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ORIGINAL ARTICLE

Effect of genistein on apoptosis of lung adenocarcinoma A549 cells and expression of apoptosis factors

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Summary

Purpose: *To investigate the effect of genistein (GEN) on the apoptosis of lung adenocarcinoma cells and the expression of Bcl-2 and Bax, so as to screen effective antitumor drugs for the clinical treatment of lung adenocarcinoma.*

Methods: *Lung adenocarcinoma A549 cells were cultured in vitro and treated with different concentrations of GEN. The untreated cells were set as control group. MTT assay and Annexin V/PI double staining were used to analyze cell proliferation and apoptosis after GEN treatment. RT-qPCR and Western blot were used to detect the expression of Bcl-2 and Bax at mRNA and protein levels, respectively.*

Results: *Cell proliferation assay showed that GEN could inhibit the proliferation of lung adenocarcinoma cell line A549 in vitro in a dose-dependent manner. Cell apoptosis assay showed that, compared with the control group, the apoptotic rate of A549 cells was significantly increased after GEN treatment. RT-qPCR and Western blot showed that the expression levels of anti-apoptotic factor Bcl-2 in A549 cells were significantly decreased at both mRNA and protein levels at 48 hrs after treatment with GEN, but the levels of pro-apoptotic Bax were significantly increased at both mRNA and protein levels.*

Conclusion: *GEN can inhibit the proliferation of A549 lung adenocarcinoma cells in vitro and induce their apoptosis. The antitumor activity of GEN is achieved by downregulating Bcl-2 and upregulating Bax. Therefore, GEN can be applied to the clinical treatment of lung adenocarcinoma.*

Key words: *apoptosis, Bax, Bcl-2, cell proliferation, genistein, lung adenocarcinoma*

Introduction

Lung cancer can be divided into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). The incidence and mortality of lung cancer is extremely high. NSCLC accounts for 80% of the total cases of lung cancer, and is a major cause of cancer-related death in men, and an important cause of cancer-related death in women [1]. There are about 220,000 new cases of lung cancer each year, with 150,000 deaths from this disease in USA [2]. The main cause of lung cancer is direct smoking or secondary smoking. In addition, air pollution, vehicle exhaust emissions and other factors can also lead to lung cancer genesis [3]. Lung cancer is often accompanied by chronic bronchitis, pulmonary heart disease, tuberculosis and other diseases, and early disease symptoms are difficult to detect. In addition, tumor metastasis and vascular factors-mediated drug resistance are common, making the treatment of lung cancer unsatisfactory [4]. Previous studies have shown that inhibition of angiogenesis and inhibition of vascular endothelial growth factor (VEGF) may

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provide a target for the treatment of lung cancer [5], but the mechanism is unclear. At present, apart from surgical operations and radiation treatment, chemotherapy is still the main treatment for lung cancer, so the development of highly effective antitumor drugs is of great clinical significance.

Studies have shown that natural flavonoids and isoflavones possess antioxidant and anticancer activities [6]. Genistein (4', 5, 7-trihydroxyisoflavones, GEN) is a rich isoflavone in soybeans. GEN can block G2/M cell cycle and the critical signal pathway in cells, inhibit the formation of tumor and induce apoptosis in a variety of animal models, including breast cancer, bladder cancer and prostate cancer [7,8]. GEN now is in phase II clinical trials of the treatment of a variety of human cancers. Studies have shown that the molecular mechanism of the antitumor and pro-apoptotic activity of GEN is achieved by competitively binding to estrogen receptors to prevent estrogen activation, so as to inhibit cancer cell growth [9]. In addition, as a tyrosine kinase and angiogenesis inhibitor [10], GEN can induce the expression of proapoptotic factors Bax and p21WAF1/CIP1, and inhibit the expression of antiapoptotic factor Bcl-2 [11]. So, GEN is a potential anticancer drug with a wide range of prospects.

The aim of this study was to investigate the effect of GEN on the proliferation and apoptosis of lung adenocarcinoma and the expression of apoptosis-related factors, so as to screen effective antitumor drugs for the clinical treatment of lung adenocarcinoma.

Methods

Cell lines

Human lung cancer cell line A549 (BNCC337696) was purchased from BeNa Culture Collection (Beijing, China). Cells were cultured with RPMI-1640 medium containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in a incubator (37 °C, 5% CO_2).

Major reagents

Genistein (GEN) and thiazolyl blue (MTT) were purchased from Sigma-Aldrich (Saint Louis, MO,USA). PRMI-1640 medium and FBS were purchased from Hyclone (New York,USA). Annexin V/PI cell apoptosis analysis kit was from eBioscience (New York,USA). TRIzol Reagent, Prime Script® RT reagent Kit with gDNA Eraser and SYBR®Premix Ex Taq ™ II were purchased from Takara (Dalian, China). Anti-Bcl-2, Bax and β-actin rabbit or mouse monoclonal antibodies were purchased from Cell Signaling Technology (Danvers,USA). Goat anti-rabbit or rabbit anti-mouse IgG/HRP secondary antibody were purchased from BioRad (New York, USA). Primers and modified BCA protein quantitative kit were purchased from Sangon (Shanghai,China). The study was approved by the ethics committee of Xiangyang No.1 People's Hospital, Hubei University of Medicine.

Cell culture and treatment

Human lung cancer cell line A549 was cultured in 6-well plate in RPMI-1640 medium containing 10% FBS for subculture. When cell density reached 5×10^3 cells/well, cells were transferred to serum-free RPMI-1640 medium to be cultured overnight. Then cells were transferred to 96-well plate with 1×10^4 cells per well. Different concentrations of GEN (0-200 μM) were added, a control group without GEN treatment was also set, and 3 replicates were set for each group.

Cell proliferation assay

A549 cells were inoculated into 96-well plates with $1x10^4$ cells per well. GEN was added to make the final concentrations of 0, 25, 50 and 100 μM. Ten μL of 5 mg/ml MTT were added into each well at 12, 24, 36, 48 and 72 hrs later, followed by incubation for another 4 hrs. Then, 200 μl of dimethylsulfoxide were added and incubated for 10 min. Optical density (OD) values at 570 nm of each group of cells were measured using a microplate reader (Tecan Group Ltd, Switzerland). IC_{50} of GEN was calculated. Then, GEN at a final concentration of 0, 25, 50, 75, 100, 150, 200 μM was used to incubate the cells. After 48 hrs, the inhibition rate was calculated: Inhibition rate = (100-At/Ac×100%), where At is the OD value of the treatment group and Ac is the OD value of the control group.

Cell apoptosis assay

Cells were transferred to 96-well plate with $1x10^6$ cells per well, and GEN at a final concentration of 0, 25, 50 and 100 μM was used for incubation with the cells for 48 hrs. Then 5 μl of Annexin V-FITC (5 μg/ml) and 5 μL of PI (1 μg/mL) were added into each well and incubated at room temperature for 10 min in the dark. The early and late apoptotic rates were analyzed by flow cytometry (BD Biosciences, New York, USA).

QRT-PCR

Cells were treated with GEN at final concentrations of 0, 25, 50 and 100 μM. Cells were harvested and total RNA was extracted from cells using Trizol kit. Two hundred ng of total RNA were reverse-transcribed to synthesize cDNA, which was used as template in PCR reaction. PCR reactions were performed on CFX-96 Detection System (Bio Rad, New York, USA). Cycle threshold (Ct) values were processed using $2^{-\Delta\Delta ct}$ method. Relative expression level of each gene was normalized to endogenous control β-actin. Primers used in PCR reaction are listed in Table 1.

Western blot

Cells were treated with GEN at final concentrations of 0, 25, 50 and 100 μM. Then cells were harvested and incubated with RIPA lysate on ice, followed by centrifu-

gation at 4°C to collect protein. After quantification, 50 μg of the protein sample was subjected to 12% SDS-PAGE electrophoresis, and then transferred to PVDF membrane. Membranes were blocked with Tris solution containing 5% skim milk and 0.1% Tween at room temperature for 2 hrs. Then, the membranes were incubated with rabbit or mouse anti-Bcl-2, Bax and β-actin primary antibodies (1:500) overnight at 4 °C. After washing with 0.05% TBST three times, the membranes were incubated with goat anti-rabbit or rabbit anti-mouse IgG secondary antibody (1:5000) at room temperature for 2 hrs. Signals were detected by ECL detection system (Thermo Fisher Scientific, Waltham, USA) and the gray scale values of the bands were calculated using ImageJ software (National Institutes of Health, Bethesda, USA). The relative expression level of each protein was expressed as gray scale of target protein/ gray scale of β-actin.

Statistics

Statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). All experiments were repeated three times and the data was expressed as mean±SD. Single factor analysis of variance and two-tailed t-values were used for comparisons between groups, and $p<0.05$ was considered to be statistically significant.

Table 1. PCR primers

Primer	*Sequence (5'-3')*
Bcl-2-F	TGAACCGGCATCTGCACAC
Bcl-2-R	CGTCTTCAGAGACAGCCAGGAG
Bax-F	AGACACCTGAGCTGACCTTGGAG
Bax-R	GTTGAAGTTGCCATCAGCAAACA
ACTB-F	ATCACCATTGGCAATGAGCG
ACTB-R	TTGAAGGTAGTTTCGTGGAT

Results

GEN inhibited the proliferation of lung cancer cell line A549 in vitro

MTT assay was performed to detect the proliferation of A549 lung cancer cell line. Figure 1A shows the proliferation of cells treated with different concentrations of GEN at different time points. Figure 1B shows the proliferation of cells treated with different concentrations of GEN for 48 hrs. Compared with the control groups, GEN could significantly inhibit the proliferation of A549 cells, and the IC_{50} of GEN was 40.14 μM.

GEN induced apoptosis of lung cancer cell line A549

The effects of GEN treatment on the apoptosis of A549 cells was detected by Annexin V/PI double staining. As shown in Figure 2, compared with the control groups, cell apoptosis was significantly increased after treatment with different concentrations of GEN for 48 hrs.

The level of Bcl-2 mRNA was decreased and the level of Bax mRNA was increased after treatment with GEN

The effect of GEN treatment on the expression of Bcl-2 and Bax mRNA was analyzed by qRT-PCR. As shown in Figure 3, compared with the control groups, treatment with different concentrations of GEN for 48 hrs significantly decreased the expression level of Bcl-2 mRNA and increased the expression level of Bax mRNA.

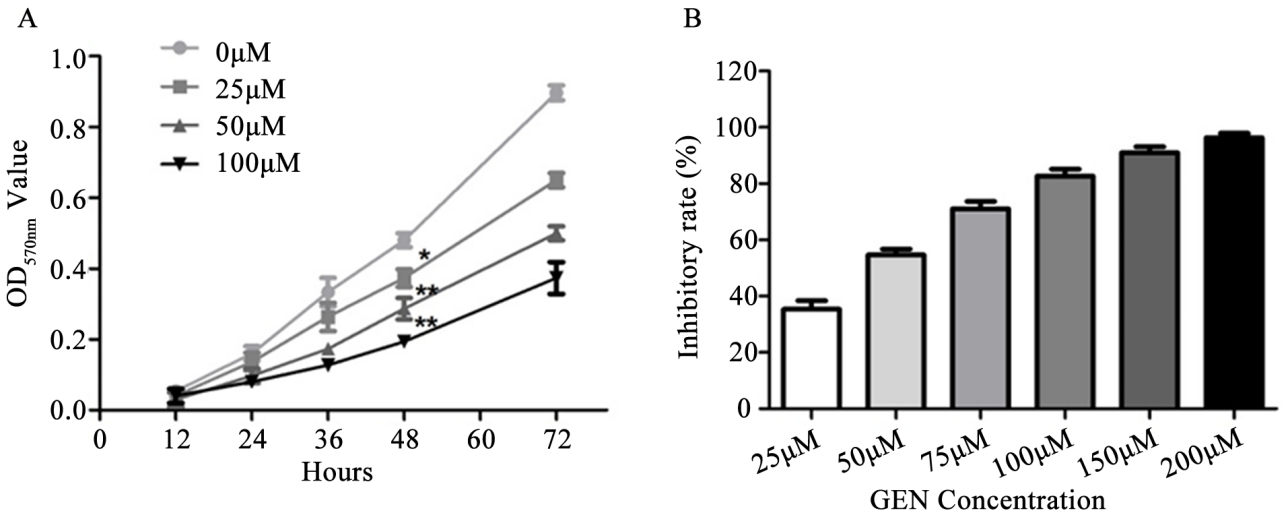

Figure 1. Effect of different concentrations of GEN on the proliferation of A549 cells. **A:** proliferation of cells treated with different concentrations of GEN at different time points; **B:** proliferation of cells treated with different concentrations of GEN for 48 hrs. MTT assay results showed that GEN had significant inhibitory effect on the proliferation of A549 cells in vitro. *compared with control cells at the same time point, $p<0.05$; **compared with control cells at the same time point, $p<0.01$.

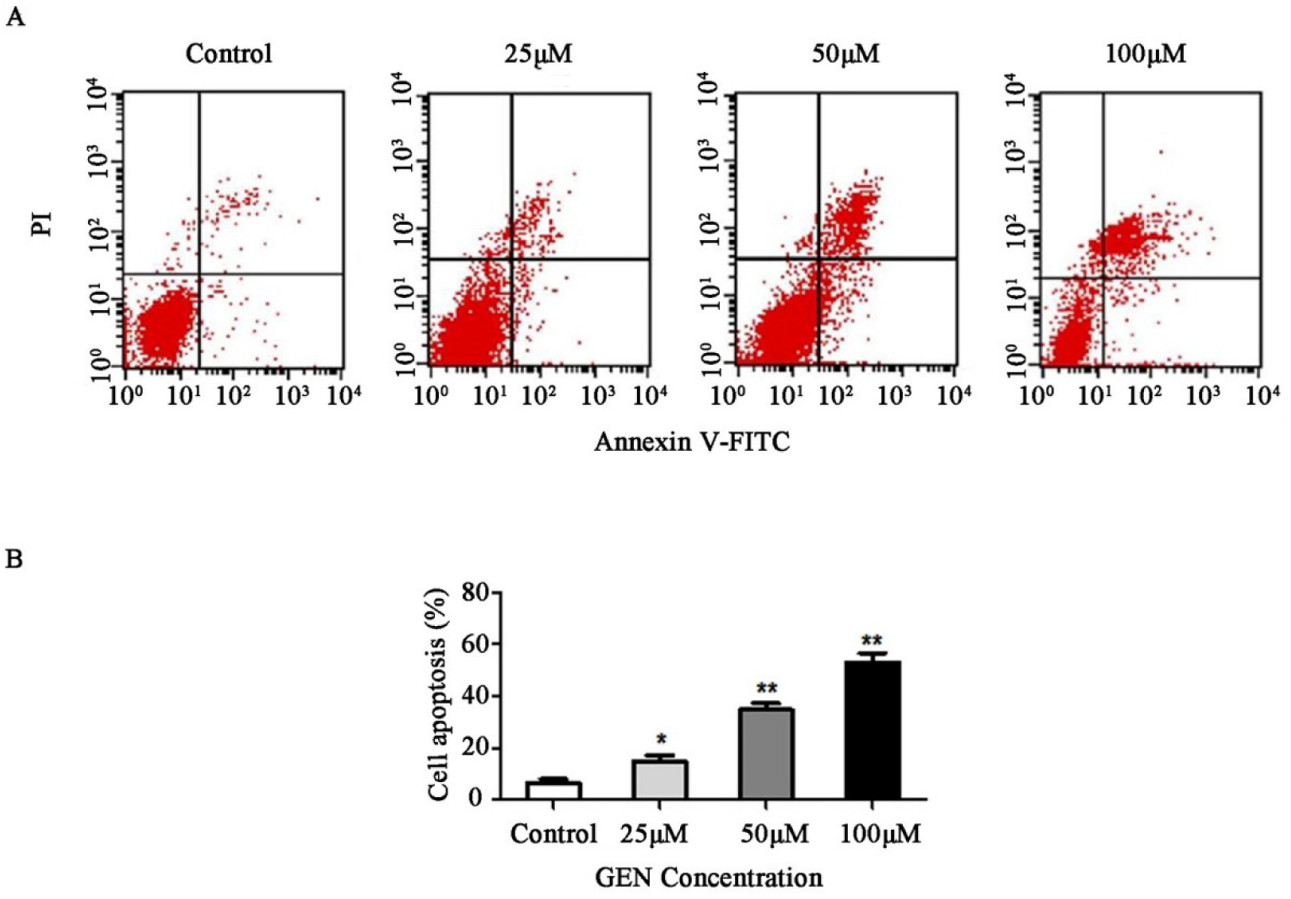

Figure 2. Effects of different concentrations of GEN on apoptosis of A549 cells. **A:** Cell apoptosis in four quadrant maps; **B:** early and late cell apoptosis rates. The results of annexinV/PI double staining showed that, compared with the control group, cell apoptosis was significantly increased after treatment with different concentrations of GEN for 48 hrs. *compared with control cells at the same time point, p<0.05; **compared with control cells at the same time point, p<0.01.

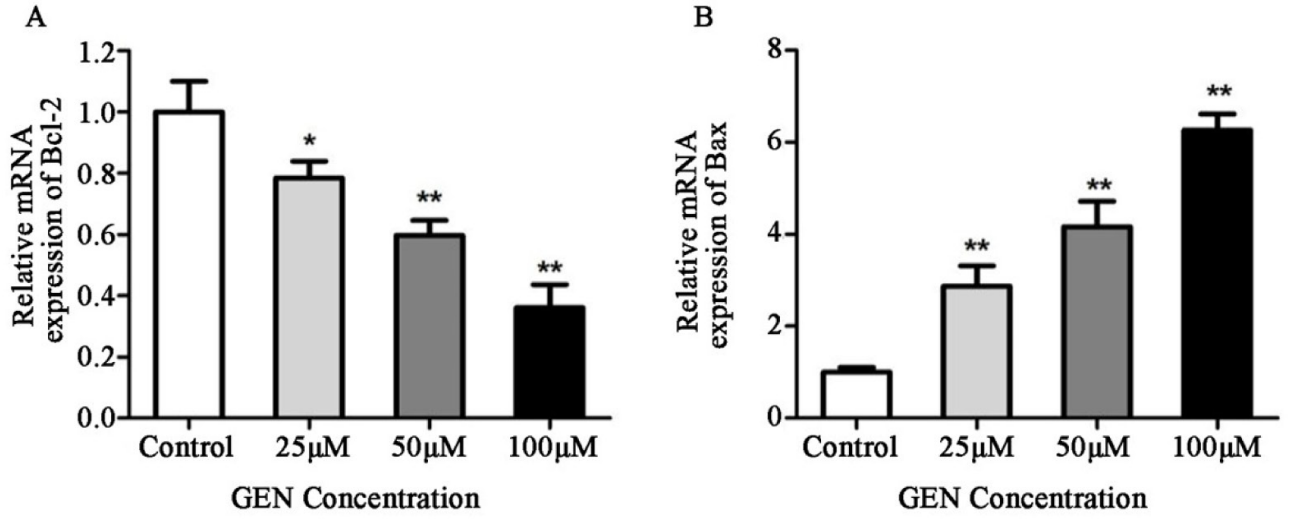

Figure 3. Effect of different concentrations of GEN on the expression of apoptotic factors in A549 cells at mRNA level. **A:** Changes in Bcl-2 mRNA level after treatment with different concentrations of GEN; **B:** Changes in Bax mRNA level after treatment with different concentrations of GEN. The results of RT-qPCR showed that the level of Bcl-2 mRNA was significantly decreased and the level of Bax mRNA was significantly increased compared with the control group. *compared with control cells at the same time point, p<0.05; **compared with control cells at the same time point, p<0.01.

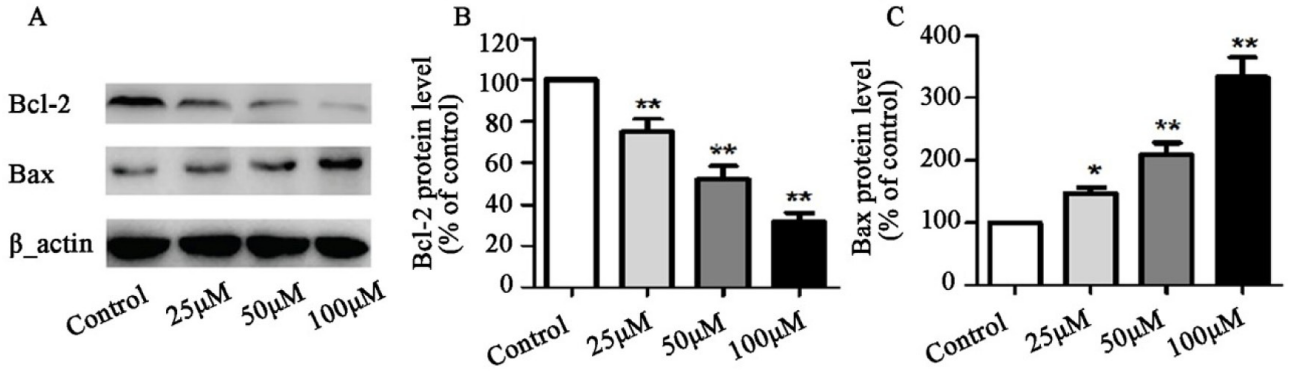

Figure 4. Effect of different concentrations of GEN on apoptotic factors in A549 cells. **A:** Representative Western blot results; **B:** Changes in Bcl-2 protein level after treatment with different concentrations of GEN; **C:** Changes in Bax protein level after treatment with different concentrations of GEN. Western blot showed that the expression of Bcl-2 protein was significantly decreased and the expression of Bax protein was significantly increased compared with the control group. *compared with control cells at the same time point, p<0.05; **compared with control cells at the same time point, p<0.

The level of Bcl-2 protein was decreased and the level of Bax protein was increased after treatment withGEN

The effect of GEN treatment on the expression of Bcl-2 and Bax proteins was analyzed by Western blot. As shown in Figure 4, compared with the control groups, treatment with different concentrations of GEN for 48 hrs significantly decreased the expression level of Bcl-2 protein and increased the expression level of Bax protein.

Discussion

Lung cancer is one of the most common types of malignant tumors. NSCLC has the highest morbidity and mortality among all types of cancer. About two thirds of patients with NSCLC are diagnosed at advanced stages or with distant metastases [12]. Therefore, the development of effective clinical treatment of lung cancer strategy is urgently needed. At present, intravenous chemotherapy assisted by thoracic radiation therapy is the main clinical treatment of lung cancer [13]. Systemic chemotherapy, biological therapy and surgical treatment have also been developed [14]. However, due to the complex development of lung cancer and the diversification of pathological processes, activation and mutation of various oncogenes, as well as defects of various tumor suppressor genes are involved. In addition, lung cancer cells are very invasive, and drug resistance can be easily developed, leading to poor treatment outcomes with these treatment methods [15]. At present, screening and developing highly effective antitumor active drugs is the primary task in lung cancer research.

GEN is a natural isoflavone extracted from leguminous plants. In vitro experiments have shown that GEN can inhibit the proliferation and metastasis of various cancer cells and induce cancer cell apoptosis in a dose-dependent manner, and it is beneficial to the activation of winter enzyme-3/9 (Caspase-3/9) [2]. Therefore, GEN is considered to possess anticancer activity, and it is now in phase II clinical trials of the treatment of a variety of human cancers [16]. Studies have shown that treatment of lung cancer cell line A549 with GEN can significantly increase the production of reactive oxygen species (ROS), extracellular diffusion of cytochrome oxidase-c and expression of Bax and AIF protein in mitochondrial apoptosis pathway [17]. In addition, GEN has been reported to inhibit cancer cell apoptosis by downregulating PI3K/AKT and HIF-1α signaling pathways, which can mediate angiogenesis to accelerate cancer cell growth [18]. Furthermore, some authors have suggested that GEN can inhibit the activity of NF-κB and COX-2 pathways to inhibit cell apoptosis [19]. In recent years, studies have shown that GEN can mediate the signal transduction of cell membrane receptors by regulating MAPK signaling pathway, so as to regulate cell apoptosis and proliferation, angiogenesis and the expression of downstream target genes [20].

Apoptosis-related factors Bcl-2 and Bax are the main genes that are currently thought to regulate cell apoptosis [21]. Bcl-2 is an upstream effector of the apoptotic pathway and can form a heterodimer with the proapoptotic factor Bax family to neutralize the proapoptotic effect, so Bcl-2 can inhibit cell apoptosis [22]. In this study, we investigated the effect of GEN on the proliferation and apoptosis of lung cancer cell A549 in vitro and the changes of expression of apoptosis-related factors after GEN treatment. The results showed that GEN could significantly inhibit the proliferation of lung cancer

cells in vitro and induce cell apoptosis. This antitumor activity may be achieved by downregulating Bcl-2 and upregulating Bax, suggesting that GEN can be used as a potential chemical drug for the clinical treatment of lung cancer. However, clinical trials are still needed to study the long-term effects of GEN, and the mechanism of function of GEN should be investigated in animal models.

Conflict of interests

The authors declare no conflict of interests.

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