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Black Tea Polyphenols Reverse Epithelial-to-Mesenchymal Transition and Suppress Cancer Invasion and Proteases in Human Oral Cancer Cells

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ABSTRACT: Epithelial-to-mesenchymal transition (EMT) in cancer cells is considered to be a prerequisite for acquiring invasive/migratory phenotype and subsequent metastasis. This study provides molecular evidence associated with the antimetastatic effect of black tea polyphenol extracts (BTE), which contain polyphenols including gallic acid, gallocatechin, catechin, epigallocatechin-3-gallate, epicatechin-3-gallate, and theaflavin 3,3′-digallate, in an an oral squamous cell culture system by showing a nearly complete inhibition on the invasion ($p < 0.001$) of SCC-4 cells via reduced activities of MMP-2 ($p < 0.001$) and u-PA ($p < 0.001$). Immunoblot was performed to find that BTE could induce up-regulation of epithelial markers such as E-cadherin and inhibit mesenchymal markers such as snail-1 and vimentin. BTE inhibited p-FAK and p-paxillin, indicating the anti-EMT effect of BTE in oral squamous cell carcinoma. BTE was evidenced by its inhibition of the tumor growth of SCC-4 cells via cancer cell xenografted nude mice mode. These results suggested that BTE could reduce invasion by reversing EMT in human oral cancer cells.

KEYWORDS: *EMT, invasion, MMP, u-PA, tea polyphenols*

INTRODUCTION

Chemopreventive properties have long been attributed to phenolic compounds present in the human diet and used as agents to postpone, inhibit, or reverse tumorigenesis. The most consistent findings on diet as a determinant of several types of cancer risk prevention is the association with consumption of fruits, vegetables, flowers, and plants.[1,2] In earlier studies, it has been shown that tea phenolic compounds may have potentially beneficial effects, including reducing the risk of cardiovascular diseases and cancers with anti-inflammatory, antioxidant, and chemoprotective properties. Tea, the most widely consumed beverage, has received considerable attention because of its polyphenolic constituents known to have strong antioxidants and repressive activity against tumorigenesis.[3] Black tea, the major form of tea consumed in India, is produced by crushing the tea leaves to promote enzymatic oxidation and subsequent polymerization of most of the tea polyphenols. Epidemiological studies on black tea and cancer are still uncertain, but several investigators have revealed that the consumption of black tea reduces the incidence of ovarian[4] and breast[5] cancers. Earlier papers have indicated that black tea polyphenols, theaflavins, could induce apoptosis in prostate cancer cells via up-regulation of p53 expression, decrement of NF-κB transcriptional activity, and inhibition of phosphorylation levels of mitogen-activated protein kinase (MAPK) pathways.[6] Black tea has been shown to protect against lung tumorigenesis in F344 rats induced by the nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in a 2 year bioassay[7] and to inhibit the invasion of human melanoma cancer A373 cells via suppression of the secretion of matrix metalloproteinase-2 (MMP-2) and the protein expression of focal adhesion kinase (FAK), epidermal growth factor receptor (EGFR), and extracellular regulated protein kinases (ERK).[8] The beneficial effects of tea polyphenols in reduction of tumor growth and cancer metastasis have been reported in many studies. Mechanisms of action of tea catechins, especially epigallocatechin-3 gallate (EGCG), the most abundant and active form of catechin, have been extensively investigated. Although it was quite clear that black tea may inhibit the growth of various cancers by inducing cancer cells toward apoptosis[6] and scavenging reactive oxygen species,[3] limited studies are available concerning the potential of black tea extracts in retarding cancer cell invasion and reversing epithelial-to-mesenchymal transition (EMT).

The metastasis of solid tumor is known to be the primary cause of human cancer death and comprises multistep processes in which tumor cells need to overcome defined barriers. Knowledge

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of the mechanism by which cancer cells succeed in invading surrounding tissues, intravasate into blood or lymphatic fluid, and grow as secondary tumors may help to identify new anticancer therapies.[9,10] EMT is thought to have an important role in promoting carcinoma invasion and metastasis.[11,12] During EMT, cancer cells lose expression of proteins that promote cell−cell contact such as E-cadherin, β-catenin, and γ-catenin and acquire mesenchymal markers such as snail, slug, vimentin, fibronectin, smooth muscle actin, and N-cadherin, which promote cell invasion and metastasis.[11] Gain of vimentin is the major hallmark of EMT. In line with a role for EMT in cancer progression, vimentin is often increased in several cancer tissues.[12] At the advanced stage, tumors also express high levels of matrix metalloproteinases (MMPs) that degrade tissue extracellular matrix (ECM) and facilitate tumor as well as endothelial cell invasion and migration.[13] The purpose of the present study was to characterize the inhibitory effects of black tea polyphenols on cancer cell invasion, migration, and EMT of squamous cell carcinoma-4 (SCC-4) cells; the changes of cell physiology and precise molecular events involved in cell invasion and EMT were also investigated.

MATERIALS AND METHODS

Materials and Chemicals. Phorbol-12-myristate-13-acetate (PMA), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's modified Eagle medium (DMEM), and F-12 Ham's were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Matrigel were purchased from BD Biosciences (Bedford, MA, USA). Monoclonal antibody against p-focal adhesion kinase (p-FAK; pY397) was purchased from BD Biosciences, and a rabbit polyclonal antibody against p-Src (Tyr 416), p-paxillin, vimentin, E-cadherin, and snail-1 were purchased from Cell Signaling Technology (Danvers, MA, USA). A rabbit polyclonal antibody against p-paxillin, gout polyclonal antibodies against RhoA, calpain-2, and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The Immobilon Western Chemiluminescent HRP substrate kit was obtained from Millipore (Burlington, MA, USA).

Preparation of Black Tea Extract (BTE). Brooke Bond Red Label Tea (Hindustan Lever Limited, Mumbai, India) was used as black tea source. The BTE were prepared as described previously.[14] Briefly, 100 g of air-dried leaves was boiled at 60 °C for 24 h with 500 mL of 50% ethanol. The extraction procedure was repeated twice. Then, solvent was removed from the combined extract with a vacuum rotary evaporator. After evaporation of the organic solvents, the resultant extract was dissolved in deionized water and then lyophilized to become powders (38.8 g) of BTE, to yield a recovery ratio of 38.8%. The filtrate was then lyophilized and stored at −20 °C. Furthermore, the chemical profile of BTE was analyzed by using a high-pressure liquid chromatograph (HPLC)−mass spectrometer (MS). Briefly, BTE was analyzed by HPLC-MS using a HPLC (Waters 600 with a 2998 photodiode array detector). Samples (10 μL) were injected into a Merck LiChrospher 100 RP-18 column (4 mm × 250 mm). The analyses were carried out using the mobile phase composed of two solvents: solvent A (0.05% acetic acid/water) and solvent B (acetonitrile). The flow rate was 1 mL/min. The elution was carried out in a programmed gradient elution as follows: 0−30 min, with 0−60% B; 30−35 min, isocratic with 100% B; 35−40 min, isocratic with 100% A. Absorbance was monitored at 280 nm. The molecular masses of the peaks were determined from electrospray ionization mass spectra using a multiply charged ion profile based on the modified method of Wong et al.[14]

Cell Culture. SCC-4, a malignant tumor derived from human tongue squamous epithelial cells, was cultured in a 1:1 mixture of DMEM and F-12 Ham's nutrient mixture (Gibco, Grand Island, NY, USA), 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 400 ng/mL hydrocortisone.

Determination of Cell Viability (MTT Assay). Cells were seeded onto 24-well plates at a density of 2×10^4 cells/well and treated with

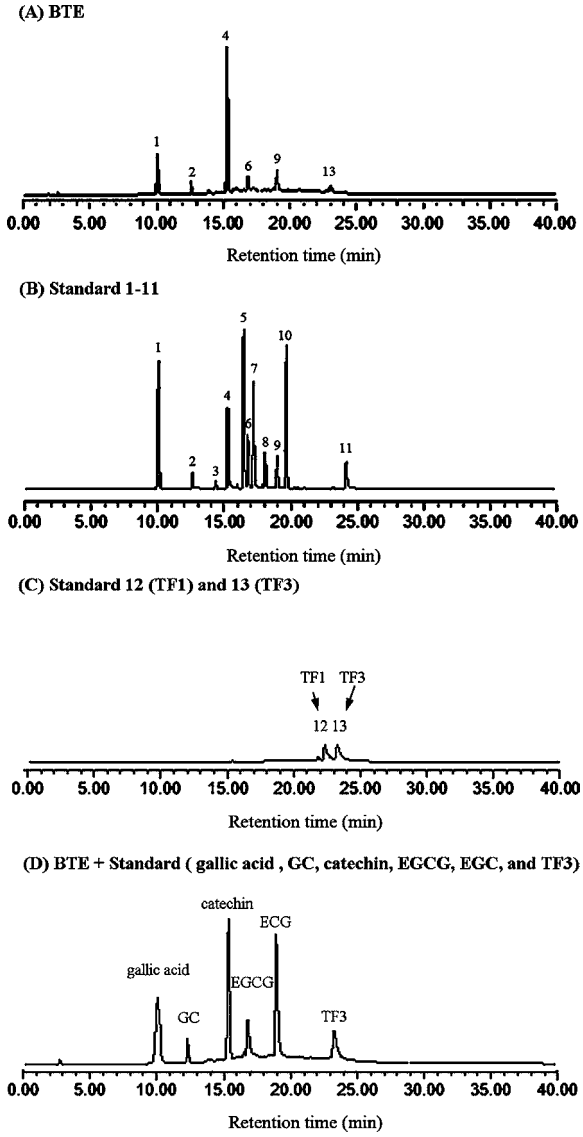

Figure 1. Chemical profile of BTE analyzed by HPLC-MS: (A) chromatographic patterns from HPLC analysis of BTE extracts showed peaks corresponding to the retention times (min); (B) HPLC chromatogram of 11 kinds of standard compounds (peaks: 1, 5 μg of gallic acid; 2, 10 μg of GC; 3, 5 μg of EGC; 4, 5 μg of catechin; 5, 5 μg of EC; 6, 5 μg of EGCG; 7, 1 μg of GCG; 8, 5 μg of rutin; 9, 5 μg of ECG; 10, 10 μg of naringin; 11, 5 μg of quercetin); (C) HPLC chromatogram of theaflavin (TF1; 30 μg) and theaflavin 3,3′-digallate (TF3; 40 μg) standard compound; (D) combination of 125 μg of BTE with six kinds of standard compound (10 μg of gallic acid; 10 μg of GC; 10 μg of catechin; 20 μg of EGCG; 20 μg of ECG; 40 μg of TF3). Absorbance was monitored at 280 nm.

Table 1. Characterization of Phenolic Compounds of BTE

peak	retention time (min)	assigned identity[a]	recovery (%)
1	10.044	gallic acid	10.49 ± 1.89
2	12.617	GC	2.82 ± 0.88
4	15.228	catechin	32.29 ± 2.32
6	16.759	EGCG	4.92 ± 1.47
9	18.969	ECG	6.21 ± 1.63
13	23.458	TE3	2.60 ± 0.34

[a]GC, gallocatechin; EGCG, epigallocatechin-3-gallate; ECG, epicatechin-3-gallate; TE3, theaflavin 3,3′-digallate.

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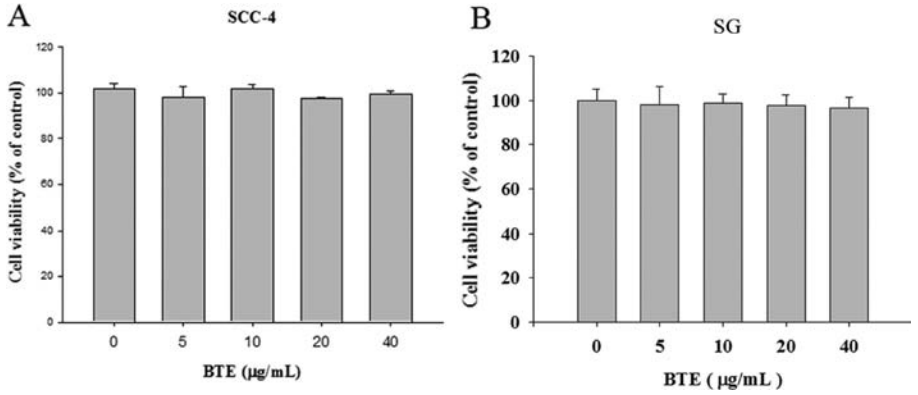

Figure 2. Effect of BTE on cell viability. Oral squamous cell lines, SCC-4 cells (A) and human gingival epithelial S-G cells (B), were treated with BTE for 24 h and then subjected to MTT assay for cell viability. Data represent the mean ± SD of at least three independent experiments. Comparisons were made by using one-way ANOVA with post hoc Dunnett's test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

BTE at a concentration of 0−40 μg/mL at 37 °C for 24 h. After the exposure period, media were removed and cells were washed with PBS followed by incubation with 0.5 mg/mL MTT in culture medium for an additional 4 h. Afterward, 1 mL of isopropyl alcohol was added to dissolve the formazan formed and then measured spectrophotometrically at 563 nm.[15]

Transwell Cell Invasion and Motility Assays. SCC-4 cells were pretreated with BTE at the indicated concentrations (0, 5, 10, 20, and 40 μg/mL) for 24 h. Afterward, cells were harvested and seeded to Millipore cell culture inserts at 5×10^4 cells/well in serum-free medium and then incubated for 48 h at 37 °C. For the invasion assay, 100 μL of Matrigel (1 mg/1 mL) was applied to 8 μm pore size polycarbonate membrane filters, with the bottom chamber of the apparatus containing standard medium. Following incubation, the filters were then air-dried for 5 h in a laminar flow hood. The invaded cells were fixed with methanol and stained with Giemsa. Cell numbers were counted using a light microscope, whereas the motility assay was carried out as described for the invasion assay, with no coating of Matrigel.[16]

Wound-Healing Migration Assay. For the cell migration assay, SCC-4 cells were seeded into a 12-well culture dish and grown in culture medium containing 10% FBS to a nearly confluent cell monolayer (>95% confluence). Wounds were introduced to the confluent monolayer of cells with a sterile 200 μL plastic pipet tip to create a denuded area. Floating cells were removed by two washings with PBS and replaced with culture medium containing 1% FBS, and then BTE was added. The cells were incubated at 37 °C, and cell movement into the wound area was photographed at 0 and 24 h using a microscope. Cell motility into a wound was expressed as percentage of cell migration.[16]

Cell-Matrix Adhesion Assay. After a 24 h treatment with BTE, cells were plated on 24-well dishes coated with type I collagen (10 μg/mL) and cultured for 60 min. Nonadherent cells were removed by PBS washes, and adherent cells were fixed in ethanol. After staining with 0.1% crystal violet, fixed cells were lysed in 0.2% Triton X-100 and the absorbance was measured at 550 nm.[17]

Cell Spreading Assay. After a 24 h treatment with BTE, cells were plated in type I collagen (10 μg/mL) coated 24-well plates and cultured in culture medium with or without BTE for 2 h. Cell morphology was photographed for morphological studies.[16]

Determination of MMPs and u-PA by Zymography. In gelatin zymography, collected media were subjected to 0.1% gelatin−8% SDS polyacrylamide gel electrophoresis (SDS-PAGE) to determine the MMPs. After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in reaction buffer for 16 h at 37 °C. Gel was then stained with Coomassie brilliant blue R-250. Visualization of u-PA activity was performed by casein zymography. Briefly, 2% w/v casein and 20 μg/mL plasminogen were added to 8% SDS-PAGE gel and then subjected to gelatin zymography.[8]

Measurement of MMP-2 and u-PA Promoter Activity. A 460 bp (−218 to +243) segment from the 5′-promoter region of the MMP-2 gene was cloned. Briefly, a 0.46 kb segment of the 5′-flanking region of the human MMP-2 gene was amplified by PCR using specific primers with restriction enzyme site from the human MMP-2 gene: 5′-GGTACCCAGATCGCGAGAGAGGCAAGTGG (forward/*Kpn*I) and 5′-AAGCTTTGGTTGGAGCCTGCTCCGCGGCG (reverse/*Hin*dIII). A 644 bp (−562 to +83) segment from the 5′-promoter region of the u-PA gene was cloned. Briefly, a 0.644 kb segment of the 5′-flanking region of the human u-PA gene was amplified by PCR using specific primers with restriction enzyme site from the human u-PA gene: 5′-GGTACCATATCTGGGGATTGCCACTG (forward/*Kpn*I) and 5′-AAGCTTGCTGCGGCAGGAGGGCGCGAG (reverse/*Hin*dIII). The pGL3-Basic vector, containing a polyadenylation signal upstream from the luciferase gene, was used to construct the expression vectors by the addition of PCR-amplified DNA with restriction enzyme site of the MMP-2 and u-PA promoter to the *Kpn*I/*Hin*dIII site of this vector. The PCR products (pGL3-MMP-2 and pGL3-u-PA) were confirmed by size, as determined by electrophoresis and DNA sequencing. pGL3-MMP-2 and -u-PA plasmid were transfected into SCC-4 cells using the PolyJet reagent (SignaGen Laboratories, Gaithersburg, MD, USA) according to the manufacturer's instructions. After incubation with BTE, cells were collected and disrupted by a Luciferase Assay System (Promega, San Diego, CA, USA). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the Luciferase Assay System. Firefly luciferase activities were standardized for β-galactosidase activity.[18]

Immunofluorescence Staining. After incubation with or without BTE (40 μg/mL), cells were cultured on sterile glass coverslips in 6-well plates. The slides were incubated overnight at 4 °C with anti-vimentin antibody, followed by incubation with TRITC-conjugated anti-rabbit Ig at room temperature for 1 h. Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and analyzed by microscopy.[16]

Western Blot. Samples of cell lysates were separated in a 12.5% polyacrylamide gel and transferred onto a nitrocellulose membrane as previously described.[8] The blot was subsequently operated with standard procedures and probed with primary and secondary antibodies. The protein expression was detected by chemiluminescence using an Immoblon Western Chemiluminescent HRP Substrate kit.

Measurement of Tumor Growth in Nude Mice. For the nude mice xenograft model, 5-week-old immunodeficient nude mice (BALB/c *nu*/*nu* mice) weighing 18−21 g were used. The mice were housed with a regular 12 h light/12 h dark cycle and ad libitum access to standard rodent chow diet (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA) and kept in a pathogen-free environment at the Laboratory Animal Unit. SCC-4 cells (5×10^6 cells/0.2 mL/mouse) were injected subcutaneously into the right front axilla. Eight days postimplantation, the mice were randomly divided into three groups

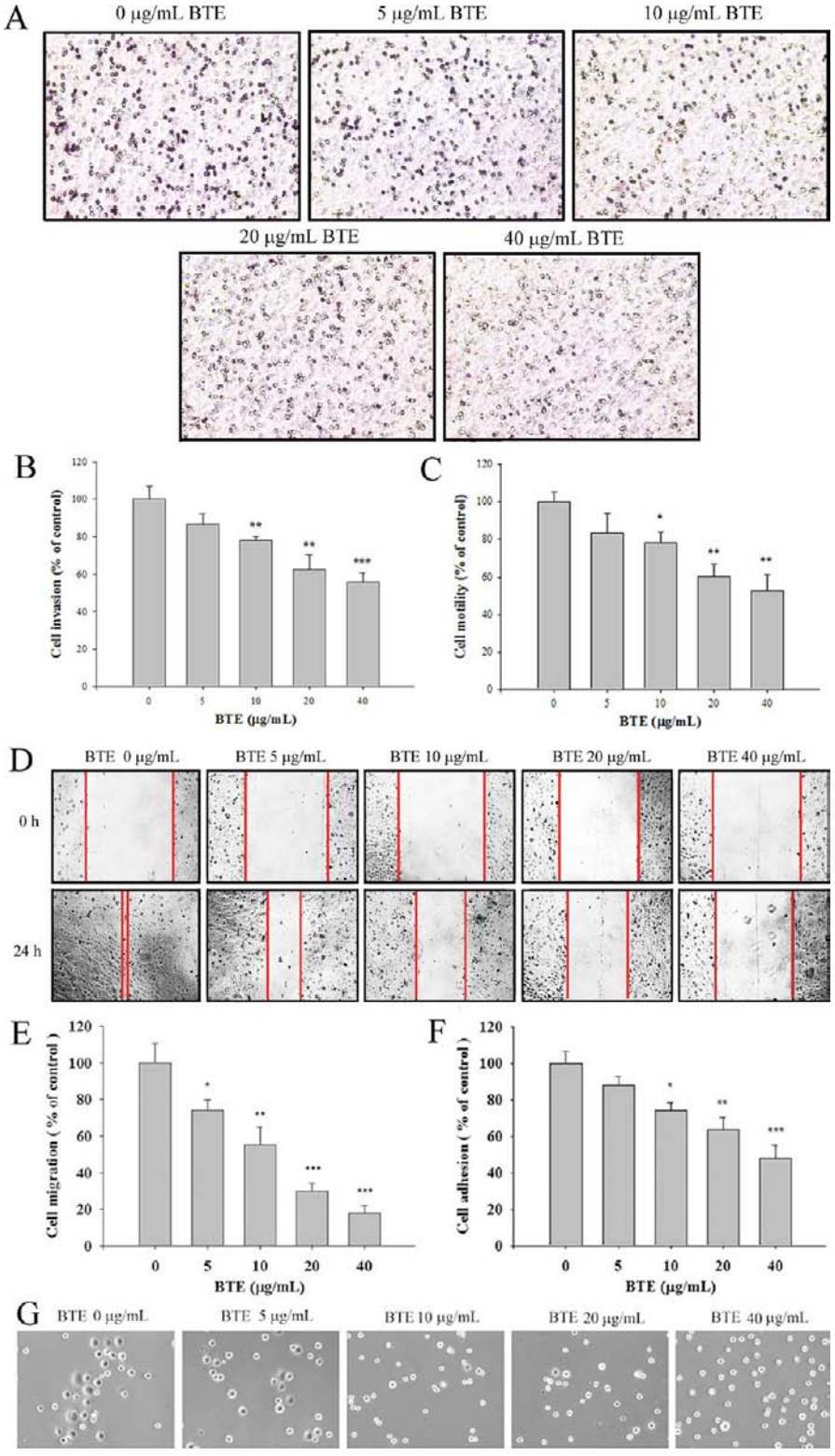

Figure 3. Effects of BTE on cell invasion, motility, migration, adhesion, and spreading. SCC-4 cells were pretreaded with BTE at the indicated concentrations for 24 h, and then equal numbers of cell were subjected to analyses for (A) cell invasion. (B) Quantification of invasion ability from (A). (C) Cell motility. (D) Cells were subjected to analysis for cell migration by wound-healing assay. (E) Determined migration ability of SCC-4 was subsequently quantified with that of control being 100% (without BTE for 24 h). Cells were treated with BTE for 24 h and then subjected to analysis for (F) cell adhesion and (G) cell spread. Data represent the mean ± SD of at least three independent experiments. Comparisons were performed by using one-way ANOVA with post hoc Dunnett's test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

($N = 5$ for each group) and fed by oral gavage with saline (control) and BTE (25 and 50 mg/day/kg) suspended in saline. The day of cell implantation was designated day 0. The average tumor volume at the start of treatment was approximately 128 mm^3, and tumors of the treated group and control group were measured every 3 days using vernier calipers to measure the long and short dimensions after

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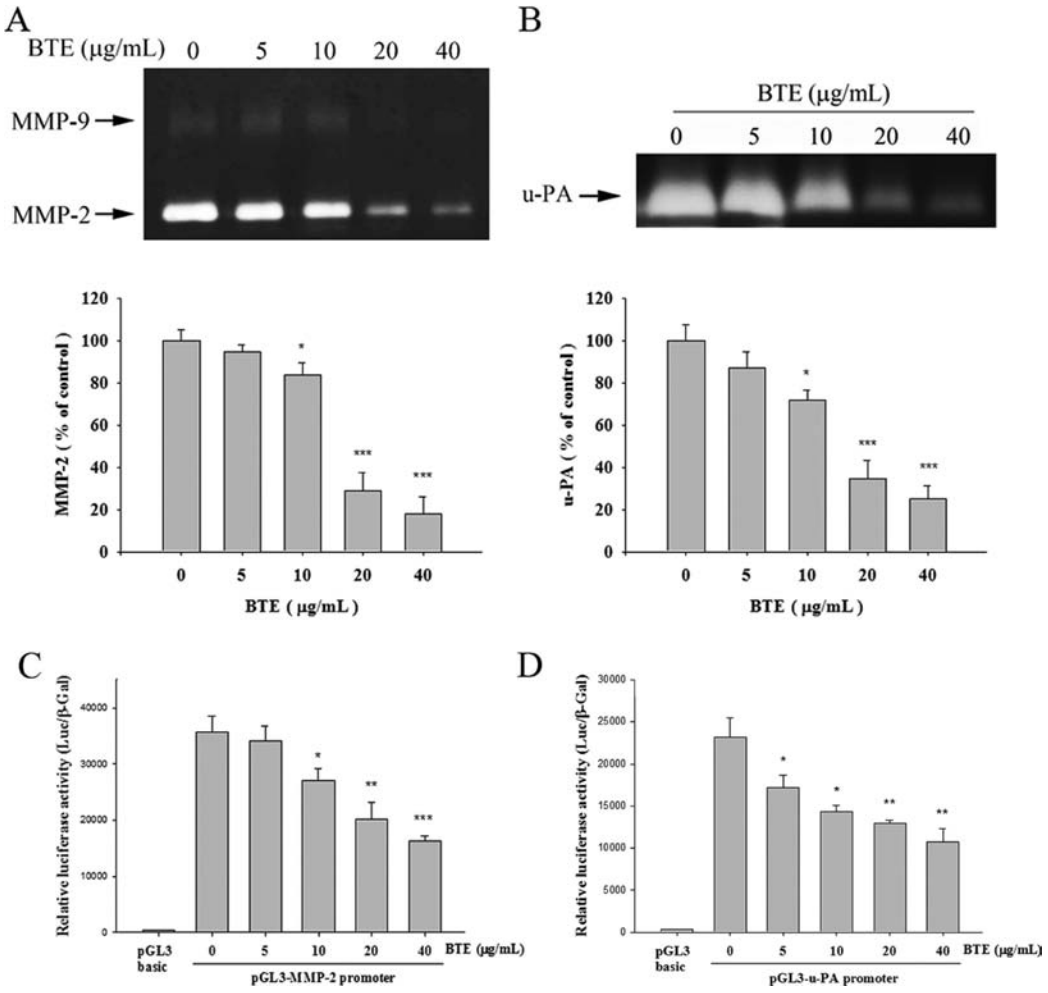

Figure 4. Inhibitory effects of BTE on the proteinase and transcription activities of MMP-2 and u-PA. Cells were treated with BTE for 24 h and then subjected to gelatin zymography and casein zymography to analyze the activities of (A) MMP-2 and (B) u-PA as described under Materials and Methods. Luciferase activity was measured in transiently transfected SCC-4 cells using (C) pGL3-MMP-2 and (D) pGL3-u-PA. Data represent the mean ± SD with that of control being 100%, and the statistical significance of results was analyzed by using one-way ANOVA with post hoc Dunnett's test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

10 days of cell injections. Body weight was assessed every 3 days after cell injection. After 46 days, the animals were euthanized, and the primary tumors were isolated and weighed.

Statistical Analysis. Statistical significances were analyzed by one-way analysis of variance (ANOVA) with post hoc Dunnett's test. A p value of <0.05 was considered to be statistically significant (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA, USA).

RESULTS

Effects of BTE on the Cell Viability of Oral Cancer Cells. To evaluate the bioactive compound of BTE, we successively extracted the BTE with 50% ethanol. Chromatographic patterns from HPLC analysis of BTE showed peaks corresponding to the retention times. Absorbance was monitored at 280 nm (Figure 1A). Figure 1 and Table 1 show that gallic acid, gallocatechin (GC), catechin, epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), and theaflavin 3,3′-digallate (TF3) are contained in the composition of BTE. The cell growth inhibitory effects of BTE on SCC-4 cells were determined. Results of the MTT assay showed that a 24 h treatment of BTE at concentrations up to 40 μg/mL has no cytotoxicity on SCC-4 cells (Figure 2A). Results from the same procedures performed on normal human gingival epithelial S-G cells revealed that this compound did not have any significant cytotoxicity on these cells (Figure 2B).

Inhibition on Cell Invasion, Motility, and Migration of SCC-4 Cells by BTE. To screen for the preventive effectors against cancer metastasis, the inhibitory effect of BTE on the invasion and motility of SCC-4 cells was examined with Transwell. The result showed that BTE significantly reduced cell invasion ($p < 0.001$) (Figure 3A), and the invasion ability of SCC-4 was subsequently quantified (Figure 3B). BTE also significantly inhibited the motility ($p < 0.001$) (Figure 3C) of SCC-4 cells in a concentration-dependent manner. Next, a wound-healing migration assay was performed to assess whether BTE affects cell migration. Incubation of SCC-4 with 1% FBS produced a marked cell migration in the wound area 24 h after wounding, whereas wounds treated with BTE showed significant delays in wound healing under the same conditions. The results showed that BTE inhibited cell migration in a dose-dependent manner (Figure 3D,E).

Inhibition on Cell Adhesion and Spreading of SCC-4 Cells by BTE. Cancer cells, invading the host tissue, break from their cell−cell contacts and make new contact with the ECM. Therefore, BTE was tested to determine their effects on the cell−matrix adhesion. The results showed that BTE

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significantly reduced the cell−matrix interactions of SCC-4 cells (Figure 3F). The majority of control (without BTE) cells exhibited spread morphology within 2 h of plating. By contrast, cells that were treated with BTE (40 μg/mL) remained round at the same time point (Figure 3G).

Inhibitory Effect of BTE on MMP and u-PA Activity. To clarify whether MMPs and u-PA were involved in inhibiting the invasion and migration of SCC-4 cells by BTE, the effects of BTE on MMP-2 and u-PA activities were investigated by gelatin and casein zymography under conditions of serum starvation, respectively. BTE reduced the activities of MMP-2 ($p < 0.001$) in gelatin zymography (Figure 4A) and the activity of u-PA ($p < 0.001$) in casein zymography (Figure 4B).

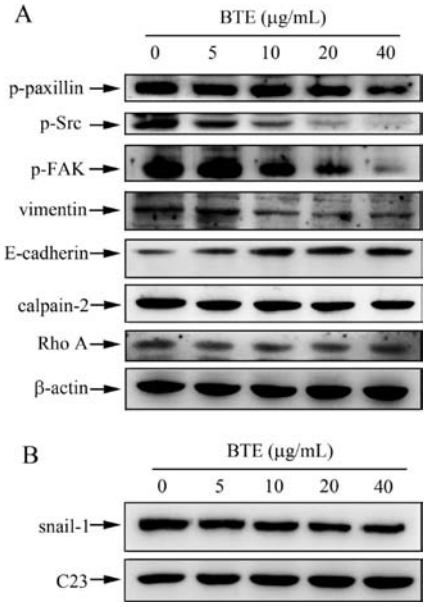

Figure 5. Effects of BTE on the cytoskeleton related protein: (A) Western blot analysis of cytoskeleton related protein with β-actin being an internal control in SCC-4 cells after 24 h of treatment with BTE; (B) nuclear extracts subjected to SDS-PAGE followed by Western blotting with anti-snail-1 antibodies with anti-C23 being an internal control. Similar results were obtained from three repeated and independent experiments.

Inhibitory Effect of BTE on the Transcriptional Activity of MMP-2 and u-PA Promoter in SCC-4 Cells. To evaluate the effects of BTE on the MMP-2 and u-PA promoter, we performed a transient transfection with the pGL3-MMP-2 and pGL3-u-PA promoter and analyzed the luciferase activities. The luciferase activities of the transfectants treated with BTE were reduced in a dose-dependent manner (Figure 4C,D).

Inhibition of p-Paxillin, p-FAK p-Src, and Vimentin and Up-regulation of E-Cadherin by BTE. BTE significantly inhibited the activation of paxillin, FAK, and Src and the expression of vementin in SCC-4 cells in a dose-dependent manner, with a slight decrease on calpain-2, whereas it has no significant effect on RhoA expression (Figure 5A). In Western blotting, BTE significantly inhibited the level of snail-1 in nuclear extracts of SCC-4 cells in a dose-dependent manner (Figure 5B). Moreover, immunofluorescence staining confirmed that vimentin expression was reduced in 40 μg/mL BTE-treated cells (Figure 6).

BTE Inhibits Phorbol-12-myristate-13-acetate (PMA)-Induced MMP-9 and u-PA Expression of SCC-4 Cells. Quantitative analyses by gelatin and casein zymography assay showed that the MMP-9 and u-PA of SCC-4 cells were increased by ∼10.3- and 5.8-fold upon PMA (a tumor promoter) treatment, respectively, and the PMA-induced MMP-9 (Figure 7A) and u-PA (Figure 7B) were significantly reduced by BTE treatment in a dose-dependent manner.

Antitumor Effects of BTE in Vivo. To verify the in vivo antitumor effects of BTE, SCC-4-bearing nude mice were treated with water or BTE. Small solid tumors were observed 10 days after the cell inoculation, and 1.71- and 2.58-fold reductions in BTE-treated (25 and 50 mg) animals were seen on day 46, respectively, as compared to control animals (Figure 8A). Moreover, by day 46, BTE feeding induced a 4.52-fold reduction in tumor weight (Figure 8B,C) without any apparent signs of toxicity as evidenced by body weight monitoring (Figure 8D) throughout the experiment.

DISCUSSION

Although of great advances have been made in cancer therapy during the past few years, most cancer deaths still result from tumor metastasis. The floor and tongue of the mouth are the most common sites of origin in Western countries. Nearly 50% of patients with oral squamous cell carcinoma present with

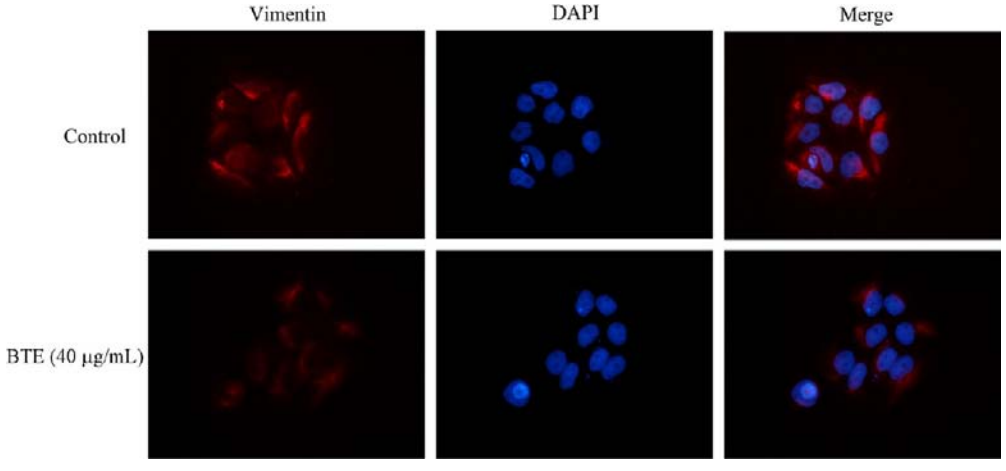

Figure 6. Effects of BTE on vimentin expression: immunofluorescence analysis of vimentin in SCC-4 cells with or without addition of 40 μg/mL BTE.

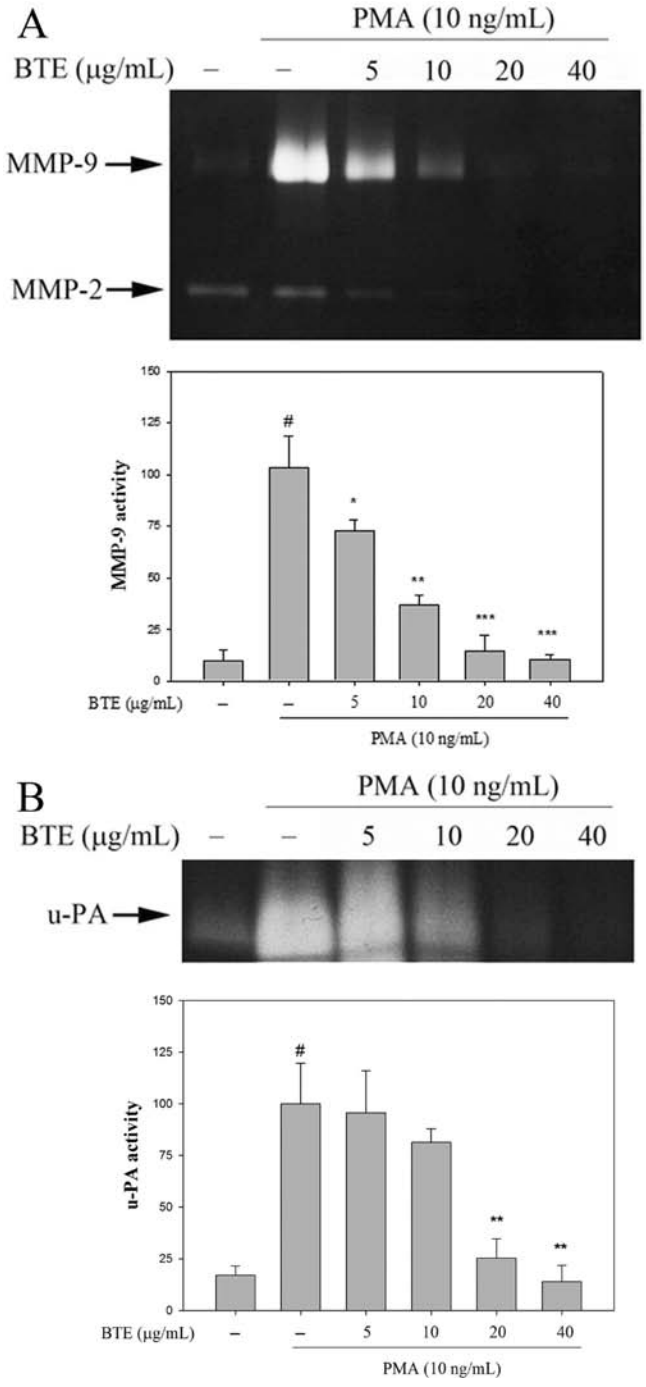

Figure 7. Concentration-dependent inhibitory effect of BTE on PMA-induced MMP-9 and u-PA activity: (A) MMP and (B) u-PA activities by gelatin zymography and casein zymography, respectively. Cells were pretreated with various concentrations of BTE for 1 h and then cultured in the presence of 10 ng/mL PMA for 24 h. The quantitative data are presented as the mean ± SD of three independent experiments (#, $p < 0.001$ compared with control; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with PMA-treated group).

pathologic or clinical evidence of lymph node metastasis and a 5-year survival rate of <50%.[19] Death as a result of oral cancer is often the result of local recurrence or regional or systemic metastasis. Thus, metastatic and invasive tumors are the major challenge in successful cancer treatment. Here, we demonstrated that BTE could significantly inhibit the invasion, motility, migration, promoter activities of MMP-2 and u-PA, and phosphorylation of FAK and Src in SCC-4 oral cancer cells, whereas this extract could reverse EMT by decrement of mesenchymal marker vimentin and up-regulation of epithelial marker E-cadherin. Black tea extracts inhibited cell−matrix adhesion, cell spreading, and PMA-induced protease activities in vitro. BTE also inhibited tumor growth in vivo via cancer cell xenografted nude mice mode.

The metastasis of solid tumor is a complicated process involving a group of proteolytic enzymes, which participate in the degradation of environmental barriers, such as ECM and basement membrane. Among these enzymes, MMPs and u-PA are deeply involved in the invasion and metastasis of various tumor cells.[20] Both proteinases MMP-2 and u-PA are abundantly expressed in various malignant tumors, including oral, breast, lung, brain, or liver cancer.[20,21] Therefore, MMPs or u-PA protein expressions and their regulatory pathways were considered as promising targets for anticancer drugs and chemopreventive agents.[21] Here, it was demonstrated that BTE markedly decreased the secretion levels and transcriptional activities of MMP-2 and u-PA. However, the regulation of BTE on the expression of MMPs in SCC-4 cells needs to be further defined in more detail. The 5′ flanking region of the MMP-9 gene contains several functional regulatory motifs that can bind with several well-characterized transcription factors, including activator protein-1, NF-κB, stimulatory protein-1, or polyoma virus enhancer activator-3.[22] Through one or more of these binding sites on the specific response elements on the promoter region of target genes, the expression of MMP-9 is regulated by various physical stimulators or chemicals, including cytokines (e.g., tumor necrosis factor-α), growth factors (e.g., epidermal growth factor), oncogenes (e.g., Ras), or diester of phorbol (e.g., PMA).[23] Among these stimulators, PMA can act as a potent tumor promoter to induce MMP-9 expression in several tumor cancer cells, including lung carcinoma, hepatocellular carcinoma, and oral squamous cell carcinoma. In the present study, BTE was sufficient to inhibit PMA-induced MMP-9 and u-PA expression in SCC-4 cells.

EMT is an embryonic program in which epithelial cells lose their characteristics and gain mesenchymal features. Several recent studies have shown that induction of EMT can lead to invasion of surrounding stroma, intravasation, dissemination, and colonization of distant sites. During EMT, tumor cells lose E-cadherin, a characteristic of epithelial cells, and express vimentin, a characteristic of mesenchymal cells.[24] This could be regulated by a transcription factor, called snail-1, which binds to the E-boxes within the E-cadherin promoter and thereby prevents its transcription. Snail-1 has been shown to increase the expression of vimentin and contribute to metastasis.[25] In the present study, BTE treatment showed the suppression of nuclear snail-1 expression. BTE also repressed the vimentin expression and increased the E-cadherin expression in SCC-4 cells.

Foacl adhesion kinase (FAK), a cytoplasmic tyrosine kinase that is involved in ECM/integrin-mediated signaling pathways, has been suggested to have an essential role in cancer metastasis. FAK is one of the Src's major binding partners, and the Src signaling pathway is an important component of cell−matrix adhesion changes associated with EMT.[26] It is reported that increased phosphorylation levels of Src were accompanied by repression of E-cadherin and up-regulation of vimentin expression in squamous carcinoma cell lines.[26] FAK has been shown to regulate cell migration and invasion through distinct pathways by promoting the dynamic regulation of focal adhesion and peripheral actin polymerization, as well as the

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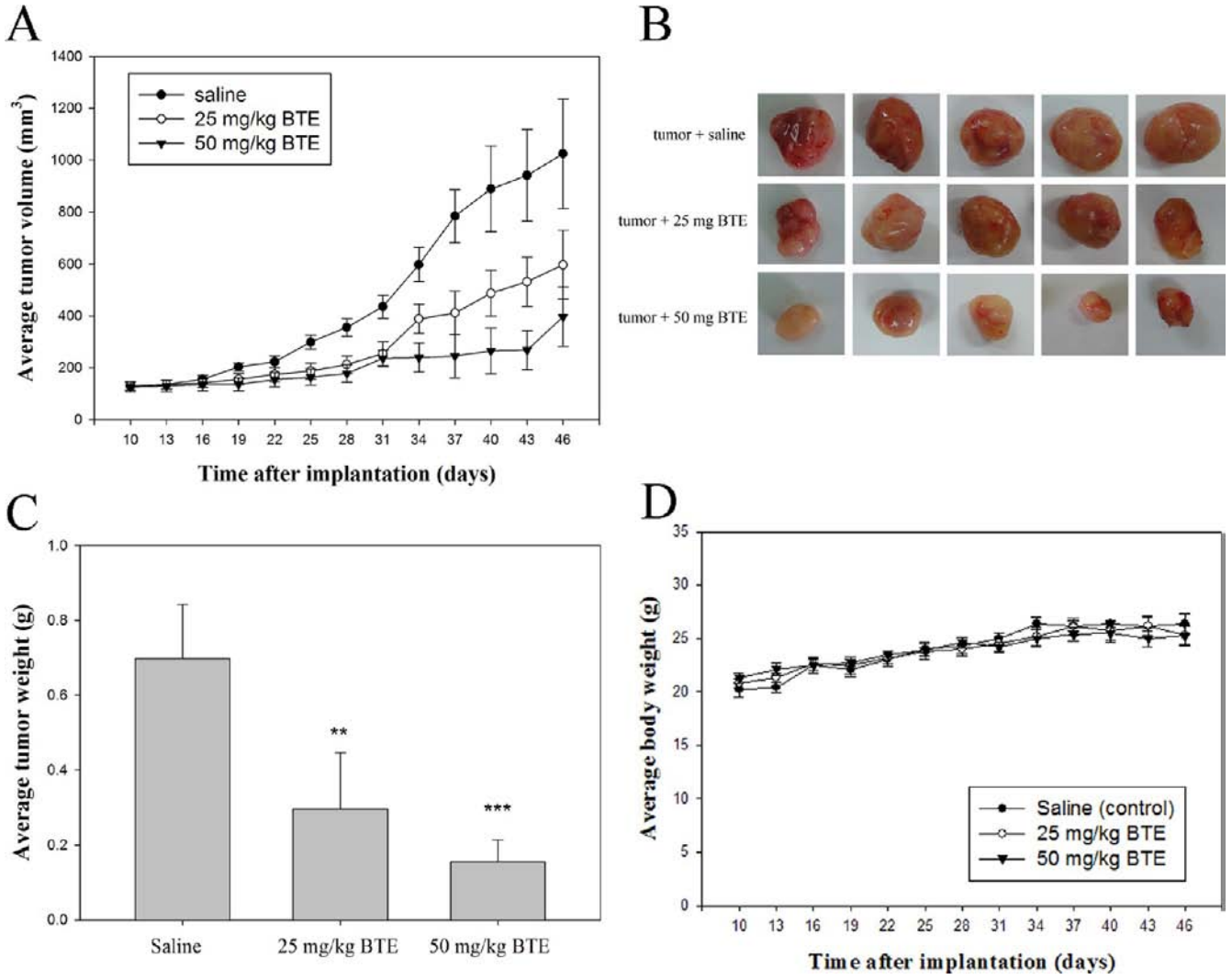

Figure 8. In vivo antitumor effects of BTE. After subcutaneous implantation of SCC-4 cells, BALB/c *nu*/*nu* mice were treated with saline or BTE and then analyzed for tumor growth: (A) average tumor volume; (B) tumor morphology on day 46; (C) average tumor weight; (D) body weight. Values represent the mean ± SD, and the statistical significance of results was analyzed by using one-way ANOVA with post hoc Dunnett's test (**, $p < 0.01$; ***, $p < 0.001$).

MMPs-mediated ECM degradation.[27] Tyrosine phosphorylation of FAK also triggers downstream signaling events, including p-paxillin, which is required for the cytoskeleton reorganization to facilitate cell metastasis.[28] Previous studies have shown that calpin-2 proteases are required for rear de-adhesion through its involvement in disassembly of focal adhesion during productive mobility whether initiated by adhesion-related signals or by growth factor (e.g., EGF).[29] Tumor cells that have high metastasized have been found to have higher levels of calpain than those that are not metastati.[30] Down-regulation of calpain-2 can limit prostate cancer cell invasiveness both in vitro and in vivo.[31] Indeed, treatment of SCC-4 cells with BTE caused a decrease in the expression of p-Src, p-FAK, p-paxillin, and calpain-2. Here, we first demonstrated that black tea extracts could significantly inhibit cell invasion via inhibition of EMT.

In summary, these findings suggest that the inhibition of invasion of oral cancer cells by BTE may be through a down-regulation of MMPs and u-PA expression of these cells and that BTE could reverse EMT in SCC-4 oral squamous cell carcinoma. These results suggest that BTE may be useful as an effector for the prevention of cancer metastasis, in addition to supporting the role of black tea as an oral cancer chemopreventive agent.

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Notes

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ABBREVIATIONS USED

EMT, epithelial-to-mesenchymal transition; PMA, phorbol-12-myristate-13-acetate; FAK, focal adhesion kinase; ECM, extracellular matrix; EGCG, epigallocatechin-3-gallate; GC, gallocatechin; ECG, epicatechin-3-gallate; MMP, matrix metalloproteinase; u-PA, urokinase-type plasminogen activator.

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