia coli* with 9 MU/g solid), sulfatase (S-9754, EC 3.1.6.1, from abalone entrails with 0.23 MU/g solid), and piperine were purchased from Sigma Chemical. All other reagents were of the highest grade commercially available. Dosing solutions of EGCG and piperine were prepared in 0.154 mol/L NaCl. For analytical purposes, a standard stock solution of EGCG, epigallocatechin, epicatechin, and epicatechin-3-gallate (10 mg/L each) was prepared in 11.4 mmol/L ascorbic acid–0.13 mmol/L EDTA (pH 3.8) and stored at −80°C.

Mice. Male CF-1 mice (30–35 g) were purchased from Charles River Laboratories and allowed to acclimate for at least 1 week prior to the start of the experiment. The mice were housed 10 per cage and maintained in air-conditioned quarters with a room temperature of 20 ± 2°C, relative humidity of 50 ± 10%, and an alternating 12-h light/dark cycle. Mice were fed Purina Rodent Chow #5001 (Research Diets) and water and were allowed to eat and drink ad libitum. Mice were deprived of food for 12 h prior to the experiment.

Microsomal glucuronidation activity. The ability of piperine (0–500 μmol/L) to inhibit EGCG glucuronidation was determined using mouse hepatic microsomes prepared as previously described (10). Small intestinal microsomes were prepared in a similar manner with the following modifications. After mice were killed, the small intestine (between the pyloric sphincter and the cecum) was removed and the contents were rinsed with 10 mL of ice-cold Tris-HCl (2 mmol/L, pH 7.4, containing 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 150 mmol/L KCl). The intestine was cut open longitudinally and placed on a wetted glass plate, and the enterocytes were gently scraped from the intestine using a metal spatula. The enterocytes were collected and homogenized in a manner analogous to liver samples. Microsomal glucuronidation assays using EGCG (50 μmol/L) as the substrate were performed using the method of Lu et al. (6). The inhibitory activity of piperine is expressed as a percentage of glucuronidation in the vehicle-treated control reactions.

Cell culture and treatment. HT-29 human colon cancer cells (American Type Tissue Culture) were maintained at subconfluence in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 kU/L penicillin, and 0.1 g/L streptomycin at 37°C in 95% humidity and 5% CO_2.

Uptake studies using 10 μmol/L EGCG were performed as previously described (11). To determine the effect of piperine on EGCG metabolism and uptake, cells were cotreated with 20 μmol/L piperine and 10 μmol/L EGCG. Cytosolic levels of EGCG and its metabolites were determined by HPLC and normalized to cytosolic protein concentrations.

Treatment of mice and sample collection. EGCG (163.8 μmol/kg, i.g.) or EGCG plus piperine (163.8 and 70.2 μmol/kg, i.g.) was administered to mice (6 per group). Mice were then killed at 60, 90, 180, and 300 min posttreatment, blood was collected via cardiac puncture, and plasma was isolated by centrifugation at 500 × *g* for 15 min. Plasma was combined with 0.1 vol of ascorbate preservative (1.14 mol/L ascorbic acid, 1.3 mmol/L EDTA) and stored at −80°C for later analysis. Feces were collected from the interior of the colon following dissection. The small intestine and colon were collected, washed in 0.154 mol/L NaCl, and frozen at −80°C for later analysis.

Quantification of EGCG and metabolites. Plasma levels of EGCG and its metabolites were analyzed as previously reported (4). Fecal samples were diluted 1:10 in 100 g/L ascorbate preservative and sonicated. A 20-μL aliquot was then hydrolyzed with β-glucuronidase/sulfatase as described previously (3). Following hydrolysis, the sample was extracted twice with ethyl acetate. The organic phase was dried under vacuum, resuspended in 10% aqueous acetonitrile, and analyzed by HPLC-electrochemical detection (ECD). Tissue samples were homogenized in 114.0 mmol/L ascorbic acid and processed as previously described (3). Duplicate samples of feces, tissues, and plasma were prepared without sulfatase/β-glucuronidase treatment to determine the unconjugated fraction of EGCG and its metabolites. Samples were analyzed by HPLC-ECD.

Sample analysis. EGCG levels were analyzed using an HPLC system consisting of 2 ESA Model 580 dual-piston pumps, a Waters Model 717plus refrigerated autosampler, and an ESA 5500 coulochem electrode array system (CEAS). The potentials of the CEAS were set at −100, 100, 300, and 500 mV. Separation was achieved using previously described methods (12). The exposure (area under the curve, AUC) and maximum concentrations (C_{max}) of EGCG were determined using Microsoft Excel. Values are means ± SE. Differences were determined using Student's *t* test or ANOVA with Tukey's test, as appropriate, and were considered significant at $P < 0.05$.

RESULTS

Piperine inhibits small intestinal but not hepatic glucuronidation. Mouse small intestinal and hepatic microsomes were both capable of rapidly glucuronidating EGCG on the B-ring and D-ring (data not shown). Piperine (50–500 μmol/L) dose-dependently inhibited the glucuronidation reaction with small intestinal microsomaes, but not with hepatic microsomes (**Fig. 2**). At 100 and 500 μmol/L, piperine inhibited small intestinal glucuronidation of EGCG by 40 and 60%, respectively. Hepatic glucuronidation of EGCG was not affected at these concentrations.

Piperine affects glucuronidation but not accumulation of EGCG by HT-29 cells. Incubation of HT-29 human colon cancer cells with 10 μmol/L EGCG resulted in intracellular levels of 90.4 ± 5.4, 15.6 ± 2.4, and 35.8 ± 4.3 nmol/mg

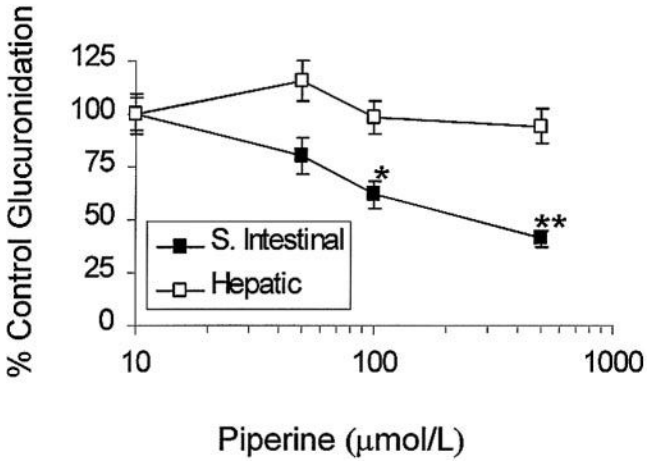

FIGURE 2 Piperine-mediated inhibition of mouse hepatic and small intestinal microsome-mediated glucuronidation of EGCG. Each point represents the mean ± SE, $n = 3$. Asterisks indicate different from control incubations performed in the absence of piperine: *$P < 0.05$, **$P < 0.01$.

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protein of EGCG, EGCG-3″-glucuronide, and 4″-*O*-methyl-EGCG, respectively (**Fig. 3**). Cotreatment with 20 μmol/L piperine reduced the concentration of EGCG-3″-glucuronide to 8.1 ± 2.1 nmol/mg, but inceased the concentration of 4″-*O*-methyl-EGCG to 48.8 ± 6.7 nmol/mg (Fig. 3). The concentration of EGCG was unchanged. The total intracellular concentration of EGCG and its metabolites was unaffected by cotreatment with piperine (141.8 ± 12.1 vs. 146.4 ± 15.8 nmol/mg, for EGCG plus piperine and EGCG alone, respectively).

Piperine increases the plasma EGCG concentration. Coadministration of piperine (70.2 μmol/kg, i.g.) and EGCG (163.8 μmol/kg, i.g.) substantially increased the plasma levels of EGCG in male CF-1 mice compared to those treated only with EGCG (**Fig. 4**A). The C_{max} of total EGCG following treatment with EGCG and piperine was 1.1-fold higher compared to treatment with EGCG only. Unconjugated EGCG was present at much lower levels in both treatment groups but was still substantially increased by cotreatment with piperine. Cotreatment with piperine increased the $AUC_{60-300min}$ by 1.2- and 1.3-fold for total EGCG and unconjugated EGCG, respectively (**Table 1**). The ratio of unconjugated to total EGCG in mice treated with EGCG plus piperine (0.18 ± 0.03) did not differ from that in mice treated with EGCG alone (0.19 ± 0.05).

Piperine affects gastrointestinal levels of EGCG in CF-1 mice. Following coadministration of piperine and EGCG to male CF-1 mice, the levels of EGCG in the small intestine reached a maximum of 43.3 ± 20.0 nmol/g tissue at 90 min and remained above 20 nmol/g until 180 min (Fig. 4*B*). In contrast, mice treated with EGCG alone had a maximal small intestinal level of 44.2 ± 26.7 nmol/g at 60 min after dosing, after which EGCG levels declined rapidly and were only 5.1 ± 1.6 nmol/g at 90 min after dosing (Fig. 4*B*). Piperine increased the small intestine EGCG AUC by 1.7-fold compared to intestinal tissue from mice treated with EGCG alone (Table 1).

The levels of EGCG in the colon following piperine coadministration were lower than that in mice treated with EGCG alone at the earliest time points but rose to equivalent levels by 300 min (Fig. 4C). The colon levels of EGCG in mice fed EGCG alone were substantially higher than that in mice cotreated with piperine (Table 1). Consistent with our previous results (3), EGCG was exclusively in the unconjugated form in both the small intestine and the colon (data not shown).

Piperine decreases excretion of EGCG in the feces. Cotreatment of mice with piperine and EGCG decreased the appearance of EGCG in the feces relative to mice treated with EGCG alone (Fig. 4*D*). At 60 min, the concentrations of EGCG in the feces were 0.04 ± 0.015 and 0.30 ± 0.22 μmol/g in mice treated with EGCG plus piperine and EGCG alone, respectively. At 5 h, the levels of EGCG in the feces of piperine-cotreated mice (0.83 ± 0.22 μmol/g) still was less than in mice treated with EGCG alone (1.76 ± 0.50 μmol/g).

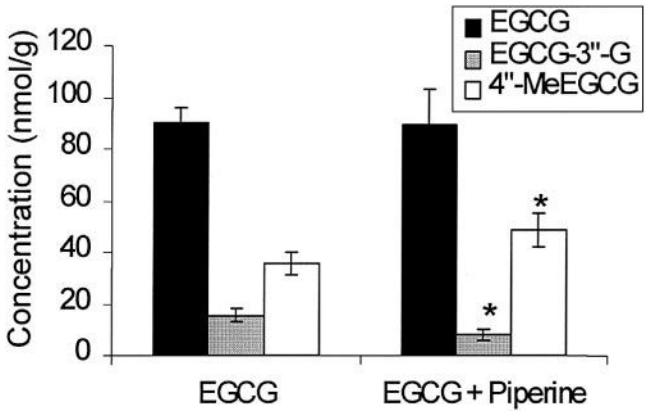

FIGURE 3 Intracellular accumulation of EGCG, EGCG-3″-glucuronide, or 4″-*O*-methyl EGCG by HT-29 human colon cancer cells treated with EGCG (10 μmol/L) or EGCG (10 μmol/L) plus piperine (20 μmol/L). Bars represent the means ± SE, $n = 3$. *Different from EGCG alone, $P < 0.05$.

DISCUSSION

In the present study, we sought to determine whether the black pepper alkaloid, piperine, could serve as a potential dietary modulator of the bioavailability of the green tea catechin, EGCG, in mice. Previously, piperine was shown to inhibit the glucuronidation of 3-hydroxybenzo[a]pyrene by small intestinal and hepatic microsomes (7,13). Moreover, coadministration of piperine and curcumin substantially enhanced the bioavailability of curcumin in humans and rats compared to treatment with curcumin only (9). Given that EGCG is subject to glucuronidation, we hypothesized that piperine could inhibit this glucuronidation and therefore increase EGCG bioavailability in mice.

In the current study piperine dose-dependently inhibited glucuronidation of EGCG by small intestinal but not hepatic microsomes (Fig. 2). This difference in sensitivity may be the result of differential expression of UGT isoforms in these 2 tissues. Piperine also inhibited EGCG glucuronidation in HT-29 human colon cancer cells. In this model, cotreatment of cells with EGCG and piperine did not increase the total cytosolic level of EGCG and its metabolites. It did, however, decrease the level of EGCG-3″-glucuronide and concomitantly increase the level of 4″-*O*-methyl-EGCG compared to cells treated only with EGCG.

Following cotreatment of male CF-1 mice with EGCG (163.8 μmol/kg, i.g.) and piperine (70.2 μmol/kg, i.g.), plasma levels of total and unconjugated EGCG increased by 1.1- and 1.4-fold, respectively, compared to mice treated with EGCG alone. Piperine also substantially increased the AUC of both total and unconjugated EGCG by 1.2- and 1.3-fold, respectively. We predicted that if piperine inhibited glucuronidation in vivo, it would increase not only the total amount of EGCG in the plasma, but also the ratio of unconjugated to total EGCG. We found, however, that cotreatment with piperine did not affect this ratio. Selective inhibition of small intestinal glucuronidation by piperine could increase the absorption of EGCG into the portal circulation, but may not affect the overall profile of EGCG glucuronidation since the compound could be subsequently glucuronidated by the liver. The results of our inhibition studies with mouse hepatic and small intestinal microsomes support this hypothesis. An additional potential confounder for the hypothesis that piperine increased EGCG bioavailability by inhibiting glucuronidation is the observed compensation by methylation in the HT-29 cells. These results suggest that any inhibition of glucuronidation is compensated for by methylation, which would negate any increase in EGCG bioavailability. However, we previously reported that the capacity for glucuronidation of EGCG is greater than the capacity for its methylation in the mouse small intestine (5,6). Based on this, we suggest that even if methylation of EGCG compensates for some of the piperine-mediated inhibition of glucuronidation, the increased amount of free EGCG may exceed methylation capacity and result in

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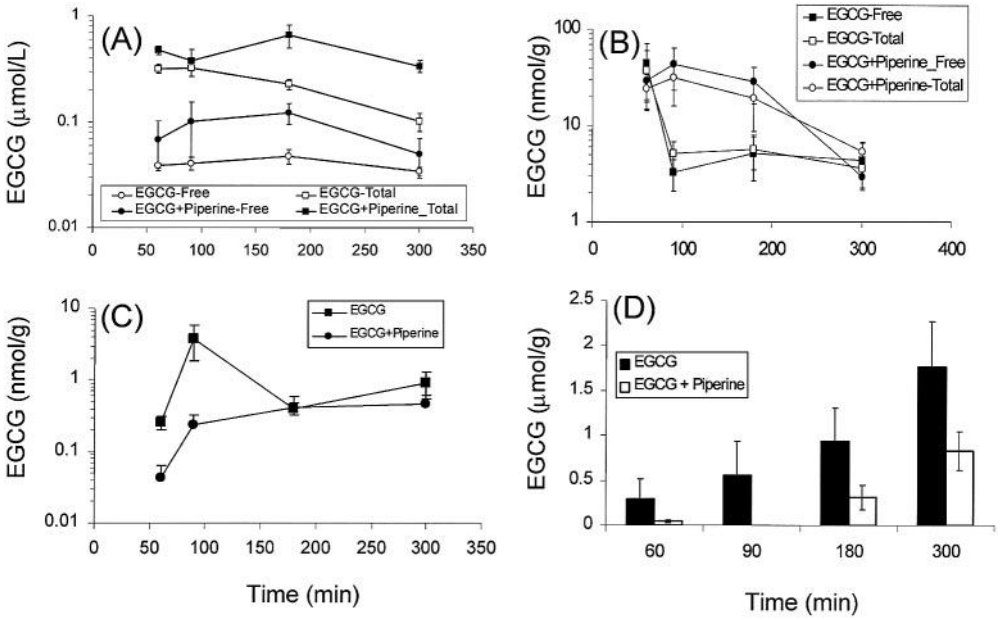

FIGURE 4 Plasma (*A*), small intestinal (*B*), colonic (*C*), and fecal (*D*) levels of EGCG following intragastric administration of either EGCG (163.8 μmol/kg) or EGCG plus piperine (70.2 μmol/kg) to male CF-1 mice. Each point represents the mean ± SE, $n = 6$.

a net increase in EGCG. The extent to which methylation compensates for glucuronidation following piperine treatment remains to be determined in vivo.

The lack of change in the ratio of total to unconjugated EGCG suggests that other factors might also play a role in the piperine-mediated increase in EGCG bioavailability. Piperine was shown to substantially inhibit gastric emptying and gastrointestinal transit in mice and rats (13). Inhibition of gastrointestinal transit was demonstrated to increase the bioavailability of many drugs (14,15).

We hypothesized that piperine, if it increased EGCG plasma levels by inhibiting gastrointestinal transit, should also increase both the levels and the residence time of EGCG in the small intestine, while simultaneously delaying the arrival of EGCG in the colon and excretion in the feces. Consistent with this hypothesis, we found that following piperine treatment, the levels of EGCG in the small intestine remained higher for a longer period of time compared to mice treated only with EGCG. The small intestine EGCG AUC was increased by 1.7-fold by cotreatment with piperine. Appearance of EGCG in the colon of mice cotreated with piperine was somewhat delayed and levels remained lower than in mice treated only with EGCG. Finally, EGCG appeared in the feces of mice cotreated with piperine at a slower rate than in mice treated only with EGCG. These changes in the gastrointestinal levels and kinetics of EGCG support the hypothesis that piperine increased plasma levels at least in part by delaying transit through the gastrointestinal (GI) tract. Alternatively, the changes in fecal and colon levels of EGCG may reflect an increase in the net overall absorption of EGCG from the upper gastrointestinal tract, thereby decreasing the pool reaching the colon and feces for absorption or excretion. Further studies are needed for clearer delineation.

Piperine was also reported to inhibit digoxin and cyclosporine A transport by *p*-glycoprotein (PGP) in Caco-2 cells; this action might also be hypothesized to affect EGCG bioavailability (16). However, we previously showed that EGCG is a substrate for the multidrug resistance related protein (MRP)-1

TABLE 1

Effect of piperine on AUC and C_{max} of EGCG in the plasma and tissues of male CF-1 mice after intragastric administration of EGCG or EGCG plus piperine[1]

	C_{max}		$AUC_{60–300}$	
	EGCG	EGCG + Piperine	EGCG	EGCG + Piperine
	μmol/L		*μmol/L · min*	
Plasma				
Unconjugated	0.05 ± 0.01	0.12 ± 0.04[a]	9.95 ± 1.09	22.67 ± 4.18[a]
Total	0.32 ± 0.05	0.66 ± 0.16[a]	53.82 ± 7.95	118.71 ± 24.99[a]
	nmol/g		*nmol/g · min*	
S. intestine, total[2]	37.50 ± 22.50	31.60 ± 15.68	1686.50 ± 757.07	4621.80 ± 1958.72[a]
Colon, total[2]	3.76 ± 1.93	0.47 ± 0.14[a]	325.10 ± 109.58	85.20 ± 25.44[a]

[1] Values are means ± SE, $n = 6$. [a] Different from the EGCG only group, Student's *t* test, $P < 0.01$.
[2] All unconjugated.

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and MRP-2 but not for PGP (11). It is likely, therefore, that this activity of piperine is unrelated to its ability to enhance EGCG bioavailability.

In conclusion, the present results demonstrate that piperine, a component of the widely consumed spice black pepper, can increase the bioavailability of EGCG, a component of green tea. Mechanistically, it appears that piperine inhibits small intestinal glucuronidation of EGCG, which may result in increased absorption, and that piperine may also slow the GI transit of EGCG, thus increasing residence time in the intestine and allowing for greater absorption. More in-depth mechanistic studies are required to fully establish the relative importance of each of these mechanisms. The increase in plasma bioavailability for EGCG may improve its cancer preventive activity in vivo. Moreover, the effect of a second dietary component on the bioavailability of EGCG suggests that variations in dietary habits may impact the outcome of epidemiological studies of the health effects of green tea. Such effects should be further studied.

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Cancer and metastasis: prevention and treatment by green tea

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Abstract

Metastasis is the most deadly aspect of cancer and results from several interconnected processes including cell proliferation, angiogenesis, cell adhesion, migration, and invasion into the surrounding tissue. The appearance of metastases in organs distant from the primary tumor is the most destructive feature of cancer. Metastasis remains the principal cause of the deaths of cancer patients despite decades of research aimed at restricting tumor growth. Therefore, inhibition of metastasis is one of the most important issues in cancer research. Several *in vitro*, *in vivo*, and epidemiological studies have reported that the consumption of green tea may decrease cancer risk. (−)-Epigallocatechin-3-gallate, major component of green tea, has been shown to inhibit tumor invasion and angiogenesis which are essential for tumor growth and metastasis. This article summarizes the effect of green tea and its major polyphenolic compounds on cancer and metastasis against most commonly diagnosed cancer sites.

Keywords

Cancer; EGCG; Green tea; Metastasis; Tumor growth

1 Introduction

Cancer is recognized worldwide to be a major health problem. A total of 1,479,350 new cancer cases and 562,340 deaths from cancer were projected to occur in the United States in 2009 [1]. The main reason for such a high mortality from cancer is due to the highly invasive behavior of cancer cells, which usually results in cancer progression and metastasis. Metastasis is the process whereby neoplastic cells spread from a primary site where the primary tumor originated to distant organs and is responsible for the majority of deaths related to cancer. The metastatic process involves tumor cell invasion from the primary tumor, intravasation, arrest, and extravasation of the circulatory system, followed by angiogenesis and growth at a distant site [2, 3]. For successful manifestation of metastasis, all steps in the metastatic cascade must be completed. Therefore, the blockade of any single step in the metastatic cascade would be expected to slow metastasis. Progression towards a metastatic phenotype requires a concerted effort between different molecules that have been implicated in advancing one or more steps of the metastatic cascade [4]. It is a complex process which is dependent on both host and tumor properties for the dissemination of malignant cells throughout the body and their survival to form secondary growths. Metastatic cancer cells require properties that allow them not only to adapt to a foreign

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microenvironment but to subvert it in a way that is conducive to their continued proliferation and survival.

Despite improvements in diagnosis, surgical techniques, patient care, and adjuvant therapies, most deaths from cancer are due to metastasis that is resistant to conventional therapies, direct organ damage by the growing lesions, paraneoplastic syndromes, or from the complications of treatment [5]. Several reasons account for treatment failure in patients with metastases. The major obstacle to effective treatment is the heterogeneity of the tumor cells which contain subpopulations of cells with different angiogenic, invasive, and metastatic properties. Although metastases can have a clonal origin, genetic instability results in rapid biological diversification and the regeneration of heterogeneous subpopulations of cells. The specific organ environment can influence the biological behavior of metastatic cells, including their response to systemic therapy as the metastases can be located in lymph nodes and different organs [2]. The outcome of cancer metastasis depends on multiple interactions between metastatic cells and homeostatic mechanisms that are unique to one or another organ microenvironment. The specific organ microenvironment determines the extent of cancer cell proliferation, angiogenesis, invasion, and survival. Therefore, the therapy of metastasis should be targeted against tumor cells and the host factors that contribute to and support the progressive growth and survival of metastatic cancer cells.

Tea, derived from the plant *Camellia sinensis*, is the most globally consumed beverage as green, black, or Oolong tea. [6, 7]. It is estimated that about 2.5 million tons of tea leaves are produced throughout the world each year with 20% produced as green tea, which is mainly consumed in Asia, some parts of North Africa, the United States, and Europe [8]. The most significant effects on human health have been attributed to green tea. It contains characteristic polyphenolic compounds, (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epicatechin (EC). [9]. Catechin, gallocatechin, epigallocatechin digallate, epicatechin digallate, 3-*O*-methyl EC and EGC, catechin gallate (CG), and gallocatechin gallate are present in smaller quantities. EGCG has an antioxidant activity about 25–100 times more effective than that of vitamins C and E and appears to be the most potent of all the catechins [10]. The anti-cancer effects of EGCG have been reported to be linked to the modulation of multiple signaling pathways, finally resulting in the downregulation of expression of proteins involved in the invasiveness of cancer cells [11]. In this review article, we discuss the modulation of signaling pathways responsible for invasive behavior and metastasis of different cancer types by green tea [Tables 1 and 2].

2 Green tea and metastasis of skin cancer

The skin cancer incidence is increasing by epidemic proportions. A trimethoxy derivative of ECG, 3-O-(3,4,5-trimethoxybenzoyl)-(−)-epicatechin (TMECG), is a prodrug that is selectively activated by the specific melanocyte enzyme tyrosinase. The treatment of melanoma cells with TMECG affected cellular folate transport and the gene expression of dihydrofolate reductase. It also inhibited tumor growth and metastasis in a mouse melanoma model, significantly enhancing the mean survival of the treated groups [12]. Treatment with a diet containing lysine, proline, arginine, ascorbic acid, and green tea extract to athymic nude mice implanted with human melanoma A2058 cells strongly suppressed tumor growth with inhibition of MMP-9 and VEGF secretion [13]. In SKH-1 hairless mice, oral administration of green tea polyphenols (GTP) reduced ultraviolet (UV)B-induced tumor incidence, tumor multiplicity, and tumor growth. The group treated with UVB and given GTP had reduced expression of the matrix metalloproteinases (MMP)-2 and -9, which have crucial roles in tumor growth and metastasis, enhanced expression of tissue inhibitor of MMP (TIMP), reduced expressions of CD31 and vascular endothelial growth factor

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(VEGF), increased expression of proliferating cell nuclear antigen (PCNA), more cytotoxic CD8(+) T cells, and increased activation of caspase-3 in the tumors as compared with group treated with UVB alone. It was concluded that administration of GTP caused inhibition of angiogenic factors and recruitment of cytotoxic T cells in the tumor microenvironment [14]. UVB-induced skin tumors with and without treatment of EGCG and age-matched skin biopsies from SKH-1 hairless mice were used to identify potential molecular targets of skin cancer prevention by EGCG. Topical application of EGCG in UV-induced tumors resulted in the inhibition of protein expression and activity of MMP-2 and -9, increased expression of TIMP, and decreased expression of CD31 and PCNA [15].

EGCG dose-dependently inhibited B16-F3m melanoma cell migration and invasion and inhibited the spread of melanoma cells on fibronectin, laminin, collagen, and Matrigel in a dose-dependent manner. EGCG significantly inhibited the tyrosine phosphorylation of focal adhesion kinase (FAK) and the activity of MMP-9. EGCG also caused reduction in the lung metastases in mice bearing B16-F3m melanomas, but a combination of EGCG and dacarbazine was more effective than EGCG alone in reducing the number of pulmonary metastases and primary tumor growths and in increasing the survival rate of melanoma-bearing mice [16]. EGCG and ECG were found to inhibit melanoma cell adhesion in the culture medium. The adhesion of murine melanoma cells to laminin was impaired on pretreatment with EGCG [17]. EGCG significantly upregulated the expression of E-cadherin time and concentration dependently in human malignant melanoma A375 cell line. EGCG inhibited the invasion of human malignant melanoma cell line which correlated with the upregulation of E-cadherin expression [18].

3 Green tea and metastasis of prostate cancer

In U.S. men, prostate cancer (PCa) is the most common non-cutaneous malignancy. Treatment of PCa PC-3 cells with Traditional Botanical Supplement-101 (TBS-101), a botanical agent containing standardized botanical extracts of *Panax ginseng*, cranberry, green tea, grape skin, grape seed, *Ganoderma lucidum*, and chamomile resulted in dose-dependent inhibition of cell growth with concomitant induction of apoptosis. On treatment with TBS-101, mice bearing moderate or large tumors showed significant inhibition of tumor growth and invasion while control group mice had significant tumor growth and lymph node metastasis. No toxicity was reported in healthy or tumor-bearing mice with high doses of TBS-101 [19]. Study from our laboratory has shown for the first time that EGCG sensitizes TRAIL-resistant PCa LNCaP cells to TRAIL-mediated apoptosis through modulation of intrinsic and extrinsic apoptotic pathways. Combination of EGCG and Apo2L/TRAIL caused induction of apoptosis accompanied by the upregulation of poly (ADP-ribose) polymerase (PARP) cleavage and modulation of pro- and antiapoptotic Bcl2 family of proteins. Pretreatment of cells with EGCG resulted in modulation of death-inducing signaling cascade complex involving DR4/TRAIL R1, Fas-associated death domain, and FLICE-inhibitory protein. There was also synergistic inhibition in the invasion and migration of PCa cells mediated through inhibition in the protein expression of VEGF, uPA, and angiopoietin-1 and -2. Additionally, on treatment of cells with a combination of EGCG and TRAIL, there was decrease in the activity and protein expression of MMP-2, -3, and -9 and upregulation of TIMP1 [20].

The effects of a diet containing lysine, proline, arginine, ascorbic acid, and green tea extract on the growth of tumors induced by implanting human PCa PC-3 cells in athymic nude mice and on the expression of MMPs, VEGF, Ki-67, and fibronectin in these tumors, as well as the production of mucin, were investigated. It was found that there was inhibition of tumor growth and inhibition of MMP-9 and VEGF secretion and mitosis in tissues of group treated with the nutrient mixture of the diet [21]. The expression of metastasis-promoting Mts1 gene

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(S100A4) was assessed in GTP-treated transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Freshly prepared 0.1% GTP solution in tap water was supplied thrice a week to experimental animals as the sole source of drinking fluid for 24 weeks, while the control group of animals received the same tap water throughout the study. The animals were sacrificed at 0, 8, 16, and 24 weeks of GTP feeding and were analyzed for S100A4 and E-cadherin. An increase in the expression of S100A4 at mRNA and protein level in dorsolateral prostate, but not in nontransgenic mice, was found with the progression of age and PCa growth in TRAMP mice. There was inhibition of PCa progression in mice fed with GTP which was associated with reduction of S100A4 and restoration of E-cadherin [22].

Our laboratory study demonstrated the role of insulin growth factor (IGF)-I/IGF binding protein (IGFBP)-3 signaling and its downstream and other associated events during chemoprevention of PCa by GTP in TRAMP mice. There were increased levels of IGF-I, phosphatidylinositol 3′-kinase (PI3K), phosphorylated Akt, and extracellular signal-regulated kinase (ERK)1/2 with concomitant decrease in IGFBP-3 in dorsolateral prostate of TRAMP mice during the course of cancer progression. By continuous GTP infusion for 24 weeks to mice, there was substantial reduction in the levels of IGF-I and significant increase in the levels of IGFBP-3 in the dorsolateral prostate. This modulation of IGF/IGFBP-3 was found to be associated with an inhibition of protein expression of PI3K, phospho-Akt, and ERK 1/2 with concomitant inhibition of markers of angiogenesis and metastasis such as VEGF, uPA, and MMP-2 and -9 [23]. The effects of EGCG were investigated on the expression and activity of PSA in PCa cells. EGCG was found to inhibit degradation of gelatin, degradation of type IV collagen in reconstituted basement membrane (Matrigel), and activation of MMP-2 but not pro-MMP-9 in a cell-free system in a dose-dependent manner at concentrations lower than the cytotoxic serine-protease inhibitor phenylmethyl sulfonyl fluoride and close to levels measured in the serum following ingestion of green tea [24]. The synergistic effects between soy and tea components on prostate tumor progression in a mouse model of orthotopic androgen-sensitive human PCa were identified. Soy phytochemical concentrate (SPC), black tea, and green tea significantly reduced tumorigenicity, while SPC and black tea also significantly reduced final tumor weights. Green tea did not reduce final tumor weight, but it elevated serum dihydrotestosterone (DHT) concentration. There was inhibition of prostate tumorigenicity, final tumor weight, and metastases to lymph nodes *in vivo* by combination of SPC and black tea while the combination of SPC and green tea inhibited final tumor weight and metastasis and significantly reduced serum concentrations of both testosterone and DHT *in vivo*. Inhibition of tumor progression was associated with reduced tumor cell proliferation and tumor angiogenesis [25].

We have reported that oral infusion of GTP at a human achievable dose, i.e., equivalent to six cups of green tea per day, significantly inhibited PCa development and increased survival in male TRAMP mice. GTP provided as the sole source of drinking fluid to TRAMP mice from 8 to 32 weeks of age caused significant delay in primary tumor incidence and tumor burden, significant decrease in prostate and genitourinary weight, significant inhibition in serum IGF-I, and restoration of IGFBP-3 levels and marked reduction in the protein expression of PCNA in the prostate compared with water-fed TRAMP mice. More importantly, GTP infusion was found to result in almost complete inhibition of distant site metastases to lymph nodes, lungs, liver, and bone. There was also significant apoptosis of PCa cells resulting in the reduced dissemination of cancer cells, thereby causing inhibition of PCa development, progression, and metastasis of PCa to distant organ sites by GTP consumption [26].

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4 Green tea and metastasis of breast cancer

Metastasis of breast cancer is the major reason for the high mortality of breast cancer patients and is directly linked to the invasive behavior of breast cancer cells. Cancer metastasis consists of several interdependent processes including cancer cell adhesion, cancer cell migration, and invasion of cancer cells. Recently, the effects of EGCG treatment on growth and invasion in a breast carcinoma cell line resistant to tamoxifen (MCF-7Tam) and parental MCF-7 were reported. Treatment with EGCG caused dose-dependent downregulation of epidermal growth factor receptor (EGFR) phoshoporylation and EGFR mRNA expression and protein level in MCF-7Tam cells. There was also decrease in ERK1/2, phospho-ERK1/2, *in vitro* cell growth, MMP-2 and -9, and extracellular MMP-inducer while increase in TIMP-1 and -2 after EGCG treatment [27]. EGC has been shown to inhibit heregulin (HRG)-β1-induced migration/invasion of MCF-7 human breast carcinoma cells to approximately the same extent as EGCG. It was found that EGCG inhibited this migration/invasion by suppressing the HRG-stimulated activation of EGFR-related protein B2 (ErbB2)/ErbB3/Akt, whereas the disruption of the HRG-stimulated activation of ErbB2/ErbB3 but not Akt was involved in the inhibition of migration/invasion by EGC. It was concluded that EGC and EGCG could play important role against the promotion of metastasis of breast cancer cells [28].

Treatment with EGCG reduced the activity, protein expression, and mRNA expression level of MMP-2. It also caused reduction on the expression of FAK, membrane type-1-MMP, nuclear factor-kappa B (NF-κB), and VEGF and reduced the adhesion of MCF-7 cells to extracellular matrix, fibronectin, and vitronectin. EGCG treatment also led to a reduction in the expression of integrin receptors α5, β1, αv, and β3 as observed by real time RT-PCR [29]. GTP has been reported to inhibit cell growth and invasive behavior of human breast cancer MDA-MB-231 cells. It also caused inhibition of constitutively active transcription factors AP-1 and NF-κB, which further suppressed secretion of uPA from breast cancer cells. GTP treatment resulted in the inhibition of formation of signaling complexes responsible for cell adhesion and migration viz., uPA, uPA-receptor, vitronectin, and integrin receptor by inhibiting the invasive behavior of breast cancer cells [30].

The effects of GTP on growth and metastasis of highly metastatic mouse mammary carcinoma 4T1 cells were examined in *in vitro* and *in vivo* systems. Treatment of metastatic mouse mammary carcinoma 4T1 cells with EGCG resulted in inhibition of cell proliferation, induction of apoptosis, decrease in the protein expression of Bcl-2, increase in Bax, cytochrome *c* release, Apaf-1, and cleavage of caspase-3 and PARP proteins. Treatment of EGCG-rich GTP in drinking water to 4T1 cells bearing BALB/c mice resulted in reduction of tumor growth, increase in Bax/Bcl-2 ratio, reduction in PCNA, and activation of caspase-3 in tumors. GTP treatment also caused inhibition of metastasis of tumor cells to lungs and increase in the survival period of animals [31]. The association between consumption of green tea prior to clinical cancer onset and various clinical parameters assessed at surgery among 472 patients with stage I, II, and III breast cancer were examined. There was decrease in the numbers of auxiliary lymph node metastases among premenopausal patients with stage I and II breast cancer and increased expression of progesterone receptor and estrogen receptor among postmenopausal patients on increased consumption of green tea. In a 7-year follow-up of stage I and II breast cancer patients, increased consumption of green tea was correlated with decreased recurrence of stage I and II breast cancer. The recurrence rate was 16.7% in those patients consuming ≥5 cups and 24.3% in patients consuming ≤4 cups/day with 0.564 relative risk of recurrence after adjustment for other lifestyle factors. This showed that increased consumption of green tea prior to clinical cancer onset was significantly associated with improved prognosis of stage I

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and II breast cancer, and this association may be related to a modifying effect of green tea on the clinical characteristics of the cancer [32].

5 Green tea and metastasis of lung cancer

Lung cancer is the most common cancer in the world and represents a major public health problem. The preventive effect of green tea catechins intake on lung tumor metastasis was examined in senescence-accelerated mice prone (SAMP) 10. Green tea catechins intake increased natural killer cell activity, which is an indicator of immune surveillance potential and was reduced in control group mice with age. Mice were given intravenous injection of the melanoma cells and the early accumulation of lung-metastatic K1735M2 melanoma cells and the subsequent experimental lung metastasis was investigated after treatment with green tea catechins. The accumulation at 6 and 24 h after injection of melanoma cells and the number of lung-metastatic colonies were significantly reduced in mice treated with green tea catechins as compared to mice in control group suggesting that green tea catechin intake prevented the experimental tumor metastasis in aged SAMP10 mice via inhibition of the reduction in immune surveillance potential with age [33]. The effect of cytokines, mitogens, and inhibitors on MMP-2 and -9 expressions in human lung cancer A549 cells malignant melanoma MSTO-211H cells was investigated. EGCG inhibited MMP-2 and -9 expressions in both cell lines [34]. It has been shown that EGCG had an inhibitory effect on bronchial tumor cells migration in 2D and 3D cell culture models. Treatment with EGCG also inhibited MMP-2 mRNA and protein expression and altered the intermediate filaments of vimentin [35].

The effects of a nutrient mixture consisting of ascorbic acid, lysine, proline, arginine, and green tea extract were investigated on lung metastasis by B16F0 melanoma cells in C57BL/6 female mice. Pulmonary metastatic colonies were counted after 2 weeks of nutrient mixture supplementation. Pulmonary colonization was reduced by 63% in mice receiving nutrient mixture in diet, whereas, it was reduced by 86% in mice receiving nutrient mixture by intraperitoneal and intravenous injections and completely inhibited in mice injected with melanoma cells pretreated with nutrient mixture [36]. This nutrient mixture supplementation to athymic nude mice implanted with human lung cancer A549 cells suppressed the tumor growth without adverse effects. It also inhibited the secretion of both MMP-2 and -9 with reduction in the invasion of human lung carcinoma cells through Matrigel in a dose-dependent fashion [37]. Treatment with EGCG reduced lung metastases in mice bearing B16-F3m melanomas while a combination of EGCG and dacarbazine was more effective in reducing the number of pulmonary metastases and primary tumor growths and increased the survival rate of melanoma-bearing mice [16]. EGCG inhibited the invasion of lung carcinoma 95-D cells in invasion assay and downregulated the expression of MMP-9 and NF-κB in a dose-dependent manner [38]. Theaflavin, theaflavin digallate, and EGCG inhibited invasion of highly metastatic mouse Lewis lung carcinoma LL2-Lu3 cells. They also inhibited MMP-2 and -9 from the culture medium of these tumor cells suggesting that these compounds inhibit tumor cell invasion by inhibiting type IV collagenases [39]. In a spontaneous metastasis system, the administration of green tea infusion reduced the number of lung colonies of mouse Lewis lung carcinoma cells which was attributed to the inhibitory effects of the green tea infusion and its constituent catechins on the penetration of the cells through the basement membrane [40].

6 Green tea and metastasis of liver cancer

Hepatocellular carcinoma is a growing health problem worldwide and only few promising treatment options are available at present, stressing the urgent need for novel therapeutic approaches. The effect of a nutrient mixture containing lysine, proline, arginine, ascorbic

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acid, and green tea extract on tumor growth and hepatic metastasis were investigated in athymic nude male mice inoculated with 10(6) B16FO melanoma cells. Metastasis was studied in C57BL/6 mice receiving melanoma cells by intrasplenic injection, as well as a regular or 0.5% nutrient mixture-supplemented diet for 2 weeks. Nutrient mixture inhibited the growth of melanoma cells, and the lesions were consistent with malignant melanoma. Mice were also injected with melanoma cells in the spleen. Control group animals developed large black spleens and livers indicating growth in the spleen and metastasis to the liver, while mice supplemented with nutrient mixture showed less growth in spleen and reduced metastasis to the liver. The survival time was also greater in mice receiving nutrient mixture supplementation than animals on the regular diet [41].

EGCG, EGC, and CG were reported as the major phenolic phytochemicals found in red pine leaves. It was found that red pine leaf extract, EGCG, and CG suppressed the invasion and the migration of human hepatocellular carcinoma cells SK-Hep-1 cells. Red pine leaf extract, EGCG, and CG suppressed the activities of both MMP-2 and MMP-9 with EGC exhibiting a lower efficacy on both MMPs with EGC exhibiting a lower efficacy [42]. There was much higher inhibition of hepatocellular carcinoma SMMC-7721 cell proliferation and migration by mixture of EGCG and ascorbic acid as compared with EGCG or ascorbic acid alone. Fluorographic analysis of oxidative stress revealed that ascorbic acid enhanced the antioxidant activity of EGCG by decreasing the intracellular oxidative stress. It was concluded in the study that the combination of EGCG and ascorbic acid strongly suppressed the proliferation and metastasis of liver cancer cells, possibly with a mechanism associated with the scavenging of reactive oxygen species [43]. The effect of the combination of theanine with doxorubicin was investigated against hepatic metastasis of M5076 ovarian sarcoma cell line transplanted subcutaneously in mice. The liver weight increased to twice the normal level because of hepatic metastasis in the control group whereas treatment with theanine and doxorubicin suppressed the increase in liver weight and hepatic metastasis. Theanine enhanced the inhibition of hepatic metastasis induced by doxorubicin as demonstrated by liver weights and metastasis scores. Theanine also increased the intracellular concentration of doxorubicin remaining in ovarian sarcoma cells proving that theanine caused enhancement of the suppressive efficacy of doxorubicin on hepatic metastasis *in vivo* [44].

7 Green tea and metastasis of colon cancer

Colorectal cancer is the second most deadly cancer in the United States. Current treatment options offer partial success when diagnosed early; however, radiation and chemotherapy are generally ineffective once metastasis occurs. Human colon cancer cell lines HCT116 and HT29 were used to examine the relationships between Met activation, EGCG treatment, and generation of H_2O_2. EGCG markedly suppressed the activation of Met in the presence of hepatocyte growth factor. The concentrations of ≤10 μM EGCG generated low amounts of H_2O_2, whereas higher H_2O_2 concentrations were required to directly increase the phosphorylation of Met. The activation of Met by EGCG occurred in the presence or absence of catalase showing that EGCG might be a beneficial therapeutic agent in the colon, inhibiting Met signaling and helping to attenuate tumor spread/metastasis, independent of H_2O_2-related mechanisms [45].

It has been reported that basic fibroblast growth factor (bFGF) protein was quickly degraded in the presence of EGCG, but proteasome inhibitor suppressed this degradation. There was increased ubiquitination of bFGF and trypsin-like activity of the 20S proteasome by EGCG, thereby resulting in the degradation of bFGF protein. EGCG was also found to inhibit intestinal tumor formation in APC (Min/+) mice, compared with vehicle-treated mice, in association with reduced bFGF expression [46]. Diet of nutrient mixture containing amino

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acids, ascorbic acid, and green tea extract inhibited growth and reduced the size of tumors in nude mice implanted with human colon HCT 116 cells. In the control group tissues, increased mitotic index and MMP-9 and VEGF secretion were found, whereas, nutrient supplementation diet inhibited MMP-9 and VEGF secretion and mitotic index in the tumor tissues [47]. EGCG treatment increased both intracellular and extracellular pro-MMP-7 protein levels in dose- and time-dependent manner HT-29 human colorectal cancer cells with a significant upregulation of its mRNA expression. EGCG also activated ERK1/2, JNK1/2, and p38 MAPK, triggered the phosphorylation of c-JUN, and induced c-JUN/c-FOS, thereby increasing the DNA-binding activity of activator protein-1 (AP-1). EGCG-induced pro-MMP-7 production was attenuated by N-Acetyl-L-cysteine, superoxide (O_2^-) dismutase and catalase, suggesting an involvement of oxidative stress in these events. EGCG treatment also induced pro-MMP-7 expression in human colorectal adenocarcinoma Caco-2 cell line [48].

8 Green tea and metastasis of pancreatic cancer

It is difficult to detect pancreatic cancer in the early stage despite the development of more sophisticated diagnostic techniques and surgical resection provides the only option. Treatment with EGCG inhibited viability, capillary tube formation, and migration of human umbilical vein endothelial cells (HUVECs). There was reduction in Ki-67, PCNA, Von Willebrand factor, VEGF, CD31, VEGFR-2, ERK1/2, JNK1/2, p38, MMP-2, MMP-7, MMP-9, and MMP-12 and induction of apoptosis, caspase-3 activity, and p21/WAF1 in tumors of athymic nude mice implanted with human pancreatic cancer AsPC-1 cells suggesting that EGCG inhibited pancreatic cancer growth, invasion, metastasis, and angiogenesis [49]. In pancreatic cancer cell line MIA PaCa-2, nutrient mixture containing green tea exhibited a dose-dependent antiproliferative effect, decreased expression of MMP-9, and inhibition of invasion through Matrigel [50].

In hamsters, the inhibitory effect of green tea extract on the process of pancreatic carcinogenesis induced by N-nitrosobis-(2-oxypropyl)amine and on tumor promotion after transplantation of N-nitrosobis-(2-hydroxypropyl)amine (BHP)-induced pancreatic cancer were investigated. In the control group, seven of the 13 hamsters were found to have pancreatic tumors, while six of the 18 hamsters had pancreatic tumors in the green tea extract group. The average number of tumors in the control group was 1.0/hamster with pancreatic cancer incidence of 54% compared with the green tea extract group which had average number of tumors of 0.5/hamster with 44% incidence of pancreatic cancer. The number of pancreatic cancers, including invasive carcinoma, carcinoma *in situ*, and incidence of atypical ductal hyperplasia, which is thought to be an early pancreatic cancer, was significantly lower in the green tea extract group than in the control group. In a different experiment, 1 mm^3 pieces of BHP-induced pancreatic cancer were transplanted into the back of hamsters and were given tap water and green tea extract. Till 11 weeks after transplantation, tumor growth was similar in both groups, but inhibition of tumor growth became evident after 11 weeks in the green tea extract treated group. The average tumor volume in the green tea extract group was significantly lower than that in the control group at 13 weeks demonstrating that green tea extract had an inhibitory effect on the process of pancreatic carcinogenesis and on tumor promotion of transplanted pancreatic cancer in hamsters [51].

9 Green tea and metastasis of miscellaneous cancers

The effects of EGCG on the methylation status of the reversion-inducing cysteine-rich protein with Kazal motifs (RECK) gene and cancer invasion in oral squamous cell carcinoma cell lines were investigated. EGCG treatment of oral cancer cells partially

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reversed the hypermethylation status of the RECK gene and significantly enhanced the expression level of RECK mRNA with inhibition of MMP-2 and MMP-9 levels. EGCG also suppressed cancer cell-invasive ability by decreasing the number of invasive foci as well as invasion depth in 3D collagen invasion model [52]. A molecular epidemiologic study was conducted at Jiangsu Province of China, on histologically confirmed esophageal squamous cell carcinoma patients to investigate the association between aberrant hypermethylation of MGMT gene and clinical characteristics as well as MTHFR C677T genetic polymorphisms in esophageal squamous cell carcinoma. The aberrant hypermethylation rate of MGMT gene was 27.2% in cancer tissues and 11.2% in precancerous normal tissues among esophageal squamous cell carcinoma patients, while no hypermethylation was found in normal esophageal tissues from healthy adult subjects. In patients with lymph node metastasis, the methylation rate of MGMT gene in cancer tissues was significantly higher than in those without lymph node metastasis. Variant allele of MTHFR C677T was found to be associated with hypermethylation of MGMT gene after adjusting by potential confounders [53]. The inhibitory effects of EGCG on ephrin-A1-mediated cell migration and angiogenesis were reported. It was shown that ephrin-A1 mediated endothelial cell migration and regulated vascular remodeling in tumor neo-vascularization *in vitro*. Treatment with EGCG inhibited ephrin-A1-mediated endothelial cell migration, tumor angiogenesis, and phosphorylation of EphA2 and ERK-1/2 in a dose-dependent manner [54].

Expression of the metastasis-associated 67-kDa laminin receptor (LR) confers EGCG responsiveness to cancer cells at physiologically relevant concentrations [55]. The 67 LR is expressed on several tumor cells, and the expression level of this protein powerfully correlates with the risk of tumor invasion and metastasis [56, 57]. The antiangiogenic activities of EGCG are linked to 67 LR by the identification of a role for the 67 LR in retinal angiogenesis and its potent upregulation in malignant mesothelioma by gene expression profiling associated with tumor endothelial cells [58]. The effects of green tea extract on cell viability, cell proliferation, cell cycle dynamics, VEGF, and expression of VEGF receptors fms-like tyrosine kinase (Flt)-1 and fetal liver kinase (Flk)-1/kinase insert domain containing receptor (KDR) were studied *in vitro* using HUVECs. Treatment of cells with green tea extract did not affect cell viability but significantly reduced cell proliferation dose-dependently and caused a dose-dependent accumulation of cells in the G1 phase. There was also decrease in the expression of Flt-1 and Flk-1/KDR/in HUVECs on treatment with green tea extract suggesting that it affects tumor angiogenesis and metastasis through reduction in the expression of VEGF receptors [59]. EGCG suppressed the gelatin-degrading activities due to MMP-2 and MMP-9 in the culture medium of human fibrosarcoma HT1080 cells which were consistent with the decreased levels of MMP-2 and MMP-9 mRNAs. The suppression of the level of MMP-9 transcript on treatment with EGCG was correlated with its suppression of MMP-9 promoter activity. EGCG treatment also caused inhibition of the phosphorylation of ERK1/2 and p38 MAPK activity showing that suppression of ERK phosphorylation by EGCG was involved in the inhibition of MMP-2 and MMP-9 mRNAs, leading to the reduction of their enzyme activities in the cancer cells [60]. The effect of EGCG on the tube formation of HUVECs on Matrigel was investigated. EGCG treatment both prior to plating and after plating endothelial cells on Matrigel caused inhibition of tube formation and reduce the migration of endothelial cells in Matrigel plug model. Zymography revealed that EGCG-treated culture supernatants modulated the gelatinolytic activities of secreted proteinases demonstrating that EGCG may be exerting its inhibitory effect by regulating proteinases. Thus, these experiments showed that EGCG acts as an angiogenesis inhibitor by modulating protease activity during endothelial morphogenesis [61].

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10 Conclusions and perspectives

The possible cancer-preventive activity of green tea constituents has been studied extensively and the amount of experimental evidence documenting the properties of green tea, which affects multiple signaling pathways (Fig. 1) against metastasis of cancer is increasing rapidly. Metastasis is responsible for most deaths due to cancer and therefore, therapeutic strategies to prevent development of metastases have potential to impact on cancer mortality. However, a better understanding of the biology and molecular events of the metastatic process is required for the development of these therapies. In successfully treating cancer, focus should be on combating metastasis formation and growth. Significant improvements in early detection of cancer and development of effective novel therapeutic strategies targeting metastasis will help improve patient outcome. A better approach for the treatment of cancer seems to be the development of strategies to treat tumor cells and to modulate the host microenvironment. For better understanding of the interaction of green tea, employment of more specific and sensitive methods with more representative models of metastasis in conjunction with the development of good predictive biomarkers are required. Well-designed clinical and intervention trials will give the clear picture about the protective effects of green tea against metastasis of cancer.

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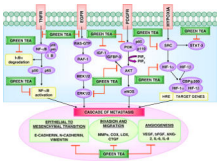

Fig. 1.
Modulation of signaling pathways and cascade of metastasis by green tea

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Table 1

Effect of green tea on metastasis of cancer in cell culture systems

Type of cancer	Mechanism	References
Skin Cancer	Inhibition of melanoma cell migration, invasion, spread of cells on fibronectin, laminin, collagen, and Matrigel, inhibition of the tyrosine phosphorylation of FAK and MMP-9 activity	[16]
	Impairment of the adhesion of murine melanoma cells to laminin	[17]
	Upregulation of the expression of E-cadherin time and concentration dependently in human malignant melanoma cell line	[18]
Prostate Cancer	Dose-dependent inhibition of cell growth with induction of apoptosis	[19]
	Combination of EGCG and Apo2L/TRAIL caused induction of apoptosis, upregulation of PARP, modulation of pro- and antiapoptotic Bcl2 family of proteins, inhibition in the invasion and migration of prostate cancer cells, inhibition of VEGF, uPA, angiopoietin-1,-2, decrease in MMP-2, -3, and -9, and upregulation of TIMP1	[20]
	Inhibition of the degradation of gelatin, type IV collagen in reconstituted basement membrane, and activation of MMP-2 but not pro-MMP-9	[24]
Breast Cancer	Dose-dependent downregulation of EGFR phoshoporylation, EGFR mRNA expression and protein level, decrease in ERK1/2, phosph-ERK1/2, *in vitro* cell growth, MMP-2 and -9, and increase in TIMP-1 and -2	[27]
	Inhibition of migration/invasion by suppressing the HRG-stimulated activation of ErbB2/ErbB3/Akt	[28]
	Decrease in MMP-2, FAK, MT1-MMP, NF-κB, VEGF, reduction in the adhesion of breast cancer cells to ECM, fibronectin, vitronectin and reduction in the expression of integrin receptors α5, β1, αv, and β3	[29]
	Inhibition of AP-1, NF-κB, uPA, uPA-R, vitronectin, and integrin receptor	[30]
Lung Cancer	Inhibition of MMP-2 and -9 and alteration of the intermediate filaments of vimentin	[31]
	Inhibition of the invasion of human lung carcinoma cells and downregulation of MMP-9 and NF-κB	[32]
	Inhibition of invasion of highly metastatic mouse Lewis lung carcinoma cells, downregualtion of MMP-2 and -9 and type IV collagenases	[39]
	Reduction in the number of lung colonies of mouse Lewis lung carcinoma cells, inhibition of penetration of the cells through the basement membrane in a spontaneous metastasis system	[40]
Liver Cancer	Suppression of the invasion and the migration of human hepatocellular carcinoma cells and activities of MMP-2 and -9	[42]
	Inhibition of the proliferation and metastasis of liver cancer cells with the scavenging of reactive oxygen species	[43]
Gastrointestinal Cancer	Suppression of the activation of Met in the presence of HGF in human colon cancer cells	[45]
	Increased ubiquitination of bFGF and trypsin-like activity of the 20S proteasome, thereby resulting in the degradation of bFGF protein in colon cancer cells	[46]
	Increase in both intracellular and extracellular pro-MMP-7 protein and mRNA expression levels, activation of ERK1/2, JNK1/2 and p38 MAPK, phosphorylation of c-JUN and induction of pro-MMP-7 production in human colorectal cancer cells	[48]
	Inhibition of viability, capillary tube formation and migration of HUVECs	[49]
	Dose-dependent antiproliferative effect, decreased expression of MMP-9 and inhibition of invasion through Matrigel in pancreatic cancer cells	[50]

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Table 2

Effect of green tea on metastasis of cancer in animal models

Type of cancer	Mechanism	References
Skin Cancer	Inhibition of tumor growth and metastasis in a mouse melanoma model, enhancement of the mean survival of the treated groups	[12]
	Suppression of tumor growth with inhibition of MMP-9 and VEGF secretion in athymic nude mice implanted with human melanoma cells	[13]
	In SKH-1 hairless mice, reduction of UVB-induced tumor incidence, tumor multiplicity, and tumor growth. Reduction of MMP-2 and -9, CD31, VEGF, and PCNA, increased activation of caspase-3, enhancement of TIMP, and inhibition of angiogenic factors and recruitment of cytotoxic T cells in the tumor microenvironment	[14]
	Inhibition of protein expression and activity of MMP-2 and -9 decreased expression of CD31 and PCNA and increased expression of TIMP	[15]
Prostate Cancer	Inhibition of tumor growth and invasion	[19]
	Inhibition of tumor growth, MMP-9, VEGF secretion and mitosis in tumors of athymic nude mice	[21]
	Inhibition of PCa progression associated with reduction of S100A4 and restoration of E-cadherin in TRAMP mice	[22]
	Reduction in IGF-I with increase in IGFBP-3 in the dorsolateral prostate in TRAMP mice. Inhibition of PI3K, p-Akt, ERK1/2, VEGF, uPA, MMP-2 and -9	[23]
	Inhibition of tumor weight and metastasis, reduction in serum concentrations of testosterone and DHT in a mouse model of orthotopic androgen-sensitive human prostate cancer	[25]
	Inhibition of prostate cancer development and increased survival in male TRAMP mice. Delay in primary tumor incidence and tumor burden, significant decrease in prostate and genitourinary weight, inhibition in serum IGF-1, increase in IGFBP-3, reduction in PCNA, and inhibition of distant site metastases to lymph nodes, lungs, liver and bone	[26]
Breast Cancer	Reduction of tumor growth, increase in Bax/Bcl-2 ratio, reduction in PCNA, activation of caspase-3, inhibition of metastasis of tumor cells to lungs and increase in the survival period of BALB/c mice	[31]
	Increased natural killer cell activity and reduction in the number of lung-metastatic colonies in SAMP10 mice	[33]
Lung Cancer	Inhibition of MMP-2 and -9 secretion, invasion of human lung carcinoma cells through Matrigel in a dose-dependent fashion in athymic nude mice	[37]
	Reduction of lung metastases, primary tumor growths and increased survival rate in mice bearing melanomas	[16]
Liver Cancer	Inhibition of metastasis of melanoma cells to the liver and increase of the survival time in C57BL/6 mice	[41]
	Suppression of the increase in liver weight and hepatic metastasis of ovarian sarcoma cells transplanted subcutaneously in mice	[44]
Gastrointestinal Cancer	Inhibition of intestinal tumor formation with reduced bFGF expression in APC (Min/+) mice	[46]
	Reduction in the size of tumors in nude mice implanted with human colon cancer cells. Inhibition of MMP-9 and VEGF secretion and mitotic index in the tumor tissues	[47]
	Reduction in Ki-67, PCNA, vWF, VEGF, CD31, VEGFR-2, ERK1/2, JNK1/2, p38, MMP-2, MMP-7, MMP-9, and MMP-12 and induction of apoptosis, caspase-3 activity, and p21/WAF1 in tumors of athymic nude mice implanted with human pancreatic cells	[49]
	Inhibition of pancreatic cancer incidence, process of pancreatic carcinogenesis, and tumor promotion of transplanted pancreatic cancer in hamsters	[51]

1. Zhongguo Shi Yan Xue Ye Xue Za Zhi. 2008 Oct;16(5):1073-8.

[Demethylation and transcription of p16 gene in malignant lymphoma cell line CA46 induced by EGCG].

[Article in Chinese]

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The study was purposed to investigate the possible mechanism of epigallocatechin-3-gallate (EGCG) induced p16 gene demethylation and transcription regulation in the malignant lymphoma cell line-CA46. The induced growth inhibition of CA46 cells was assayed by growth curve and MTT; the DNA content of CA46 cells was analyzed by flow cytometry after being exposed to EGCG; the methylation status of the p16 gene in CA46 cell line before and after treatment with EGCG was detected by the nested-methylation specific PCR and DNA sequencing; the mRNA of p16 and DNA methyltransferases (DNMT3A and DNMT3B) gene
were determined by RT-PCR. The results showed that in comparison with the control, all the 3 different concentration of EGCG were able to inhibit the growth of malignancy cell lines and increase the cell number in G(0)/G(1) phase. After treatment with EGCG for 48 hours, the methylation level was apparently attenuated in a concentration-dependent manner. Expression of p16 gene in untreated group was mild while in the treated groups it had been greatly strengthened, as compared with untreated group, the gray scale ratio of p16 to beta-actin 1 treated with EGCG (6, 12, 24) microg/ml was increased from (0.05 +/- 0. 01) to (0.19 +/- 0.03), (0.39 +/- 0.10), (0.85 +/- 0.09) respectively, exhibiting a significant difference ($p < 0.05$); as compared with the untreated group, after treatment with EGCG for 48 hours, the expressions of DNMT3A and DNMT3B were obviously down-regulated. It is concluded that EGCG can activate and up-regulate the expression of p16 gene mRNA which inhibits the proliferation of CA46 cell through inducing the G(0)/G(1) arrest by demethylation and/or by inhibiting DNMT3A and DNMT3B gene.

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1. Anticancer Res. 2009 Jun;29(6):2025-30.

Promoter demethylation of WIF-1 by epigallocatechin-3-gallate in lung cancer cells.

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BACKGROUND: Aberrant promoter methylation of Wnt inhibitory factor-1 (WIF-1) is a fundamental mechanism of epigenetic silencing in human cancers. Epigallocatechin-3-gallate (EGCG) has been reported to directly reactivate several methylation-silenced genes. The promoter demethylation and reactivation of WIF-1 has not previously been reported.
MATERIALS AND METHODS: Methylation-specific PCR, sequencing analysis and RT-PCR
analysis were performed to evaluate promoter demethylation of WIF-1 and WIF-1 expression, Western blot analysis and luciferase reporter assay were performed to evaluate expression of cytosolic beta-catenin protein and Tcf/Lef reporter activity.
RESULTS: Promoter demethylation of WIF-1 and restoration of WIF-1 expression after EGCG treatment are demonstrated in H460 and A549 cell lines. EGCG also decreased cytosolic beta-catenin protein level and inhibited Tcf/Lef reporter activity.
CONCLUSION: These results suggest the potential therapeutic use of EGCG for the reversal of WIF-1 promoter methylation.

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