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EGCG Inhibits the Invasion of Highly Invasive CL1-5 Lung Cancer Cells through Suppressing MMP-2 Expression via JNK Signaling and Induces G2/M Arrest

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ABSTRACT: Tumor metastasis is the main obstacle to the treatment of lung cancer. According to previous findings, matrix metalloproteinase-2 (MMP-2) is closely correlated with metastatic potential in lung cancer. This study showed that epigallocatechin-3-gallate (EGCG), a natural polyphenol in green tea, is a potent inhibitor of MMP-2 expression. EGCG effectively suppressed the invasion and migration of highly invasive CL1-5 lung cancer cells. Gelatin zymography, Western blot analysis, and RT-PCR were used to investigate the effects of EGCG on MMP-2 expression. The effects of EGCG on cell cycle and apoptosis were determined by flow cytometry analysis. To investigate the effects of EGCG on cell migration and cell invasion, Transwell migration/invasion assays were used. EGCG downregulated MMP-2 expression at the transcriptional level in CL1-5 cells. Moreover, the treatment of CL1-5 cells with EGCG caused downregulation of c- Jun N-terminal kinase (JNK), resulting in repression of the translocation of transcriptional factors, Sp1, and NF-κB, from the cytosol into the nucleus. In addition, EGCG significantly and synergistically enhanced the antitumor effects of the clinical drug, docetaxel, in CL1-5 cells. Further, EGCG induced G2/M arrest at dosages higher than those of suppression in cell invasion in CL1-5 cells. These results reveal that EGCG might decrease MMP-2 mRNA expression through JNK signaling, further suggesting that a combination of EGCG and docetaxel may be a promising strategy to help increase the efficacy of docetaxel in suppressing metastasis in lung cancer cells. In addition, EGCG may suppress cell proliferation in CL1-5 cells through inducing G2/M arrest.

KEYWORDS: EGCG, metastasis, lung cancer, G2/M arrest

INTRODUCTION

Epidemiological studies have shown that green tea consumption decreases the risk of human cancers.[1] Green tea is thought to exert inhibitory effects against carcinogenesis and proliferation resulting from the biological activities of its polyphenol components. EGCG (Figure 1A) is the major polyphenol component in green tea, which is a potential component for antiproliferation,[2] antioxidation,[3] anti-inflammation,[4,5] and antimutagenesis.[6] The main impediment to treating lung cancer is tumor invasion. Therefore, investigating whether EGCG could inhibit tumor invasion in lung cancer and the underlying mechanisms could be interesting avenues of exploration in the treatment of lung cancer. To date, previous studies show that the effects of EGCG on the treatment of lung cancer contribute to inhibit cell growth[7−9] or induce apoptosis.[10−12] However, the main impediment to treat lung cancer is tumor invasion. Therefore, to investigate whether EGCG could inhibit tumor invasion in lung cancer and the underlying mechanisms might be exploits toward the treatment of lung cancer.

Lung cancer has the highest rate of cancer mortality worldwide, exceeding the mortality rates of colorectal, breast, and prostate cancers combined.[13] The high mortality of this disease is due to the high potential of invasion and metastasis to distant organs. The average 5-year survival rate is only 8−14% and has improved only slightly during the past 25 years.[14] Lung cancer is highly resistance to radiation and chemotherapy, and more than 90% of patients diagnosed with lung cancer die from their disease.[15] Tumor metastasis is the main obstruction on the treatment of lung cancer. Therefore, blocking metastasis might be a potential approach to improve cancer therapy in this deadly disease.

Metastasis is a fundamental property of highly malignant cancer cells with poor clinical outcome. One important step is the degradation of extracellular matrix (ECM) which exerts mechanical barriers to cell invasion to tissues. Excess ECM degradation is one of the hallmarks of tumor metastasis. In addition, MMPs may stimulate cell proliferation by releasing growth factors[16] and contribute to other steps in the metastatic cascade, such as angiogenesis, differentiation, and apoptosis.[17] In general, MMPs are expressed at very low levels in normal adult tissues, and the levels become raised only when there is a challenge to the system, such as a wound that is required to heal over.[18] It is well established that MMPs play an important role in tumor invasion and metastasis.[17] MMPs are divided into different types based on their substrate specificity and structural homology.[19] In particular, MMP-2 and MMP-9 have a unique ability to degrade collagen, a major component of the basement membrane, and are strongly implicated in the invasion and metastasis of malignant tumors.[20] Further, MMP-2 and MMP-9 are the major proteases in lung cancer and are closely related to invasion in patients with lung carcinoma.[21,22] MMP-2 was first mentioned by Liotta.[23] The proteolytic activity of MMP-2 alters affinity and avidity for their ECM, which facilitates the migration of endothelial cells toward the source of angiogenic stimulus. The activity of MMP-2 is highly regulated at four levels, including transcriptional level, stability of mRNA, proenzyme activation, and posttranslational level through interaction with the tissue inhibitor

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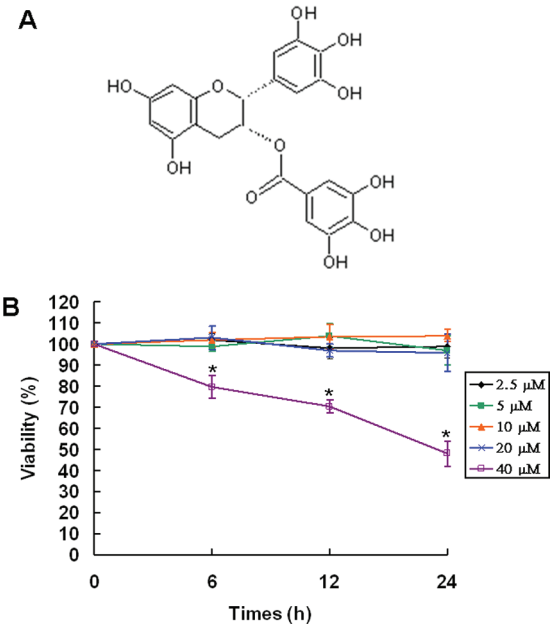

Figure 1. Effects of EGCG on the cell viability of CL1-5 cells. (A) The chemical structure of EGCG. (B) CL1-5 cells were treated with different concentrations (2.5, 5, 10, 20, and 40 μM) of EGCG for the indicated time periods, and subsequent cell viability was measured by an MTT assay, as described in Materials and Methods. The number of viable cells after treatment is expressed as a percentage of 0 h control. The results represent the mean ± SE of three independent experiments. *Indicates the values significantly differ from the control ($^*P < 0.05$).

of metalloproteinases, TIMP-2.[24] In addition, MMP-2 is overexpressed in lung cancer and is closely correlated with metastatic potential.[21,25] Thus, repression of MMP-2 might affect the treatment of lung cancer.

The cell cycle is the event leading to cell duplication and division. The cell cycle contains four major phases, including the G1 (gap) phase, the S (synthesis) phase, the G2 (gap) phase, and the M (mitosis) phase. The G1 phase ensures that everything ready for DNA synthesis. DNA duplication is performed in the S phase. The G2 phase ensures that everything is ready for cell division. Cell division is performed in the M phase. When the cell cycle stops at the G1 or G2 phase in the cell, proliferation of the cell will decrease.

This study evaluated the inhibitory effects of EGCG on the invasion of the highly invasive human lung adenocarcinoma cell line, CL1-5. We showed that EGCG suppresses MMP-2 expression at the transcriptional level. Further, we demonstrated that downregulation of MMP-2 resulted from decreasing translocation of NF-κB and Sp1 and inactivation of JNK. We also found that EGCG could induce G2/M arrest at higher doses than suppress the invasion in CL1-5 cells. Thus, EGCG might be a chemopreventive agent against highly invasive human lung cancer.

MATERIALS AND METHODS

Materials. EGCG, bovine serum albumin (BSA), docetaxel and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). SP600125 and antibodies against MMP-2 and MMP-9 were obtained from Calbiochem (San Diego, CA). Antibodies against ERK 1 (extracellular signal-regulated protein kinase) MAPK (mitogen-activated protein kinase), p38 MAPK, JNK1, Akt, phospho-p44/p42 MAPK, phospho-p38 MAPK, phospho-JNK, phospho-Akt, Bcl-2, Bax, p21 and p27 were purchased from Cell Signaling Technology (Beverly, MA). Antibody against FAK was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against phospho-FAK was purchased from BD Biosciences (Los Angeles, CA). Antibody against β-actin was obtained from Abcam Inc. (Cambridge, MA). Anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase and were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture. The highly invasive human lung adenocarcinoma cell line, CL1-5 cells, and less invasive CL1-0 human lung cancer cells were kindly provided by Dr. M. S. Lee (National Taiwan University, Taipei, Taiwan). CL1-5 cells were established previously.[26] Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin−streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. These cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

Cell Viability Assay. MTT assay was used to determine cell viability. Briefly, cells were seeded in 24-well plates (2×10^4 cells/well) overnight, and then treated with various concentrations of EGCG for the indicated times. Then, 300 μL of MTT solution (2 mg/mL in PBS) was added to each well and incubated for 2−4 h at 37 °C. The supernatant was aspirated, and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 500 μL of DMSO. Finally, the absorbance was monitored by a microplate reader at a wavelength of 550 nm.

Cell Invasion and Migration Assays. The invasive ability of the cells treated with the indicated concentrations of EGCG was examined by Boyden chambers (Millipore, Bedford, MA) invasion assay.[27] Briefly, the upper surface of the Transwell membrane was coated with 40 μL of Matrigel (1 mg/mL; BD Biosciences, Bedford, MA). For invasion assay, 2×10^5 cells were seeded onto the upper well of each chamber. The bottom chamber contained the same medium with 10% FBS. Cells were then treated with the indicated concentrations of EGCG. After incubation for 24 h, cells were fixed with methanol for 10 min and stained with 0.25% Coomassie Brilliant Blue G. The invaded cells were counted under a light microscope (magnification, ×200). The method for the Transwell migration assay was the same as that for the Transwell invasion assay, except that the Transwell membrane was not coated with Matrigel and treated with EGCG for 16 h.

Zymography Assay for MMP-2 and MMP-9. The amount of gelatinase (MMP-2, MMP-9) in the conditioned medium was estimated by gelatin zymography assay.[28] Then, conditioned media were concentrated 50-fold and mixed with loading buffer containing 0.01% SDS but not β-mercaptoethanol. The samples were not boiled before loading. Sixteen microliters of each prepared sample was subjected to electrophoresis in the presence of SDS in 10% polyacrylamide gels containing 0.1% (w/v) gelatin. After SDS−PAGE, the gel was washed twice, each time for 30 min at room temperature in a solution containing 2.5% (v/v) Triton X-100 to remove SDS, and then washed twice, each time for 20 min at room temperature in ddH_2O. The gel was subsequently transferred to a substrate buffer containing 50 mM Tris-HCl/5 mM $CaCl_2$/0.02% NaN_3, pH 8.0 at 37 °C with shaking for 18 h. Then the gel was stained with 0.25% (w/v) Coomassie blue in 10% (v/v) acetic acid and 45% (v/v) methanol and destained with 10% (v/v) acetic acid and 5% (v/v) methanol. Clear bands of gelatinolytic activity were visualized.

Western Blot Analysis. Cells were treated with various agents as indicated in figure legends. After treatment, cells were placed on ice, washed twice with cold PBS (pH 7.4), and lysed in lysis buffer [1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 25 mM NaF, 0.5 mM sodium orthovanadate, 1 mM dithiothreitol, 1 μg/mL pepstanin, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.1 mg/mL phenylmethylsulfonyl fluoride]. After vigorous vortexing, cell lysates were centrifuged at 12,000 rpm for 30 min. The concentrations of protein extracts were

estimated with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard. Equal amounts of proteins (50 μg) were mixed with 5× sample buffer, boiled at 95 °C for 5 min, and resolved by 10% SDS−PAGE. Proteins then transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked with a blocking buffer containing 1% bovine serum albumin in PBS at room temperature for 1 h. It was further incubated with indicated primary antibodies at 4 °C overnight followed by washing with 1× PBST (1× PBS, 0.02% Tween-20, v/v). The membrane was continuously incubated with appropriate secondary antibodies conjugated with horseradish peroxidase at room temperature for 1 h. Immunoreactivity was detected with an enhanced chemiluminescence system (Amersham, Piscataway, NJ).

RT-PCR. Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer's instructions. Then, 5 μg of total RNA was reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase and oligo (dT_{18}) primer at 37 °C for 90 min. PCR amplification was performed in a final volume of 50 μL of reaction mixture containing 2 μL of cDNA, deoxynucleotide triphosphates each with 200 μM, 1× reaction buffer, 1 μM of each primer (MMP-2, forward 5′- GGCCCTGTCACTCCTGA-GAT-3′, reverse 5′-GGCATCCAGGTTATCGGGGA-3′; MMP-9, forward 5′-AGTGGCACCACCACAACAT-3′, reverse 5′-TCCTGGGTGTAGAG-TCTCTCG-3′; GAPDH, forward 5′-GAGTCAACGGATTTGGTCGT-3′, reverse 5′-TGTGGTCATGAGTCCTTCCA-3′), and 50 units/mL of Taq DNA polymerase. The reaction mixtures were incubated at 95 °C for 5 min to denature, and then 30 cycles (95 °C for 30 s, 58 °C for 1 min, 72 °C for 2 min) of PCR amplification were performed, followed by 72 °C for 10 min. Each 5 μL of the PCR product was separated on a 2% agarose gel and visualized by staining with ethidium bromide.

Preparation of Cytosolic and Nuclear Lysate. The collected cells were suspended in ice-cold hypotonic buffer and incubated on ice for 50 min. The extracts were centrifuged at 12,000 rpm for 1 min, and the supernatant was collected (cytosolic fraction). The pellets were then washed with ice-cold hypotonic buffer and resuspended in RIPA lysis buffer followed by sonication. The extracts were centrifuged at 12,000 rpm for 30 min, and the supernatant was collected (nuclear fraction).

Immunocytochemistry. Cells were washed with PBS, fixed with 4% formaldehyde and blocked with 1% BSA/PBS for 1 h at room temperature. Then, primary antibodies were incubated with cells for 1 h at room temperature and secondary antibodies conjugated with Alexa Fluor 555 were employed for 1 h at room temperature. DAPI was used to stain the nuclei, and images were captured by Leica confocal microscopy (Leica Microsystems GmbH, Wetzlar, Germany).

Transient Transfection and Luciferase Reporter Assay. Plasmid pGL3 containing the full-length of the human MMP-2 promoter and pGL3-Renilla luciferase control reporter plasmid were kind gifts from Dr. S. W. Tang (National Taiwan University, Taipei, Taiwan). Luciferase activities were estimated by a dual luciferase assay kit (Promega, Madison, WI). Cells were seeded in 12-well plates overnight and then cotransfected with 0.5 μg of pGL3 plasmid containing the full-length of the human MMP-2 promoter and 0.05 μg of pGL3-Renilla as an internal control for normalization. pGL3 plasmid containing the full-length of the human MMP-2 promoter and pGL3-Renilla were mixed with OPTI-MEM for 5 min. Furthermore, Lipofectamine 2000 was also mixed with OPTI-MEM for 5 min. Then, plasmid and Lipofectamine 2000 were mixed and incubated for 25 min. The mixture was added to the cells to perform the transfection process. Following transfection, cells were incubated in culture media to recover overnight. Cells were then treated with or without the indicated concentrations of EGCG for 9 h, and the luciferase activities were determined using a dual luciferase assay kit (Promega, Madison, WI) following the manufacturer's instructions and detected by a Berthold luminometer (Berthold, Oak Ridge, TN).

Flow Cytometry Analysis. CL1-5 cells (1×10^6) were cultured in 10 cm dishes and incubated for 24 h, and they were then treated with the indicated concentrations of EGCG for 24 h. Then, the cells were harvested and fixed in iced 100% ethanol at −20 °C. Cell pellets were resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 mg/mL RNase) and incubated at 37 °C for 1 h. Then, 1 μL of propidium iodide solution (50 mg/mL) was added. Fluorescence emitted from the propidium iodide−DNA complex was quantitated by FACScan cytometry (Becton Dickinson, San Jose, CA).

In Vivo Studies. Female BALB/c nude mice (6−8 weeks of age) were purchased from the National Animal Center and maintained in a pressurized ventilated cage according to institutional regulations. CL1-5 cells (5×10^5) in 100 μL of PBS were injected into the tail vein of nude mice. Mice were randomly divided into two groups. Each group contained 5 mice. The first group only received PBS as a vehicle. The other group was given an ip injection containing EGCG (50 mg/kg) twice a week for 6 weeks. After 6 weeks, the mice were sacrificed and the lungs were removed. The lungs were rinsed with PBS and then placed in formalin. After 24 h, an H&E stain was performed.

Statistical Analysis. All values were expressed as mean ± SD. Each value is the mean of at least three separate experiments in each group. The significant difference (P value) between experimental groups was found with Student's t tests. $P < 0.05$ was considered significant (*, $P < 0.05$).

RESULTS

EGCG Induced G2/M Arrest but Not Apoptosis at Higher Dose in CL1-5 Cells. Adenocarcinoma is a subgroup of non-small cell lung cancer, and it is the most common type of lung cancer in the U.S. and Asia.[29] Patients diagnosed with lung adenocarcinoma are not candidates for surgical resection because they are in an advanced stage resulting from its highly invasive properties. Thus, we used a special lung cancer cell line, the highly invasive CL1-5 human lung adenocarcinoma cell line, to estimate the effects of EGCG on human lung cancer. Previous studies revealed that EGCG could inhibit cell growth[7−9] or induce apoptosis[10−12] in lung cancer. CL1-5 is a highly invasive human lung cancer cell line,[26] therefore, we used the CL1-5 cell line to investigate the effects of EGCG in lung cancer. In order to test the effects of EGCG on human lung cancer CL1-5 cells, we first determined whether EGCG could influence the cell viability of CL1-5 cells. Cells were treated with the indicated concentrations of EGCG for the indicated time periods. After treatment, the number of live cells was detected using MTT assay as described in Materials and Methods. In the MTT assay (Figure 1B), we found that EGCG could suppress cell proliferation at doses higher than 20 μM. We then determined whether EGCG could induce cell cycle arrest or apoptosis at doses higher than 20 μM in CL1-5 cells. Cells were treated with EGCG (30, 40, and 50 μM) for 6, 12, and 24 h and analyzed by flow cytometry. EGCG at 30, 40, and 50 μM could not induce apoptosis, but did promote G2/M arrest in CL1-5 cells (Figure 2A). To further confirm these results, Western blotting was used to detect the protein expression of apoptosis-related proteins. Apoptosis-related proteins such as PARP, Bcl-2, and Bax did not change under EGCG treatment (Figure 2B). Thus, EGCG could not induce apoptosis at 30, 40, and 50 μM. In addition, EGCG could not change the protein expression of G1 arrest-related proteins such as CDK2, CDK4, cyclin E, p21 and p27. However, EGCG can decrease M phase-promoting protein, cyclin B1, and induce cell arrest in the G2 phase, but it cannot decrease M phase-promoting protein, CDK1 (Figure 2C). Therefore, EGCG can induce cell cycle arrest at G2 phase at doses higher than 20 μM.

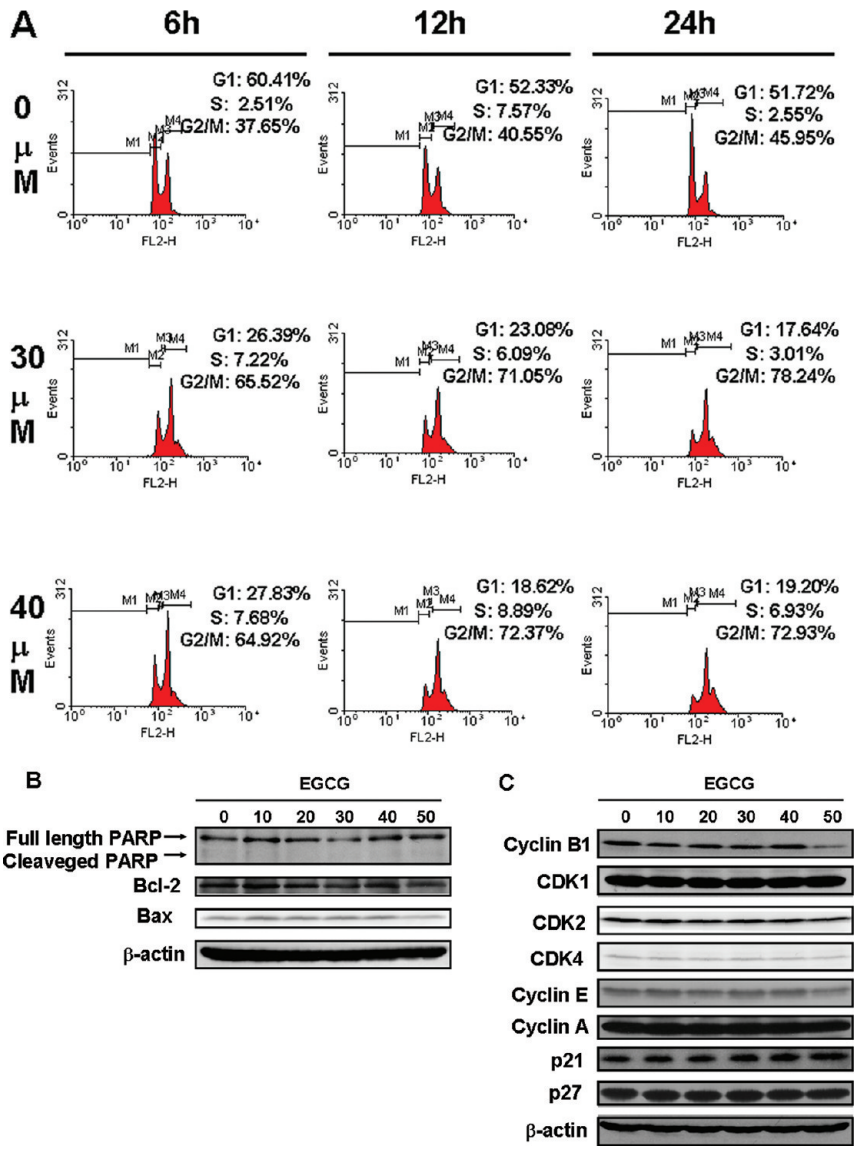

Figure 2. Effects of EGCG at higher doses in CL1-5 cells. (A) CL1-5 cells were treated with the indicated concentrations (30, 40, and 50 μM) of EGCG for 6, 12, or 24 h. Cells were then analyzed by flow cytometry. (B) Cells were treated with the indicated concentrations of EGCG for 24 h and analyzed by Western blotting to detect protein expression of apoptosis-related proteins. (C) Cells were treated with the indicated concentrations of EGCG for 24 h and analyzed by Western blotting to detect protein expression of cell cycle-related proteins.

EGCG Suppressed Cell Invasion and Migration in CL1-5 Cells. As shown in Figure 1B, EGCG did not affect the cell viability of CL1-5 cells at 2.5, 5, 10, and 20 μM under 24 h. EGCG only decreased the cell viability of CL1-5 cells at higher doses. EGCG appeared not to repress cell proliferation of highly invasive human lung cancer CL1-5 cells at doses lower than 20 μM.

The main obstruction to treating lung cancer is tumor invasion. Thus, we wanted to determine whether EGCG had effects on the inhibition of cell invasion and migration in highly invasive CL1-5 cells.[26] Since EGCG reduced cell viability over the concentration of 20 μM (Figure 1B), the effects of EGCG on cell invasion and migration in CL1-5 were performed below 20 μM using Transwell invasion/migration assays. EGCG (5−20 μM) reduced the invasive ability of CL1-5 in a dose-dependent manner (Figure 3A,B). Similar results were shown in the Transwell migration assay. EGCG (5−20 μM) also decreased the migratory ability of CL1-5 cells in a dose-dependent manner (Figure 3C,D). These results suggested that EGCG significantly suppressed cell invasion and migration but not cell viability in CL1-5 cells below 20 μM. To further confirm that EGCG could indeed suppress metastasis of CL1-5 cells, in vivo animal studies were used. A detailed description is given in Material and Methods. In Figure 3E, most of the tumor colonies were found on the surface of the lungs. The injection of EGCG showed a significantly reduced number of tumor nodules in the lungs compared to the control group. The H&E staining was further used to confirm this data (Figure 3E). EGCG can suppress cell invasion at low doses (5, 10, and 20 μM) in CL1-5 cells.

Effects of EGCG on MMP-2 and MMP-9 Activities in Highly Invasive CL1-5 Cells and Less Invasive CL1-0 Cells. Excess ECM degradation is one of the hallmarks of tumor invasion. MMP-2 and MMP-9 play important roles to degrade collagen, a major component of the basement membrane, and are strongly implicated in invasion and metastasis of malignant tumors.[20]

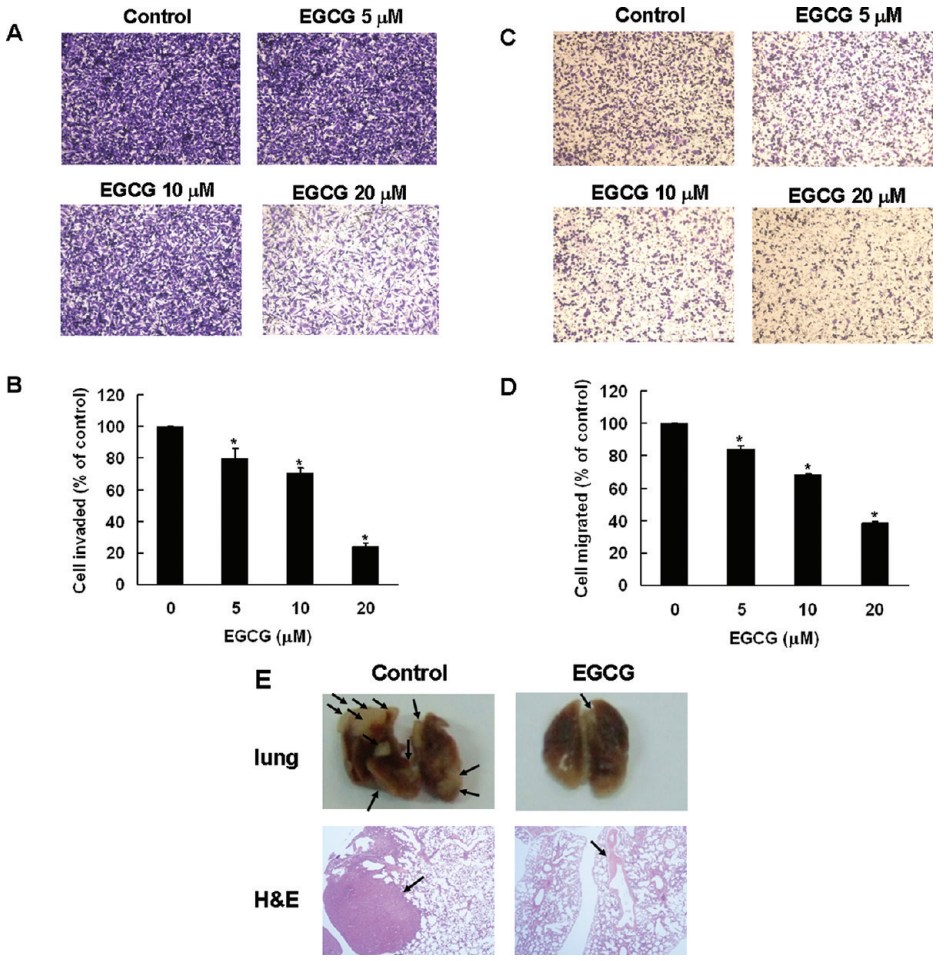

Figure 3. Effects of EGCG on cell invasion and migration in CL1-5 cells. (A, B) 2×10^5 CL1-5 cells were seeded in the upper chamber of a Transwell coated with Matrigel (40 μg/well) and treated with the indicated concentrations (5, 10, and 20 μM) of EGCG for 24 h. Cells on the upper surface of the Transwell membrane were wiped away, and cells on the lower surface of the Transwell membrane were stained and counted under a microscope. Photographs of cells that invaded the Matrigel (A) and quantification of the three independent experiments (B) are illustrated. (C, D) 2×10^5 CL1-5 cells were seeded in the upper chamber of the Transwell not coated with Matrigel and treated with the indicated concentrations (5, 10, and 20 μM) of EGCG for 16 h. Photographs of cells that invaded the Matrigel (C) and quantification of the three independent experiments (D) are illustrated. (E) EGCG suppressed cell invasion in vivo. Mice were randomly divided into two groups. Each group contained 5 mice. CL1-5 (5×10^5) cells were injected into the tail vein of nude mice. The first group only received PBS as a vehicle. The other group was given an ip injection containing EGCG (50 mg/kg). After 6 weeks, the mice were sacrificed and the lungs were removed and placed in formalin. After 24 h, H&E staining was performed. *Indicates the values significantly differ from the control ($^*P < 0.05$).

In addition, MMP-2 and MMP-9 are the major proteases in lung cancer and are closely related to invasion in patients of lung carcinoma.[21,22] To establish whether EGCG has any effect on MMP-2 and MMP-9 in human lung adenocarcinoma, we determined the effects of EGCG on the activities of MMP-2 and MMP-9 in highly invasive CL1-5 cells and less invasive CL1-0 human lung cancer cells. Conditioned media of CL1-5 cells and CL1-0 cells treated with EGCG (0−20 μM) for 24 h were analyzed by gelatin zymographic assay. The gelatin degraded activity in the zymography was significantly decreased in the presence of EGCG at 10 μM in highly invasive CL1-5 cells, however, it was only slightly reduced at an EGCG concentration of 20 μM in less invasive CL1-0 cells (Figure 4A). These data suggested that EGCG could repress the activities of MMP-2 and MMP-9 in human lung adenocarcinoma cells, especially cells showing highly invasive ability.

Effects of EGCG on the Protein and mRNA Levels of MMP-2 and MMP-9. To explore whether downregulation of the gelatinase activity was due to diminished amounts of MMP-2 and MMP-9, the protein levels of MMP-2 and MMP-9 were analyzed by Western blot in the present of EGCG (0−20 μM) for 24 h in CL1-5 and CL1-0 cells. As shown in Figure 4B, the protein levels of MMP-2 were significantly repressed in the presence of EGCG (5−20 μM) in highly invasive CL1-5 cells in a dose-dependent manner, however, MMP-2 protein only showed a slight decrease at an EGCG concentration of 20 μM in less invasive CL1-0 cells. At the same time, the protein levels of MMP-9 showed no significant change in the presence of EGCG, neither in CL1-5 nor in CL1-0 cells (Figure 4B). Further, to examine whether the suppressed protein level of MMP-2 in CL1-5 cells by EGCG was due to the reduced mRNA level of MMP-2, RT-PCR analysis was performed in CL1-5 cells. As shown in Figure 3C, the mRNA level of MMP-2 was dramatically reduced in a dose-dependent manner in the presence of EGCG (5−20 μM) for 9 h in CL1-5 cells. Nevertheless, EGCG could not influence the mRNA level of MMP-9 in CL1-5 cells (Figure 4C). These results demonstrated that EGCG

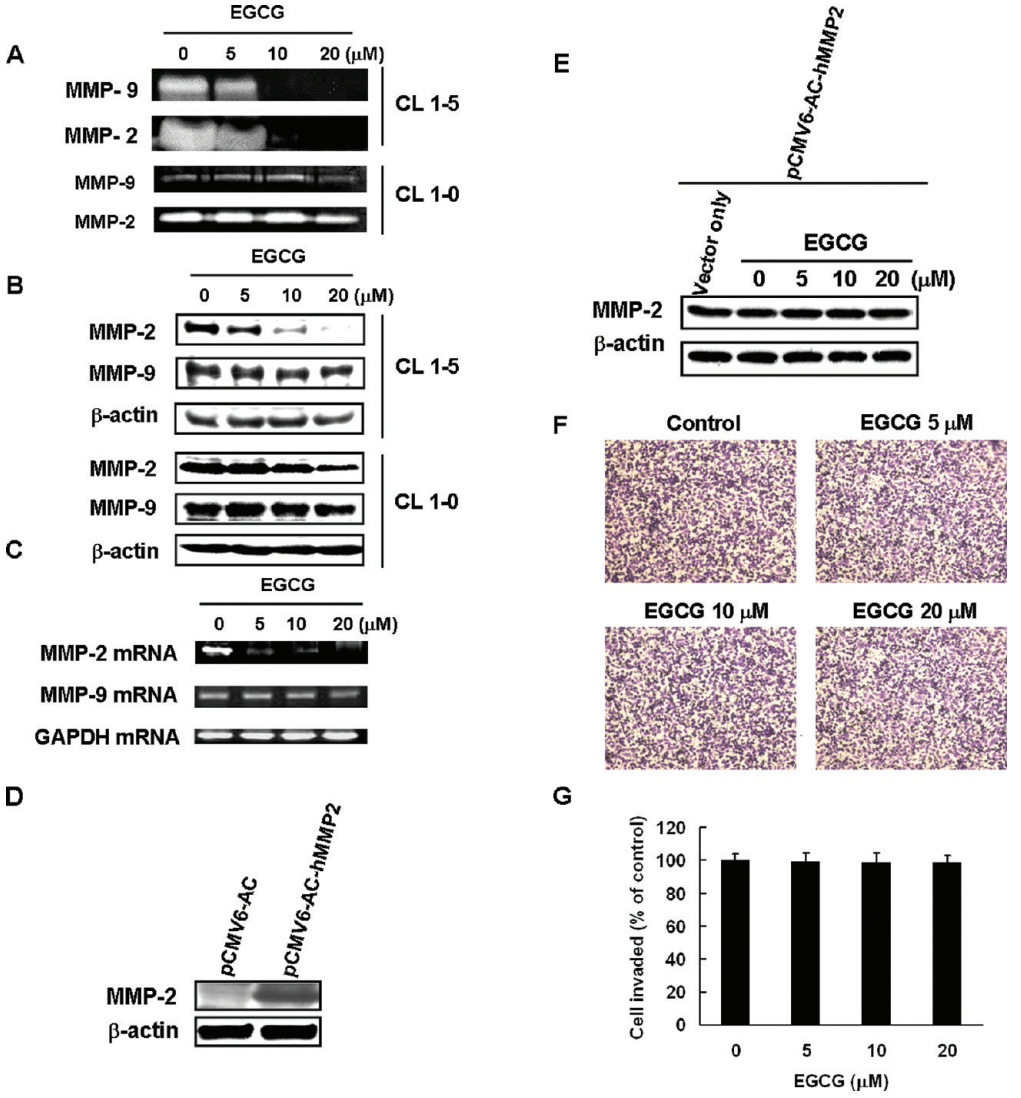

Figure 4. Effects of EGCG on the activities and expression of MMP-2 in lung cancer cells. (A) Effects of EGCG on the MMP-2 activities in highly invasive CL1-5 cells and less invasive CL1-0 cells. CL1-5 and CL1-0 cells were treated with the indicated concentrations (0, 5, 10, and 20 μM) of EGCG for 24 h. Cell lysates were subjected to 10% SDS−PAGE containing 0.1% gelatin and incubated in reaction buffer as described in Materials and Methods. (B) Effects of EGCG on the expression of protein levels of MMP-2 in CL1-5 and CL1-0 cells. Cells were treated with the indicated concentrations of EGCG for 24 h, and the cell lysates were subjected to Western blot analysis. Protein expression of MMP-2 and MMP-9 was determined by using specific antibodies. The Western blot data presented was representative of that obtained in at least three separate experiments. The expression of β-actin was used as the internal control for equivalent loading. (C) Suppression of MMP-2 mRNA by EGCG in CL1-5 cells. Cells were treated with different concentrations of EGCG for 9 h, and RT-PCR analysis was performed to determine the mRNA levels of MMP-2 and MMP-9. GAPDH was used as the internal control for equivalent loading. (D) Overexpressed MMP-2 in CL1-5 cells. Plasmid pCMV6-AC-hMMP2 was transfected into CL1-5 cells for 24 h to overexpress MMP-2 in CL1-5 cells. (E) Effects of EGCG on CL1-5 cells overexpressed MMP-2. CL1-5 cells transfected with plasmid pCMV6-AC-hMMP2 were treated with or without the indicated concentrations (0, 5, 10, and 20 μM) of EGCG for 24 h, and the total cell lysates were subjected to Western blot analysis. (F, G) CL1-5 cells transfected with plasmid pCMV6-AC-hMMP2 for 24 h, and then 2×10^5 cells were seeded in the upper chamber of the Transwell coated with Matrigel (40 μg/well) and treated with the indicated concentrations of EGCG for 24 h. Photographs of cells that invaded the Matrigel (F) and quantification of the three independent experiments (G) are illustrated.

modulated MMP-2 but not MMP-9 expression in human lung adenocarcinoma cells at the transcriptional level. To further confirm the important role of MMP-2 in CL1-5 cells, plasmid pCMV6-AC-hMMP2 was transfected into CL1-5 cells, the cells were treated with EGCG (0−20 μM) for 24 h, and then Western blot was used to determine the protein level of MMP-2. If MMP-2 plays an important role in the effects of EGCG on CL1-5 cells, EGCG slightly decreases the expression level of MMP-2 upon MMP-2 overexpressed in CL1-5 cells. The protein level of MMP-2 was indeed overexpressed in CL1-5 cells when plasmid pCMV6-AC-hMMP2 was transfected into CL1-5 cells (Figure 4D). EGCG could not decrease MMP-2 expression in CL1-5 cells whose MMP-2 was overexpressed (Figure 4E). Further, EGCG also could not repress cell invasion in CL1-5 cells whose MMP-2 was overexpressed (Figure 4F,G). These results indicated that EGCG repressed invasion of CL1-5 cells through decreasing MMP-2 expression and MMP2 indeed played important roles in the invasion of CL1-5 cells.

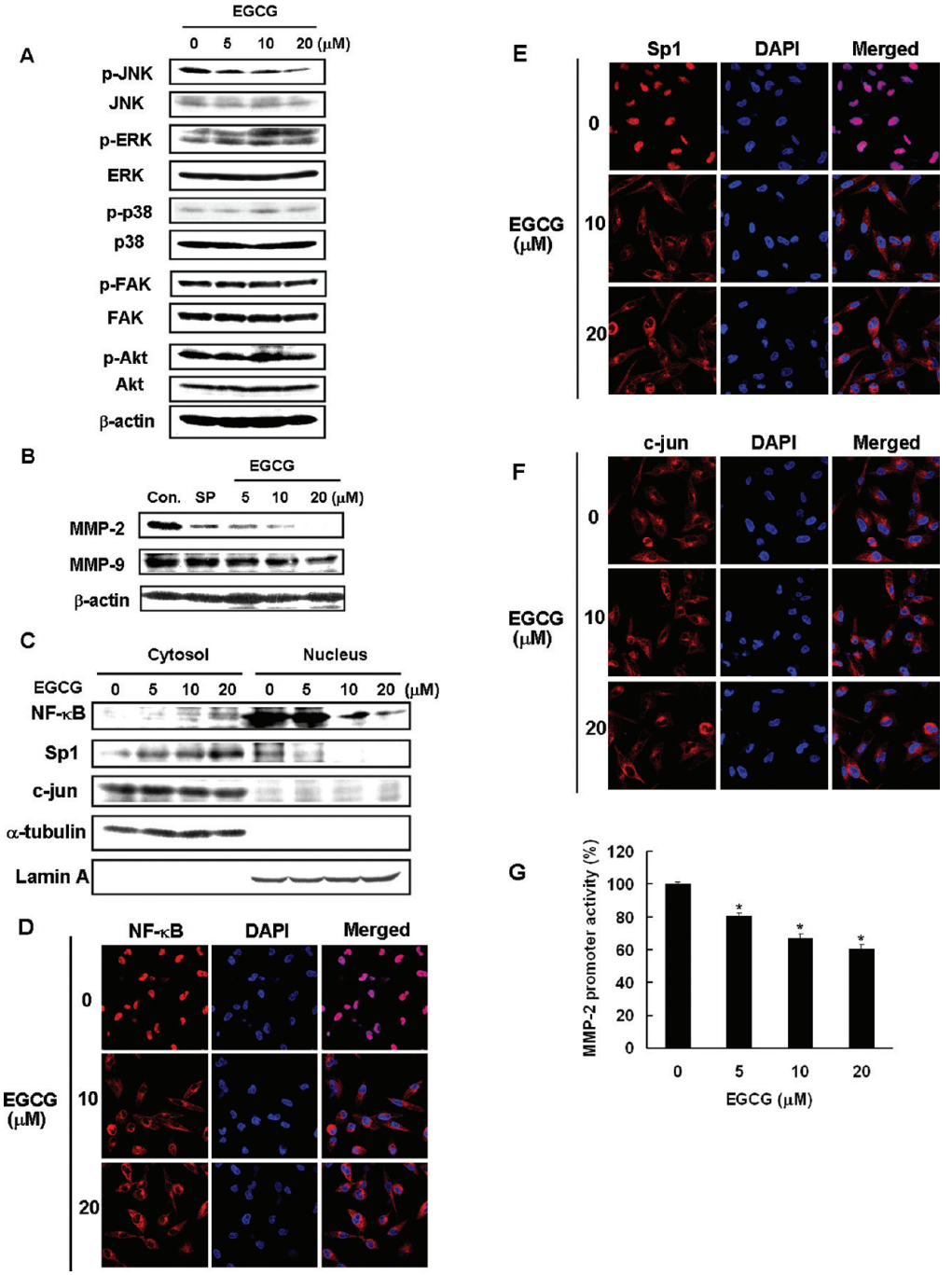

Figure 5. EGCG suppressed JNK signaling in CL1-5 cells. (A) Phosphorylation level of JNK, ERK1/2, p38 MAPK, FAK, and Akt in CL1-5 cells treated with EGCG. CL1-5 cells were treated with the indicated concentrations (5, 10, and 20 μM) of EGCG for 9 h, and the cell lysates were subjected to Western blot analysis. The levels of activated JNK, ERK1/2, p38 MAPK, FAK, and Akt were detected using phosphospecific antibodies. The levels of JNK, ERK1/2, p38 MAPK, FAK, and Akt were also detected using the indicated antibodies. (B) CL1-5 cells were treated with the JNK inhibitor, SP600125 (SP; 30 μM), or the indicated concentrations (5, 10, and 20 μM) of EGCG for 24 h, and the protein levels of MMP-2 and MMP-9 were detected by Western blot analysis. (C) EGCG inhibited NF-κB and Sp1 translocation from cytosol to nucleus in CL1-5 cells. CL1-5 cells were treated with the indicated concentrations (5, 10, and 20 μM) of EGCG for 9 h. Nuclear extracts were prepared and Western blot was carried out using anti-Sp1, anti-NF-κB, anti-c-Jun antibodies. α-Tubulin and lamin A were used as internal controls. (D,E,F) CL1-5 cells were treated with different concentrations of EGCG (0, 10, and 20 μM) for 9 h. Then, immunocytochemistry was used. All images were demonstrated by Leica confocal microscopy. (G) CL1-5 cells were cotransfected with 0.5 μg of pGL3 plasmid containing the full length of the human MMP-2 promoter and 0.05 μg of pGL3-Renilla as an internal control for normalization. After transfection, cells were treated with the indicated concentrations (5, 10, and 20 μM) of EGCG for 9 h, and the luciferase activities were determined.

EGCG Suppressed JNK Signaling in CL1-5 Cells. ERK,[30] JNK,[31] p38 MAPK,[32] Akt,[33] and FAK[34] have been shown to be involved in the induction of MMP-2 in many types of cancer. We explore whether the activities of these signaling pathways help

decrease expression of MMP-2 by EGCG in CL1-5 cells. Cells treated with EGCG (0−20 μM) for 9 h were analyzed by Western blot. As shown in Figure 5A, EGCG reduced the phosphorylation of JNK, whereas the phosphorylation of ERK, p38, Akt, and FAK was not affected by EGCG in CL1-5 cells. To further confirm that JNK performed an important role in reducing the expression of MMP-2 in CL1-5 cells, the inhibitor of JNK, SP600125, was used. Cells were treated with the indicated concentrations of EGCG (0−20 μM) or 30 μM SP600125 for 24 h, and then total cell lysates were subjected to Western blot analysis. Cells treated with EGCG or the inhibitor of JNK showed decreased expression of MMP-2 (Figure 5B). This data revealed that EGCG suppressed the expression of MMP-2 through downregulating JNK signaling in CL1-5 cells.

EGCG Reduced Nuclear Translocation of Transcription Factor NF-κB and Sp1. There are several transcription factor binding sites on the MMP-2 promoter, including NF-κB, Sp1, and AP-1. Therefore, signaling pathways that induce translocation of these transcription factors from the cytoplasm into the nucleus may contribute to the transcription of MMP-2 and enhance tumor invasion. In addition, NF-κB, Sp1, and AP-1, which are composed of c-Jun and c-Fos, are MAPKs-responsive elements. Thus, the JNK signaling pathway may play an important role to elicit the expression of MMP-2. To determine whether EGCG could affect the translocation of these transcription factors from the cytoplasm into the nucleus, cell lysates of the cytosolic and nuclear fraction were prepared from cells treated with EGCG (0−20 μM) for 9 h and were then subjected to Western blot analysis. As shown in Figure 5C, translocation of NF-κB and Sp1 into the nucleus was suppressed in the presence of EGCG, however, EGCG did not have a similar effect on c-Jun. To further confirm these results, we used the immunocytochemistry method. Cells were incubated with 0, 10, or 20 μM of EGCG for 9 h and immunocytochemistry was performed as described in Materials and Methods. As shown in Figure 5D−F, similar results were revealed as indicated in Figure 5C. These data suggested that EGCG reduced translocation of transcription factors, NF-κB and Sp1, into the nucleus, resulting in suppressed transcription of MMP-2 and invasion of CL1-5 cells.

MMP-2 Promoter Activity Was Influenced by EGCG in CL1-5 Cells. To further confirm that EGCG played an important role in regulating transcription of MMP-2, luciferase reporter assay was used to estimate whether the activity of MMP-2 promoter was affected in the presence of EGCG. CL1-5 cells were cotransfected with plasmid containing the full-length MMP-2 promoter and control vector. The cells were then treated with the indicated concentrations of EGCG for 9 h followed by measuring MMP-2 promoter activity as described in Materials and Methods. These results demonstrated that EGCG suppressed MMP-2 promoter activity in a dose-dependent manner, as demonstrated in Figure 5G in CL1-5 cells.

Enhancing the Anticancer Effects of Docetaxel by Combining Docetaxel and EGCG. Docetaxel is an effective agent recently used in the treatment of lung cancer, but it has serious side effects, such as low white blood cell count, anemia, diarrhea, hair loss, fatigue and weakness, nausea, and so on. To extend the study of synergistic effects, we examined whether the anticancer effect of docetaxel could be enhanced by combining docetaxel with EGCG in CL1-5 cells. After 24 h of incubation with 2.5 μM EGCG alone, the number of viable cells was 96.43% of the control, and the number of viable cells with 50 nM docetaxel alone was 92.88%. Cotreatment with 2.5 μM EGCG and 12.5, 25, and 50 nM docetaxel showed cell viability of 84.75%, 83.91%, and 72.84%, respectively (Figure 6A). These results indicated that EGCG and docetaxel synergistically affect the inhibition of CL1-5 cell growth. To further examine the synergistic effect of EGCG and docetaxel on the expression level of MMP-2, CL1-5 cells were treated with 2.5 μM EGCG or 50 nM docetaxel alone, or cotreated with 2.5 μM EGCG and the indicated concentrations of docetaxel for 24 h. The cell lysates were then subjected to Western blot analysis. As shown in Figure 6B, cotreatment with EGCG and docetaxel significantly decreased the expression of MMP-2. These data demonstrated that docetaxel could enhance the anticancer activity of EGCG in CL1-5 cells.

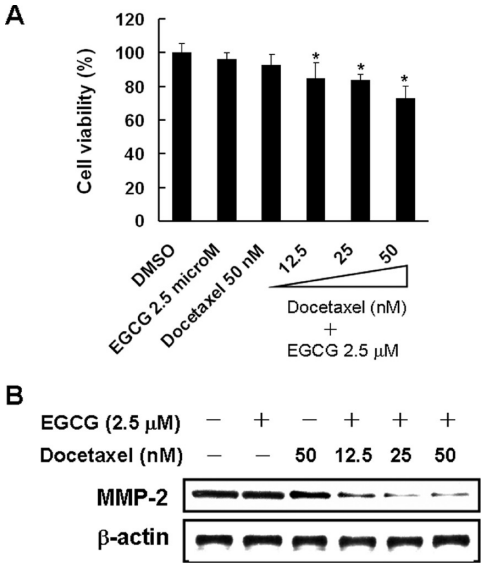

Figure 6. Effects of EGCG and docetaxel on cell proliferation and MMP-2 expression in CL1-5 cells. (A) CL1-5 cells were treated with EGCG or docetaxel as indicated for 24 h. Cell growth inhibition was determined by MTT assays. The number of viable cells after treatment is expressed as a percentage of the DMSO-only control. Data are the means of three independent experiments. Bars represent the SE. *$P < 0.05$. (B) CL1-5 cells were treated with EGCG or docetaxel as indicated for 24 h. Total cell lysates were subjected to Western blot analysis and immunodetected with anti-MMP-2 antibody. β-Actin was used as an internal control.

DISCUSSION

Phytochemicals from certain vegetables, fruits, and tea in the daily diet provide effective cancer prevention.[3] These effects contribute to the flavonoids and related flavonols in these plants. Green tea is thought to have anticarcinogenesis activities, which mainly occur through EGCG.[2,6] However, the effect of EGCG on highly invasive human lung cancer is largely unknown. MMP-2 is overexpressed in lung cancer and is closely correlated with metastatic potential.[21,25] We hypothesized, when MMP-2 was downregulated, that the invasive ability of highly invasive lung adenocarcinoma might be suppressed. The present study demonstrated, for the first time, that EGCG plays an important role in suppressing MMP-2 expression and reducing invasion of highly invasive human lung adenocarcinoma cells through inhibiting the JNK signaling pathway. Inhibition of MMP-2 expression resulting in reducing invasive ability of highly invasive lung adenocarcinoma cells indicated that MMP-2 might show an important role in invasion of these cells via the JNK signaling pathway. Indeed, blockage

of activated JNK in highly invasive CL1-5 adenocarcinoma cells significantly suppressed MMP-2 expression. Thus, our results suggested that downregulated JNK was involved in the repression of MMP-2 in CL1-5 cells.

Proteolytic activity to degrade ECM is required for a cancer cell to invade to other tissues or organs. Among these proteinases, MMPs were reported to play important roles in degrading ECM, which were correlated with invasion and metastasis of the tumors.[35] Studies performed over several decades have suggested that growth factors and cytokines secreted by tumor cells will induce the production of MMPs. Since MMP-2 was pointed out to be closely related to metastatic potential of lung cancer, we wanted to investigate whether EGCG could affect the expression of MMP-2 in highly invasive lung cancer cells. In our data, EGCG could decrease the protein level of MMP-2 at concentrations of 5, 10, and 20 μM for 24 h. Furthermore, we demonstrated that the mRNA level of MMP-2 was reduced under treatment with EGCG. These results indicated that EGCG repressed the expression of MMP-2 at the transcriptional level.

Numerous signaling pathways, including ERK, JNK, p38, Akt, and FAK,[30] were reported to participate in inducing expression of MMP-2 and play important roles in metastasis. Therefore, we examined the effects of EGCG on these signaling pathways. CL1-5 cells treated with EGCG (5−20 μM) showed decrease in activation of JNK. To further confirm this result, the inhibitor of JNK, SP600125, was used. These data suggested that JNK might play an important role in the effect of EGCG on highly invasive CL1-5 human adenocarcinoma cells. Several transcription factor binding sites had been shown on MMP-2 promoter, including NF-κB, Sp1, and AP-1. Thus, we determined which transcription factor played an important role in the effects of EGCG on CL1-5 cells. The results showed that EGCG could repress the translocation of NF-κB and Sp1 from cytoplasm to nucleus. We and others showed that NF-κB and Sp1 might play critical roles in the expression of MMP-2. We now expanded these finding and suggested that NF-κB and Sp1 might play important roles in the expression of MMP-2 via JNK signaling pathway under the treatment with EGCG in highly invasive CL1-5 cells. EGCG could repress the translocation of NF-κB and Sp1 from the cytoplasm to the nucleus. Therefore, the suppression effect of EGCG on the invasion and migration in CL1-5 cells might occur through downregulating JNK and repressing the translocation of NF-κB and Sp1 transcription factors into the nucleus, resulting in reduced expression of MMP-2. In addition to suppressing cell invasion, EGCG can induce G2/M arrest at higher doses (30, 40, and 50 μM) in CL1-5 cells. The novel findings in the present studies are (a) EGCG decreases the expression of MMP-2 at the transcriptional level through JNK pathways in highly invasive CL1-5 lung cancer cells; (b) EGCG suppresses the invasion ability of CL1-5 cells; (c) the combination of EGCG and docetaxel potentiated the inhibition of MMP-2 expression; (d) EGCG can induce G2/M arrest at higher doses (30, 40, and 50 μM) to decrease cell proliferation in CL1-5 cells. These findings highlight the potential use of EGCG as a chemotherapeutic modulator in preventing or treating highly invasive human CL1-5 lung cancer cells.

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ABBREVIATIONS USED

EGCG, epigallocatechin-3-gallate; MMP-2, matrix metalloproteinase-2; JNK, c- Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated protein kinase 1/2

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