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EGCG inhibited bladder cancer SW780 cell proliferation and migration both *in vitro* and *in vivo via* down-regulation of NF-κB and MMP-9

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Abstract

Epigallocatechin-3-gallate (EGCG), the bioactive polyphenol in green tea, has been demonstrated to have various biological activities. Our study aims to investigate the antiproliferation and antimigration effects of EGCG against bladder cancer SW780 cells both *in vitro* and *in vivo*. Our results showed that treatment of EGCG resulted in significant inhibition of cell proliferation by induction of apoptosis, without obvious toxicity to normal bladder epithelium SV-HUC-1 cells. EGCG also inhibited SW780 cell migration and invasion at 25–100 μM. Western blot confirmed that EGCG induced apoptosis in SW780 cells by activation of caspases-8, -9 and -3, Bax, Bcl-2 and PARP. Besides, animal study demonstrated that EGCG [100 mg/kg, intraperitoneal (i.p.) injection daily for 3 weeks] decreased the tumor volume significantly in mice bearing SW780 tumors, as well as the tumor weight (decreased by 68.4%). In addition, EGCG down-regulated the expression of nuclear factor-kappa B (NF-κB) and matrix metalloproteinase (MMP)-9 in both protein and mRNA level in tumor and SW780 cells. When NF-κB was inhibited, EGCG showed no obvious effect in cell proliferation and migration. In conclusion, our study demonstrated that EGCG was effective in inhibition SW780 cell proliferation and migration, and presented first evidence that EGCG inhibited SW780 tumor growth by down-regulation of NF-κB and MMP-9.

Keywords: EGCG; Bladder cancer; SW780; NF-κB; MMP-9

1. Introduction

Despite significant advances in the front lines of cancer research, cancer remains a worldwide health problem, with an upward incidence and mortality rate. Bladder cancer is one of the most malignant types of cancer, which ranks ninth of world cancers and caused 165,000 deaths in 2012 [1]. In the United States, bladder cancer is the fourth most common type of cancer in men, and approximately 66,000 persons are diagnosed with the disease each year. Globally, bladder cancer resulted in 170,000 deaths in 2010 [2]. Therefore, bladder cancer still takes a tremendous toll, and novel therapeutic strategies and more effective agents for advanced disease are still urgently needed.

Green tea is the most popular and well-known beverage worldwide, especially in China and Asian countries, which is obtained from the dried leaves of the plant *Camellia sinensis*. Green tea contains as many as 200 bioactive compounds, including tea polyphenols, caffeine, theanine, vitamin and minerals. The largest group of tea components is tea polyphenol, and epigallocatechin-3-gallate (EGCG) is found to be the most abundant ingredient in green tea. Green tea (*C. sinensis*) is demonstrated to have various biological activities, including antiobesity, antioxidation, cardiovascular protection and anticancer effects. Epidemiological studies have shown that consumption of green tea extract was associated with increased weight loss [3]. Clinical and research studies indicated that green tea extract intake could help reduce the risk of cardiovascular diseases and then lower the rate of heart diseases [4]. Besides, EGCG was well documented to be effective in prevention and treatment for many cancers, such as prostate, breast, bladder and colon cancers [5]. Regular consumption of green tea could lower the risks of several cancer types, such as stomach, lung, colon, rectum, liver, breast and pancreas cancers [6]. Our previous studies have demonstrated that green tea aqueous extract had potent antitumor and antimetastasis effects in mice bearing breast tumor and could protect the bone from breast-cancer-induced bone destruction [7], and high-performance liquid chromatography analysis showed that EGCG was the most abundant ingredient in green tea water extract. EGCG, the bioactive polyphenol in green tea, has been demonstrated to have various biological

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activities, including antiobesity, cardiovascular protection and anti-cancer effects. A number of literatures reported the anticancer effect of EGCG. It was demonstrated that EGCG was effective in lowering the risk of several cancer types, including stomach, prostate and lung cancers [8]. Treatment with EGCG resulted in significant inhibition of tumor growth and remarkable reduction in the levels of growth factor of EGFR and IGF in serum in breast and prostate cancers [9,10]. EGCG was also shown to be effective in inducing apoptosis, inhibition of metastasis or angiogenesis in a variety of cancer cell lines including leukemia, breast, prostate, liver and lung cancer cells through production of H_2O_2, induction of cell-cycle arrest or activation of the mitogen-activated protein kinase cascade [11]. Recently, a variety of studies investigated the effects of EGCG in bladder cancer. A clinical study showed that bladder cancer patients who received tea polyphenol including EGCG were associated with a lower level of biomarker of PCNA which is related to cell proliferation and metastasis [12]. *In vivo* study showed that intravesical treatment of EGCG resulted in the inhibition of the growth of AY-27 tumor in rats [13]. EGCG in nanogold particles form was effective in inhibition of tumor growth by means of apoptosis [14]. In addition, *in vitro* studies demonstrated that EGCG inhibits cell growth and migration in bladder cancer T24, MBT-2 and SW780 cells, and decreased the Bcl-2 expression level [14–16]. However, reports seldom evaluated the *in vivo* effects and underlying mechanisms of EGCG in bladder cancer SW780 cells.

During the process of cancer propagation, nuclear factor-kappa B (NF-κB) plays an important role. NF-κB is a ubiquitously expressed transcription factor that plays a critical role in cancer proliferation and metastasis [17]. Usually, NF-κB is expressed as a dimer protein form in the cytoplasm as p50/p65. When NF-κB is activated, it would translocate into the nucleus and bind to κB DNA elements, thereby regulating the expression of target genes [18]. NF-κB has shown to up-regulate the expression level of matrix metalloproteinases (MMPs) including MMP-9, which is postulated to play a vital role in cancer migration and invasion by degradation of extracellular matrix (ECM) [19,20]. EGCG was also demonstrated to be effective in down-regulation the NF-κB and/or MMP-9 expression in breast, cervical, gastric and colon cancer cells [21,22].

The present study aimed to investigate the ant-cancer effect of tea polyphenol EGCG in bladder cancer SW780 cell line both *in vitro* and *in vivo*. Also, the role of EGCG in different mechanisms of action would be discussed. Here, we assessed the apoptosis-induction, antimigration and anti-invasion abilities of EGCG *in vitro* and then further evaluated the antitumor activities of EGCG in nude mice bearing SW780 tumors. Besides, the involvement of NF-κB and MMP-9 was also evaluated both *in vitro* and *in vivo* after EGCG treatment.

2. Materials and methods

2.1. Cells and reagents

The SW780 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum and 1% penicillin–streptomycin (Life Technology, USA) at 37°C in 5% CO_2 humidified incubator. Annexin V-FITC kit and caspase-3 enzyme-linked immunosorbent assay kit were purchased from BD Pharmingen, USA. MTT was obtained from Sigma, USA. Transwell plate assays were from Corning Incorporated, USA. Caspase-3, -8 and -9 (9915s); MMP-9; Bax (5023s); Bcl-2 (2870s); PARP (9542s); NF-κB (8242s) and phosphorylated NF-κB p65 (3033s) were purchased from Cell Signaling Technology, USA. Polymerase chain reaction (PCR) kits were purchased from TOYOBO, Japan. Creatine kinase (CK), alanine transaminase (ALT) and aspartate transaminase (AST) kits were purchased from Stanbio, USA. NF-κB inhibitor (SC75741) was provided by Selleck, USA.

2.2. Cell viability assay

Cells (1×10^4/well) were seeded in 96-well plates (Corning, USA) and incubated with different concentrations of EGCG for 24 and 48 h. Following incubation, 30 μl of MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well, and the plate was incubated at 37°C for another 4 h. Then, the medium was discarded, and 150 μl of DMSO was added to dissolve the formazan crystals. The absorbance of each sample was read at 540 nm using a microplate reader (Thermo Multiskan GO, USA).

2.3. Annexin V-FITC/PI double staining

After treatment with EGCG, SW780 cells were collected, washed with ice-cold PBS and then stained in solution containing Annexin V-FITC and PI for 15 min in the dark at room temperature. The fluorescent signal in the cells was detected by flow cytometry (FACSARIA II, Becton Dickinson). Positioning of quadrants on Annexin-V/PI plots was performed to distinguish living cells (FITC−/PI−), early apoptotic cells (FITC+/PI−) and late apoptotic or necrotic cells (FITC+/PI+).

2.4. Caspase-3 activity assay

Cells treated with EGCG for 24 h were collected and lysed in lysis buffer to determine the caspase-3 activity using the caspase-3 activity assay kit (BD, USA). The lysates was collected and tested for protease activity. After being normalized, the sample lysates were added to microplate together with reaction buffer and substrate solution, and then the microplate was incubated at 37°C for 30 min. The plate was then read at 450 nm in a microplate reader (Thermo Multiskan GO, USA).

2.5. Cell migration and invasion assays

The efficacy of EGCG against SW780 cell migration and invasion *in vitro* was assessed using scratch wound and Transwell migration assays.

In the scratch wound assay, (1×10^5/well) SW780 cells were seeded in 24-well plates. After being starved in medium without FBS for 24 h, SW780 cells were scraped with crosses, and then the medium was replaced with fresh medium with EGCG. SW780 cells were incubated for 24 h, and each well was photographed under a microscope (Olympus IX73). The percentages of open wound area were measured and calculated using the TScratch software [23].

During the Transwell migration assay, (2×10^4/well) SW780 cells were added into Transwell chambers with 1% v/v FBS together with 100 μl medium containing various concentrations of EGCG (with 1% v/v FBS). Then, 500 μl complete DMEM (with 10% v/v FBS), which served as chemoattractant medium, was added in the lower well. After being incubated at 37°C for 24 h, cells were fixed with methanol and stained with 0.1% crystal violet. Stained filters were photographed under microscope (Olympus IX73). The migrated cells were quantified by manual counting and represented as a percentage of control values [24].

2.6. Western blot analysis

SW780 cells treated with EGCG were lysed in lysis buffer. After the lysate boiled, protein samples (20 μg) were fractionated in 10% sodium dodecyl sulfate polyacrylamide gel. Proteins on the gel were transferred to polyvinylidene difluoride membrane (Millipore, USA). After blocking with 10% nonfat milk, the membranes were washed with PBS-T and then incubated with primary antibodies (dilution at 1:1000) for 2 h at 4°C. After washing with PBS-T, the membrane was incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h. Finally, visualization of protein bands was performed using the ECL substrate reagent kit (GE Healthcare) on a Gel Doc XR imaging system (Bio-RAD, USA).

2.7. Real-time quantitative PCR (qPCR)

Total RNA extracted from SW780 cells or the tumor samples used TRIzol reagent (Invitrogen, USA). The mRNA levels of NF-κB and MMP-9 were determined by real-time qPCR following the instructions. The GAPDH mRNA was used as an internal control to normalize the amount of the mRNA in each sample. Our preliminary results have shown that GAPDH is suitable for normalization purposes, although metabolic differences have been described in bladder cancer TCCSUP cells [25]. The primers designed were as follows: GAPDH forward: 5′-AAG GTG AAG GTC GGA GTC AAC-3′, reverse: 5′-GGG GTC ATT GAT GGC AAC AAT A-3′; NF-κB forward: 5′-GTC ACT GCC CAG ACT TTA CT-3′, reverse: 5′-GCT TCT CCA CTG AAA ATC CT-3′; MMP-9 forward: 5′-CTT TGA CAG CGA CAA GAA GTG G-3′, reverse: 5′-GGC ACT GAG GAA TGA TCT AAG C-3′. The reactions were performed in triplicate by using the ABI VII7 Fluorescent Quantitative PCR System (Thermo, USA). The average value in each triplicate was used to calculate the relative amount of NF-κB and MMP-9 using the comparative ΔCt method.

2.8. Nude mice tumor model

Female BALB/c mice (6–8 weeks of age) were provided by Vital River Laboratory Animal Technology Co. Ltd., Beijing, and were housed under pathogen-free conditions in Shenzhen Institutes of Advanced Technology (license: SYXK 2012-0119), Chinese Academy of Sciences. SW780 cells (3×10^6) resuspended in 0.2 ml PBS were subcutaneously inoculated at the back of each mouse. After SW780 cell implantation, the tumor-bearing mice were randomly assigned into four groups ($n=7$): control group (saline, i.p. injected every day), EGCG low-dose group (25 mg/kg EGCG, i.p. injected every day), EGCG medium-dose group (50 mg/kg EGCG, i.p. injected every day) and

EGCG high-dose (EGCG-H) group (100 mg/kg EGCG, i.p. injected every day). Treatments were initiated 1 week after cancer cell implantation and lasted for 3 weeks. During EGCG treatment, the body weight and tumor volume of each mouse were measured twice a week. At day 28, mice were sacrificed, and the tumors were removed for quantification of tumor burden. The tumors in EGCG high dose group were also lysed for analysis of protein and mRNA expression. The effect of EGCG (100 mg/kg) on hematobiochemical markers was assessed by measuring the activities of liver- or heart-related enzymes (ALT, AST and CK) in the plasma using assay kits purchased from Stanbio Co. Ltd.

2.9. Statistical analysis

All data were expressed as mean±S.D./S.E.M. Statistical analysis was performed using one-way analysis of variance, with $P<.05$ as regarded statistically significant.

3. Results

3.1. EGCG inhibited bladder cancer SW780 cell proliferation

Treatment with EGCG for 24 and 48 h resulted in inhibition of cell proliferation in a time- and dose-dependent manner. As shown in Fig. 1, EGCG inhibited the growth of SW780 cells with an IC_{50} of 70.2 and 113.6 μM at 24 and 48 h, respectively. Besides, the cytotoxicity of EGCG on normal human bladder epithelium SV-HUC-1 cells was also tested. The results showed that EGCG was much more sensitive in SW780 cells than in SV-HUC-1 cells. EGCG at 100 μM induced over 70% cell inhibition in SW780 cell at 24 h, and only resulted in 7.8% inhibition in SV-HUC-1 cells (Fig. 1B).

3.2. EGCG induced apoptosis in SW780 cells

Annexin-V FITC/PI staining and caspase-3 activity assays were performed to determine whether EGCG induced apoptosis in SW780 cells. Annexin-V FITC/PI staining showed that when SW780 cells were incubated with increasing dose of EGCG from 0 to 50 μM, the rates of cell apoptosis were increased in a dose-dependent manner. The percentage of apoptotic cells upon treatment with 0, 25 and 50 μM of EGCG was found to be 3.5%, 8.3% and 12.7% after 24-h incubation (Fig. 2). Furthermore, EGCG-induced SW780 cell apoptosis was concomitant with a dose-related increase in caspase-3 activity. Significant difference was shown between untreated control and EGCG (50 μM) treatment group on caspase-3 activity (Fig. 2C).

3.3. EGCG inhibited SW780 cell migration and invasion

To determine the efficacy of EGCG against cancer cell metastasis *in vitro*, the scratch wound and Transwell migration assays were introduced. As shown in Fig. 3A, EGCG significantly inhibited cell migration in SW780 from 25 μM after 24-h incubation, and the inhibition was enlarged when the concentration increased (Fig. 3B). Besides, the result from Transwell migration assay was in line with the data from scratch assay. In Fig. 3C, EGCG inhibited SW780 cell invasion efficiently with the increase of EGCG concentration. In the presence of 50 μM, EGCG inhibited cell invasion of SW780 cells significantly by 32.4% (Fig. 3D).

3.4. EGCG regulated the protein expressions

Treatment with various concentrations of EGCG for 24 h resulted in the change of protein expression level (Fig. 4). The EGCG-treated SW780 cells induced the cleavage of protein caspase-8, -9 and -3 and PARP, and significant differences were shown between control and EGCG-treated group, indicating the apoptosis induction effects of EGCG in SW780 cells (Fig. 4A–D). EGCG also up-regulated the Bax expression, down-regulated Bcl-2 expression and resulted in significant increase of the ratio of Bax/Bcl-2 (Fig. 4F). Besides, EGCG down-regulated NF-κB and activated p65 in a dose-dependent manner, and significant difference was shown between untreated control and EGCG treatment groups (100 μM) (Fig. 4G). In addition, EGCG decreased MMP-9 expression in a dose-dependent manner (Fig. 4H), indicating the inhibitory effect of EGCG on cell migration and invasion.

3.5. EGCG down-regulated the mRNA expressions of NF-κB and MMP-9

In order to confirm whether EGCG was effective in regulating the mRNA expression level of NF-κB and MMP-9, the RT qPCR was performed. As shown in Fig. 5, EGCG treatment resulted in significant decreasing of NF-κB expression at doses of 50 and 100 μM in SW780 cells. Besides, SW780 cells treated with EGCG induced a significant down-regulation of MMP-9, which was downstream of NF-κB. The results were consistent with the effects of EGCG shown in protein expression of NF-κB and MMP-9.

3.6. EGCG decreased tumor burden in nude mice without obvious toxicity to the hosts

To investigate the activity of EGCG on tumor growth *in vivo*, a subcutaneous tumor model in nude mice was employed, in which cells were injected into the subcutis of BALB/c nude mice. It was observed that no significant body weight loss was found in EGCG-treated groups during the treatment (Fig. 6A). Besides, hematobiochemical markers test showed that no significant difference was shown on plasma activities of liver-related (AST, and ALT) and heart-specific (CK) enzymes between control and EGCG-H treatment groups (Fig. 6B). As shown in Fig. 6C, the tumor volume was decreased in EGCG treatment groups, and significant differences were shown from day 22 in EGCG-H group (Fig. 6D). In addition, tumors were excised from each animal for examination of tumor weight. Tumor weights were decreased in all EGCG treatment groups, and significant difference was shown in

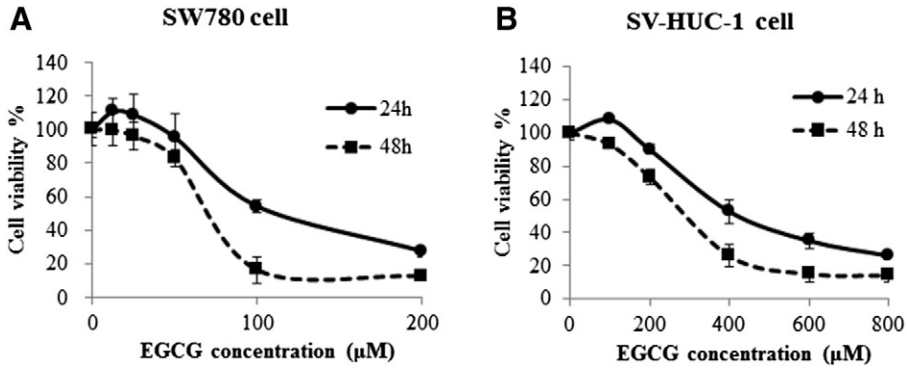

Fig. 1. Cytotoxicity of EGCG on bladder cancer SW780 (A) and normal bladder epithelium SV-HUC-1 (B) cells after 24- and 48-h incubation. Data were expressed as mean±S.D.

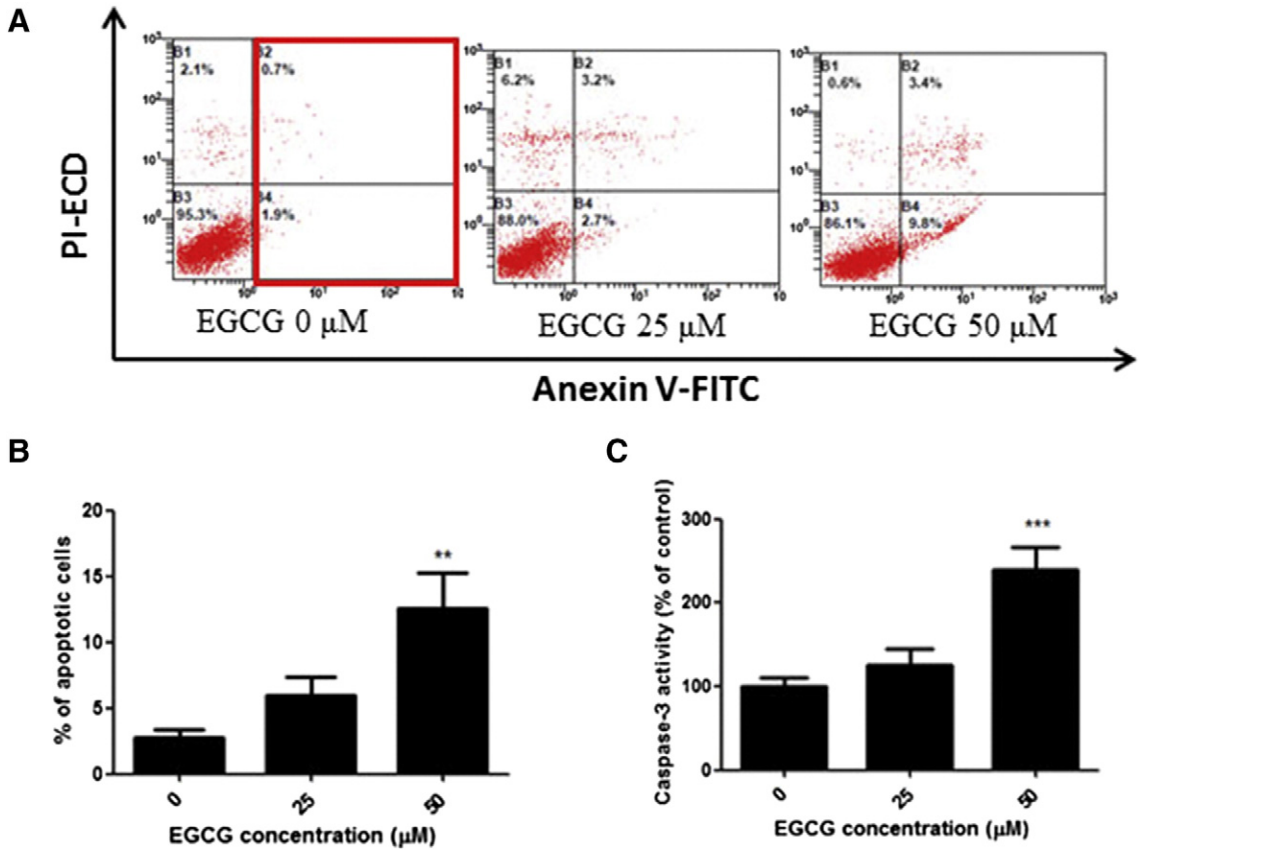

Fig. 2. Induction of apoptosis on SW780 cells by EGCG. (A) Flow cytometry images. (B) Quantitative analysis of the percentage of apoptotic cells of EGCG after 24-h incubation. The percentage of total apoptotic cells was defined as the sum of early and late apoptotic cells. (C) Caspase-3 activity test of SW780 cells after EGCG treatment. Data were presented as mean±S.D. (n=3). ** P<.01 and *** P<.001, as compared with untreated control.

EGCG-H treatment group (by 68.4%) when compared with control (Fig. 6 E and F).

3.7. EGCG inhibited NF-κB and MMP-9 expression in tumor

In order to assess the protein and gene expression of NF-κB and MMP-9 in tumor, the Western blot and qPCR were performed. The protein and mRNA were extracted from tumors obtained from control and EGCG-H treatment groups. As shown in Fig. 7A, treatment of EGCG (100 mg/kg) in nude mice resulted in obvious inhibition of NF-κB p65 and MMP-9 in protein. EGCG also induced activation of caspase-3 and Bcl-2 in tumor (Fig. 7A). In addition, similar results were also shown in qPCR that NF-κB and MMP-9 were down-regulated in EGCG-H group, and significant difference was shown in MMP-9 expression between control and EGCG-H treatment groups (Fig. 7B and C). The protein and gene expression of NF-κB and MMP-9 in tumor was in line with the results *in vitro*.

3.8. EGCG showed no obvious effect on proliferation and migration when NF-κB was inhibited

In order to confirm the important role of NF-κB in EGCG-induced proliferation and migration inhibition, 10 μg/ml of NF-κB inhibitor SC75741 (Selleck) was added in SW780 cells, and then the cells were collected for MTT and Transwell assays. As shown in Fig. 8A, the NF-κB was totally suppressed when the inhibitor SC75741 was added. In Fig. 8B, EGCG inhibited cell proliferation in normal SW780 cells but showed no obvious effect in NF-κB-inhibited SW780 cells. Similar result was also present in Transwell assay that EGCG treatment resulted in no difference in NF-κB-inhibited SW780 cells when compared with untreated control (Fig. 8C).

4. Discussion

Throughout the world, tea polyphenol especially EGCG has been widely consumed as a health-promoting food ingredient. Given the increasing popularity and commercial development of EGCG in cancer treatment, there is an urgent need to study the comprehensive protection of EGCG against bladder cancer. In this study, we aim to investigate the antiproliferation and antimetastasis effects of EGCG in bladder carcinoma SW780 cell line both *in vitro* and *in vivo*.

In the present study, we found that the treatment of EGCG resulted in dose- and time-dependent inhibition of cell viability on SW780 cells *in vitro* without obvious toxicity to the normal human bladder epithelium SV-HUC-1 cells (Fig. 1). To determine whether the antiproliferative effect of EGCG was associated with apoptosis induction, AV/PI double staining and caspase-3 assays were employed. The results showed that EGCG induced apoptosis in SW780 cells in a dose-dependent manner (Fig. 2). The findings were in line with the effect of EGCG on TCCSUP cells, which inhibited cell proliferation in a dose-dependent manner [26]. EGCG was also demonstrated to be effective in inducing apoptosis in bladder cancer T24 and MBT-2 cells by activation of Bcl-2 family proteins [14,15]. Apart from the antiproliferation and apoptosis induction effects, EGCG was also found to be effective in inhibiting SW780 cell migration and invasion in a dose-dependent manner as assessed by wound healing and Transwell migration assays (Fig. 3). The results were comparable to previous reports that EGCG could reduce the adhesion, migration and

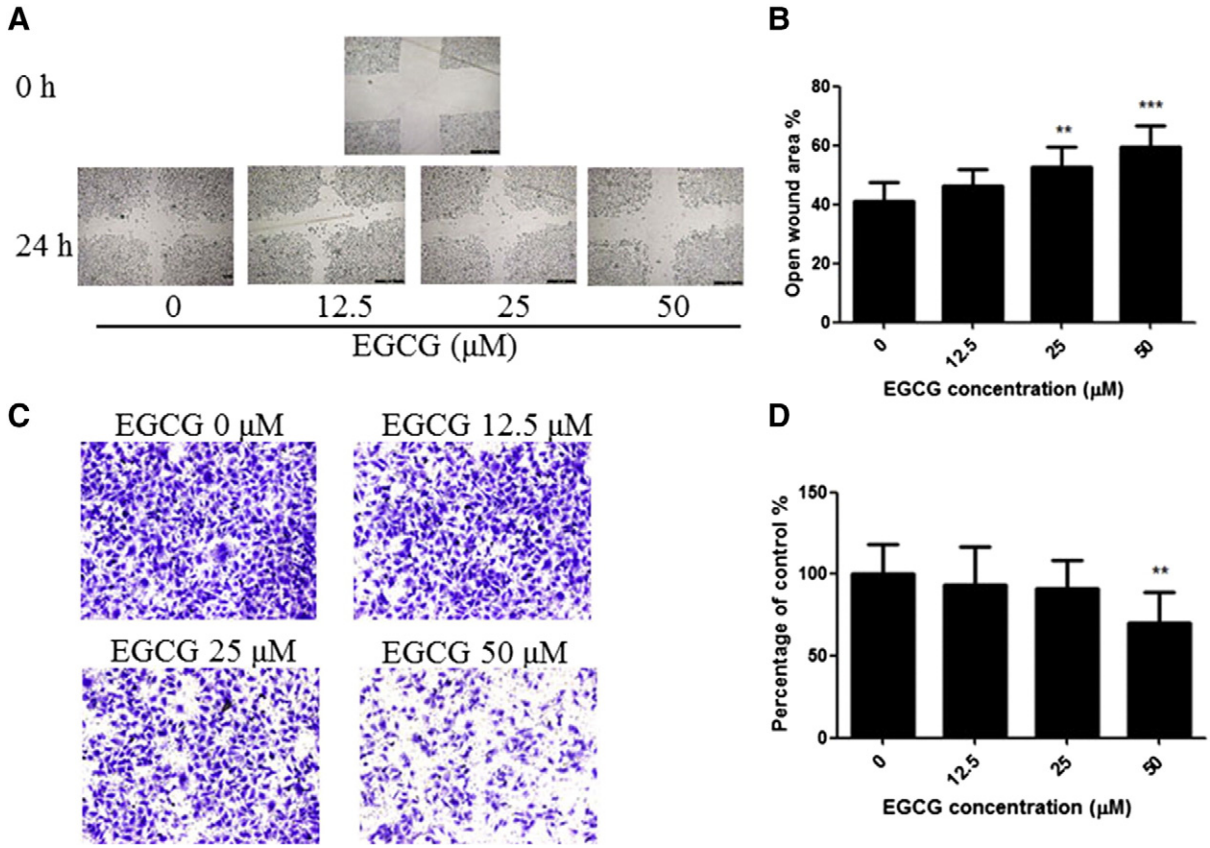

Fig. 3. Effect of EGCG on SW780 cell migration and invasion activities. (A) Representative images of the wounded cell monolayers of SW780 cells. (B) Quantitative analysis of the antimigration activity of EGCG after 24-h incubation. Data were expressed as the percentage of open wound area from baseline cultures without treatment. (C) Representative images of the stained SW780 cells. (D) Quantitative analysis of the anti-invasion activity of EGCG. Data were presented as mean±S.D. (n=3). ** P<.01 and *** P<.001, as compared with untreated control.

invasion of T24 human bladder cancer cells [27]. EGCG was also found to be effective in inhibition of proliferation and migration in human breast cancer MDA-MB-231, MCF-7 cells and colon cancer SW620 cells [28,29]. The selected doses of EGCG for *in vitro* studies were set according to the MTT results. The EGCG doses for migration and invasion assays were noncytotoxic doses (≤50 μM), and the cell viability was over 80%; these noncytotoxic doses of EGCG showed significant antimigration and anti-invasion effects to SW780 cells. That means that EGCG could be added as a nutritional supplement for bladder cancer prevention and treatment. EGCG not only has antimetastasis and apoptosis-induction effects but also has other health benefits such as antioxidation, cardiovascular protection and antiobesity effects. Besides, tea polyphenol EGCG is healthy to be used in a low amount without obvious toxicity to normal bladder cells.

To gain insight into the underlying mechanism of EGCG-induced apoptosis and migration inhibition, several proteins were tested, including caspases-8, -9 and -3; PARP; Bcl-2; NF-κB and MMP-9 (Fig. 4). EGCG was effective in activation of caspases-8, -9 and -3 and PARP, indicating that EGCG induced apoptosis in SW780 cells through both intrinsic and extrinsic pathways. Besides, treatment of EGCG resulted in significant increase of proapoptotic protein Bax, and the expression of antiapoptotic protein Bcl-2 was significantly inhibited. The ratio of Bax/Bcl-2 was significantly up-regulated with the increasing concentration of EGCG, indicating the crucial role Bax/Bcl-2 played in transducing the apoptotic signal in SW780 cells. The result was in complete agreement with Qin's finding that EGCG treatment resulted in down-regulation of caspase-3 with a concomitant activation in PARP in T24 cells [15]. Similar findings that EGCG decreased Bcl-2 or increased Bax expression were also found in human breast, prostate and cervical cancer cells [8]. The results suggested that EGCG-induced apoptosis played a vital role in the inhibition of SW780 cells, as apoptosis was considered as a protective mechanism against cancer development. In addition, we demonstrated that NF-κB and MMP-9 were significantly decreased by EGCG in a dose-dependent manner. NF-κB is a heterodimer consisting of p65 and p50; when activated, NF-κB is activated as p65. We found that the expression of both NF-κB and phosphorylated NF-κB p65 was decreased by EGCG. In order to confirm whether EGCG regulates NF-κB in mRNA level, RT qPCR was conducted, and results showed that EGCG down-regulated NF-κB significantly in mRNA expression (Fig. 5A). The data were comparable to previous reports that showed that EGCG down-regulated NF-κB p65 expression in T24 cell line [27] and decreased the mRNA level of AKT and NF-κB in hepatocellular carcinoma [30]. Furthermore, both the protein and mRNA expressions of MMP-9 were significantly suppressed by EGCG in a dose-dependent manner (Figs. 4H and 5B), indicating the potent antimetastasis effect of EGCG in SW780 cells. The result was in complete agreement with Fang's finding that EGCG decreased the MMP-9 expression in protein level in nasopharyngeal carcinoma cells [31]. Similar findings were also shown in T24 cells that EGCG down-regulated MMP-9 expression in protein and mRNA level [24]. Treatment of EGCG in SW780 cells resulted in the activation of NF-κB, which regulated the expression of caspases, Bcl-2 and MMP-9 expression, and lastly contributed to the inhibition of migration and induction of apoptosis.

Apart from the *in vitro* studies, the antitumor and antimetastasis effects of EGCG were investigated in a subcutaneous tumor model.

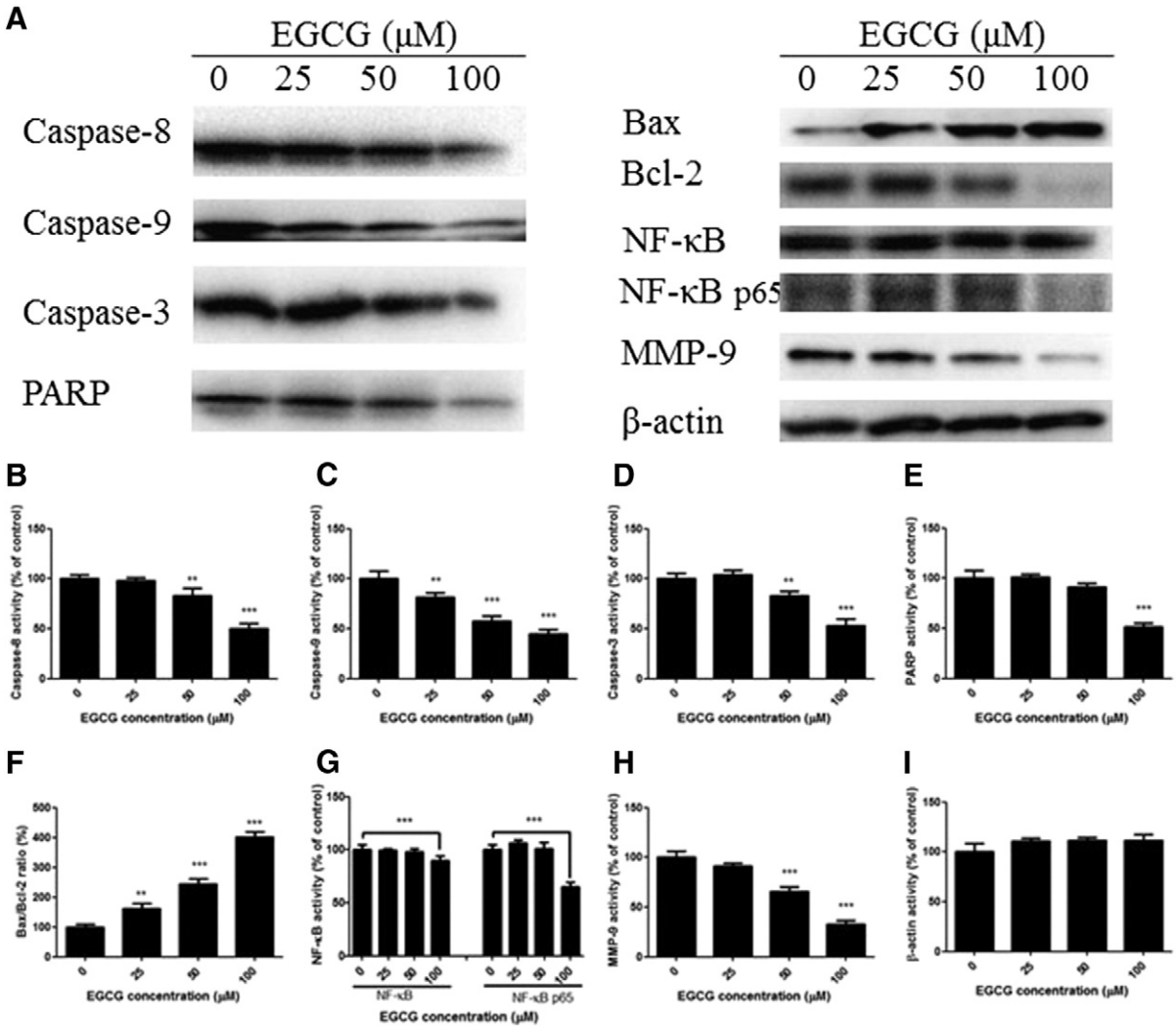

Fig. 4. Effect of EGCG on protein expression. (A) Representative images of Western blot of proteins after treatment with EGCG in SW780 cells. (B–I) Statistical analysis of caspase-8, -9 and -3; PARP; ratio of Bax/Bcl-2; NF-κB and phosphorylated NF-κB p65; MMP-9 and β-actin protein expressions in SW780 cells after EGCG treatment. Data were shown as mean±S.D. (n=3). ** P<.01 and *** P<.001, as compared with untreated control.

After treatment, no significant difference was shown on body weight and hematobiochemical markers (AST, ALT and CK), indicating no obvious toxicity of EGCG to the hosts (Fig. 6A and B). The administration of EGCG (100 mg/kg) was able to inhibit the tumor volume and tumor weight in mice bearing SW780 tumors (Fig. 6C–F), which was in line with Kemberling's finding that instillation of Fisher

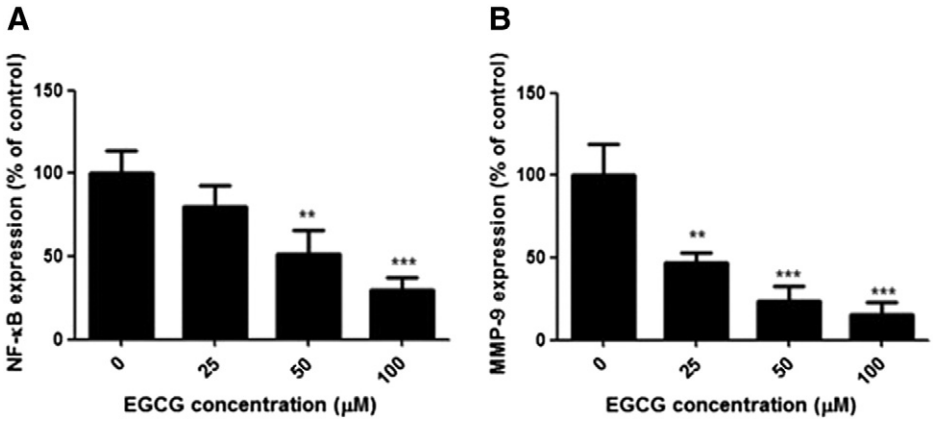

Fig. 5. Expression changes of NF-κB (A) and MMP-9 (B) after treatment with EGCG. The relative expression level was evaluated using RT qPCR. Data were shown as mean±S.D. (n=3). **P<.01 and *** P<.001, as compared with untreated control.

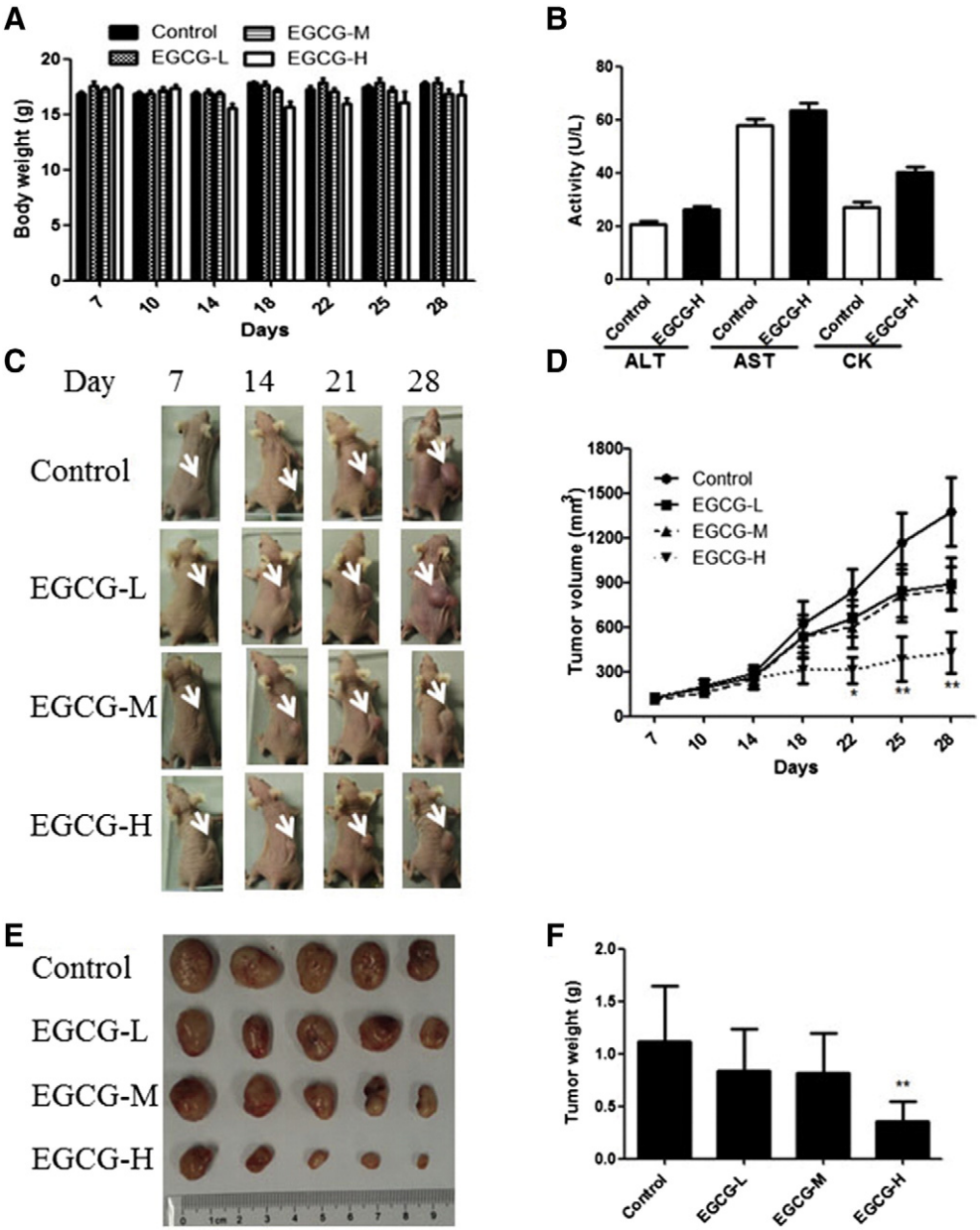

Fig. 6. *In vivo* antitumor effect of EGCG in SW780 nude mice xenograft tumor model. (A) No significant body weight loss of mice was found during EGCG treatment period. (B) Evaluation of the hematobiochemical markers (ALT, AST and CK) of mice plasma after treatment with EGCG. (C) Representative images of tumor burden obtained from each group at different time points. (D) Graph showed the tumor volume in each group, which was assessed by caliper and calculated as the length*width*width*0.5. (E) Representative images of tumor from each group at the termination of the experiment. (F) Graph showed the tumor weight from different group. High-dose EGCG treatment resulted in significant decrease in tumor weight. Data were expressed as mean±S.E.M., $n=7$. * $P<.05$ and ** $P<.01$, as compared with control.

rats with EGCG resulted in significant inhibition of AY-27 bladder tumor growth [13]. Hsieh et al. also demonstrated that oral, i.p. or intratumor injection of EGCG in nanoparticle form resulted in significant reduction of tumor weight in C3H/He nice with MBT-2 bladder tumor [14]. In addition, a recent preclinical study revealed that EGCG prevented intravesical tumor growth with efficacy equivalent to clinical drug of mitomycin C (used to prevent tumor cell implantation) in 344 Fischer female rats [32]. A clinical study from Liu et al. demonstrated that intravesical irrigation of EGCG in patients diagnosed with interstitial cystitis resulted in remission of symptoms, such as attenuation of the expression of purinergic receptors and up-regulation of iNOS and phosphorylated NF-κB [33]. Clinical studies from prostate and breast cancer also demonstrated that treatment with EGCG or tea polyphenol catechin resulted in significant inhibition of tumor growth in patients [34,35]. Besides, treatment with EGCG (100 mg/kg) in nude mice resulted in obvious inhibition of NF-κB p65 and MMP-9 in tumor in both protein and mRNA levels. The pro-caspase-3 and Bcl-2 were also suppressed in tumor after treatment with EGCG (Fig. 7). The *in vivo* findings in tumor were completely in line with the *in vitro* result that EGCG significantly suppressed NF-κB p65 and MMP-9 expression. Furthermore, when NF-κB was inhibited, EGCG showed no obvious effect in SW780 cell proliferation and migration (Fig. 8), indicating the important role NF-κB played in EGCG-induced inhibition. NF-κB is a ubiquitously expressed transcription factor that could translocate into the nucleus and then regulate the expression of a large number of genes, including TNF-α,

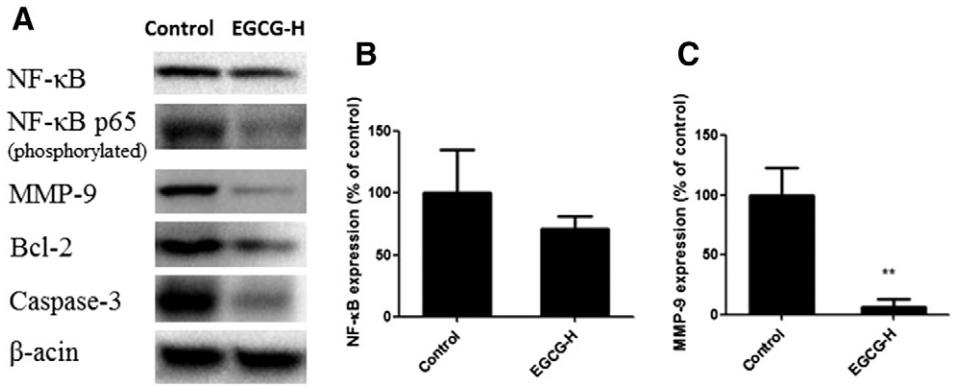

Fig. 7. Effect of EGCG on protein and mRNA expression in tumor. (A) Protein expressions in tumor. (B–C) Expression (mRNA) changes of NF-κB (B) and MMP-9 (C) in tumor after treatment with EGCG. Data were expressed as mean±S.D., $n=3$. ** $P<.01$, as compared with control.

Bcl-2 family proteins and MMP-9 [16]. MMP-9 is a zinc- and calcium-dependent endopeptidase which could degrade ECM and allow transitional cancer cells to migrate and invade into distant locations [36,37]. Our results have clearly demonstrated that EGCG decreased the NF-κB and MMP-9 expression in both protein and mRNA levels, and showed significant antiproliferation and antimigration effects both *in vitro* and *in vivo*. This revelation sheds light on the underlying mechanisms of EGCG on tumor inhibition in bladder cancer and provides clear directions for cancer treatment and drug combination. Besides, the effective dose of EGCG showed potent antitumor effect in mice bearing bladder tumors and demonstrated no obvious toxicity to the hosts, indicating that EGCG could be used as a safe and natural supplement for cancer prevention and treatment. In addition, EGCG also has other benefits, such as antioxidation, antiobesity, antidiabetes and cardiovascular protective effects. That means that EGCG may be a safe and effective healthy supplement not only for cancer treatment but also for comprehensive protection.

In our study, the effective dose of EGCG was 100 mg/kg for the mice study, which was equivalent to a single dose of 487 mg EGCG powder for a 60-kg adult. This dosage of EGCG was effective in inhibition of tumor progress in tumor-bearing mice and did not result in body weight loss and great change of hematobiochemical markers (AST, ALT and CK) in mice, indicating no obvious toxicity of EGCG to liver and heart. However, this high concentration of EGCG was unlikely to be consumed from tea beverage because EGCG accounts for about 8.5% in green tea water extract [7]. It would be achievable for human beings to consume the effective dose of EGCG as a powder capsule in concentrated form. Similar effect of EGCG was demonstrated by Henning *et al.* that a single dose of green tea extract (618 mg of EGCG) or purified EGCG (518 mg) was healthy for individuals and had significant antioxidant activities [10]. Besides, EGCG in nanoparticle forms could improve the absorption efficiency. Hsieh *et al.* showed that EGCG conjugated into nanogold particles was more effective than free EGCG in inhibiting bladder tumor in model mice because of the improved delivery efficacy [14]. Similar finding was also shown in Debnath's study that the nanoparticle form of EGCG was more powerful in prevention and curing protein-aggregation-derived diseases [38]. This metabolomics-based approach of EGCG may greatly improve the distribution efficacy and play crucial roles in cancer treatment.

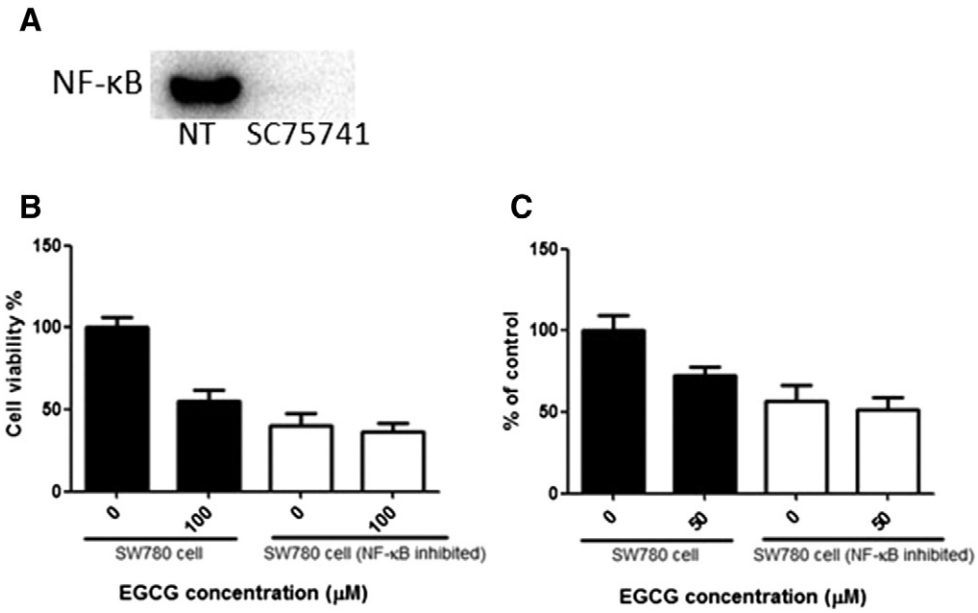

Fig. 8. Antiproliferation and antimigration effects of EGCG in normal and NF-κB-inhibited SW780 cells. (A) NF-κB was inhibited in SW780 cells after treatment with SC75741. Antiproliferation (B) and antimigration (C) effects of EGCG in normal and NF-κB-inhibited SW780 cells. Data were expressed as mean±S.D., $n=3$.

In summary, our results present the first evidence on the antitumor and antimigration effects of EGCG against SW780 cells by down-regulating the expression of NF-κB and MMP-9 without obvious toxicity to the hosts. This revelation sheds light on the underlying mechanisms of EGCG on tumor inhibition in bladder cancer and suggests that EGCG may be a safe and natural healthy supplement for cancer treatment and comprehensive health protection. However, more detailed molecular mechanisms, for instance, genomic and proteomic responses underlying the EGCG-induced bladder cancer cell apoptosis and antimetastasis, remain to be elucidated. Besides, further investigation is needed to determine the clinical efficacy and safety of EGCG in human subjects with bladder cancer. The pharmacokinetics of EGCG in human beings also needs investigation. Our observation holds promise for further studies to examine the efficacy of EGCG and develop EGCG as a potential anticancer supplement against bladder cancer.

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