

# Combined effect of green tea and *Ganoderma lucidum* on invasive behavior of breast cancer cells

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**Abstract.** Epidemiological studies have suggested that consumption of green tea may decrease the risk of a variety of cancers. In addition, mushroom *Ganoderma lucidum* has been used for the promotion of health, longevity and treatment of cancer in traditional Chinese medicine. In the present study we show that extract from green tea (GTE) increased the anti-cancer effect of *G. lucidum* extract (GLE) on cell proliferation (anchorage-dependent growth) as well as colony formation (anchorage-independent growth) of breast cancer cells. This effect was mediated by the down-regulation of expression of oncogene c-myc in MDA-MB-231 cells. Although individual GTE and GLE independently inhibited adhesion, migration and invasion of MDA-MB-231 cells, their combination demonstrated a synergistic effect, which was mediated by the suppression of secretion of urokinase plasminogen activator (uPA) from breast cancer cells. Our study suggests the potential use of combined green tea and *G. lucidum* extracts for the suppression of growth and invasiveness of metastatic breast cancers.

## Introduction

Breast cancer is the leading cause of cancer death among women of 20-59 years old and contributed to one third of newly diagnosed cases of breast cancer in the United States in 2005 (1). The low incidence of breast cancers among Asian women was associated with dietary behavior, suggesting the preventative effect of nutrition against cancer (2). Although inconclusive, epidemiological studies suggested the importance of different nutritional/natural products for cancer prevention. However, while important, these studies are highly correlative

because they are usually focused only on one nutritional product. For example, the consumption of green tea was correlated to the prevention of a variety of cancers (3), and an inverse correlation between mushroom intake and risk of gastric cancer was described (4).

Carcinogenesis is a multistage overlapping process consisting of initiation, promotion and progression phases. Initiation starts when normal cells are exposed to a carcinogen, which results in genomic DNA damage (5). Promotion is characterized as clonal proliferation of damaged cells and formation of preneoplastic focal lesions. Finally, progression produces a new clone of tumor cells with increased proliferative capacity, invasive behavior and metastatic potential (5). Therefore, some natural/dietary compounds demonstrated strong potential in the inhibition of different stages of carcinogenesis and their effectiveness is currently being evaluated as non-toxic chemopreventative agents with possible chemotherapeutic activity (6). Nevertheless, the combination or mixture of different natural/dietary products could enhance the effect of isolated compounds, and could reflect dietary behavior.

Extracts from an oriental medicinal mushroom *Ganoderma lucidum* has been used in traditional Chinese medicine for the prevention or treatment of a variety of diseases. *G. lucidum* is currently consumed in the form of tea, powder or extract as a dietary supplement. Furthermore, *in vivo* animal studies demonstrated inhibition of hepatoma, sarcoma, and lung and colon cancers in mice by *G. lucidum* extracts containing a variety of biologically active compounds, including  $\beta$ -glucan-based polysaccharides and lanostane-type triterpenes (7-11). In addition, we have recently demonstrated *in vitro* that an extract from *G. lucidum* inhibits the proliferation and invasive behavior of highly metastatic breast cancer cells through the suppression of Akt/NF- $\kappa$ B signaling, which resulted in the down-regulation of expression of cyclin D1, Cdk4 and urokinase-plasminogen activator (uPA) in breast cancer cells (12-14). Tea, from the plant *Camellia sinensis*, belongs to the most globally consumed beverages (3). Moreover, green tea and especially its major biologically active compound (-)-epigallocatechin-3-gallate (EGCG) also demonstrated cancer chemopreventative effects in different cancer models (15,16). The biological mechanism of the chemopreventative effects of EGCG was linked to the modulation of multiple signaling pathways finally resulting in the down-regulation of expression

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of AP-1 and NF- $\kappa$ B regulated genes (3). In addition, we have recently demonstrated that green tea extract containing 50% of EGCG induced cell cycle arrest and suppressed the invasive behavior of breast cancer cells through the inhibition of AP-1 and NF- $\kappa$ B signaling (17). The present study was undertaken to evaluate the effect of combination of green tea and *G. lucidum* extracts on the growth and invasive behavior of breast cancer cells.

## Materials and methods

**Materials.** *G. lucidum* extract (GLE, ReishiMax<sup>GLP</sup>), containing 13.5% polysaccharides and 6% triterpenes, and green tea leaf extract (GTE, Tegreen<sup>97</sup>), containing 97% polyphenols (38% EGCG) were obtained from Pharmanex LLC (Provo, UT). These extracts were dissolved in sterile water at a concentration of 50 mg/ml and stored at 4°C.

**Cell culture.** The human breast cancer cells MDA-MB-231 were obtained from ATCC (Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (50 U/ml), streptomycin (50 U/ml), and 10% fetal bovine serum (FBS). Media and supplements were from Invitrogen (Grand Island, NY). FBS was obtained from Hyclone (Logan, UT).

**Cell proliferation (anchorage-dependent growth).** MDA-MB-231 cells ( $2.5 \times 10^3$ ) were cultured in a 96-well plate and treated for 24 and 48 h with GLE (0-125  $\mu$ g/ml), GTE (0-120  $\mu$ g/ml) or the combination of GLE and GTE as indicated in the text. Cell proliferation was determined by the tetrazolium salt method, as described (18). Data points represent mean  $\pm$  SD in one experiment repeated at least twice.

**Colony formation (anchorage-independent growth).** MDA-MB-231 cells were harvested and seeded in 6-well plates coated with 1% agarose. Colony formation was assessed after incubation for 10-14 days with culture media with GLE (0-60  $\mu$ g/ml), GTE (0-60  $\mu$ g/ml) or the combination of GLE and GTE, which was replaced every 4 days. Plates were stained with 0.005% Crystal Violet, and the colonies were counted manually under a microscope and photographed (13).

**Cell adhesion, migration, and invasion assays.** MDA-MB-231 cells were treated with GLE (0-500  $\mu$ g/ml), GTE (0-125  $\mu$ g/ml) or the combination of GLE and GTE. Cell adhesion was performed with Cytomatrix adhesion strips coated with human vitronectin (Chemicon International, Temecula, CA) as previously described (19). Cell migration of MDA-MB-231 cells was assessed in Transwell chambers in the DMEM medium containing 10% FBS, as previously described (13). Invasion of MDA-MB-231 cells treated with GLE (0-500  $\mu$ g/ml) and 30  $\mu$ g/ml GTE was assessed in Transwell chambers coated with 100  $\mu$ l of Matrigel<sup>TM</sup> (BD Biosciences, Bedford, MA) diluted 1:4 with DMEM, after 72 h of incubation as described (13).

**uPA secretion.** DMEM media from MDA-MB-231 cells treated with GLE (0-250  $\mu$ g/ml), GTE (0-20  $\mu$ g/ml) or the combination of GLE and GTE for 24 h were collected and concentrated,

and the secretion of uPA was detected by Western blot analysis with anti-uPA antibody (Oncogene Research Products, Cambridge, MA), as described (20).

**Western blot analysis.** Whole cell extracts were prepared from MDA-MB-231 cells treated with GLE (0-8  $\mu$ g/ml), GTE (0-60  $\mu$ g/ml) or the combination of GLE and GTE for 24 h as previously described (14). Equal amounts of proteins (20  $\mu$ g/lane) were separated on NuPAGE 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to a PVDF membrane (Millipore, Bedford, MA). The protein expression was detected with anti-c-myc antibody and anti- $\beta$ -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and visualized using the ECL Western blotting detection system (Amersham Biosciences, Buckinghamshire, UK).

**Statistical analysis.** Data are presented as means  $\pm$  SD. Statistical comparison between the control group (0  $\mu$ g/ml of GLE and GTE) and groups with different GLE and GTE doses were analyzed by Student's t-tests. The value of  $p < 0.05$  was considered to be significant.

## Results

**Green tea extract increases anti-proliferative effect of *G. lucidum*.** The inhibition of proliferation of a variety of cancer cells by green tea or its major polyphenolic constituent EGCG has been demonstrated in numerous studies (3). In the present study we evaluated GTE containing 97% of polyphenols with 38% of EGCG on highly invasive breast cancer cells. MDA-MB-231 cells were treated with GTE (0-120  $\mu$ g/ml) for 24 and 48 h and cell proliferation was determined as described in Materials and methods. As seen in Fig. 1A, GTE markedly suppressed the proliferation of MDA-MB-231 cells in a dose- and time-dependent manner. Since we have also recently demonstrated that *G. lucidum* inhibits the proliferation of MDA-MB-231 cells in a dose-dependent manner (0-250  $\mu$ g/ml), we hypothesized that GTE would enhance the effect of GLE on breast cancer cells. Therefore, MDA-MB-231 cells were treated with GLE (0-125  $\mu$ g/ml) in the presence of 0, 30 and 60  $\mu$ g/ml of GTE for 24 and 48 h. Although GLE itself suppressed the proliferation of MDA-MB-231 cells by 23% (30  $\mu$ g/ml GLE), 44% (60  $\mu$ g/ml GLE) and 47% (125  $\mu$ g/ml GLE), addition of 30  $\mu$ g/ml of GTE increased this inhibition by 51, 68 and 70%, and addition of 60  $\mu$ g/ml of GTE further increased the inhibition of proliferation by 60, 73 and 77%, respectively (Fig. 1B, only data for 24 h are shown). Finally, the low concentration (0.25-8  $\mu$ g/ml) of GLE inhibits the proliferation of MDA-MB-231 cells by 6-39% (Fig. 1C), and addition of 30  $\mu$ g/ml GTE further increased this inhibition by 19-51%, respectively (Fig. 1D).

**Combined effects of green tea and *G. lucidum* on colony formation.** The formation of colonies of cancer cells *in vitro* (anchorage-independent growth) is comparable with tumorigenesis *in vivo* (21). Because our previous study demonstrated that *G. lucidum* (250-500  $\mu$ g/ml) suppresses the colony formation of highly invasive breast cancer cells (13), we assessed the effect of GTE, GLE and combination of GTE and GLE on anchorage-independent growth of MDA-MB-

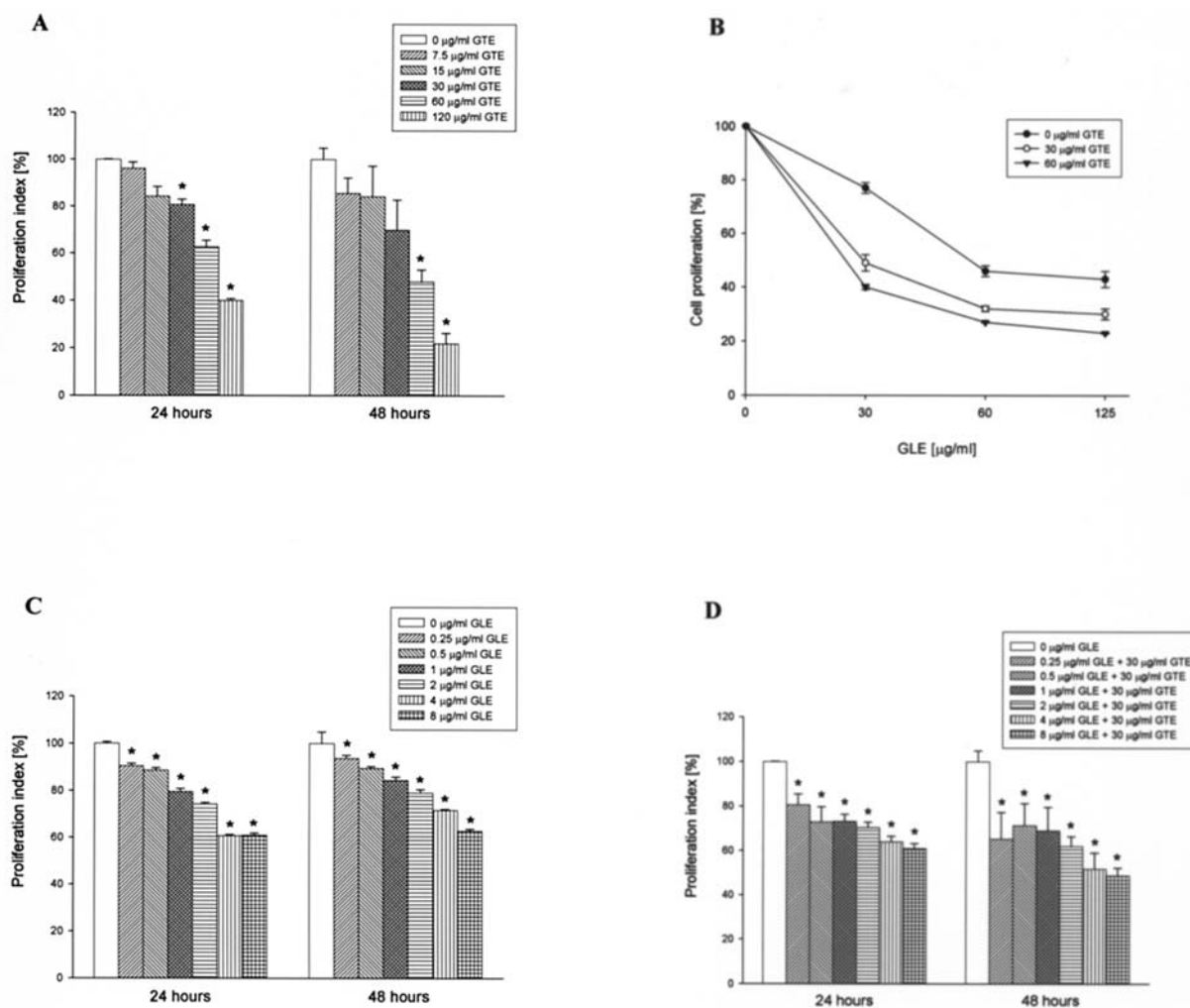


Figure 1. Green tea extract and *G. lucidum* extract inhibited the proliferation of breast cancer cells. MDA-MB-231 cells were treated with (A) GTE (0-120  $\mu\text{g/ml}$ ) for 24 and 48 h, (B) GLE (0-125  $\mu\text{g/ml}$ ) and GTE (0-60  $\mu\text{g/ml}$ ) for 24 h, (C) GLE (0-8  $\mu\text{g/ml}$ ) for 24 and 48 h, and (D) GLE (0-8  $\mu\text{g/ml}$ ) and 30  $\mu\text{g/ml}$  GTE for 24 and 48 h. Proliferation was assessed, as described in Materials and methods. Each bar represents the mean  $\pm$  SD of three experiments. \* $p < 0.05$  versus control.

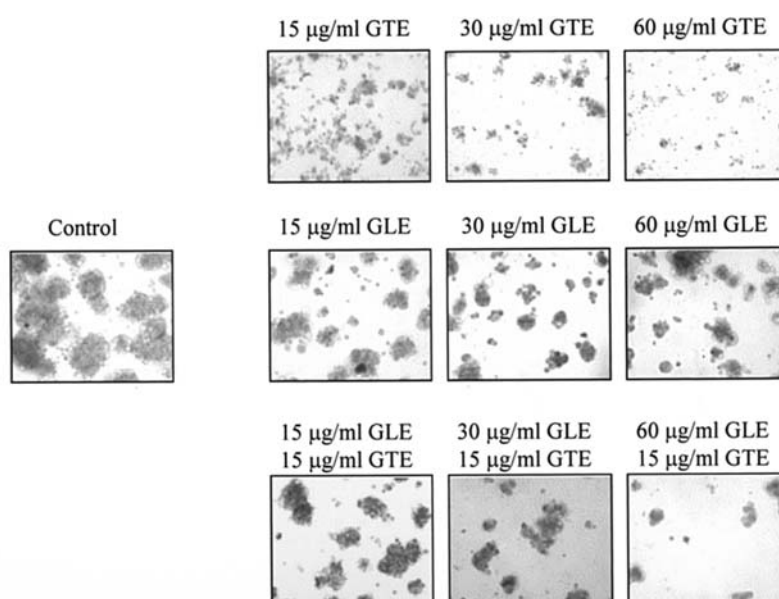


Figure 2. Green tea extract and *G. lucidum* extract suppressed the colony formation of MDA-MB-231 cells. MDA-MB-231 cells were harvested and seeded in 6-well plates coated with agarose in the presence of GTE (0-60  $\mu\text{g/ml}$ ), GLE (0-60  $\mu\text{g/ml}$ ), or the combination of GLE (0-60  $\mu\text{g/ml}$ ) and 15  $\mu\text{g/ml}$  GTE. Anchorage-independent growth was assessed as described in Materials and methods.



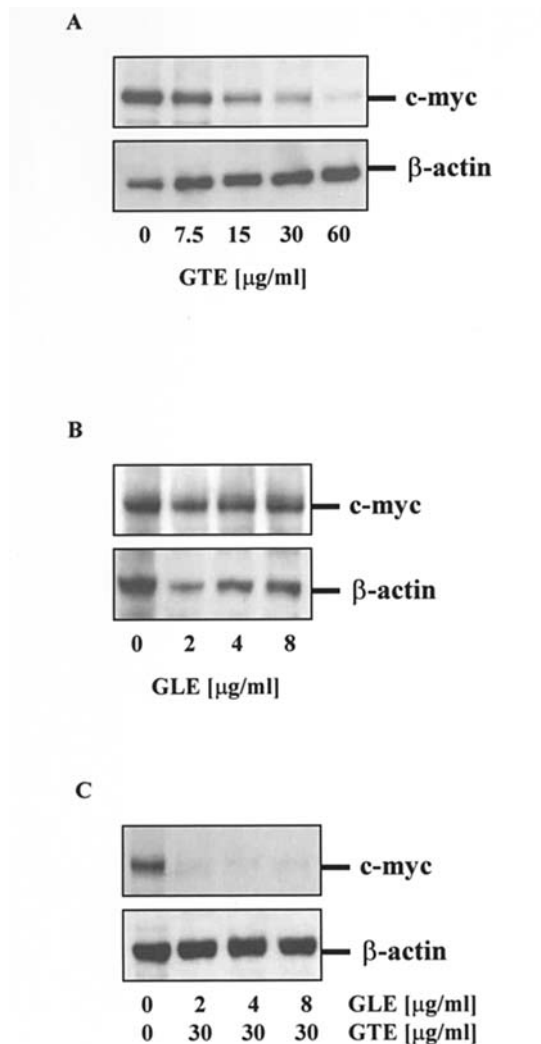


Figure 3. Effect of GTE and GLE on the expression of c-myc in breast cancer cells. MDA-MB-231 cells were treated with (A) GTE (0-60 µg/ml), (B) GLE (0-8 µg/ml), and (C) the combination of GLE (0-8 µg/ml) and 30 µg/ml GTE for 24 h. Whole cell extracts were subjected to Western blot analysis with anti-c-myc antibody. The equal protein loading was verified with anti-β-actin as described in Materials and methods. The results are representative of three separate experiments.

Table I. Adhesion of MDA-MB-231 cells to vitronectin.

	GTE (0 µg/ml)	GTE (30 µg/ml)	GTE (60 µg/ml)
GLE (0 µg/ml)	100±4.8	24±1.1 <sup>a</sup>	7±1.7 <sup>a</sup>
GLE (125 µg/ml)	55±4.8 <sup>a</sup>	20±3.7 <sup>a</sup>	2±1.1 <sup>a</sup>
GLE (250 µg/ml)	45±2.4 <sup>a</sup>	13±9.2 <sup>a</sup>	1.5±0.6 <sup>a</sup>
GLE (500 µg/ml)	27±9.8 <sup>a</sup>	10±6.2 <sup>a</sup>	0.3±0.2 <sup>a</sup>

Adhesion is expressed as % of cells which bind to vitronectin coated strips. <sup>a</sup>p<0.05 versus control (0 µg/ml GLE, 0 µg/ml GTE).

whereas GTE (15, 30 and 60 µg/ml) markedly suppressed colony formation. Although GLE (15, 30 and 60 µg/ml) only slightly decreased the colony formation of MDA-MB-231 cells, the combination of GLE and GTE demonstrated a distinctly more inhibitory effect than separated GLE or GTE (Fig. 2). Therefore, green tea extract and *G. lucidum* extract synergistically inhibited the colony formation of invasive human breast cancer cells.

*Combination of green tea and G. lucidum down-regulate expression of c-myc in breast cancer cells.* High proliferative potential of breast cancer cells is associated with the increased expression of the oncogene c-myc (22,23). As recently demonstrated, EGCG suppressed c-myc mRNA, and *G. lucidum* down-regulated expression of c-myc protein in MDA-MB-231 cells, respectively (24,25). Therefore, we hypothesized that the suppression of proliferation and colony formation by GTE and GLE in breast cancer cells is modulated by the down-regulation of expression of c-myc. MDA-MB-231 cells were treated with GTE (0-60 µg/ml) for 24 h and the expression of c-myc was evaluated by Western blot analysis as described in Materials and methods. As seen in Fig. 3A, GTE markedly decreased expression of c-myc in a dose-response manner. Since we have recently demonstrated the down-regulation of c-myc expression with 250 µg/ml of *G. lucidum* extract (25), and as GLE started to inhibit proliferation at the lower concentration (Fig. 1C), we evaluated if GLE (0-8 µg/ml) would also down-regulate expression of c-myc in MDA-MB-231 cells. However, GLE at the low concentration did not significantly decrease expression of c-myc (Fig. 3B), whereas the combination of GTE and GLE clearly down-regulated expression of c-myc in MDA-MB-231 cells (Fig. 3C).

*Combined effects of green tea and G. lucidum on invasive behavior of breast cancer cells.* We have previously demonstrated that *G. lucidum* and green tea polyphenols inhibit adhesion, migration and invasion of highly invasive breast cancer cells (13-17). In order to investigate whether invasive behavior could be suppressed by the combination of *G. lucidum* and green tea, cell adhesion, cell migration and cell invasion of MDA-MB-231 cells was assessed in the presence of GLE and GTE. Although the sole GLE (0-500 µg/ml) or GTE (0-60 µg/ml) suppresses adhesion of MDA-MB-231 cells to the extracellular matrix protein vitronectin in a dose-response manner, combination of GLE and GTE demonstrated a synergistic effect in the inhibition of cell adhesion (Table I). Next we evaluated the effect of GLE and GTE on migration of MDA-MB-231 cells. As expected the individual GTE suppresses migration of MDA-MB-231 cells in a dose-response manner by 13, 25 and 38% for 30, 60 and 125 µg/ml of GTE, respectively, and GLE inhibits migration by 48, 63 and 74% for 125, 250 and 500 µg/ml, respectively. Nevertheless, the combination of GLE and GTE synergistically inhibits the migration of invasive breast cancer cells (Table II). Because the proteolytic activity of cancer cells reflects their invasiveness by the degradation of extracellular matrix (ECM) components, we investigated whether the addition of GTE enhances the inhibitory effect of GLE on invasion of MDA-MB-231 cells through Matrigel. As seen in Fig. 4, although GLE markedly suppressed the invasion of MDA-MB-231

231 cells. As seen in Fig. 2, MDA-MB-231 cells formed colonies on 1% agarose after 14 days of incubation (control),

Table II. Combined effect of GLE and GTE on migration of MDA-MB-231 cells.

	GTE (0 $\mu\text{g/ml}$ )	GTE (30 $\mu\text{g/ml}$ )	GTE (60 $\mu\text{g/ml}$ )	GTE (125 $\mu\text{g/ml}$ )
GLE (0 $\mu\text{g/ml}$ )	100 $\pm$ 2.4	87 $\pm$ 2.4 <sup>a</sup>	75 $\pm$ 2.5 <sup>a</sup>	62 $\pm$ 4.3 <sup>a</sup>
GLE (125 $\mu\text{g/ml}$ )	54 $\pm$ 3.9 <sup>a</sup>	46 $\pm$ 1.9 <sup>a</sup>	43 $\pm$ 2.0 <sup>a</sup>	38 $\pm$ 4.0 <sup>a</sup>
GLE (250 $\mu\text{g/ml}$ )	37 $\pm$ 1.3 <sup>a</sup>	36 $\pm$ 2.0 <sup>a</sup>	31 $\pm$ 4.6 <sup>a</sup>	22 $\pm$ 3.3 <sup>a</sup>
GLE (500 $\mu\text{g/ml}$ )	26 $\pm$ 2.8 <sup>a</sup>	21 $\pm$ 1.3 <sup>a</sup>	19 $\pm$ 1.8 <sup>a</sup>	16 $\pm$ 2.4 <sup>a</sup>

Migration is expressed as % of cells, which migrated through the filters in Boyden chambers. <sup>a</sup>p<0.05 versus control (0  $\mu\text{g/ml}$  GLE, 0  $\mu\text{g/ml}$  GTE).

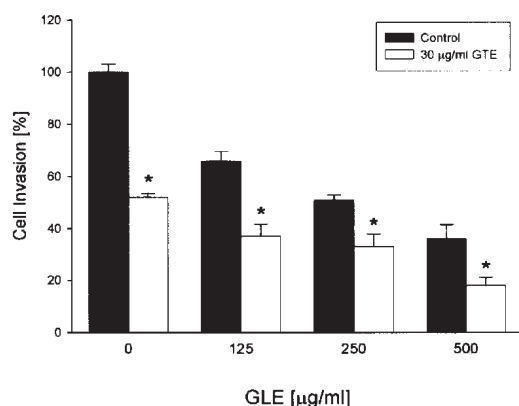


Figure 4. Combined effect of GLE and GTE on invasion of MDA-MB-231 cells. MDA-MB-231 cells were harvested and treated with GLE (0-500  $\mu\text{g/ml}$ ) and 30  $\mu\text{g/ml}$  GTE. Invasion through Matrigel was assessed after 72 h of incubation as described in Materials and methods. Data are the means  $\pm$  SD of triplicate determinations. Similar results were obtained in at least two additional experiments. \*p<0.05 versus control.

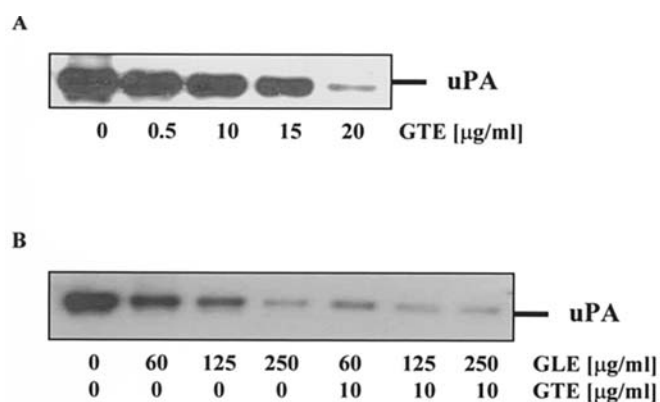


Figure 5. Combined effect of GLE and GTE on uPA secretion. Medium from MDA-MB-231 cells treated with (A) GTE (0-20  $\mu\text{g/ml}$ ), (B) GLE (0-250  $\mu\text{g/ml}$ ) and GTE (0, 10  $\mu\text{g/ml}$ ) for 24 h was concentrated as described in Materials and methods. Secretion of uPA was detected by Western blot analysis with anti-uPA antibody. The results are representative of three separate experiments.

cells, the addition of 30  $\mu\text{g/ml}$  of GTE further increased the inhibition of invasion of MDA-MB-231 cells. Taken together, green tea extract increases the anti-invasive effect of *G. lucidum* extract on breast cancer cells.

*Combination of green tea and G. lucidum suppressed the secretion of uPA from breast cancer cells.* Since uPA plays a crucial role in the cell adhesion, migration and invasion (26), we evaluated if the combination of GLE and GTE suppresses secretion of uPA from highly invasive breast cancer cells. Conditioned medium from MDA-MB-231 cells treated with GTE (0-20  $\mu\text{g/ml}$ ) for 24 h was collected and subjected to Western blot analysis with anti-uPA antibody. As seen in Fig. 5A, GTE suppresses secretion of uPA in a dose-response manner. uPA secretion was also inhibited from cells treated with GLE (0-125  $\mu\text{g/ml}$ ), and this effect was further enhanced by the addition of 10  $\mu\text{g/ml}$  of GTE (Fig. 5B). In summary, green tea extract enhances *G. lucidum*-dependent inhibition of secretion of uPA from highly invasive breast cancer cells.

## Discussion

Uncontrolled proliferation and increased invasive potential of cancer cells usually result in cancer metastasis. Therefore, natural/dietary compounds with anti-proliferative and anti-invasive capacity are suitable agents for cancer prevention. Several *in vitro* and *in vivo* studies demonstrated the chemopreventative effect of green tea, and its major compound, EGCG, was identified as one of the major natural modulators of signaling pathways involved in cancer progression (3). Other studies demonstrated the anticancer activity of medicinal mushroom *G. lucidum*, and its extracts, containing triterpenes and polysaccharides, suppressed signaling responsible for the invasive behavior of cancer cells (27). In the present study, we evaluated the combined effect of green tea extract (GTE, a green tea leaf extract standardized to contain 97% polyphenols with 38% of EGCG) and *G. lucidum* extract (GLE, a *G. lucidum* powdered extract with cracked spores standardized to 13.5% polysaccharides and 6% triterpenes) on highly invasive and metastatic breast cancer cells.

GTE increased the anti-cancer effect of GLE on the anchorage-dependent (cell proliferation) as well as anchorage-independent (colony formation) growth of breast cancer cells. This effect can be mediated, in part, by the inhibition of oncogene c-myc because GTE, and especially the combination of GTE and GLE, down-regulated the expression of c-myc in MDA-MB-231 cells. Although individual GTE and GLE independently inhibited the adhesion, migration and invasion of MDA-MB-231 cells, their combination demonstrated a synergistic effect, which was mediated by the suppression of secretion of uPA from breast cancer cells.

Our observation is in agreement with the generally accepted concept that the combination of different bioactive agents and/or food factors could increase the cancer preventative effects of a single constituent (28,29). As previously demonstrated, the combination of EGCG with a non-steroidal anti-inflammatory drug (NSAID) sulindac (30), or the combination of EGCG with phytic acid from grain (31), synergistically suppressed preneoplastic lesions in a rat colon carcinogenesis model. Furthermore, the combination of grape skin extract with green tea infusion (containing 50% EGCG) inhibited the growth of 4T1 mouse tumors *in vivo*, and suppressed the viability of human cervical cancer cells (HeLa) *in vitro* (32). Finally, the combination of soy phytochemical concentrate with green tea infusion (containing 38% EGCG) inhibited the growth of human breast cancer cells in mice through the suppression of cell proliferation (33).

In this study we demonstrate, for the first time, that the combination of green tea and *G. lucidum* extracts inhibits the growth of breast cancer cells. This inhibition of proliferation of MDA-MB-231 is mediated by the down-regulation of expression of c-myc by the combined effect of GLE and GTE. In agreement with a recent report describing decrease of c-myc RNA levels by EGCG (24), GTE also markedly suppressed expression of c-myc in MDA-MB-231 cells. Although a GLE concentration of 250 µg/ml previously inhibited expression of c-myc in breast cancer cells (25), a low concentration of GLE (0-8 µg/ml) did not affect its expression (Fig. 3B). Nevertheless, the combination treatment of a low concentration of GLE and GTE markedly suppressed expression of c-myc in MDA-MB-231 cells.

The combination of GLE and GTE inhibited cell invasiveness as assessed by the suppression of cell adhesion, migration and invasion of breast cancer cells. This effect was caused by the suppression of secretion of uPA from MDA-MB-231 cells. Since the increased levels of uPA correspond to highly metastatic cancers in breast cancer patients, and are associated with poor prognosis and outcome (34), the inhibition of secreted uPA from invasive breast cancer cells by GLE and GTE has potential clinical application. Although *G. lucidum* and green tea extracts can inhibit invasiveness through the suppression of uPA secretion independently (12,17), their synergistic effect requires markedly low doses of individual compounds.

In conclusion, the present study demonstrated the synergistic effect of GLE and GTE in the inhibition of growth and invasive behavior of highly metastatic breast cancer cells. Further studies are necessary to elucidate if the combination of *G. lucidum* and green tea extracts as a dietary supplement is suitable for breast cancer chemoprevention and/or treatment.

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