

Genistein decreases A549 cell viability via inhibition of the PI3K/AKT/HIF-1 α /VEGF and NF- κ B/COX-2 signaling pathways

JUAN ZHANG¹, HONGZHENG SU², QINGFENG LI¹, JING LI¹ and QIANFENG ZHAO¹

¹Department of Oncology, Xiangyang Central Hospital,

The Affiliated Hospital of Hubei College of Arts and Science, Xiangyang, Hubei 441021;

²Department of Infectious Disease, Zaoyang First People's Hospital, Zaoyang, Hubei 441200, P.R. China

Received November 12, 2015; Accepted November 24, 2016

DOI: 10.3892/mmr.2017.6260

Abstract. Genistein is an important chemopreventive agent against atherosclerosis and cancer. However, whether genistein is effective in the treatment of lung cancer, and its underlying mechanism, remains to be determined. The present study demonstrated that genistein treatment of A549 lung cancer cells decreased viability in a dose- and time-dependent manner, and induced apoptosis. Additionally, A549 cells exhibited significantly increased reactive oxygen species formation and cytochrome-c leakage, and activated caspase-3, B-cell lymphoma 2-associated X protein and apoptosis inducing factor expression levels, which are involved in the mitochondrial apoptosis pathway. Furthermore, the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT)/hypoxia-inducible factor-1 α (HIF-1 α) and nuclear factor- κ B (NF- κ B)/cyclooxygenase-2 (COX-2) signaling pathways were significantly downregulated by genistein treatment. In conclusion, reduced proliferation and increased apoptosis in A549 lung cancer cells was associated with inhibition of the PI3K/AKT/HIF-1 α / and NF- κ B/COX-2 signaling pathways, which implicates genistein as a potential chemotherapeutic agent for the treatment of lung cancer.

Introduction

Lung cancer, additionally known as bronchial cancer, is the leading cause of cancer-associated mortality in males, and is among the most common types of female malignancies (1,2). In China, approximately two-thirds of adult males are smokers, representing one-third of all smokers worldwide (3). Cigarette smoking and second-hand smoke inhalation are the

primary causes of lung cancer (4). For this reason, proven population-based tobacco prevention strategies used in the US should be implemented in China, to reduce the lung cancer incidence. Despite advances in surgical, radiotherapeutic and chemotherapeutic strategies, lung cancer is an aggressive and heterogeneous disease, and therefore the long-term survival rate remains low (5-7). Lung cancer treatment is complex for numerous reasons, including hard-to-detect early symptoms, early metastasis and vascular factors that mediate drug-resistance and disease progression (8-10). Previous studies have investigated inhibiting angiogenesis and antagonizing vascular endothelial growth factors as potential therapeutic targets for the treatment of lung cancer (11,12); however, the potential of this strategy remains unclear.

Genistein, a natural phytoestrogen found in soy, has demonstrated the potential to inhibit numerous types of cancer, including breast, pancreatic and colorectal cancers (13-15). Previous studies have indicated that this effect may be due to its ability to induce cancer cell apoptosis, arrest the cell cycle and inactivate critical signaling pathways in human cancer cells (16,17). However, the underlying mechanisms of genistein, and its potential therapeutic effects in lung cancer, remain to be determined. The present study therefore investigated the anti-tumor effects of genistein on the A549 lung cancer cell line and its underlying molecular mechanisms.

Materials and methods

Cell culture. A549 human lung carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA), maintained in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal calf serum (HyClone; GE Healthcare Life Sciences), 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in 5% CO₂ and passaged twice a week. Cells were cultured at a density of 5 \times 10³ cells/well in 6-well culture plates for 24 h. Following this, A549 cells were transferred to serum-free RPMI-1640 medium for overnight serum starvation prior to each experiment.

Assessment of cell viability. The cytotoxicity of the genistein was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described

Correspondence to: Dr Qingfeng Li, Department of Oncology, Xiangyang Central Hospital, The Affiliated Hospital of Hubei College of Arts and Science, 136 Jinzhou Road, Xiangyang, Hubei 441021, P.R. China
E-mail: ely_28467@163.com

Key words: genistein, apoptosis, nuclear factor- κ B, protein kinase B, A549 cells

by Mossman (18). Cells were seeded at 1×10^5 cells/ml in 96-well plates and treated with 0–200 $\mu\text{mol/l}$ genistein for 24, 48 and 72 h. Following incubation, 10 μl 5 mg/ml MTT dye (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was added to the cells for 4 h followed by incubation with dimethyl sulfoxide for 10 min. The absorbance at a wavelength of 570 nm was measured using a microplate reader (Asys UVM340; Bichrom, Ltd., Cambridge, UK). Cell viability was determined as the ratio of the signal obtained from treated and control cultures.

Analysis of apoptosis. Cells were cultured and harvested by trypsinization, then washed twice with cold PBS and centrifuged at room temperature for 8 min, at $800 \times g$. About 1×10^5 – 1×10^6 cells were then resuspended in 300 μl 1X binding buffer and centrifuged again at 1,000 rpm for 5 min. Cells were resuspended in 300 μl 1X binding buffer and transferred to a sterile flow cytometry glass tube. A total of 10 μl Annexin V-FITC Annexin was added and incubated in a dark at room temperature for 30 min. Then cells were incubated in the dark with 5 μl propidium iodide. Cells were analyzed by a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA). Cellular apoptosis was determined using the Annexin V-FITC Apoptosis Detection kit I (Clontech Laboratories Inc., Mountainview, CA, USA).

Detection of intracellular reactive oxygen species (ROS). ROS detection was performed using the ROS assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. A549 cells were treated with 0, 25, 50 and 100 $\mu\text{mol/l}$ genistein for 24, 48 and 72 h. Following this, 5×10^6 cells were incubated with 10 $\mu\text{mol/l}$ 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA; Beyotime Institute of Biotechnology) at 37°C for 30 min, and washed three times with PBS to remove the residual dye. DCF-DA fluorescence was detected using a flow cytometer (BD Biosciences) and the results were analyzed using Quantity One software version 4.62 (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Western blot analysis. Cells were centrifuged at $125 \times g$ for 10 min at 4°C and washed twice with ice-cold PBS. They were subsequently lysed with Triton X-100 in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Sigma-Aldrich; Merck Millipore). Lysed cells were sonicated and centrifuged for 5 min at room temperature, at a speed of $6,000 \times g$. The total protein concentration measurement was performed using the Bradford method. Total protein (50 μg) was loaded onto gels and separated by 10% SDS-PAGE. The proteins were subsequently transferred onto a polyvinylidene difluoride membrane using a Bio-Rad apparatus (Bio-Rad Laboratories, Inc.) for 2 h at 4°C and 100 V. Following this, membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature. They were subsequently incubated at 4°C overnight with the following primary antibodies: Mouse monoclonal anti-B-cell lymphoma 2-associated X protein (Bax; 1:400; catalog no. sc-20067; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); mouse monoclonal anti-apoptosis inducing factor (AIF; 1:400; catalog no. sc-13116; Santa Cruz Biotechnology, Inc.); mouse monoclonal anti-cytochrome c

(cyto-c; 1:400; catalog no. sc-13561; Santa Cruz Biotechnology, Inc.); rabbit monoclonal anti-caspase-3 (1:400; catalog no. 9664; Cell Signaling Technology, Inc., Danvers, MA, USA); mouse monoclonal anti-total (t)-protein kinase B (AKT; 1:400; catalog no. sc-377457; Santa Cruz Biotechnology, Inc.); rabbit polyclonal anti-phosphorylated (p)-AKT (1:400; catalog no. sc-135650; Santa Cruz Biotechnology, Inc.); mouse monoclonal anti-hypoxia-inducible factor-1 α (HIF-1 α ; 1:400; catalog no. sc-53546; Santa Cruz Biotechnology, Inc.) and mouse monoclonal anti- β -actin (1:400; catalog no. sc-47778; Santa Cruz Biotechnology, Inc.). Following this, the membranes were incubated with horseradish peroxidase-conjugated polyclonal goat anti-mouse (catalog no. sc-2005) and goat anti-rabbit (catalog no. sc-2004) IgG secondary antibodies (1:5,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature and the resulting protein bands were visualized using an Enhanced Chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Densitometry was performed using the Quantity One software version 4.62 (Bio-Rad Laboratories, Inc.).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNase-free DNase I was used in order to eliminate genomic DNA contamination in the RNA samples. The 260/280 absorbance ratio was measured for verification of the purity of RNA. The sequences of the nuclear factor- κB (NF- κB), cyclooxygenase (COX-2) and GAPDH genes were obtained from the GenBank database and specific primers were designed using Primer Premier software version 5.0 (Premier Biosoft International, Palo Alto, CA, USA). The primer sequences were as follows: Forward, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' and reverse, 5'-CGGAGTCAACGGATTGTCGTAT-3', for GAPDH; forward, 5'-CTGAACCAGGGCATACTGT-3' and reverse, 5'-GAGAAGTCCATGTCCGCAAT-3' for NF- κB ; and forward, 5'-TGAAACCCACTCCAAACACA-3' and reverse, 5'-TGGAACAACCTGCTCATCACC-3' for COX-2. PCR reactions were performed with PrimeScript[™] RT-PCR kit RR014A (Takara Bio, Inc, Otsu, Japan), using a GeneAmp[®] PCR system 9700 (PerkinElmer, Inc., Waltham, MA, USA) and amplified. The reaction conditions were as follows: 94°C for 4 min; 94°C for 40 sec, 50°C for 45 sec, and 72°C for 45 sec, for 35 cycles; and followed by extension at 72°C for 10 min before ending.

The amplified products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. IPLab software, version no: 2.8 (Scanalytics, Fairfax, VA, USA) was used for densitometry and image density was quantified using a FluoroImager[™] SI scanner (GE Healthcare Life Sciences, Chalfont, UK).

Statistical analysis. Data are expressed as the mean \pm standard error. The significance of differences between groups was assessed by one-way analysis of variance. Data was analyzed using SPSS software version 18 (SPSS, Inc., Chicago, IL, USA). Individual comparisons were subsequently performed using the Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of genistein on the apoptosis of A549 cells. Cell viability assays were performed to assess the inhibition of cell growth by genistein. A549 cells were treated with various doses of genistein (0-200 $\mu\text{mol/l}$) for 24, 48 and 72 h. Cell viability assays revealed that genistein inhibited cell growth in a dose- and time-dependent manner (Fig. 1A). Furthermore, genistein induced apoptosis most effectively at a concentration of 200 $\mu\text{mol/l}$, however, concentrations that induced a reversible level of apoptosis were selected for experimentation.

A549 cells incubated with 50 $\mu\text{mol/l}$ genistein for 48 h lost their original morphological shape and additional floating cells appeared, as observed by the CX22 microscope (Olympus Corporation, Tokyo, Japan; Fig. 1B; $P<0.01$). To examine genistein-induced apoptosis, western blotting was performed to detect the expression levels of the apoptosis-associated proteins AIF, cyto-*c*, Bax and caspase-3. As presented in Fig. 1C and D, genistein markedly upregulated the protein expression levels of cleaved caspase-3, Bax, cyto-*c* and AIF in A549 cells ($P<0.01$). These data indicate that genistein inhibited cell viability via the induction of apoptosis in A549 cells.

Effects of genistein on intracellular ROS production in A549 cells. Apoptosis is, at least partially, mediated by oxidative stress (19). Thus, intracellular ROS levels in A549 cells were examined by DCF-DA staining. A549 cells were treated with 0, 25, 50 and 100 $\mu\text{mol/l}$ genistein for 48 h, and intracellular ROS levels were detected by flow cytometry. As presented in Fig. 2, these results demonstrated that ROS production increased following genistein treatment in a dose-dependent manner ($P<0.01$).

Effects of genistein on the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT and HIF-1 α signaling pathways in A549 cells. The PI3K/AKT and HIF-1 α signaling pathways have been demonstrated to be involved in cell viability and tumor angiogenesis, and mediate cell survival (20,21). To investigate the underlying molecular mechanisms by which genistein exerts its apoptotic effects, the PI3K/AKT and HIF-1 α signaling pathways were examined. A549 cells were treated with 0 or 50 $\mu\text{mol/l}$ genistein for 48 h and apoptosis was determined by flow cytometry. Flow cytometry assays revealed a marked increase in apoptosis in cells treated with genistein ($P<0.01$; Fig. 3A). Additionally, p-AKT, t-AKT and HIF-1 α protein expression levels were determined by western blot analysis (Fig. 3B). Following exposure to 50 $\mu\text{mol/l}$ genistein for 48 h, p-AKT and HIF-1 α protein expression levels decreased compared with the control ($P<0.01$), while t-AKT levels were not significantly altered. These results suggested that genistein induced cell apoptosis by suppressing the PI3K/AKT and HIF-1 α signaling pathways.

Effects of genistein on the NF- κB /COX-2 signaling pathway in A549 cells. The NF- κB and COX-2 signaling pathways serve important roles in cancer cell growth, tumor angiogenesis and invasion (22,23). To investigate the underlying molecular mechanisms by which genistein contributes to these malignant features, the present study examined the effect of genistein on the NF- κB and COX-2 signaling pathways. A549 cells

were treated with 0 or 50 $\mu\text{mol/l}$ genistein for 48 h, and the mRNA expression levels of NF- κB and COX-2 were detected by RT-PCR. As presented in Fig. 4, treatment with genistein decreased the mRNA expression levels of NF- κB and COX-2 in A549 cells, compared with the control ($P<0.01$). Therefore, this indicates that genistein-induced A549 cell apoptosis was partly dependent on the inhibition of the NF- κB /COX-2 signaling pathways.

Discussion

Lung cancer is the most common type of malignant tumor, yet effective treatments remain to be developed. Intravenous chemotherapy supplemented with chest radiation is the most common method of treatment, with surgery only rarely performed (24). However, genetic mutations in cancer cells and multidrug resistance, in addition to insensitivity to radiotherapy, often result in poor outcomes (25).

The lungs are supplied with blood by the systemic and pulmonary circulations. The blood supply of lung cancer is primarily provided by the bronchial artery; however, whether the pulmonary blood supply is involved remains to be determined. A previous study addressed this issue using clinical pulmonary angiography, and indicated that the pulmonary artery does not supply lung cancer with blood (26). However, the dual blood supply of non-small cell lung cancer may depend on tumor size and histological subtype (27). A previous study demonstrated that the blood supply of the pulmonary artery serves important roles in the nourishment, development, metastasis, prognosis and angiogenesis of lung cancer (28). Furthermore, a previous tumor study suggested that tumor cell proliferation and angiogenesis are closely associated (29). Growth factors, including vascular endothelial, fibroblast, transforming and platelet-derived growth factors, have been demonstrated to induce tumor angiogenesis (30).

Previous reports have indicated that genistein, a naturally occurring isoflavonoid, possesses anticancer properties (13-17). However, the underlying mechanisms of inhibition remain unclear. The present study demonstrated that genistein decreased A549 cell viability in a dose- and time-dependent manner, and induced apoptosis. Treatment of A549 cells with genistein significantly increased ROS formation, activation of caspase-3, cyto-*c* leakage and protein expression levels of Bax and AIF, which are involved in the mitochondrial apoptosis pathway. Additionally, the current study investigated the underlying mechanisms of the anti-cancer effects of genistein. Previous studies have demonstrated that the PI3K/AKT and HIF-1 α signaling pathways may regulate critical steps in apoptosis and cancer cell survival (29,31). Therefore, activation of these pathways may mediate angiogenesis, resulting in accelerated cancer cell growth. Genistein was demonstrated to significantly inhibit cell apoptosis via downregulation of the PI3K/AKT and HIF-1 α signaling pathways. Furthermore, due to the importance of the NF- κB and COX-2 signaling pathways in apoptosis (32,33), the present study hypothesized that genistein may inhibit these pathways in A549 cells and antagonize apoptosis. These results revealed that genistein significantly inhibited apoptosis via suppressing the NF- κB and COX-2 signaling pathways in lung cancer cells.

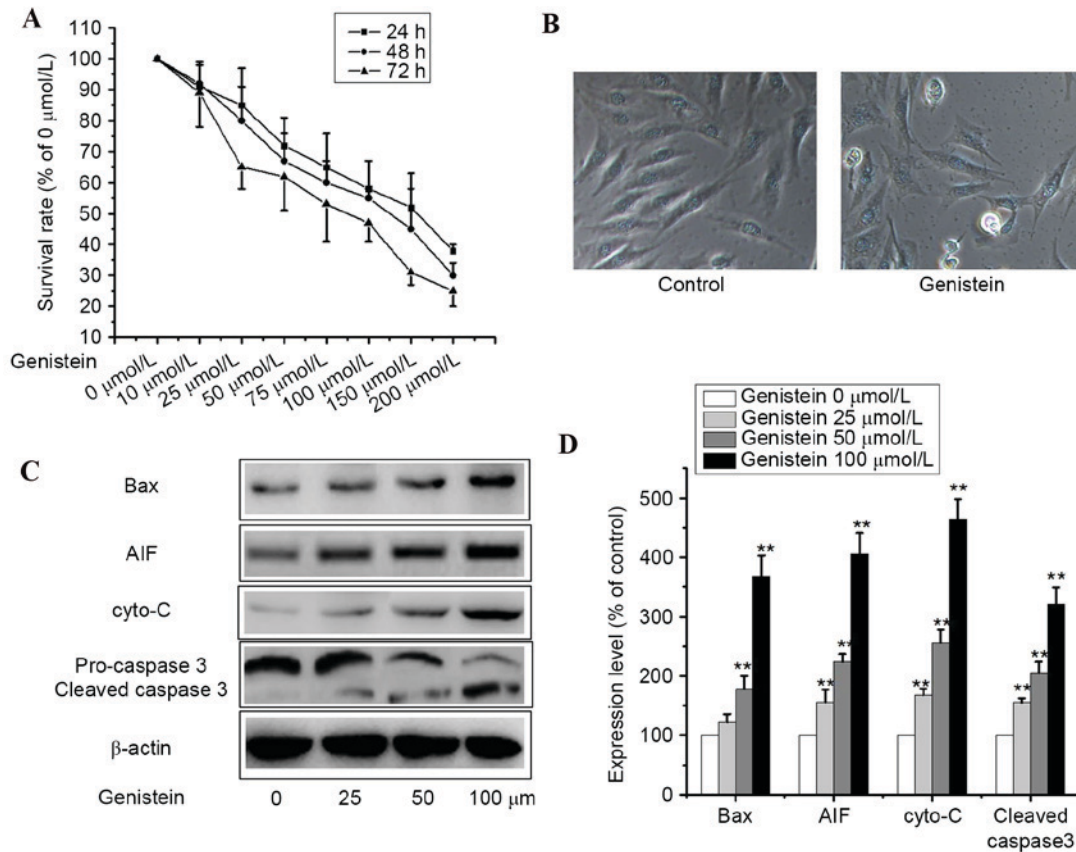


Figure 1. Genistein induces A549 cell apoptosis. (A) A549 cells were treated with 0-200 μmol/l genistein for 24, 48 and 72 h and cell viability was analyzed by MTT assay. (B) Effect of genistein on cell morphology 48 h post-treatment with 0 (control) or 50 μmol/l genistein. A549 cells were treated with 0, 25, 50 and 100 μmol/l genistein for 48 h and the protein expression levels of Bax, AIF, cyto-*c* and pro- and cleaved caspase-3 were detected by (C) western blotting, with β-actin serving as a loading control. (D) Quantification of western blotting revealed that treatment with genistein significantly increased expression levels of all proteins measured. Data are presented as the mean ± standard error (n=3). **P<0.01 vs. 0 μmol/l genistein. Bax, B-cell lymphoma 2-associated X protein; AIF, apoptosis inducing factor; cyto-*c*, cytochrome *c*.

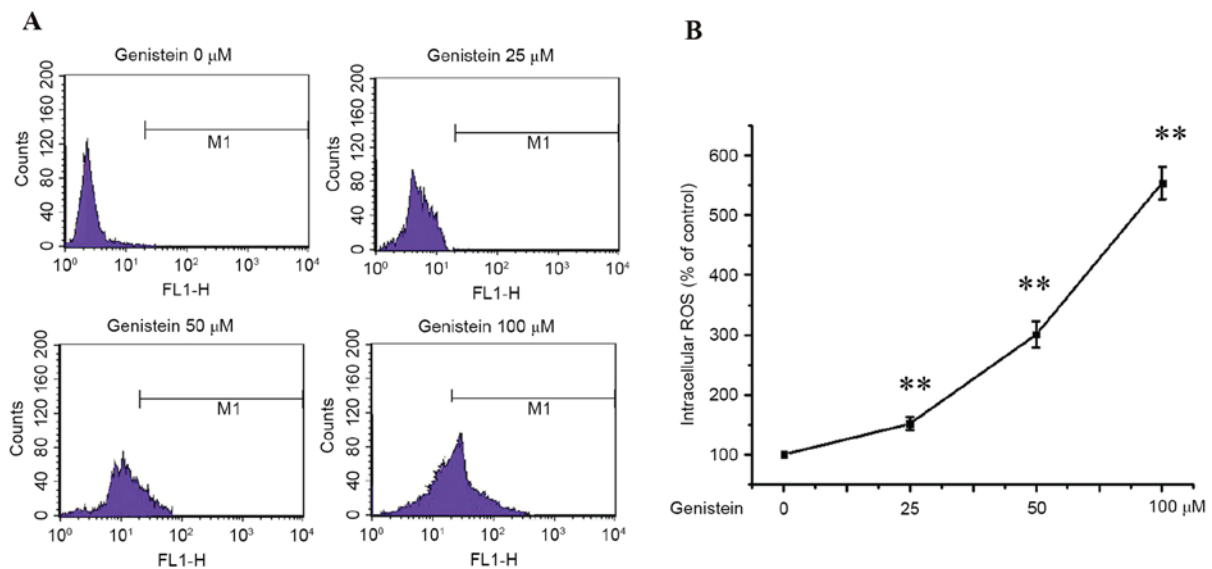


Figure 2. Effect of genistein on ROS production in A549 cells. (A) A549 cells were treated with 0, 25, 50 and 100 μmol/l genistein for 48 h and stained with 2',7'-dichlorodihydrofluorescein diacetate to detect intracellular ROS production. (B) Treatment with genistein increased intracellular ROS levels in a dose-dependent manner. Data are presented as the mean ± standard error (n=3). **P<0.01 vs. control. ROS, reactive oxygen species.

The mitogen-activated protein kinase (MAPK) signaling pathway serves important roles in tumorigenesis, cell growth,

differentiation, proliferation, apoptosis, migration and angiogenesis. It has been demonstrated in previous studies that the

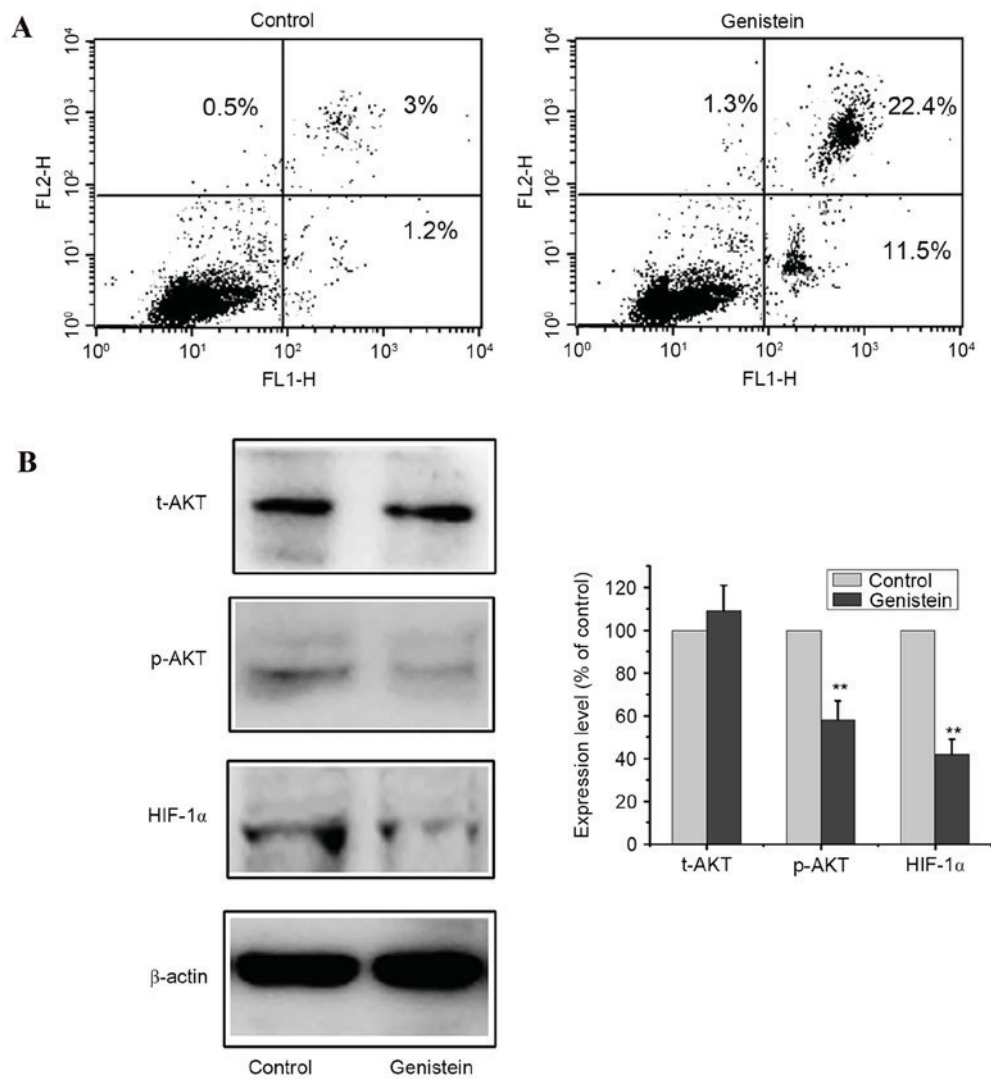


Figure 3. Effect of Genistein on the phosphatidylinositol-4,5-biphosphate 3-kinase/AKT and HIF-1 α signaling pathways. A549 cells were treated with 0 (control) or 50 μ mol/l genistein for 48 h. (A) Apoptosis was determined by flow cytometry followed by annexin V-propidium iodide double staining. (B) The protein expression levels of t-AKT, p-AKT and HIF-1 α were detected by western blotting. Data are presented as the mean \pm standard error (n=3). **P<0.01 vs. control. p, phosphorylated; AKT, protein kinase B; HIF-1 α , hypoxia-inducible factor-1 α ; t, total.

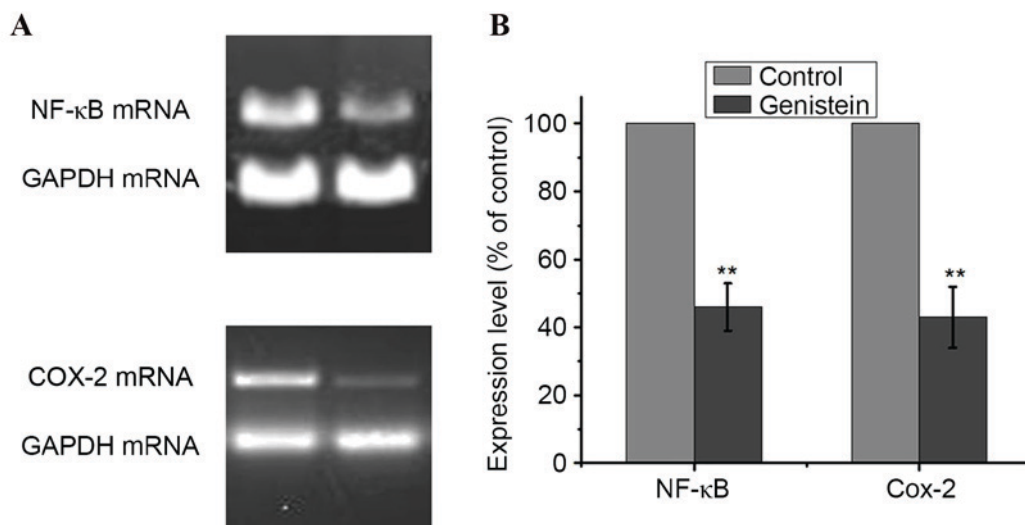


Figure 4. Effect of genistein on the NF- κ B/COX-2 signaling pathways. (A) A549 cells were treated with 0 (control) or 50 μ mol/l genistein for 48 h and mRNA expression levels of NF- κ B and COX-2 were detected by reverse transcription-polymerase chain reaction. (B) mRNA expression levels of NF- κ B and COX-2 were significantly decreased following genistein treatment, compared with the control group. Data are presented as the mean \pm standard error (n=3). **P<0.01 vs. control. NF- κ B, nuclear factor- κ B; COX-2, cyclooxygenase-2.

MAPK signaling pathway is overactive in cancer, and that its activation is associated with angiogenesis (34). Additionally, previous studies have revealed that genistein has effects on the MAPK signaling pathway (35,36). The MAPK and PI3K/AKT signaling pathways are important for cell membrane receptor signal transduction, which regulates apoptosis, cell growth and the expression of numerous genes. Computer simulations have demonstrated that interactions between the two pathways are context-dependent, and that they may activate or inhibit each other (37). Typically, NF- κ B and COX-2 appear as downstream pathways (38). The interaction of these pathways suggests that the effects of genistein on signal transduction requires further investigation.

In conclusion, the present study demonstrated that genistein induced apoptosis in A549 cells. Genistein appeared to exert its pro-apoptotic effects via inhibition of the PI3K/AKT/HIF-1 α and NF- κ B/COX-2 signaling pathways. This implicates genistein as a potential chemopreventive agent for the treatment of lung cancer. Therefore, future clinical studies investigating the long-term benefits of genistein are required, in addition to investigation into the mechanism of action of genistein and *in vitro* animal studies.

Acknowledgements

The present study was supported by the Xiangyang Science and Technology Bureau (grant no. 20136811).

References

- Henley SJ, Richards TB, Underwood JM, Ehemann CR, Plescia M and McAfee TA: Centers for Disease Control and Prevention (CDC): Lung cancer incidence trends among men and women-United States, 2005-2009. *MMWR Morb Mortal Wkly Rep* 63: 1-5, 2014.
- Chen W, Zheng R, Zeng H and Zhang S: The updated incidences and mortalities of major cancers in China, 2011. *Chin J Cancer* 34: 502-207, 2015.
- Guo H and Sa Z: Socioeconomic differentials in smoking duration among adult male smokers in China: Result from the 2006 China Health and Nutrition Survey. *PLoS One* 10: e0117354, 2015.
- Lubin JH, Caporaso N, Wichmann HE, Schaffrath-Rosario A and Alavanja MC: Cigarette smoking and lung cancer: Modeling effect modification of total exposure and intensity. *Epidemiology* 18: 639-648, 2007.
- Stinchcombe TE: Recent advances in the treatment of non-small cell and small cell lung cancer. *F1000Prime Rep* 6: 117, 2014.
- Hensing T, Chawla A, Batra R and Salgia R: A personalized treatment for lung cancer: Molecular pathways, targeted therapies, and genomic characterization. *Adv Exp Med Biol* 799: 85-117, 2014.
- Molina JR, Yang P, Cassivi SD, Schild SE and Adjei AA: Non-small cell lung cancer: Epidemiology, risk factors, treatment, and survivorship. *Mayo Clin Proc* 83: 584-594, 2008.
- Izbicki JR, Passlick B, Hosch SB, Kubuschock B, Schneider C, Busch C, Knoefel WT, Thetter O and Pantel K: Mode of spread in the early phase of lymphatic metastasis in non-small-cell lung cancer: Significance of nodal micrometastasis. *J Thorac Cardiovasc Surg* 112: 623-630, 1996.
- Guldbrandt LM: The effect of direct referral for fast CT scan in early lung cancer detection in general practice. A clinical, cluster-randomised trial. *Dan Med J* 61: B5027, 2015.
- Shen H, Feng G, Cui J, Du Q, Qin Y, Cai J, Shen L and Zhu Y: Clinical implications of serum hypoxia inducible factor-1 α and vascular endothelial growth factor in lung cancer. *Tumori* 101: 404-411, 2015.
- Nakade J, Takeuchi S, Nakagawa T, Ishikawa D, Sano T, Nanjo S, Yamada T, Ebi H, Zhao L, Yasumoto K, *et al*: Triple inhibition of EGFR, Met, and VEGF suppresses regrowth of HGF-triggered, erlotinib-resistant lung cancer harboring an EGFR mutation. *J Thorac Oncol* 9: 775-783, 2014.
- Fu L, Chen W, Guo W, Wang J, Tian Y, Shi D, Zhang X, Qiu H, Xiao X, Kang T, *et al*: Berberine targets AP-2/hTERT, NF- κ B/COX-2, HIF-1 α /VEGF and cytochrome-c/caspase signaling to suppress human cancer cell growth. *PLoS One* 8: e69240, 2013.
- Pons DG, Nadal-Serrano M, Blanquer-Rossello MM, Sastre-Serra J, Oliver J and Roca P: Genistein modulates proliferation and mitochondrial functionality in breast cancer cells depending on ERalpha/ERbeta ratio. *J Cell Biochem* 115: 949-958, 2014.
- Pavese JM, Krishna SN and Bergan RC: Genistein inhibits human prostate cancer cell detachment, invasion, and metastasis. *Am J Clin Nutr* 100: 431S-436S, 2014.
- Xiao X, Liu Z, Wang R, Wang J, Zhang S, Cai X, Wu K, Bergan RC, Xu L and Fan D: Genistein suppresses FLT4 and inhibits human colorectal cancer metastasis. *Oncotarget* 6: 3225-3239, 2015.
- Kim IG, Kim JS, Lee JH and Cho EW: Genistein decreases cellular redox potential, partially suppresses cell growth in HL-60 leukemia cells and sensitizes cells to γ -radiation-induced cell death. *Mol Med Rep* 10: 2786-2792, 2014.
- Prietsch RF, Monte LG, da Silva FA, Beira FT, Del Pino FA, Campos VF, Collares T, Pinto LS, Spanevello RM, Gamaro GD and Braganhol E: Genistein induces apoptosis and autophagy in human breast MCF-7 cells by modulating the expression of proapoptotic factors and oxidative stress enzymes. *Mol Cell Biochem* 390: 235-242, 2014.
- Mosmann T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55-63, 1983.
- Kannan K and Jain SK: Oxidative stress and apoptosis. *Pathophysiology* 7: 153-163, 2000.
- Joshi S, Singh AR, Zulcic M and Durden DL: A macrophage-dominant PI3K isoform controls hypoxia-induced HIF1 α and HIF2 α stability and tumor growth, angiogenesis, and metastasis. *Mol Cancer Res* 12: 1520-1531, 2014.
- Lee H, Jung KH, Jeong Y, Hong S and Hong SS: HS-173, a novel phosphatidylinositol 3-kinase (PI3K) inhibitor, has anti-tumor activity through promoting apoptosis and inhibiting angiogenesis. *Cancer Lett* 328: 152-159, 2013.
- Yang M, Zou J, Zhu H, Liu S, Wang H, Bai P and Xiao X: Paris saponin II inhibits human ovarian cancer cell-induced angiogenesis by modulating NF- κ B signaling. *Oncol Rep* 33: 2190-2198, 2015.
- Ma JX, Sun YL, Wang YQ, Wu HY, Jin J and Yu XF: Triptolide induces apoptosis and inhibits the growth and angiogenesis of human pancreatic cancer cells by downregulating COX-2 and VEGF. *Oncol Res* 20: 359-368, 2013.
- Maguire J, Khan I, McMenamin R, O'Rourke N, McNee S, Kelly V, Peedell C and Snee M: SOCCAR: A randomised phase II trial comparing sequential versus concurrent chemotherapy and radical hypofractionated radiotherapy in patients with inoperable stage III non-small cell lung cancer and good performance status. *Eur J Cancer* 50: 2939-2949, 2014.
- Sun FF, Hu YH, Xiong LP, Tu XY, Zhao JH, Chen SS, Song J and Ye XQ: Enhanced expression of stem cell markers and drug resistance in sphere-forming non-small cell lung cancer cells. *Int J Clin Exp Pathol* 8: 6287-6300, 2015.
- Xiao XS, Yu H, Li HM, Liu SY, Li CZ and Liu J: Impact of multi-layer spiral CT angiography of bronchial artery and pulmonary artery in assessment of the main blood supply to the primary lung cancer. *Zhonghua Zhong Liu Za Zhi* 28: 302-305, 2006 (In Chinese).
- Nguyen-Kim TD, Frauenfelder T, Strobel K, Veit-Haibach P and Huellner MW: Assessment of bronchial and pulmonary blood supply in non-small cell lung cancer subtypes using computed tomography perfusion. *Invest Radiol* 50: 179-186, 2015.
- Yang X, Zhang Y, Hosaka K, Andersson P, Wang J, Tholander F, Cao Z, Morikawa H, Tegner J, Yang Y, *et al*: VEGF-B promotes cancer metastasis through a VEGF-A-independent mechanism and serves as a marker of poor prognosis for cancer patients. *Proc Natl Acad Sci USA* 112: E2900-E2909, 2015.
- Yao L, Nie X, Shi S, Song S, Hao X, Li S and Zhu D: Reciprocal regulation of HIF-1 α and 15-LO/15-HETE promotes anti-apoptosis process in pulmonary artery smooth muscle cells during hypoxia. *Prostaglandins Other Lipid Mediat* 99: 96-106, 2012.
- Raica M, Mogoantă L, Kondylis A and Cîmpean AM: Angiogenesis in the human thymoma assessed by subclassification of tumor-associated blood vessels and endothelial cells proliferation. *Rom J Morphol Embryol* 51: 627-631, 2010.

31. Zhang HB, Lu P, Guo QY, Zhang ZH and Meng XY: Baicalein induces apoptosis in esophageal squamous cell carcinoma cells through modulation of the PI3K/Akt pathway. *Oncol Lett* 5: 722-728, 2013.
32. Mohankumar K, Sridharan S, Pajaniradje S, Singh VK, Ronsard L, Banerjee AC, Somasundaram DB, Coumar MS, Periyasamy L and Rajagopalan R: BDMC-A, an analog of curcumin, inhibits markers of invasion, angiogenesis, and metastasis in breast cancer cells via NF- κ B pathway-A comparative study with curcumin. *Biomed Pharmacother* 74: 178-186, 2015.
33. Mena MP, Papiewska-Pajak I, Przygodzka P, Kozaczuk A, Boncela J and Cierniewski CS: NFAT2 regulates COX-2 expression and modulates the integrin repertoire in endothelial cells at the crossroads of angiogenesis and inflammation. *Exp Cell Res* 324: 124-136, 2014.
34. Gao JH, Wang CH, Tong H, Wen SL, Huang ZY and Tang CW: Targeting inhibition of extracellular signal-regulated kinase kinase pathway with AZD6244 (ARRY-142886) suppresses growth and angiogenesis of gastric cancer. *Sci Rep* 16: 16382, 2015.
35. Jin CY, Park C, Kim GY, Lee SJ, Kim WJ and Choi YH: Genistein enhances TRAIL-induced apoptosis through inhibition of p38 MAPK signaling in human hepatocellular carcinoma Hep3B cells. *Chem Biol Interact* 180: 143-150, 2009.
36. Huang X, Chen S, Xu L, Liu Y, Deb DK, Platanias LC and Bergan RC: Genistein inhibits p38 map kinase activation, matrix metalloproteinase type 2, and cell invasion in human prostate epithelial cells. *Cancer Res* 65: 3470-3478, 2005.
37. Aksamitiene E, Kiyatkin A and Kholodenko BN: Cross-talk between mitogenic Ras/MAPK and survival PI3K/Akt pathways: A fine balance. *Biochem Soc Trans* 40: 139-146, 2012.
38. N'Guessan PD, Hippenstiel S, Etouem MO, Zahlten J, Beermann W, Lindner D, Opitz B, Witzernath M, Rosseau S, Suttrop N and Schmeck B: Streptococcus pneumoniae induced p38 MAPK- and NF-kappaB-dependent COX-2 expression in human lung epithelium. *Am J Physiol Lung Cell Mol Physiol* 290: L1131-L1138, 2006.