

# Epigallocatechin-3-gallate inhibits nicotine-induced migration and invasion by the suppression of angiogenesis and epithelial-mesenchymal transition in non-small cell lung cancer cells

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**Abstract.** Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea extract, has been found to have anticancer activities in various types of cancer. However, the underlying mechanisms are not completely clear. In the present study, the effects of EGCG on migration, invasion, angiogenesis and epithelial-mesenchymal transition (EMT) induced by nicotine in A549 non-small cell lung cancer (NSCLC) cells were investigated, and the underlying molecular mechanisms were preliminarily examined. The results showed that different concentrations of EGCG significantly inhibited nicotine-induced migration and invasion. Moreover, EGCG reversed the upregulation of HIF-1 $\alpha$ , vascular endothelial growth factor (VEGF), COX-2, p-Akt, p-ERK and vimentin protein levels and the downregulation of p53 and  $\beta$ -catenin protein levels mediated by nicotine in A549 cells, but had no significant effect on their mRNA levels. Furthermore, EGCG markedly inhibited HIF-1 $\alpha$ -dependent angiogenesis induced by nicotine *in vitro* and *in vivo*, and suppressed HIF-1 $\alpha$  and VEGF protein expression induced by nicotine in A549 xenografts of nude mice. Taken together, the results indicated that EGCG inhibited nicotine-induced angiogenesis and EMT, leading to migration and invasion in A549 cells. The results of the present study suggested that

EGCG can be developed into a potential agent for the prevention and treatment of smoking-associated NSCLC.

## Introduction

Lung cancer is a leading cause of cancer-related mortality worldwide. Non-small cell lung cancer (NSCLC) comprises ~80% of all lung cancers (1). Cigarette smoking is the most important risk factor of NSCLC. Nicotine, the principal chemical component in tobacco smoke associated with addiction, is involved in the progression and metastasis of NSCLC (2). Accumulating experimental evidence has demonstrated that nicotine and its metabolites promotes migration and invasion in lung cancer cells (3-5). Angiogenesis, the formation of new blood vessels from a pre-existing vascular network, is essential for tumor migration, invasion and metastasis (6). Epithelial-mesenchymal transition (EMT), a process by which epithelial cells lose the epithelial phenotype and gain the mesenchymal phenotype, is largely thought to play a key role in invasion and metastasis (7). Nicotine has also been found to enhance angiogenesis (8,9) and EMT (4,5) in NSCLC cells. Angiogenesis and EMT play a crucial role in the development and progression of NSCLC (6,10). Thus the inhibitors of angiogenesis and EMT mediated by nicotine may be developed into chemopreventive agents of smoking-associated NSCLC.

Green tea, widely consumed in Asia, contains polyphenolic compounds that account for 30% of the dry weight of the leaves. Previous epidemiological studies and meta-analysis have revealed that green tea consumption reduces the risk of lung cancer (11,12). The chemopreventive effects of green tea are mediated by polyphenolic compounds known as catechins including (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epicatechin (EC), with EGCG being the most abundant and powerful catechin in cancer prevention and treatment (13,14). A growing body of evidence has demonstrated that EGCG inhibits migration and invasion in a variety of cancer cells (15-17). In a previous study, we found that EGCG inhibited angiogenesis by downregulating hypoxia- and serum-induced hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) protein accumulation and vascular endothelial growth factor (VEGF) expression in

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*Abbreviations:* EGCG, epigallocatechin-3-gallate; NSCLC, non-small cell lung cancer; EMT, epithelial-mesenchymal transition

*Key words:* epigallocatechin-3-gallate, nicotine, non-small cell lung cancer, angiogenesis, epithelial-mesenchymal transition, migration, invasion

human cervical carcinoma and hepatoma cells (18). Previously, we demonstrated that EGCG inhibits human papillomavirus (HPV)-16 oncoprotein- and IGF-1-induced angiogenesis in NSCLC by targeting HIF-1 $\alpha$  (19,20). Furthermore, nicotine induces HIF-1 $\alpha$  protein expression in NSCLC cells through the nicotinic acetylcholine receptor-mediated signaling pathway (8). However, the effect of EGCG on angiogenesis induced by nicotine in NSCLC cells has not been reported.

EMT promotes the invasion and metastasis of various types of cancers including NSCLC (7,10). EGCG has been found to inhibit EMT mediated by a variety of factors (21-23). Liu *et al* have reported that EGCG inhibited transforming growth factor- $\beta$  (TGF- $\beta$ )-mediated EMT in NSCLC cells via inhibition of the Smad2 and Erk1/2 signaling pathways (21). Ko *et al* also found that EGCG negatively affected TGF- $\beta$ 1-mediated EMT by suppressing the acetylation of Smad2 and Smad3 in A549 NSCLC cells (22). However, the effect of EGCG on nicotine-induced EMT in NSCLC cells remains to be determined.

In the present study, we determined the effect of EGCG on nicotine-induced migration, invasion, angiogenesis and EMT in NSCLC cells. To the best of our knowledge, the results of the present study showed for the first time that, EGCG inhibited nicotine-induced angiogenesis and EMT in A549 NSCLC cells, leading to the suppression of migration and invasion. Furthermore, we found that EGCG suppressed Akt and ERK1/2 activation induced by nicotine. These findings provide further evidence with regard to the underlying molecular mechanisms involved in the anticancer effect of EGCG on smoking-associated NSCLC.

## Materials and methods

**Drug and reagents.** EGCG was purchased from Sigma (St. Louis, MO, USA), dissolved in distilled water at a concentration of 100 mM and stored at -80°C as a stock solution. Nicotine was also obtained from Sigma. Complete protease inhibitor cocktail was from Roche (Mannheim, Germany). Mouse anti-human HIF-1 $\alpha$  monoclonal antibody was purchased from BD Transduction Laboratories (San Diego, CA, USA). Vimentin,  $\beta$ -catenin, total and phosphorylated Akt (Ser473) or ERK1/2 (Thr202/Tyr204) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody for  $\beta$ -actin was purchased from Beyotime Biotechnology Corporation (Shanghai, China). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology. The human VEGF ELISA Development kit was purchased from Wuhan Boster Bio-Engineering Co., Ltd. (Wuhan, China). One-Step SYBR<sup>®</sup> PrimeScript<sup>®</sup> RT-qPCR (no. DRR086A) was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Transfection reagent (Lipofectamine<sup>™</sup> 2000) was obtained from Invitrogen Corporation (Carlsbad, CA, USA). An In Vitro Angiogenesis Assay kit (ECM625) was obtained from Millipore (Temecula, CA, USA). An In Vivo Angiogenesis Assay kit (no. 354248) was obtained from BD Biosciences (Bedford, MA, USA). HiCN Hemoglobin Detection kit was purchased from Shanghai Rongsheng Biotech Co., Ltd. (Shanghai, China).

**Cell culture and treatment with EGCG.** The human A549 NSCLC cell line (adenocarcinoma cell line) and human umbil-

ical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Exponentially growing cells (~80% confluence) in complete media were pretreated for 1 h with different concentrations of EGCG, followed by exposure to nicotine (5  $\mu$ M) for the indicated time points according to the purpose of the experiment.

**Wound-healing assays.** A549 cells were plated and grown to 80% confluence in a 24-well plate (Corning Inc.). The cells were starved in 1% FBS for 24 h and then washed with phosphate-buffered saline (PBS). The cells were scratched with a sterile 200  $\mu$ l pipette tip in each well and medium containing 10% FBS and 5  $\mu$ M nicotine in the presence or absence of EGCG. After 24 and 48 h, the wounds were observed and images were captured. The data were representative of three independent experiments.

**Cell invasion assays.** The invasive ability of A549 cells was assayed using 24-well invasion chambers (Corning Inc.) with filters coated with Matrigel (BD Biosciences) on the upper surface with 8.0- $\mu$ m pores. Briefly, Matrigel was applied to the upper surface of the chambers. A549 cells (5 $\times$ 10<sup>4</sup>) in 0.2 ml medium were added into the upper chamber, and 0.5 ml 10% FBS medium in the presence or absence of 5  $\mu$ M nicotine or different concentrations of EGCG (10, 25, 50 and 100  $\mu$ M) was added into the lower chamber. After incubation at 37°C with 5% CO<sub>2</sub> for 36 h, non-migrating A549 cells on the upper surface of the chambers were removed by wiping with cotton swabs. The filters were stained with hematoxylin for 30 min. The number of cells migrating on the other side of the filters was counted. The data are representative of three independent experiments.

**Western blot analysis.** Nicotine- or EGCG-treated A549 cells were lysed with cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100 and sodium pyrophosphate,  $\beta$ -glycerophosphate, EDTA, Na<sub>3</sub>VO<sub>4</sub>, leupeptin phenylmethyl-sulfonyl fluoride (PMSF), and complete protease inhibitor cocktail (Roche) followed by incubation at 4°C for 1 h. The lysates were ultra-sonicated and centrifuged at 12,000  $\times$  g for 10 min. Protein concentrations were determined by BCA methods. Protein (100  $\mu$ g) was separated on 10% polyacrylamide-SDS gel and electroblotted onto PVDF membranes (Millipore). After blocking with 5% skim milk, the membrane was incubated overnight at 4°C with primary antibody against HIF-1 $\alpha$ , vimentin,  $\beta$ -catenin, total-Akt (t-Akt), phosphorylated Akt (p-Akt), total-ERK1/2 (t-ERK1/2), or phosphorylated-ERK1/2 (p-ERK1/2), followed by incubation with HRP-conjugated secondary antibodies (1:1,000). As a loading control, the blots were stripped and re-probed with anti- $\beta$ -actin antibody.

**RT-qPCR analysis.** Total RNA was isolated from cells using TRIzol<sup>®</sup> reagent (Invitrogen). RT-qPCR analysis of HIF-1 $\alpha$  and VEGF mRNA levels was performed using One-Step SYBR<sup>®</sup> PrimeScript<sup>®</sup> RT-PCR (Biotechnology Co., Ltd.) according to the manufacturer's instructions. The primers

designed for RT-qPCR were as follows: HIF-1 $\alpha$ , forward: 5'-TCTGGGTTGAACTCAAGCAACTG-3' and reverse: 5'-CAACCGGTTTAAGGACACATTCTG-3' (GenBank, NM\_001243084.1); human VEGF, forward: 5'-TGCTTCTGAGTTGCCAGGA-3' and reverse: 5'-TGGTTTCAATGGTGTGAGGACATAG-3' (GenBank, NM\_003376.5); p53, forward: 5'-GAGGCCTTGGAACTCAAGGATG-3' and reverse: 5'-TCAGGCCCTTCTGTCTTGAACA-3' (GenBank, NM\_000546.4); COX-2, forward: 5'-CTGTAACCAAGATGGATGCAA-AGA-3' and reverse: 5'-GTCAGTGACAATGAGATGTGGAA-3' (GenBank, NM\_000963.2);  $\beta$ -actin, forward: 5'-TGGCACCCAGCACAATGAA-3' and reverse: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' (GenBank, NM\_001101.3). The primers were produced by Takara Biotechnology Co., Ltd. The thermocycling conditions used were: 42°C for 5 min, 95°C for 10 sec, followed by 40 cycles at 95°C for 5 sec, and 60°C for 31 sec. The size of the PCR product of HIF-1 $\alpha$ , VEGF, p53, COX-2 and  $\beta$ -actin was 150, 176, 137, 195 and 186 bp, respectively. The relative HIF-1 $\alpha$ , VEGF, p53 and COX-2 mRNA levels were normalized to  $\beta$ -actin. The experiment was repeated in triplicate.

**Enzyme-linked immunosorbent assay (ELISA).** The concentration of VEGF protein in the conditioned media derived from nicotine- or EGCG-treated A549 cells was determined using a human VEGF ELISA development kit according to the manufacturer's instructions. The results were normalized to the cell number ( $5 \times 10^3$  cells). The experiments were repeated in triplicate.

**RNA interference.** HIF-1 $\alpha$ -siRNA (sense strand, 5'-GCCGCUC AAUUUAUGAAUATT-3') and non-specific (NS)-siRNA (sense strand, 5'-UUCUCCGAACGUGUCACGUTT-3') duplexes were generated by Shanghai GenePharma Co., Ltd. (Shanghai, China). Transfection was performed using the Lipofectamine™ 2000 transfection reagent according to the manufacturer's instructions.

**In vitro angiogenesis assay.** An *in vitro* angiogenesis assay kit was employed according to the manufacturer's instructions (Millipore). HUVECs ( $5 \times 10^3$  cells/well) were seeded onto the surface of 96-well cell culture plates pre-coated with polymerized ECMatrix™ and then incubated at 37°C for 6-8 h in the conditioned media derived from nicotine-treated A549 cells transfection with HIF-1 $\alpha$ -siRNA or NS-siRNA. In parallel studies, HUVECs were cultured in the conditioned media derived from nicotine-treated A549 cells pretreated with 100  $\mu$ M of EGCG. Tubule formation was observed under a phase-contrast microscope. The total tube length in three random view-fields/wells was measured by Scion Image software and the average value was calculated. The experiment was repeated in triplicate.

**In vivo angiogenesis assay.** Male nude mice (BALB/C nude) (6-8 weeks old) were purchased from the Animal Center of Southern Medical University (Guangzhou, China). Experiments with animals were undertaken in accordance with the institution guidelines of the Institutional Animal Care and Use Committee of Southern Medical University and Guangdong Medical College. Nicotine- or EGCG-treated

A549 cells were re-suspended in serum-free media at a density of  $8.0 \times 10^6$  cells/ml and 0.25 ml ( $2.0 \times 10^6$  cells in total) of cell suspension was mixed with the same volume of BD Matrigel Matrix (0.25 ml). The BD Matrigel mixture was subcutaneously injected into the two flanks of nude mice. On day 11, the mice were euthanized and Matrigel plugs were harvested, with half of each Matrigel plug fixed with 10% neutral formalin for immunohistochemical studies and the other half weighed for the determination of hemoglobin content as previously described (20).

**Immunohistochemistry.** The expression of HIF-1 $\alpha$  and VEGF proteins in Matrigel plugs was analyzed by immunohistochemistry as previously described (20). Briefly, formalin-fixed Matrigel plugs were paraffin-embedded and sectioned at 4  $\mu$ m. The sections were then deparaffinized in xylene, rehydrated through serial dilutions of alcohol and washed in PBS (pH 7.2). The sections were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0) and then incubated overnight at 4°C with primary antibody, followed by incubation with streptavidin/HRP-conjugated secondary antibodies. The conventional streptavidin/peroxidase method (Histostain™-Plus kits, SP0023; Beijing Biosynthesis Biotechnology, Co., Ltd., Beijing, China) was used to develop signals, and the cells were counterstained with hematoxylin. The sections incubated with secondary antibodies in the absence of primary antibodies served as negative controls.

**Statistical analysis.** Data are presented as the mean  $\pm$  SD for three separate experiments. One-way ANOVA, Bonferroni and Dunnett-T3 were employed for statistical analysis using SPSS 19.0 for windows software.  $P < 0.05$  was considered to indicate a statistically significant result.

## Results

**EGCG inhibits nicotine-induced migration and invasion in A549 cells.** A wound-healing assay was carried out to assess whether EGCG affects the migratory properties of A549 cells induced by nicotine. Similar to recent results (3), we found that nicotine enhanced A549 cell migration (Fig. 1A). However, EGCG significantly inhibited the migration of A549 cells induced by nicotine in a dose-dependent manner, with the maximal efficiency being observed at 100  $\mu$ M EGCG treatment (Fig. 1A). The effect of EGCG on invasion induced by nicotine in A549 cells was examined. As shown in Fig. 1B and C, treatment of cells with nicotine promoted cell invasive property. However, EGCG inhibited the invasive property of A549 cells induced by nicotine in a dose-dependent manner, and the maximum effect was observed at 100  $\mu$ M EGCG treatment. Taken together, the results indicated that EGCG inhibited nicotine-induced migration and invasion in A549 cells.

**EGCG reverses the upregulation of HIF-1 $\alpha$ , VEGF, COX-2, p-Akt, p-ERK and vimentin protein levels and the down-regulation of p53 and  $\beta$ -catenin protein levels mediated by nicotine in A549 cells.** To determine the effects of EGCG on angiogenesis and EMT mediated by nicotine in A549 cells, the expression of angiogenesis- and EMT-associated proteins was analyzed. Western blot analysis was carried out to assess

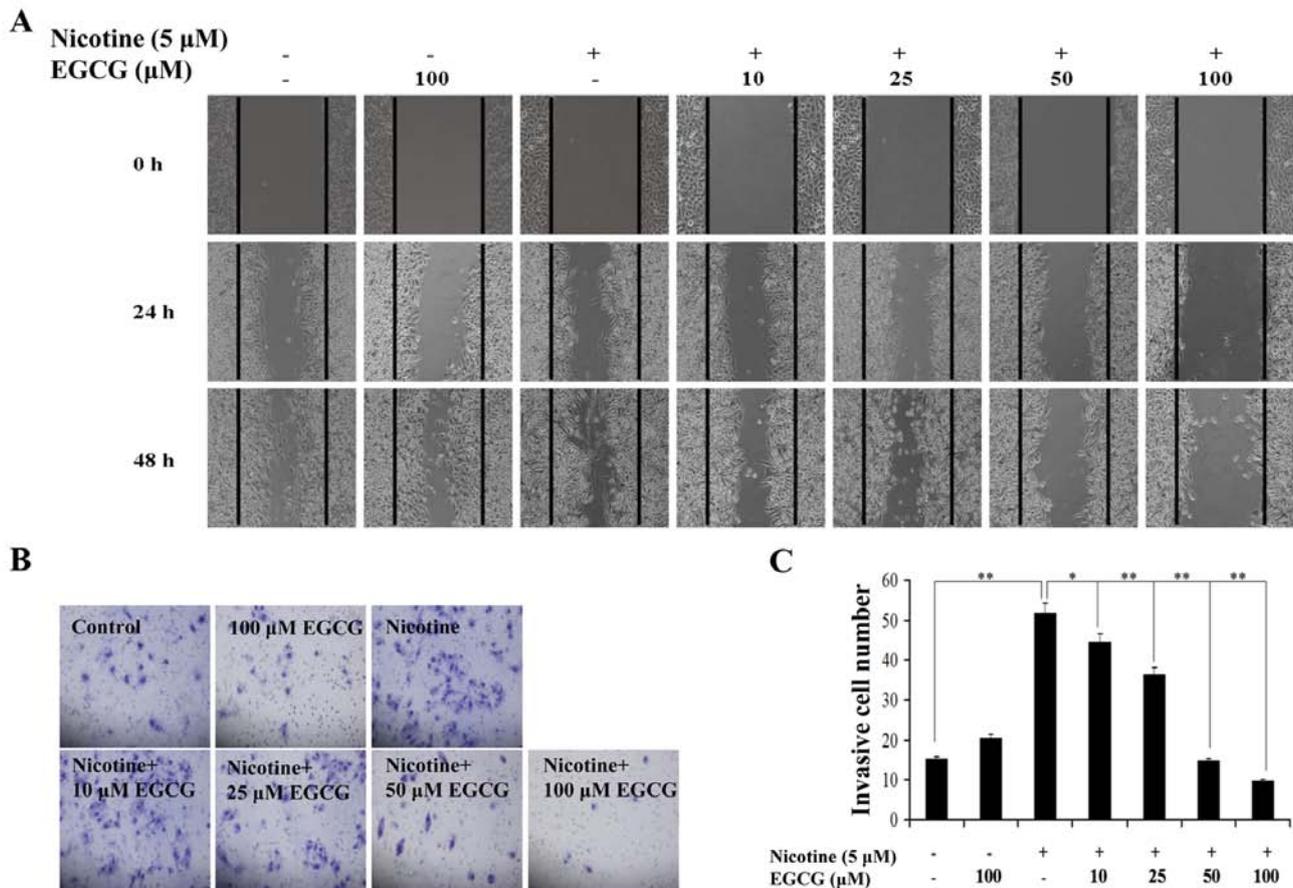


Figure 1. EGCG inhibits nicotine-induced migration and invasion in A549 NSCLC cells. (A) The migration of A549 cells exposed to nicotine in the presence or absence of EGCG was analyzed by a wound-healing assay. (B and C) The invasion of A549 cells exposed to nicotine in the presence or absence of EGCG was detected by the Matrigel invasion chamber. (B) Images are representative of results from three independent experiments. (C) The number of invasive cells from (B). The data are presented as the mean  $\pm$  SD from three replicate experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ . EGCG, epigallocatechin-3-gallate; NSCLC, non-small cell lung cancer.

the expression of HIF-1 $\alpha$ , p53, COX-2, vimentin,  $\beta$ -catenin, p-Akt and p-ERK, and ELISA was performed to determine VEGF concentrations in the conditioned media derived from differentially treated cells. The results showed that nicotine significantly upregulated the expression of HIF-1 $\alpha$ , VEGF, COX-2 and vimentin proteins in A549 cells, but downregulated the expression of  $\beta$ -catenin and p53 proteins in A549 cells (Fig. 2A, B and D). However, EGCG significantly reversed the effects of nicotine on the expression of these proteins in A549 cells (Fig. 2A, B and D). We also assessed whether EGCG affects the activation of Akt and ERK induced by nicotine in A549 cells. The results showed that EGCG inhibited the expression of p-Akt and p-ERK induced by nicotine in A549 cells (Fig. 2C). Taken together, our results suggested that EGCG inhibited angiogenesis and EMT mediated by nicotine in A549 cells.

*EGCG has no significant effect on the expression of HIF-1 $\alpha$ , VEGF, COX-2 and p53 mRNA mediated by nicotine in A549 cells.* Since EGCG affected the expression of HIF-1 $\alpha$ , VEGF, p53 and COX-2 proteins induced by nicotine in A549 cells, to assess whether the alteration of HIF-1 $\alpha$ , VEGF, p53 and COX-2 expression can be regulated at the transcriptional levels, we measured mRNA levels by RT-qPCR. As shown in Fig. 3, treatment of cells with nicotine upregulated HIF-1 $\alpha$ , VEGF

and COX-2 mRNA levels and downregulated the p53 mRNA level as compared to the controls, whereas EGCG treatment had no obvious effects on HIF-1 $\alpha$ , VEGF, p53 and COX-2 mRNA levels mediated by nicotine. These results suggested that EGCG affected the expression of HIF-1 $\alpha$ , VEGF, p53 and COX-2 proteins mediated by nicotine in A549 cells possibly through translational or post-translational mechanisms.

*EGCG inhibits HIF-1 $\alpha$ -dependent angiogenesis induced by nicotine in vitro and in vivo, and suppressed HIF-1 $\alpha$  and VEGF protein expression induced by nicotine in A549 xenografts of nude mice.* To determine the effects of EGCG on nicotine-induced angiogenesis in A549 cells, an *in vitro* angiogenesis model was employed to evaluate the capillary tube formation of HUVECs stimulated by the conditioned media derived from A549-treated cells. Since VEGF is one of the downstream genes of HIF-1 $\alpha$  (24,25), to investigate whether the increased expression of VEGF and angiogenesis induced by nicotine in A549 cells was mediated by HIF-1 $\alpha$ , A549 cells were transfected with HIF-1 $\alpha$ -specific siRNA (HIF-1 $\alpha$ -siRNA) or HIF-1 $\alpha$  non-specific siRNA (NS-siRNA). The results showed that A549 cells treated with nicotine enhanced the ability to stimulate the formation of capillary tube-like structures by HUVECs as compared to the controls. Furthermore, A549 cells treated with EGCG or transfection

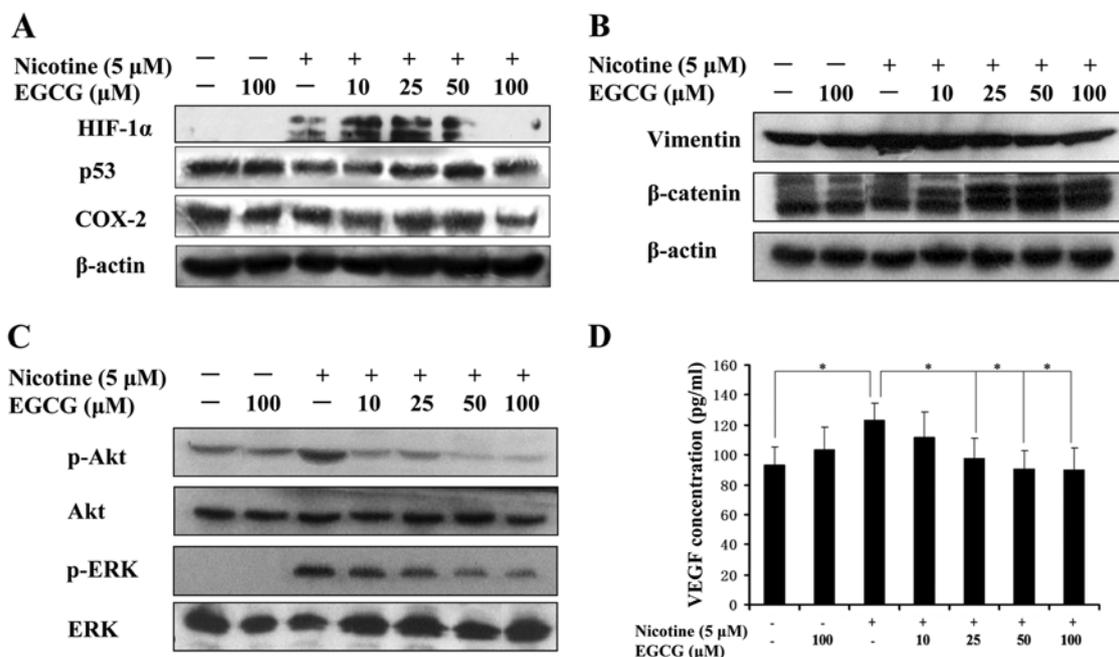


Figure 2. EGCG reverses the upregulation of HIF-1 $\alpha$ , VEGF, COX-2, p-Akt, p-ERK and vimentin protein levels and the downregulation of p53 and  $\beta$ -catenin protein levels mediated by nicotine in A549 cells. Exponentially growing cells in complete media were pretreated for 1 h with different concentrations of EGCG, followed by exposure to nicotine (5  $\mu$ M) for 16 h. (A-C) Western blot analysis was performed to determine the expression of HIF-1 $\alpha$ , p53, COX-2, vimentin,  $\beta$ -catenin, p-Akt and p-ERK proteins. (D) VEGF concentration in the conditioned media derived from A549-treated cells was determined by ELISA. The data are presented as the mean  $\pm$  SD from three replicate experiments. \* $P$ <0.05. EGCG, epigallocatechin-3-gallate; VEGF, vascular endothelial growth factor.

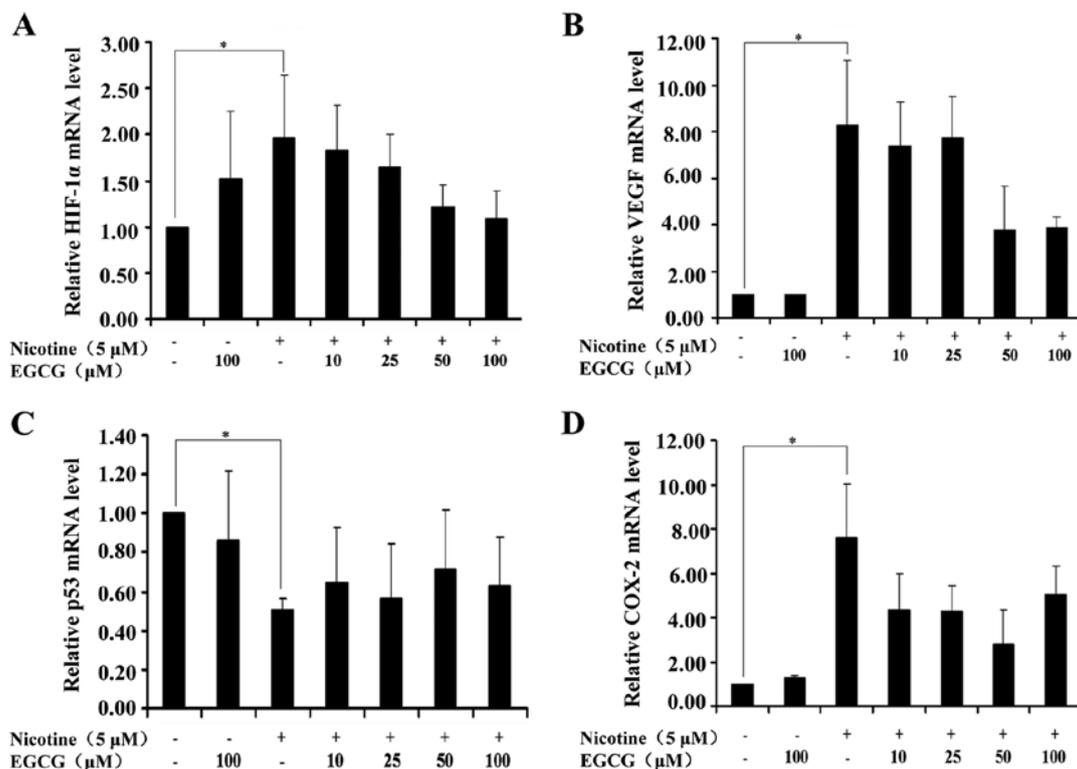


Figure 3. EGCG has no significant effects on the expression of HIF-1 $\alpha$ , VEGF, p53 and COX-2 mRNA mediated by nicotine in A549 cells. Exponentially growing cells in complete media were pretreated for 1 h with different concentrations of EGCG, followed by exposure to nicotine (5  $\mu$ M) for 16 h. The expression levels of (A) HIF-1 $\alpha$ , (B) VEGF, (C) p53 and (D) COX-2 mRNA were determined by RT-qPCR. The data are presented as the mean  $\pm$  SD from three replicate experiments. \* $P$ <0.05. EGCG, epigallocatechin-3-gallate; VEGF, vascular endothelial growth factor.

with HIF-1 $\alpha$ -siRNA inhibited the nicotine-induced ability to stimulate the formation of capillary tube-like structures by

HUVECs as compared with nicotine-treated cells (Fig. 4A), which was further confirmed by quantification of the total

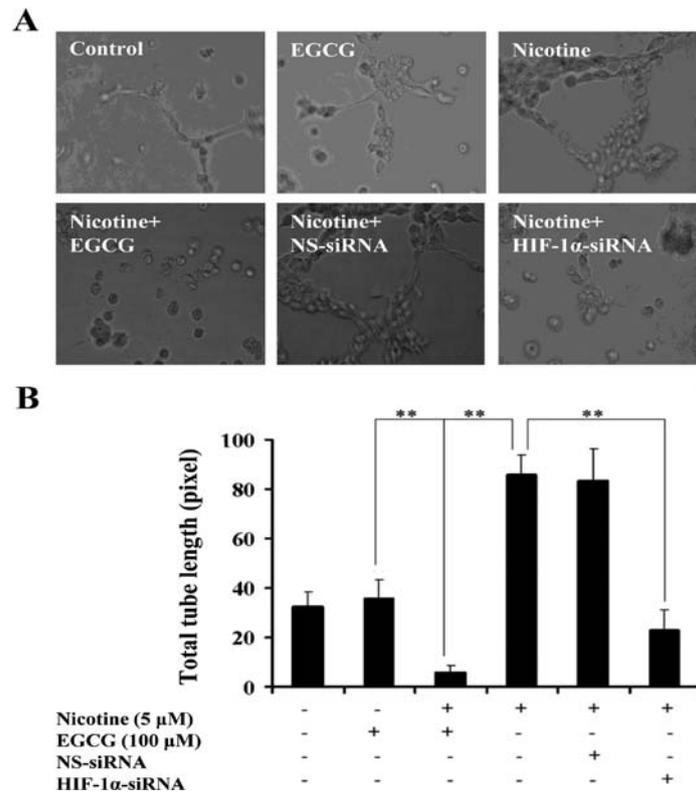


Figure 4. Treatment with EGCG and transfection with HIF-1 $\alpha$ -siRNA inhibit nicotine-induced angiogenesis *in vitro* in A549 cells. (A) Tube formation was observed under a phase-contrast microscope (magnification,  $\times 200$ ). The data presented are representative of three separate experiments. (B) The total tube length from (A) in three random view-fields/well was measured by Scion Image software and the average value was calculated. The data are presented as mean  $\pm$  SD of three independent experiments. \*\* $P < 0.01$ . EGCG, epigallocatechin-3-gallate.

tube length pixel values ( $P < 0.05$ ; Fig. 4B). However, such inhibition of angiogenic abilities was not observed in NS-siRNA-transfected cells ( $P > 0.05$ ; Fig. 4A and B).

We performed a Matrigel plug angiogenesis assay using BALB/C nude mice to observe the effect of EGCG on nicotine-induced tumor angiogenesis *in vivo*. The results showed that Matrigel plugs mixed with nicotine-treated cells obviously induced angiogenesis *in vivo* (Fig. 5A), showing much higher hemoglobin levels than those in the controls ( $P < 0.05$ ; Fig. 5B). Moreover, Matrigel plugs mixed with EGCG or HIF-1 $\alpha$ -siRNA-transfected cells significantly inhibited angiogenesis *in vivo* stimulated by nicotine (Fig. 5A), showing barely detectable or low hemoglobin levels ( $P < 0.05$ ; Fig. 5B). However, Matrigel plugs mixed with NS-siRNA-transfected cells did not inhibit angiogenesis *in vivo* induced by nicotine (Fig. 5A and B).

In addition, immunohistochemical results showed that xenografts of A549 cells treated with nicotine exhibited a significantly enhanced expression of HIF-1 $\alpha$  and VEGF proteins as compared to the controls (Fig. 5C). The enhanced expression of HIF-1 $\alpha$  protein was inhibited by transfection with HIF-1 $\alpha$ -siRNA, but not by transfection with NS-siRNA (Fig. 5C). The increase of VEGF expression induced by nicotine was abrogated by transfection with HIF-1 $\alpha$ -siRNA, but not by transfection with NS-siRNA. These results indicated that the upregulation of VEGF protein expression induced by nicotine was HIF-1 $\alpha$ -dependent in the xenografts of A549 cells. Taken together, these results indicated EGCG markedly inhibited HIF-1 $\alpha$ -dependent angiogenesis induced by nicotine *in vitro*

and *in vivo*, and downregulated HIF-1 $\alpha$  and VEGF protein expression in A549 xenografts of nude mice.

## Discussion

Cancer progression is associated with the abrogation of normal biological characteristics that limit cell proliferation, migration and invasion, eventually leading to metastasis (26-28). Since metastases are a major cause of recurrence and mortality in cancer patients, the development of new drugs that reduce invasion and metastasis is extremely important for the prevention and treatment of cancer. In the present study, we demonstrate that EGCG obviously inhibited the migration and invasion induced by nicotine in A549 cells, suggesting that EGCG holds great potential as a chemopreventive agent in nicotine-associated NSCLC.

EMT is a critical normal process during development and wound healing. However, the properties of EMT were shown to be involved in human pathology, including fibrosis and cancer metastasis (29,30). EMT facilitates tumor cell migration, invasion and metastasis, and therefore may also be a major mechanism of tumor progression (26-28). The results presented in this study showed that nicotine upregulated the expression of vimentin but downregulated the expression level of  $\beta$ -catenin, an extracellular matrix protein marker. However, treatment with EGCG reversed the expression of vimentin and  $\beta$ -catenin mediated by nicotine in A549 cells. Previous findings have demonstrated that the decrease in E-cadherin and  $\beta$ -catenin levels with a concurrent increase in

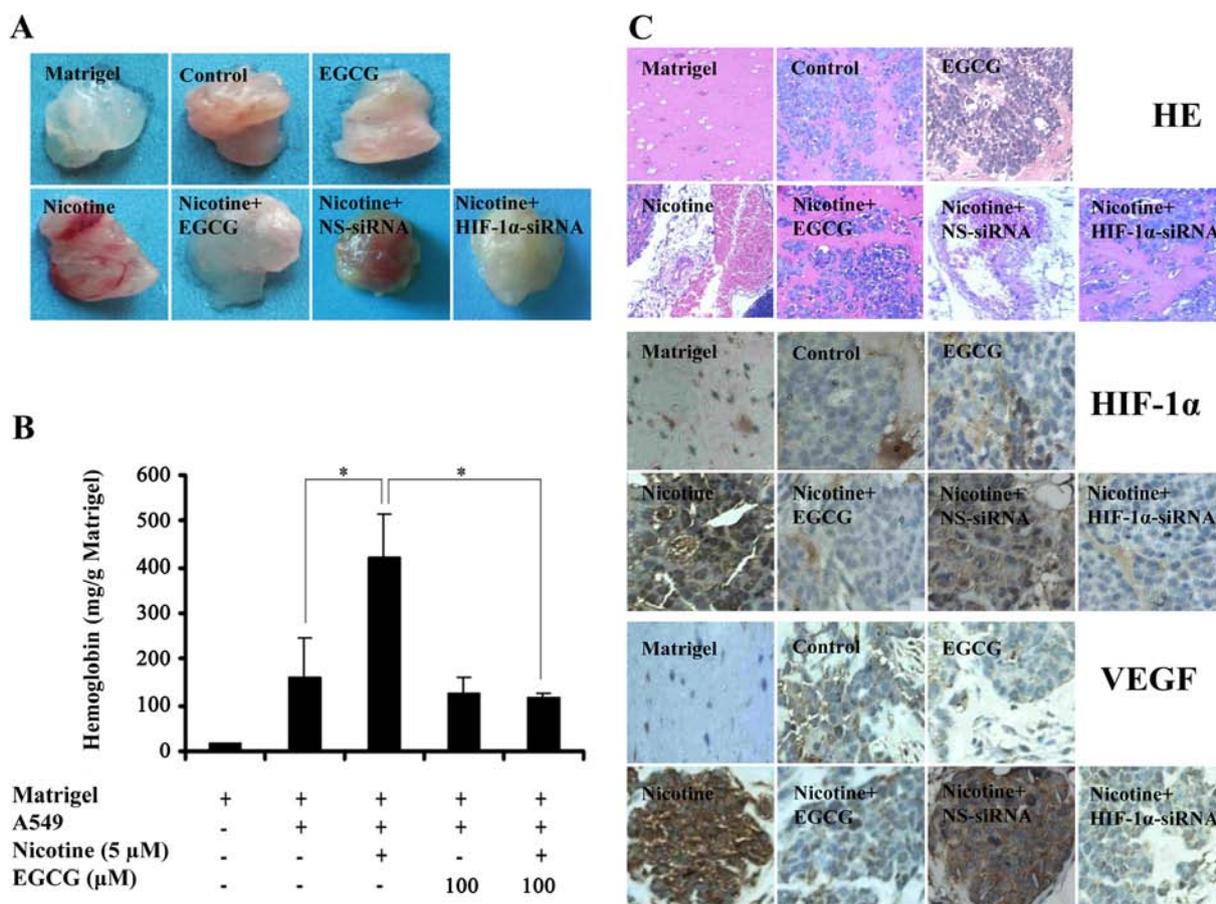


Figure 5. EGCG inhibits nicotine-induced angiogenesis *in vivo*, and downregulated HIF-1 $\alpha$  and VEGF protein expression in A549 xenografts of nude mice. (A) Representative Matrigel plugs (magnification, x200). (B) Hemoglobin levels in Matrigel plugs. Hemoglobin content was expressed as (mg/g) of Matrigel plug. The data are presented as the mean  $\pm$  SD of three independent experiments. \* $P < 0.05$ . (C) Immunohistochemical studies on the expression of HIF-1 $\alpha$  and VEGF proteins in A549 xenografted tumors (magnification, x200). The results are representative of three independent experiments. EGCG, epigallocatechin-3-gallate; VEGF, vascular endothelial growth factor; HE, hematoxylin and eosin.

the vimentin level is one of the hallmarks of EMT in lung cancer cells (31-33). Therefore, EGCG may be important in suppression of EMT mediated by nicotine in A549 NSCLC cells. Angiogenesis is required for tumor migration, invasion and metastasis (6). In the present study, we found that EGCG also inhibited nicotine-induced angiogenesis *in vitro* and *in vivo*. Thus, the results suggest that EGCG-inhibited nicotine-induced migration and invasion may at least occur in part, through the suppression of EMT and angiogenesis.

HIF-1 $\alpha$  and VEGF play an important role in tumor invasion and metastasis, particularly in tumor angiogenesis (34). It has been reported that the expression of HIF-1 $\alpha$  and VEGF was related to a poor prognosis and a worse overall survival (34-36). It has been previously demonstrated that hypoxia-stabilized HIF-1 $\alpha$  promoted EMT through increasing SNAI1 transcription in hepatocellular carcinoma cells (37), and that vascular endothelial growth factor receptor-1 (VEGFR-1) activation enhanced migration and invasion of breast cancer cells through EMT (38). To identify the mechanisms by which EGCG inhibited nicotine-induced angiogenesis and EMT, we determined the expression of HIF-1 $\alpha$  and VEGF. Our results show that EGCG significantly inhibited nicotine-induced HIF-1 $\alpha$  and VEGF protein expression in A549 cells and in A549 xenografts of the nude mice, indicating that EGCG-inhibited

nicotine-induced angiogenesis and EMT may be associated with the inhibition of HIF-1 $\alpha$  and VEGF protein expression.

HIF-1 $\alpha$ , a key transcriptional factor, governs the expression of a large array of hypoxia-responsive genes, including *VEGF*, *COX-2* and *p53* (39-42). *COX-2*, a key regulator of inflammation-producing prostaglandins, can promote tumor cell proliferation and growth. *p53*, an important tumor suppressor, holds a crosstalk with HIF-1 (42). In the present study, we found that nicotine-induced angiogenesis *in vitro* and *in vivo* and VEGF protein expression in A549 cells were HIF-1 $\alpha$ -dependent. Additionally, we analyzed the expression of other downstream protein of HIF-1 $\alpha$ , including *COX-2* and *p53*. We found that EGCG inhibited nicotine-induced *COX-2* protein expression, and concomitantly upregulated the *p53* protein level. However, EGCG had no obvious effects on the expression of HIF-1 $\alpha$ , VEGF, *COX-2* and *p53* mRNA mediated by nicotine in A549 cells. These results suggest that EGCG altered the expression of HIF-1 $\alpha$ , VEGF, *COX-2* and *p53* proteins mediated by nicotine through a post-transcriptional mechanism.

The PI3K/Akt and ERK1/2 signaling pathways have been found to be involved in tumor angiogenesis and EMT stimulated by various factors (43,44). These signaling pathways are important in the development and progression of NSCLC (45,46). Therefore, targeting PI3K/AKT and ERK1/2

signaling pathways may be an emerging treatment strategy for NSCLC (47,48). In the present study, to the best of our knowledge we have for the first time demonstrated that, EGCG suppressed nicotine-induced Akt and ERK1/2 activation, providing the new evidence of a novel molecular mechanism for the anticancer action of EGCG on smoking-associated NSCLC.

In conclusion, to the best of our knowledge, we have demonstrated for the first time that, EGCG significantly inhibited nicotine-induced EMT and angiogenesis *in vitro* and *in vivo*, leading to the suppression of migration and invasion in A549 cells. Our findings suggest that EGCG is a potential agent for the prevention and treatment of smoking-associated NSCLC.

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### References

- Pesch B, Kendzia B, Gustavsson P, Jöckel KH, Johnen G, Pohlabein H, Olsson A, Ahrens W, Gross IM, Brüske I, *et al*: Cigarette smoking and lung cancer - relative risk estimates for the major histological types from a pooled analysis of case-control studies. *Int J Cancer* 131: 1210-1219, 2012.
- Schaal C and Chellappan SP: Nicotine-mediated cell proliferation and tumor progression in smoking-related cancers. *Mol Cancer Res* 12: 14-23, 2014.
- Nair S, Bora-Singhal N, Perumal D and Chellappan S: Nicotine-mediated invasion and migration of non-small cell lung carcinoma cells by modulating *STMN3* and *GSPT1* genes in an ID1-dependent manner. *Mol Cancer* 13: 173, 2014.
- Dasgupta P, Rizwani W, Pillai S, Kinkade R, Kovacs M, Rastogi S, Banerjee S, Carless M, Kim E, Coppola D, *et al*: Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell lines. *Int J Cancer* 124: 36-45, 2009.
- Wu SQ, Lv YE, Lin BH, Luo LM, Lv SL, Bi AH and Jia YS: Silencing of periostin inhibits nicotine-mediated tumor cell growth and epithelial-mesenchymal transition in lung cancer cells. *Mol Med Rep* 7: 875-880, 2013.
- Jackson AL, Zhou B and Kim WY: HIF, hypoxia and the role of angiogenesis in non-small cell lung cancer. *Expert Opin Ther Targets* 14: 1047-1057, 2010.
- Creighton CJ, Gibbons DL and Kurie JM: The role of epithelial-mesenchymal transition programming in invasion and metastasis: A clinical perspective. *Cancer Manag Res* 5: 187-195, 2013.
- Zhang Q, Tang X, Zhang ZF, Velikina R, Shi S and Le AD: Nicotine induces hypoxia-inducible factor-1 $\alpha$  expression in human lung cancer cells via nicotinic acetylcholine receptor-mediated signaling pathways. *Clin Cancer Res* 13: 4686-4694, 2007.
- Ma X, Jia Y, Zu S, Li R, Jia Y, Zhao Y, Xiao D, Dang N and Wang Y:  $\alpha 5$  nicotinic acetylcholine receptor mediates nicotine-induced HIF-1 $\alpha$  and VEGF expression in non-small cell lung cancer. *Toxicol Appl Pharmacol* 278: 172-179, 2014.
- Nurwidya F, Takahashi F, Murakami A and Takahashi K: Epithelial mesenchymal transition in drug resistance and metastasis of lung cancer. *Cancer Res Treat* 44: 151-156, 2012.
- Wang L, Zhang X, Liu J, Shen L and Li Z: Tea consumption and lung cancer risk: A meta-analysis of case-control and cohort studies. *Nutrition* 30: 1122-1127, 2014.
- Lin IH, Ho ML, Chen HY, Lee HS, Huang CC, Chu YH, Lin SY, Deng YR, He YH, Lien YH, *et al*: Smoking, green tea consumption, genetic polymorphisms in the insulin-like growth factors and lung cancer risk. *PLoS One* 7: e30951, 2012.
- Ravindranath MH, Ramasamy V, Moon S, Ruiz C and Muthugounder S: Differential growth suppression of human melanoma cells by tea (*Camellia sinensis*) epicatechins (ECG, EGC and EGCG). *Evid Based Complement Alternat Med* 6: 523-530, 2009.
- Du GJ, Zhang Z, Wen XD, Yu C, Calway T, Yuan CS and Wang CZ: Epigallocatechin gallate (EGCG) is the most effective cancer chemopreventive polyphenol in green tea. *Nutrients* 4: 1679-1691, 2012.
- Wang F, Chang Z, Fan Q and Wang L: Epigallocatechin-3-gallate inhibits the proliferation and migration of human ovarian carcinoma cells by modulating p38 kinase and matrix metalloproteinase-2. *Mol Med Rep* 9: 1085-1089, 2014.
- Zhang W, Yang P, Gao F, Yang J and Yao K: Effects of epigallocatechin gallate on the proliferation and apoptosis of the nasopharyngeal carcinoma cell line CNE2. *Exp Ther Med* 8: 1783-1788, 2014.
- Li H, Li Z, Xu YM, Wu Y, Yu KK, Zhang C, Ji YH, Ding G and Chen FX: Epigallocatechin-3-gallate induces apoptosis, inhibits proliferation and decreases invasion of glioma cell. *Neurosci Bull* 30: 67-73, 2014.
- Li X, Feng Y, Liu J, Feng X, Zhou K and Tang X: Epigallocatechin-3-gallate inhibits IGF-I-stimulated lung cancer angiogenesis through downregulation of HIF-1 $\alpha$  and VEGF expression. *J Nutrigenet Nutrigenomics* 6: 169-178, 2013.
- Zhang Q, Tang X, Lu Q, Zhang Z, Rao J and Le AD: Green tea extract and (-)-epigallocatechin-3-gallate inhibit hypoxia- and serum-induced HIF-1 $\alpha$  protein accumulation and VEGF expression in human cervical carcinoma and hepatoma cells. *Mol Cancer Ther* 5: 1227-1238, 2006.
- He L, Zhang E, Shi J, Li X, Zhou K, Zhang Q, Le AD and Tang X: (-)-Epigallocatechin-3-gallate inhibits human papillomavirus (HPV)-16 oncoprotein-induced angiogenesis in non-small cell lung cancer cells by targeting HIF-1 $\alpha$ . *Cancer Chemother Pharmacol* 71: 713-725, 2013.
- Liu LC, Tsao TC, Hsu SR, Wang HC, Tsai TC, Kao JY and Way TD: EGCG inhibits transforming growth factor- $\beta$ -mediated epithelial-to-mesenchymal transition via the inhibition of Smad2 and Erk1/2 signaling pathways in non-small cell lung cancer cells. *J Agric Food Chem* 60: 9863-9873, 2012.
- Ko H, So Y, Jeon H, Jeong MH, Choi HK, Ryu SH, Lee SW, Yoon HG and Choi KC: TGF- $\beta$ 1-induced epithelial-mesenchymal transition and acetylation of Smad2 and Smad3 are negatively regulated by EGCG in human A549 lung cancer cells. *Cancer Lett* 335: 205-213, 2013.
- Takahashi A, Watanabe T, Mondal A, Suzuki K, Kurusu-Kanno M, Li Z, Yamazaki T, Fujiki H and Suganuma M: Mechanism-based inhibition of cancer metastasis with (-)-epigallocatechin gallate. *Biochem Biophys Res Commun* 443: 1-6, 2014.
- Nilsson I, Shibuya M and Wennström S: Differential activation of vascular genes by hypoxia in primary endothelial cells. *Exp Cell Res* 299: 476-485, 2004.
- Li G, He L, Zhang E, Shi J, Zhang Q, Le AD, Zhou K and Tang X: Overexpression of human papillomavirus (HPV) type 16 oncoproteins promotes angiogenesis *via* enhancing HIF-1 $\alpha$  and VEGF expression in non-small cell lung cancer cells. *Cancer Lett* 311: 160-170, 2011.
- Skinner HD, Zheng JZ, Fang J, Agani F and Jiang BH: Vascular endothelial growth factor transcriptional activation is mediated by hypoxia-inducible factor 1 $\alpha$ , HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/AKT signaling. *J Biol Chem* 279: 45643-45651, 2004.
- Wang L, Gu F, Ma N, Zhang L, Bian JM and Cao HY: CIP2A expression is associated with altered expression of epithelial-mesenchymal transition markers and predictive of poor prognosis in pancreatic ductal adenocarcinoma. *Tumour Biol* 34: 2309-2313, 2013.
- Zhao D, Yang K, Tang XF, Lin NN and Liu JY: Expression of integrin-linked kinase in adenoid cystic carcinoma of salivary glands correlates with epithelial-mesenchymal transition markers and tumor progression. *Med Oncol* 30: 619, 2013.

29. Balanis N, Wendt MK, Schiemann BJ, Wang Z, Schiemann WP and Carlin CR: Epithelial to mesenchymal transition promotes breast cancer progression via a fibronectin-dependent STAT3 signaling pathway. *J Biol Chem* 288: 17954-17967, 2013.
30. Xing Y, Qi J, Deng S, Wang C, Zhang L and Chen J: Small interfering RNA targeting ILK inhibits metastasis in human tongue cancer cells through repression of epithelial-to-mesenchymal transition. *Exp Cell Res* 319: 2058-2072, 2013.
31. López-Novoa JM and Nieto MA: Inflammation and EMT: An alliance towards organ fibrosis and cancer progression. *EMBO Mol Med* 1: 303-314, 2009.
32. Pirozzi G, Tirino V, Camerlingo R, Franco R, La Rocca A, Liguori E, Martucci N, Paino F, Normanno N and Rocco G: Epithelial to mesenchymal transition by TGF $\beta$ -1 induction increases stemness characteristics in primary non small cell lung cancer cell line. *PLoS One* 6: e21548, 2011.
33. Kim H, Yoo SB, Sun P, Jin Y, Jheon S, Lee CT and Chung JH: Alteration of the E-cadherin/ $\beta$ -catenin complex is an independent poor prognostic factor in lung adenocarcinoma. *Korean J Pathol* 47: 44-51, 2013.
34. Zhan H, Liang H, Liu X, Deng J, Wang B and Hao X: Expression of Rac1, HIF-1 $\alpha$ , and VEGF in gastric carcinoma: Correlation with angiogenesis and prognosis. *Onkologie* 36: 102-107, 2013.
35. Simonetti O, Lucarini G, Rubini C, Goteri G, Zizzi A, Staibano S, Campanati A, Ganzetti G, Di Primio R and Offidani A: Microvessel density and VEGF, HIF-1 $\alpha$  expression in primary oral melanoma: Correlation with prognosis. *Oral Dis* 19: 620-627, 2013.
36. Kang FW, Gao Y, Que L, Sun J and Wang ZL: Hypoxia-inducible factor-1 $\alpha$  overexpression indicates poor clinical outcomes in tongue squamous cell carcinoma. *Exp Ther Med* 5: 112-118, 2013.
37. Zhang L, Huang G, Li X, Zhang Y, Jiang Y, Shen J, Liu J, Wang Q, Zhu J, Feng X, *et al*: Hypoxia induces epithelial-mesenchymal transition via activation of SNAIL by hypoxia-inducible factor -1 $\alpha$  in hepatocellular carcinoma. *BMC Cancer* 13: 108-117, 2013.
38. Ning Q, Liu C, Hou L, Meng M, Zhang X, Luo M, Shao S, Zuo X and Zhao X: Vascular endothelial growth factor receptor-1 activation promotes migration and invasion of breast cancer cells through epithelial-mesenchymal transition. *PLoS One* 8: e65217, 2013.
39. Semenza GL: Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene* 29: 625-634, 2010.
40. Jiang Y, Cukic B, Adjeroh DA, Skinner HD, Lin J, Shen QJ and Jiang BH: An algorithm for identifying novel targets of transcription factor families: Application to hypoxia-inducible factor 1 targets. *Cancer Inform* 7: 75-89, 2009.
41. Kaidi A, Qualtrough D, Williams AC and Paraskeva C: Direct transcriptional up-regulation of cyclooxygenase-2 by hypoxia-inducible factor (HIF)-1 promotes colorectal tumor cell survival and enhances HIF-1 transcriptional activity during hypoxia. *Cancer Res* 66: 6683-6691, 2006.
42. Obacz J, Pastorekova S, Vojtesek B and Hrstka R: Cross-talk between HIF and p53 as mediators of molecular responses to physiological and genotoxic stresses. *Mol Cancer* 12: 93, 2013.
43. Zhang E, Feng X, Liu F, Zhang P, Liang J and Tang X: Roles of PI3K/Akt and c-Jun signaling pathways in human papillomavirus type 16 oncoprotein-induced HIF-1 $\alpha$ , VEGF, and IL-8 expression and in vitro angiogenesis in non-small cell lung cancer cells. *PLoS One* 9: e103440, 2014.
44. Yu H, Zhang L and Liu P: CXCR7 signaling induced epithelial-mesenchymal transition by AKT and ERK pathways in epithelial ovarian carcinomas. *Tumour Biol*: Oct 31, 2014 (Epub ahead of print).
45. Wojtalla A and Arcaro A: Targeting phosphoinositide 3-kinase signalling in lung cancer. *Crit Rev Oncol Hematol* 80: 278-290, 2011.
46. Zhang J, Cao J, Ma S, *et al*: Tumor hypoxia enhances non-small cell lung cancer metastasis by selectively promoting macrophage M2 polarization through the activation of ERK signaling. *Oncotarget* Oct 5: 9664-9677, 2014.
47. Beck JT, Ismail A and Tolomeo C: Targeting the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway: An emerging treatment strategy for squamous cell lung carcinoma. *Cancer Treat Rev* 40: 980-989, 2014.
48. Stinchcombe TE and Johnson GL: MEK inhibition in non-small cell lung cancer. *Lung Cancer* 86: 121-125, 2014.