Study of EGCG induced apoptosis in lung cancer cells by inhibiting PI3K/Akt signaling pathway

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Abstract. – **OBJECTIVE:** To investigate the role of phosphatidylinositol-3-kinase protein kinase B (PI3K/Akt) signaling pathway in the apoptosis of H1299 lung cancer cells induced by epigallocatechin gallate (EGCG).

MATERIALS AND METHODS: H1299 lung cancer cells were treated with EGCG at a dose of 10 μM, 20 μM, and 40 μM, respectively. Cell culture was performed for 72 h and then: 1, cell proliferation was detected by MTT assay; 2, cell apoptosis rate was detected by flow cytometry; 3, expression of Caspase-3, Bax, and Bcl-2 was detected by Western blot; 4, expression of PI3K, p-PI3K, Akt, and p-Akt was detected by Western blot.

RESULTS: The proliferation of H1299 cells was significantly inhibited 72 h after treatment with different doses of EGCG, and cell apoptosis rate was significantly increased ($p<0.05$). Compared with those in the control group, expression of PI3K and Akt in the lung cancer cells H1299 after EGCG treatment showed no significant differences ($p>0.05$), while expression levels of p-PI3K and p-Akt were significantly reduced ($p<0.05$).

CONCLUSIONS: EGCG can inhibit the proliferation and induce apoptosis of H1299 lung cancer cells, and the effect is related to the inhibition of the activation of PI3K/Akt signaling pathway.

Key Words:
EGCG, Lung cancer cells, PI3K/Akt, Apoptosis.

Introduction

Lung cancer is a malignant tumor which derives from the bronchial mucosa and alveolar cells. With the increased number of smokers and aggregated environmental pollution, incidence of lung cancer in recent years increased significantly[1]. Symptoms of lung cancer in the early stage are not evident, and most patients with lung cancer are diagnosed in advanced stages, which could hinder effective treatment[2]. Lung cancer is one of the malignant tumors with the highest incidence of morbidity and mortality in China[3]. There are two main types of lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), of which NSCLC accounts for about 80% of all cases[4,5]. PI3K is an intracellular phosphatidylinositol kinase that is associated with the expression of oncogenes and consists of a regulatory subunit p85 and a catalytic subunit p110. PI3K can be activated in two ways: one is to interact with a growth factor receptor or connexin which has a phosphorylated tyrosine residue, the dimeric conformation is changed therefore activated; and the other way is the binding of Ras to p110. Studies have shown that the occurrence and development of NSCLC are closely correlated with the disorders of phosphatidylinositol-3-kinase-protein kinase B (PI3K/Akt) signaling pathway[6]. In addition, the tyrosine kinase inhibitor BIBW2992 is effective in overcoming T790M-EGFR-mediated erlotinib resistance in H1975 human lung cancer cells by inhibiting the PI3K/AKT signaling pathway[7].

Epigallocatechin gallate (EGCG) is a compound extracted from green tea, which is the main component of tea polyphenols. It has antioxidant, anti-inflammatory and anti-tumor effects[8,9]. Previous studies have shown that EGCG can induce apoptosis in human pancreatic cancer cells by inhibiting the activation of PI3K/Akt signaling pathway[10]. NSCLC cells H1299 originated from neuroendocrine cells with a homozygous deletion of p53 protein. A549 cells were constructed by Giard et al[11] in 1972 by culturing lung cancer tissue, and can synthesize lecithin-rich unsaturated fatty acids through cytidine phosphatidylcholine pathway.

In this study, effects of EGCG on the proliferation and apoptosis of lung cancer cells was observed by treating HSC99 cells and A549 cells using different doses of EGCG, and the

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possible mechanism was also explored. Our study provided the theoretical basis for the clinical application of EGCG in the treatment of lung cancer.

Materials and Methods

Cell Lines

NSCLC cells H1299 were purchased from the Peking Union Medical College cell bank. Lung adenocarcinoma cell line A549 was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. The study was approved by the Ethics Committee of the Fourth People's Hospital of Zibo (Shandong, China).

Main Reagents

Roswell Park Memorial Institute 1640 (RPMI 1640) medium (GE Healthcare Life Sciences, HyClone Laboratories, South Logan, UT, USA), fetal bovine serum (FBS; Sijiqing, Hangzhou, China), trypsin (Sigma-Aldrich, St. Louis, MO, USA), penicillin, streptomycin (Shanghai Pharmaceuticals, China), rabbit anti-human Caspase-3, rabbit anti-human Bax, rabbit anti-human Bcl-2, rabbit anti-human β-actin (Millipore, Billerica, MA, USA), Annexin V Cell Apoptosis Detection Kit, rabbit anti-human PI3K, rabbit anti-human p-PI3K, rabbit anti-human Akt, rabbit anti-human p-Akt, tetramethylazo-azole blue (Sigma-Aldrich, St. Louis, MO, USA), HRP-labeled anti-sheep/rabbit (BD Biosciences, Franklin Lakes, NJ, USA), radioimmunoprecipitation assay (RIPA) lysis buffer (Applygen Genetic Technology Co., LTD, Beijing, China), bicinchoninic acid (BCA) Protein Concentration Assay Kit (Thermo Scientific, Waltham, MA, USA).

Main Instrument

Microplate reader (Thermo Scientific, Waltham, MA, USA), cell incubator (Thermo Scientific, Waltham, MA, USA), inverted phase contrast microscope (Nikon, Tokyo, Japan), ultra clean bench (Suzhou Hengda Purification Equipment Co., Ltd, Suzhou, China), flow cytometry (BD, San Josè, CA, USA), PCR Amplifier (Bio-Rad, Hercules, CA, USA), micro-pipettes (Eppendorf, Hamburg, Germany).

H1299 and A549 Cell Culture and Grouping

H1299 and A549 lung cancer cells were cultured with RPMI 1640 medium (containing 10% FBS) at 37ºC (5% CO_2) for 48-72 h, until the cells covered the bottom of the flask. Cells were digested with 0.25% trypsin to remove the wall and passaged at ratio of 1:4. Cell density was adjusted to 4×10^4/ml, and then inoculated on a 96 well plate. Cells were divided into control group (0 μM), low dose treatment group (10 μM), middle dose group (20 μM), and high dose treatment group (40 μM) according to EGCG concentrations. Complete medium containing certain concentration of EGCG was added to the 96 well plate, cells were cultured at 37ºC (5% CO_2) for 72 h.

H1299 and A549 Cell Proliferation Inhibition Rate Detection

Cells were treated with EGCG for 72 h, and the culture medium was discarded after centrifugation. After washing with PBS, complete medium containing 5 mg/ml MTT was added. Cell culture was performed for 4 h and supernatant was discarded. Then, 150 μL dimethyl sulfoxide (DMSO) was added into each well. Plates were oscillated at low speed on the shaker for 10 min until the crystal was completely dissolved. The absorbance (A) of each well was measured using a microplate reader at the wavelength of 570 nm. Inhibition rate (%) = (1 – treatment group A value/control group A value) × 100%.

Detection of Apoptosis in H1299 and A549 Cells

After treatment with EGCG for 72 h, cells were digested by trypsin. Adherent cells were collected, washed twice with PBS and centrifuged at 1000 r/min for 10 min. Cells were mixed with PBS to prepare single cell suspension. Apoptosis detection kit dye (Annexin V) was added and cells were incubated for 15 min at room temperature in dark. Cell apoptotic rate was measured by flow cytometry.

The Expression Levels of Caspase-3, Bax, tBcl-2, PI3K, p-PI3K, Akt, and p-Akt in H1299 and A549 Cells

Expression levels of Caspase-3, Bax, and Bcl-2 in H1299 and A549 cells were measured by Western blot. β-actin was used as endogenous control. Cells were incubated with lysate at 4ºC for 30 min, then total protein was extracted. Protein concentration was measured by
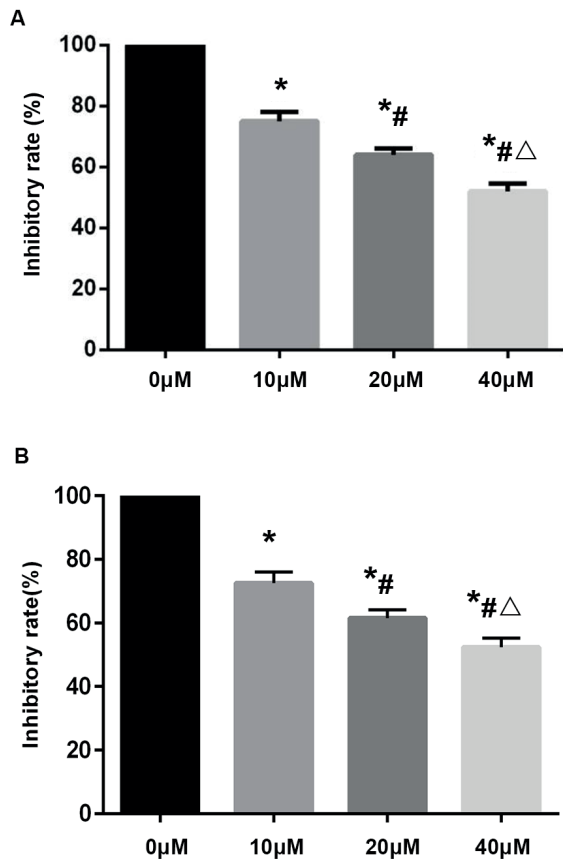
A

B

Figure 1. EGCG on the proliferation of H1299 and A549 cells in each group. MTT assay showed that ECGC could inhibit the cell proliferation and showed a significant dose-effect relationship. *A,* H1299 cells; *B,* A549 cells. Notes: *: Compared with that in the control group, the inhibition rate of cell proliferation in low dose group was significantly decreased (*p*<0.05). #: Compared with that in the low-dose treatment group, the inhibitory rate of cell proliferation was decreased in the medium-dose group, *p*<0.05. Δ: Compared with that in the medium-dose group, the proliferation inhibition rate of cells was decreased in the high dose group, *p*<0.05.

BCA kit and the protein curve was plotted. SDS polyacrylamide gel was prepared in advance and electrophoresis was performed after loading 40 μg of total protein from each sample. Protein was transferred onto PVDF membrane (150 min under 100V). Membrane were blocked for 120 min at room temperature using 5% skim milk, and incubated with primary antibodies of caspase, bax, bcl-2, PI3K, p-PI3K, Akt, p-Akt, and β-actin at 4°C overnight. After washing with TBS-T, membranes were incubated with secondary antibody. Chemiluminescence reac-

tion was added to detected signals, and Image J software was used for quantitative analysis. Each experiment was performed 3 times.

Statistical Analysis

Data were collected and analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and the data were compared by one-way ANOVA and the post-hoc test was SNK test. *p*<0.05 indicates that the difference was statistically significant.

Results

H1299 and A549 Cell Proliferation Inhibition Rate

After treated with different concentrations of EGCG for 72 h, the proliferation of H1299 and A549 cell was significantly decreased, EGCG inhibited the proliferation of H1299 and A549 cells in a dose-dependent manner, wherein the high-dose EGCG treatment group had the strongest inhibitory effect on H1299 and A549 cell proliferation (*p*<0.05) (Figure 1).

H1299 and A549 Cell Apoptosis Rate

The apoptosis rate of H1299 and A549 cells was significantly increased after EGCG treatment, EGCG induced the highest apoptosis rate of H1299 and A549 cells in high dose group (40 μM), EGCG induced H1299 and A549 cell apoptosis rate in dose-dependent, and the comparisons between the groups were statistically significant (*p*<0.05) (Figure 2).

Caspase-3, Bax, and Bcl-2 Expression in H1299 and A549 cells

The expression of Caspase, bax, and bcl-2 in H1299 and A549 cells were significantly increased after treatment with different concentrations of EGCG. With the increase of EGCG concentration, expression levels of Caspase, bax, and bcl-2 were significantly increased (*p*<0.05) (Figure 3).

The expression of PI3K, p-PI3K, Akt, and p-Akt in H1299 and A549 cells

The expression of PI3K and Akt in lung cancer H1299 and A549 cells treated with different
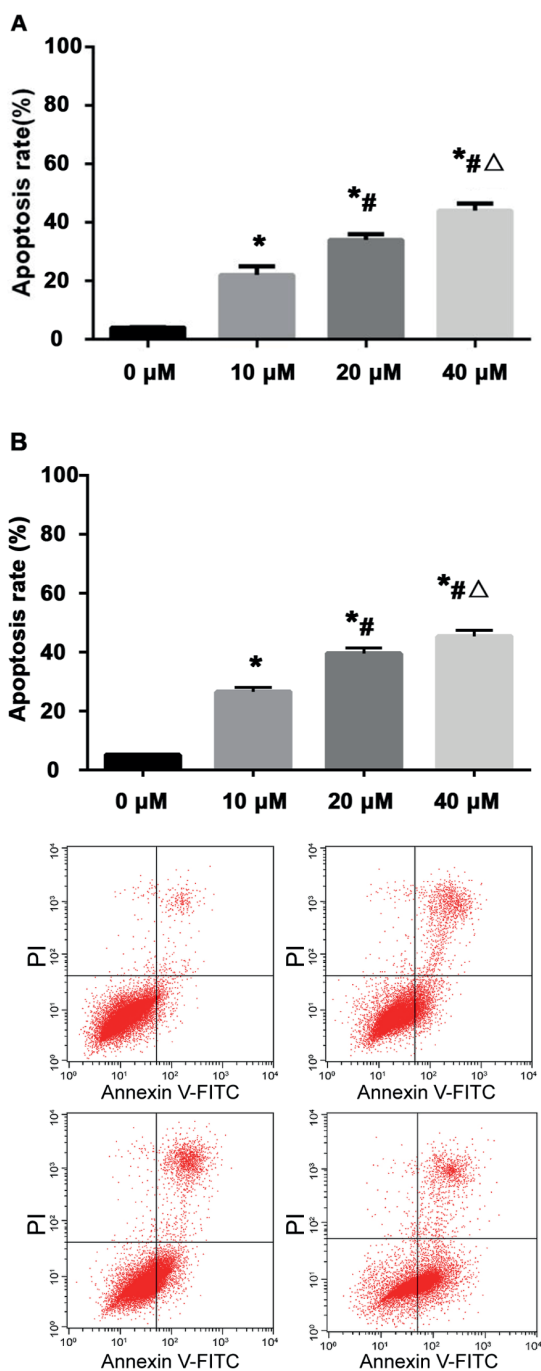
Figure 2. Apoptosis rate of H1299 and A549 cells after EGCG treatment. **A,** H1299 cells; **B,** A549 cells. Notes: *: Compared with that in the control group, the apoptotic rate of cells was increased in low dose group (p <0.05). #: The apoptotic rate of cells was higher in the medium dose group than that in the low dose group ($p<0.05$). Δ: Compared with that in the medium dose group, the apoptotic rate of cells increased in high dose group ($p<0.05$).

concentrations of EGCG was not significantly different from that of the control group ($p>0.05$), while expression levels of p-PI3K and p-Akt were significantly lower than those of control group ($p<0.05$). The expression level of p-PI3K and p-Akt was significantly down-regulated by EGCG in the high-dose treatment group (40 μM) ($p<0.05$) (Figure 4).

Discussion

Lung cancer is a malignant disease that can seriously threaten human health and life expectancy due to the rapid development of tumor metastasis and poor prognosis. The average 5-year survival rate of patients with lung cancer was less than 15%[12]. At present, the clinical treatment for lung cancer is mainly radiotherapy, chemotherapy, and surgery. However, treatment outcomes are usually unsatisfactory. Some patients with tumor metastasis are not sensitive to radiotherapy and chemotherapy in the early stage, and drug resistance may occur. At present, effective treatment remains lacking. Therefore, it is of great importance to develop novel treatments for this disease[13-15].

PI3K/Akt signaling pathway is involved in the regulation of cell proliferation, differentiation, and apoptosis, and its abnormal activation is usually closely related to the formation of a malignant tumor. PI3K/Akt signaling pathway also plays an important role in the invasion and metastasis of tumor cells[16,17]. In the development and progression of lung cancer, abnormal activation of PI3K/Akt signaling pathway can lead to normal cell malignancy, so as to promote lung cancer cell proliferation, and reduce the sensitivity to radiotherapy and chemotherapy, and induce the development of drug resistance[18]. Thus, PI3K/Akt signaling pathway may serve as a key target in the treatment of lung cancer[19-21]. Activated Akt regulates cell proliferation, differentiation, apoptosis, and migration by inhibiting a series of downstream substrates such as Bad, caspase9, NF-κB, GSK[23], etc.

In this study, we investigated the effect of EGCG on H1299 and A549 lung cancer cells by administrating EGCG at different doses. Results showed that EGCG could inhibit the proliferation of H1299 lung cancer cells in a dose-dependent manner. At the same time, Western blot showed that EGCG could upregulate the expres-
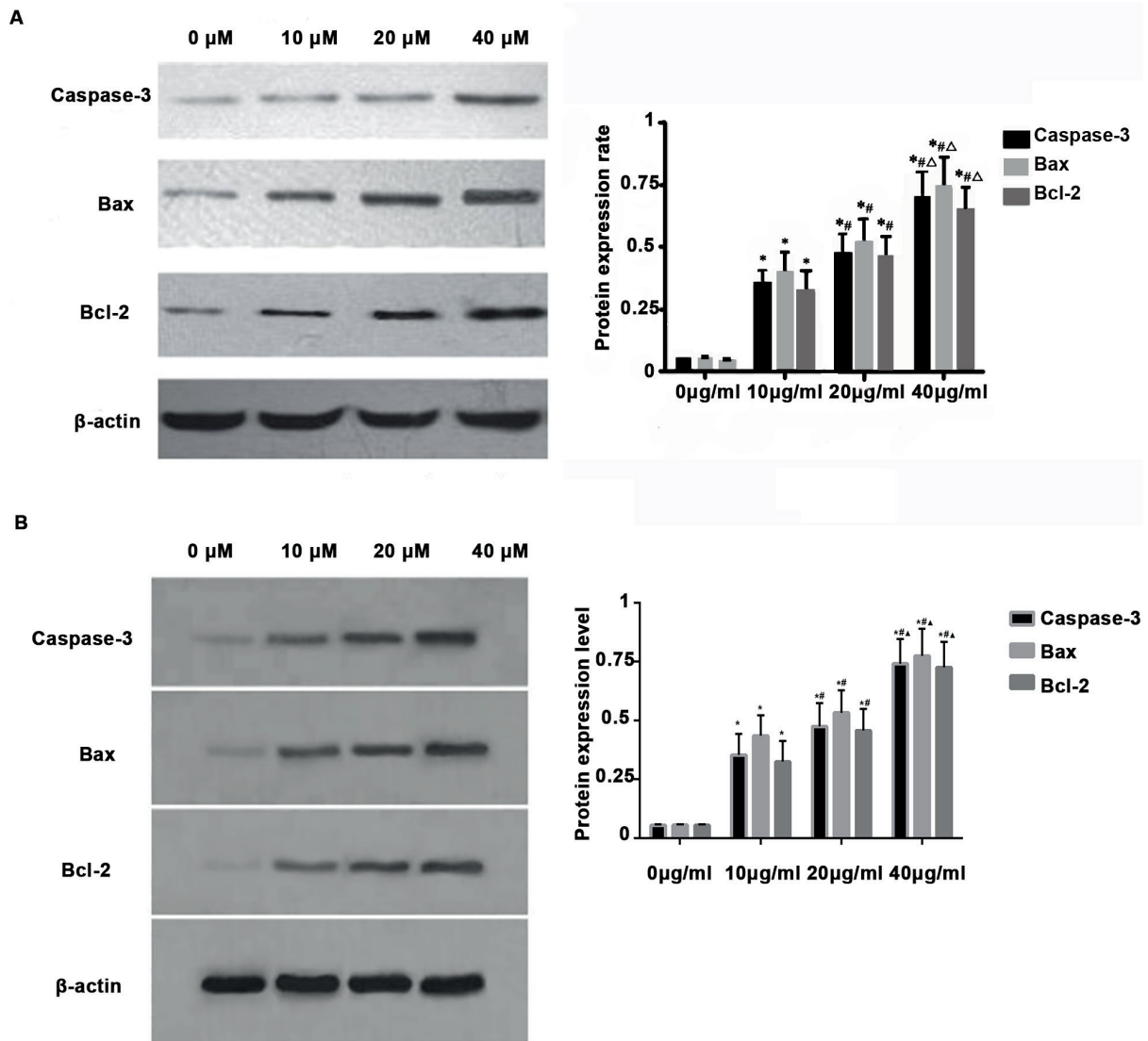
Figure 3. The expression of Caspase-3, Bax, and Bcl-2 in H1299 and A549 cells. **A,** H1299 cells; **B,** A549 cells. Notes: *Compared with that in the control group, the expression of Caspase-3, Bax, and Bcl-2 in the low dose group was higher ($p<0.05$). #: The expression of Caspase-3, Bax, and Bcl-2 in medium dose group was higher than that in low dose group ($p<0.05$). Δ: Compared with that in the medium dose group, the expression of Caspase-3, Bax, and Bcl-2 in the high dose group increased ($p<0.05$). Each experiment was performed 3 times.

sion of Caspase-3, Bax, and Bcl-2 protein in H1299 and A549 lung cancer cells in a dose-dependent manner. The apoptotic process of lung cancer cells is regulated by multiple signaling pathways, in which PI3K/Akt signaling pathway plays a key role. The apoptosis of lung cancer cells can be induced by using drugs to inhibit the activation of PI3K/Akt signaling pathway[22-24]. Our results showed that EGCG could down-regulate the expression of p-PI3K and p-Akt in lung cancer cells, but had no significant effect on the expression of PI3K and Akt, which indicated that EGCG could induce the apoptosis of H1299 lung cancer cells by inhibiting the activation of PI3K/Akt signaling pathway in a dose-dependent manner.

Conclusions

We observed that EGCG is a safe and efficient natural drug that may have great potentiality in
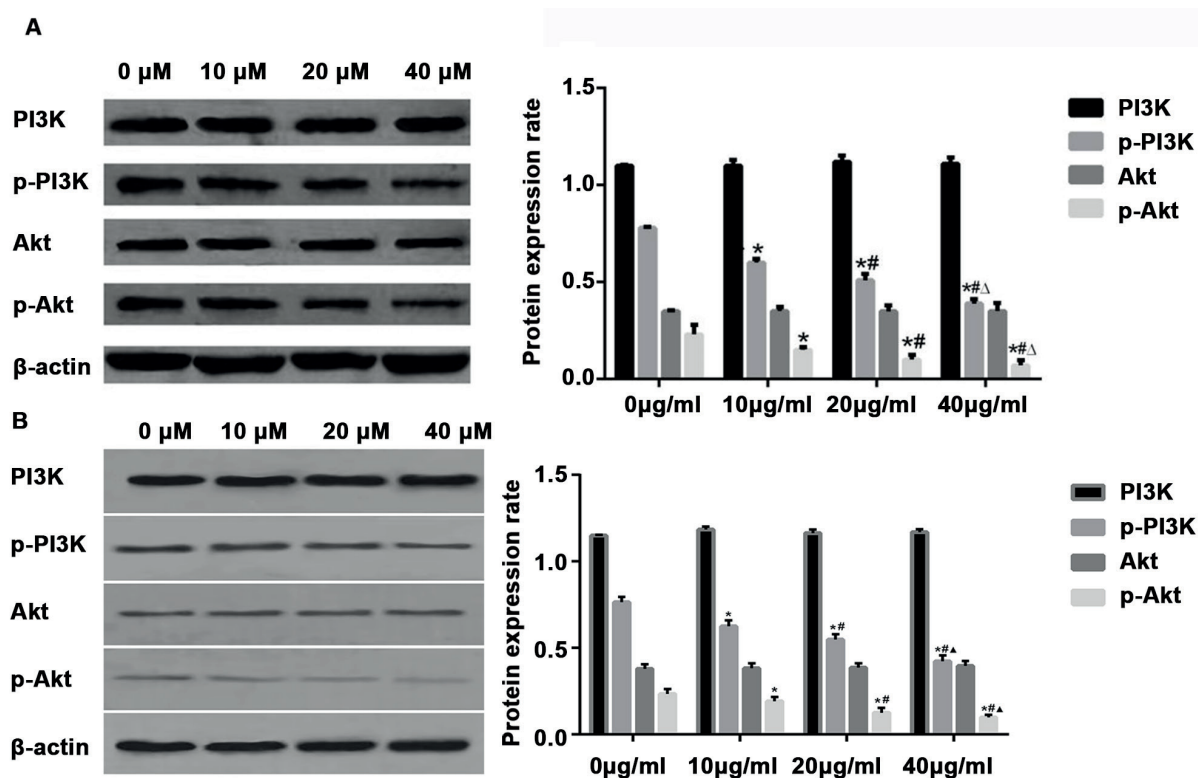
Figure 4. The expression of PI3K, p-PI3K, Akt, and p-Akt in H1299 and A549 cells. *A,* H1299 cells; *B,* A549 cells. Notes: *: Compared with that in the control group, the expression of PI3K, p-PI3K, Akt, and p-Akt in the low dose group was higher ($p<0.05$). #: The expression levels of PI3K, p-PI3K, Akt, and p-Akt in the medium dose group were significantly higher than those in the low dose group ($p<0.05$). Δ: Compared with that in the medium dose group, the expression of PI3K, p-PI3K, Akt, and p-Akt increased in the high dose group ($p <0.05$). Each experiment was performed 3 times.

the lung cancer treatment[25,26]. We found that EGCG inhibited the proliferation inhibition and apoptosis of lung cancer cells, and that the function of EGCG may be achieved through PI3K/Akt signaling pathway. However, the specific molecular mechanism still needs to be further studied.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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