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

JUAN ZHANG[1], HONGZHENG SU[2], QINGFENG LI[1], JING LI[1] and QIANFENG ZHAO[1]

[1]Department of Oncology, Xiangyang Central Hospital,
The Affiliated Hospital of Hubei College of Arts and Science, Xiangyang, Hubei 441021;
[2]Department of Infectious Disease, Zaoyang First People's Hospital, Zaoyang, Hubei 441200, P.R. China

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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-biphosphate 3-kinase (PI3K)/protein kinase B (AKT)/hypoxia-inducible factor-1α (HIF-1α) and nuclear factor-κB (NF-κB)/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-1α/ and NF-κB/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 μg/ml streptomycin at 37˚C in 5% CO_2 and passaged twice a week. Cells were cultured at a density of $5x10^3$ 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

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by Mossman (18). Cells were seeded at $1x10^5$ cells/ml in 96-well plates and treated with 0-200 μmol/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 x *g*. About $1x10^5$-$1x10^6$ 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 μmol/l genistein for 24, 48 and 72 h. Following this, $5x10^6$ cells were incubated with 10 μmol/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 x *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 x *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-1α (HIF-1α; 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'-CGGAGTCAACGGATTTGGTCGTAT-3', for GAPDH; forward, 5'-CTGAACCAGGGCATACCTGT-3' and reverse, 5'-GAGAAGTCCATGTCCGCAAT-3' for NF-κB; and forward, 5'-TGAAACCCACTCCAAACACA-3' and reverse, 5'-TGGAACAACTGCTCATCACC-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 sued 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 ± 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 μmol/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 μl mol/l, however, concentrations that induced a reversible level of apoptosis were selected for experimentation.

A549 cells incubated with 50 μmol/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 μmol/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-biphosphate 3-kinase (PI3K)/AKT and HIF-1α signaling pathways in A549 cells. The PI3K/AKT and HIF-1α 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-1α signaling pathways were examined. A549 cells were treated with 0 or 50 μmol/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-1α protein expression levels were determined by western blot analysis (Fig. 3B). Following exposure to 50 μmol/l genistein for 48 h, p-AKT and HIF-1α 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-1α 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 μmol/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-1α 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-1α 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.

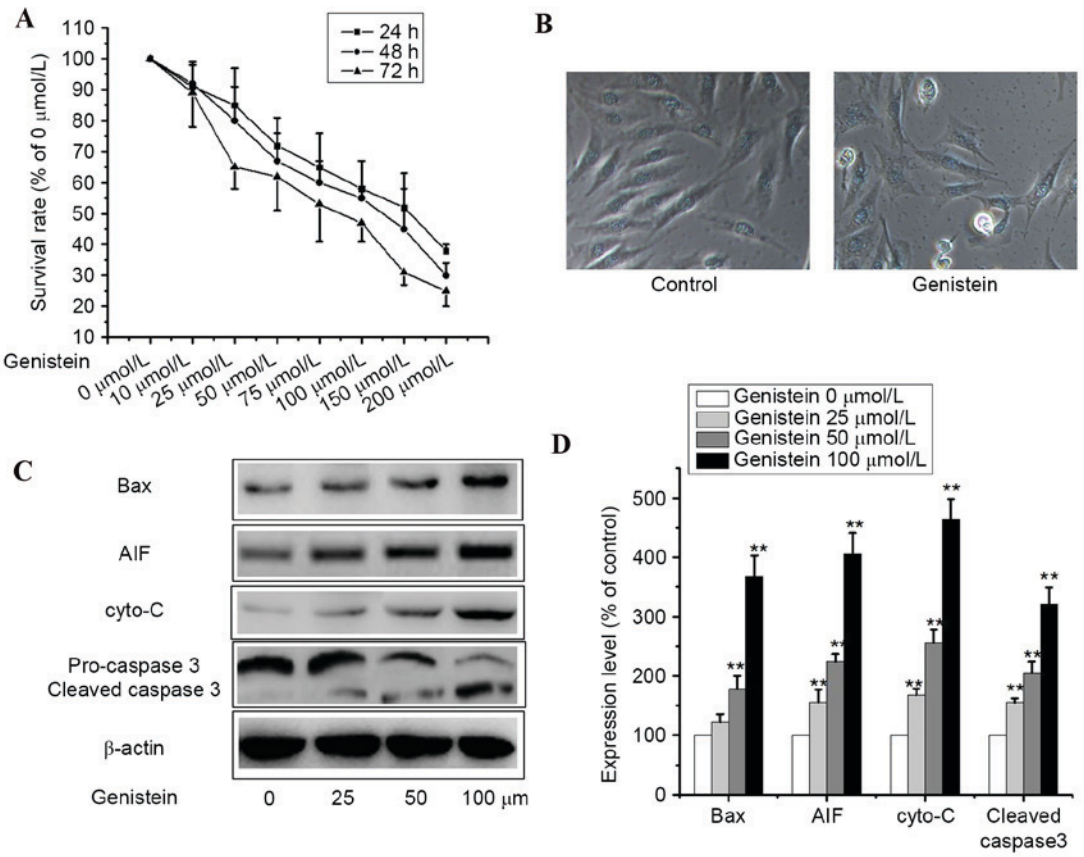

Figure 1. Genistein induces A549 cell apoptosis. (A) A549 cells were treated with 0-200 μmol/l genistein for 24, 48 and 72 h and cell viability was analyzed by MTT assay. (B) Effect of genistein on cell morphology 48 h post-treatment with 0 (control) or 50 μmol/l genistein. A549 cells were treated with 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*.

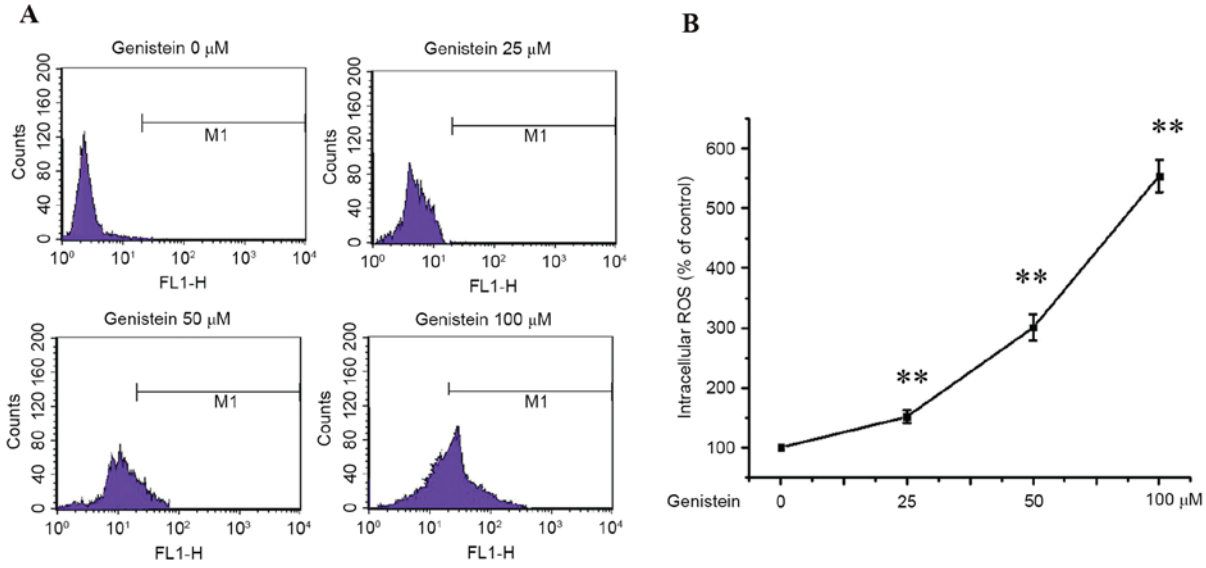

Figure 2. Effect of genistein on ROS production in A549 cells. (A) A549 cells were treated with 0, 25, 50 and 100 μmol/l genistein for 48 h and stained with 2',7'-dichlorodihydrofluorescein diacetate to detect intracellular ROS production. (B) Treatment with genistein increased intracellular ROS levels 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

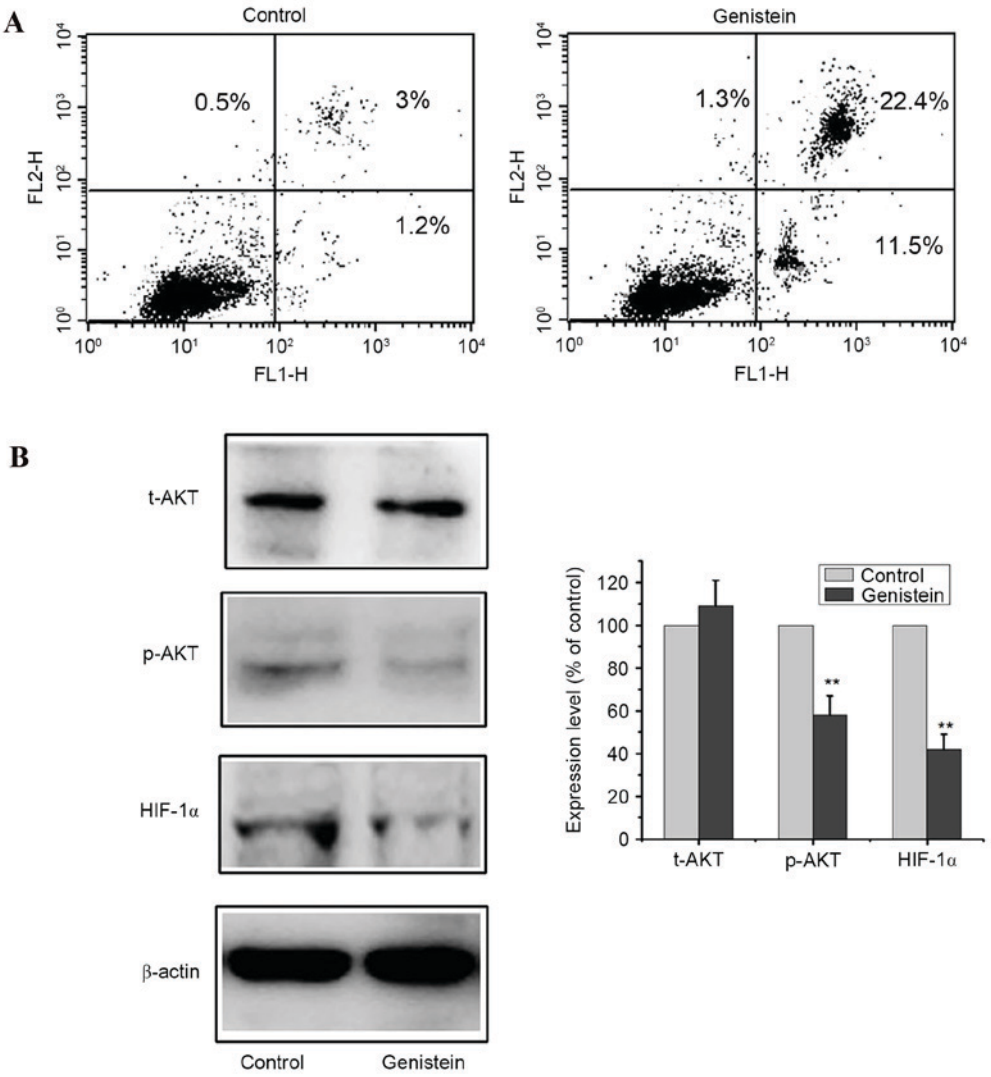

Figure 3. Effect of Genistein on the phosphatidylinositol-4,5-biphosphate 3-kinase/AKT and HIF-1α signaling pathways. A549 cells were treated with 0 (control) or 50 μmol/l genistein for 48 h. (A) Apoptosis was determined by flow cytometry followed by annexin V-propidium iodide double staining. (B) The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

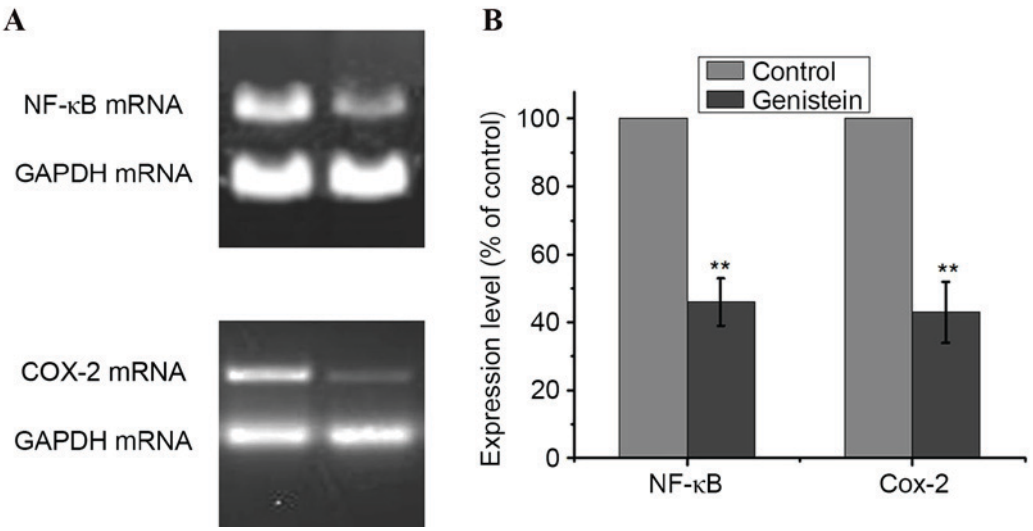

Figure 4. Effect of genistein on the NF-κB/COX-2 signaling pathways. (A) A549 cells were treated with 0 (control) or 50 μmol/l genistein for 48 h and mRNA expression levels of NF-κB and COX-2 were detected by reverse transcription-polymerase chain reaction. (B) mRNA expression levels of NF-κB and COX-2 were significantly decreased following genistein treatment, compared with the control group. Data are presented as the mean ± standard error (n=3). **P<0.01 vs. control. NF-κB, nuclear factor-κB; 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-κB 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-1α and NF-κB/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.

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