

Original Research Article

Apigenin-7-glucoside induces apoptosis and ROS accumulation in lung cancer cells, and inhibits PI3K/Akt/mTOR pathway

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Abstract

Purpose: To investigate how apigenin-7-glucoside (AGL) affects the proliferation, migration, invasion, and reactive oxygen species (ROS) accumulation in lung cancer cells, and to evaluate the potential of AGL as a therapeutic target.

Methods: Human bronchial epithelial cells (BEAS-2B), human lung carcinoma (A549), and non-small cell lung cancer cells (H1975), were treated with 25, 50, 100, and 200 μ M AGL. Cell viability curves and EdU assays were used to evaluate their proliferation, while apoptosis was assessed by flow cytometry. Cell migration and invasion were evaluated by Transwell assays, and western blot was performed to determine the expression level of cytochrome C and phosphorylation of PI3K, AKT, and mTOR. Furthermore, ELISA was used to quantify the level of malondialdehyde (MDA) and glutathione (GSH). Indirect immunofluorescent assay (IFA) was performed by staining with DCFH-DA to evaluate ROS level.

Results: Proliferation of A549 and H1975 cells was suppressed by AGL in a dose-dependent manner. AGL significantly reduced proliferation, promoted cell apoptosis, and attenuated the migration and invasion of A549 or H1975 cells. It also elevated the levels of cytochrome C and MDA but reduced the production of GSH in A549 and H1975 cells. AGL enhanced the accumulation of ROS and weakened phosphorylation of AKT, PI3K, and mTOR in A549 and H1975 cells.

Conclusion: AGL represses proliferation, promotes apoptosis, suppresses migration and invasion, and induces ROS accumulation in lung cancer cells by repressing PI3K/Akt/mTOR pathway, thus indicating the potential of AGL as a target in lung cancer treatment.

Keywords: Apigenin-7-glucoside, Apoptosis, Lung cancer, Phosphorylation, Reactive oxygen species

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INTRODUCTION

Lung cancer, characterized by uncontrolled cell growth in lung tissues, is threatening human health. It is the most common cause of cancer-

related death, as there are nearly 2 million deaths from lung cancer annually. [1,2]. The common treatments include chemotherapy and radiation therapy, in combination with newer targeted molecular therapies and immune

checkpoint inhibitors [3]. However, lung cancer is still the carcinoma with the highest mortality rate. Therefore, it is exigent to develop safe and effective drugs for lung cancer treatment.

Apoptosis occurs through one of two typical pathways, including death receptor-mediated and mitochondrial pathways [4]. In the mitochondrial pathway, the generated ROS initiates the mitochondrial permeability transition, reducing mitochondrial membrane potential and releasing Cytochrome C [2,5,6]. Reactive oxygen species also initiate PI3K/Akt signaling pathway in order to regulate cell growth and survival [7]. The PI3K/Akt pathway might be inactivated when cells are stimulated by anticancer drugs, eventually inducing cell apoptosis.

Phytochemicals are of importance for the treatment and prevention of various malignancies [3]. A large amount of experimental evidence shows that flavonoids exert anticancer potential in many cancer cells [8]. Apigenin is a flavonoid compound extracted from chamomile, which has antibacterial and non-steroidal anti-inflammatory properties [9]. In gastric cells, AGL induces apoptosis and promotes ROS production by suppressing STAT3/JAK2 signaling pathways [10]. In addition, AGL treatment promotes the apoptosis of cervical cancer cells and represses cell migration by regulating the PTEN/PI3K/AKT pathway [11]. However, the effect of AGL on lung cancer is still unclear. In this work, A549 and H1975 cells treated with AGL were used to evaluate the efficacy of AGL on proliferation, apoptosis, ROS accumulation and phosphorylation of AKT, PI3K and mTOR.

EXPERIMENTAL

Cell culture

Human bronchial epithelial cell (BEAS-2B) were cultured in DMEM and supplemented with 10 % fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). Human lung carcinoma (A549) cells and non-small cell lung cancer cells (H1975) were cultivated in RPMI-1640, supplemented with 10 % FBS. All the cells were kept in a 37 °C incubator and aerated with 5 % CO₂.

MTT assay

BEAS-2B, A549 and H1975 cells in 96-well plates were treated with 25, 50, 100, and 200 μM AGL. After 48 h, the medium was removed and replaced with fresh medium supplemented with 100 μL of MTT (5 mg/mL) followed by another 4 h incubation. During this time, the MTT was metabolized by viable cells to form purple

formazan crystals. Then the MTT reagent was removed, and 100 μL of DMSO was added to dissolve the formazan crystals. After incubation for 15 min, the absorbance was measured at 570 nm using a microplate reader. The absorbance is directly proportional to the number of viable cells in the well.

5-Ethynyl-2'-deoxyuridine (EdU) assay

A549 or H1975 cells in 96-well plates were respectively treated with 25, 50, 100 μM AGL, and cultured for 48 h. Then, the cells were co-cultivated with 50 μM EdU for 2 h, and then fixed with 4 % paraformaldehyde, followed by 0.5 % Triton X-100 for permeabilization. The fixed cells were cultivated with Apollo staining reaction solution for 30 min followed by DAPI staining (28718-90-3, Sigma-Aldrich, Shanghai, China) for 5 min in the dark room. The washed cells were observed under a fluorescence microscope, and the captured pictures were analyzed, as well as the cell count intended for calculating the EdU/DAPI ratio.

Flow cytometry

Apoptosis was measured using an apoptosis detection kit (556547, BD Biosciences, NJ, USA) based on Annexin V-fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) according to the manufacturer's manual guides. The A549 or H1975 cells were treated with 25, 50, 100 μM AGL, stained with FITC and PI, and then analyzed using a FACS Calibur. The data was analyzed using FlowJo software.

Transwell assay

The AGL-treated A549 or H1975 cells were seeded into an invasion chamber, in which the lower chamber was provided with media containing 10 % FBS but the upper one was filled with serum-free medium. Then the cells which passed through polycarbonate membrane were stained with 0.1 % crystal violet. For each sample, the mean number of stained cells were counted from more than 10 fields.

Western blot assay

Total cellular protein was extracted using RIPA lysis buffer (89901, Thermo Scientific, Carlsbad, California, USA). The lysates were processed for immunoblot with the primary antibodies (Table 1), and then incubated with HRP-conjugated goat anti-rabbit IgG (B900210, ProteinTech Group; 1:5000). Finally, the target bands were visualized with ECL reagents (Solarbio Life Sciences, Beijing, China), and the relative intensity of each

band was quantified using ImageJ software, and normalized to β -actin.

Enzyme-linked immunosorbent assay (ELISA)

The levels of MDA and GSH was evaluated using ELISA kits of MDA (ab238537, Abcam, Cambridge, MA, USA) and GSH (MBS265674, MyBioSource Inc., San Diego, CA, USA). 100 μ L sample was added into an ELISA well and incubated for 2 h, followed by incubation with 100 μ L detected antibody for 1 h. Then each well was incubated with 100 μ L enzyme reagent, 100 μ L TMB reagent for 30 min, and terminated by 50 μ L stop solution. The absorbance value was measured at 450 nm by a microplate reader.

Indirect immunofluorescent assay (IFA)

AGL-treated A549 or H1975 cells were fixed with 4 % paraformaldehyde and permeabilized with 0.1 % Triton X-100. The cells were stained with DCFH-DA (2044-85-1, Sigma-Aldrich, Shanghai, China) and observed under a fluorescence microscope. The quantity of stained cells was calculated from at least 10 fields and normalized to control group.

Statistical analysis

Data are presented as mean \pm standard error of the mean (SE, $n = 3$), and differences between any two groups were calculated using unpaired t -tests. Multiple group comparisons were analyzed by ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

AGL inhibits the proliferation but promotes apoptosis of lung cancer cells

The structure of AGL is shown in Figure 1 A. To evaluate the effect of AGL on lung cancer cells proliferation, the viability of A549, H1975 and BEAS-2B cells were evaluated at AGL doses ranging from 0 to 200 μ M. The data showed that AGL repressed the cell proliferation of A549 and

H1975. Specifically, the reduction of proliferation was significant in the dose above 25 μ M. Although 200 μ M AGL had a better inhibitory effect on lung cancer cells, it damaged normal human lung epithelial cells. Therefore, the concentrations of 25, 50 and 100 μ M AGL were used in subsequent experiments (Figure 1 B). Moreover, EdU assay was performed on A549 and H1975 treated with AGL, which revealed that AGL at the dose of 25, 50 or 100 μ M significantly reduced the quantity of Edu positive cells, compared to the control group (Figure 1 C). Meanwhile, flow cytometry was conducted to study the apoptosis of A549 and H1975 treated with AGL. The data showed that AGL at the concentration of 25, 50 or 100 μ M significantly promoted cell apoptosis (Figure 1 D). To sum up, AGL repressed the proliferation and promoted the apoptosis of lung cancer cells.

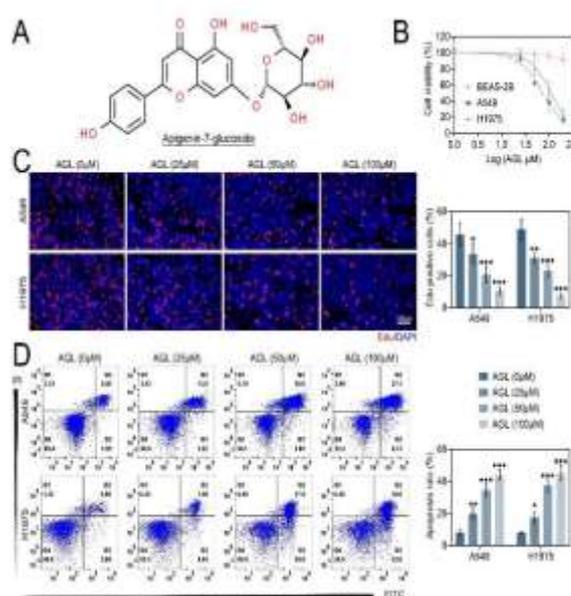


Figure 1: AGL inhibits the proliferation of lung cancer cells. (A) Chemical structure of AGL; (B): Viability curves for A549, H1975 and BEAS-2B cells treated with AGL on the dose of 0 to 200 μ M; (C): EdU assay result for A549 and H1975 treated with AGL; (D): Apoptosis of A549 and H1975 treated with AGL. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 1: Information on antibodies used

Protein	Cat no.	Manufacturer	Dilution
Cytochrome C	4280	Cell Signaling Technology, MA, USA	1:1000
p-AKT	ab38449	Abcam, Cambridge, MA, USA	1:2000
AKT	ab8805	Abcam, Cambridge, MA, USA	1:1000
p-PI3K	ab278545	Abcam, Cambridge, MA, USA	1:2000
PI3K	ab154598	Abcam, Cambridge, MA, USA	1:3000
p-mTOR	5536	Cell Signaling Technology, MA, USA	1:1000
mTOR	2983	Cell Signaling Technology, MA, USA	1:2000
β -actin	20536-1-AP	ProteinTech Group, Rosemont, IL, USA	1:5000

AGL suppressed the migration and invasion of lung cancer cells

Given that AGL inhibited the proliferation of lung cancer cells, it was necessary to investigate the effect of AGL on migration and invasion which were evaluated using Transwell assays. The data showed that 25, 50 or 100 μM AGL significantly attenuated the migration of A549 and H1975 cells than the control group. Similarly, the invasion properties of A549 and H1975 cells were weakened by AGL (Figure 2). Therefore, AGL played a key role in repressing the migration and invasion of lung cancer cells.

AGL induces the accumulation of ROS

The effect of AGL on the accumulation of ROS was investigated here. The expression of Cytochrome C was determined using western blot, and the data demonstrated that Cytochrome C significantly increased in A549 or H1975 cells treated with AGL (Figure 3 A). The MDA concentration was promoted by 25, 50 or 100 μM AGL in A549 or H1975 cells, while GSH

production was declined in A549 or H1975 cells treated with similar dose of AGL (Figure 3 B). In addition, A549 or H1975 cells were stained with DCFH-DA to determine the ROS level. The data showed that 25, 50 or 100 μM AGL treatment elevated ROS formation in lung cancer cells. Thus, AGL enhanced oxidative stress and induces ROS accumulation in lung cancer cells.

AGL inhibits PI3K/Akt/mTOR pathway

The results revealed that AGL reduced the phosphorylation levels of AKT, PI3K and mTOR. Specifically, the phosphorylation of AKT and PI3K was significantly reduced by 25, 50 or 100 μM AGL in A549 or H1975 cells. The mTOR phosphorylation declined 25, 50 or 100 μM AGL in A549 cells. Despite the fact that there is no significant reduction of mTOR phosphorylation was observed in H1975 cells treated with 25 μM AGL, 50 or 100 μM AGL decreased mTOR phosphorylation to a considerably low level (Figure 4). In summary, AGL suppressed the PI3K/Akt/mTOR pathway in lung cancer cells.

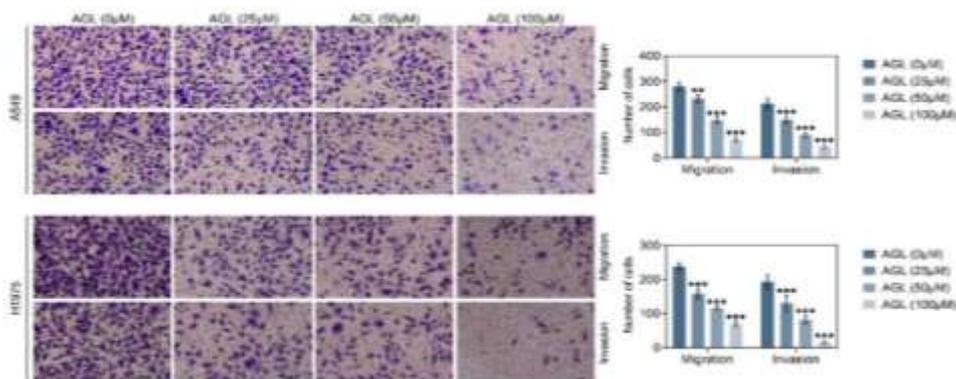


Figure 2: AGL suppresses the migration and invasion of lung cancer cells. Migration and invasion of A549 and H1975 cells treated with 25, 50 or 100 μM AGL. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$

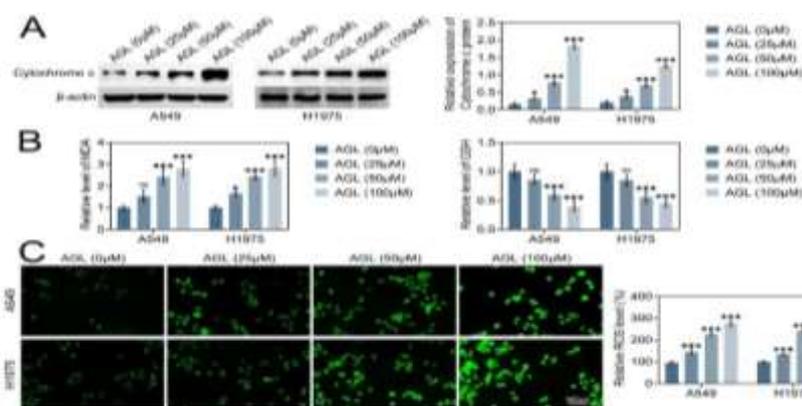


Figure 3: AGL induces ROS accumulation (A): The production of Cytochrome C was significantly increased in A549 or H1975 cells treated with AGL; (B): The level of MDA and GSH in AGL-treated A 549 or H1975 cells; (C): A549 or H1975 cells treated with 25, 50 or 100 μM AGL were stained with DCFH-DA to determine the ROS level. ns, non-significant. * $P < 0.05$, *** $p < 0.001$

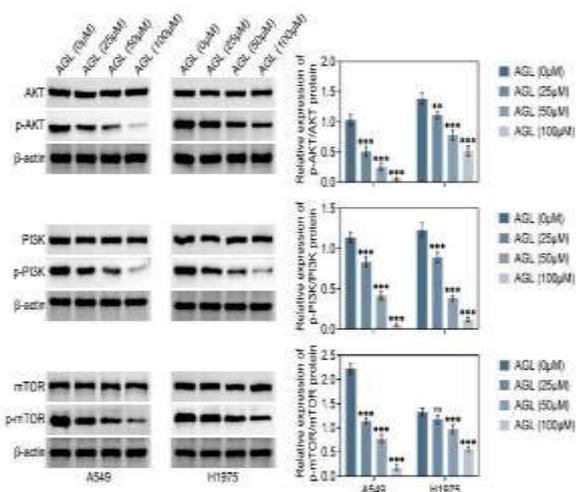


Figure 4: AGL inhibits PI3K/Akt/mTOR pathway. Western blot was performed to evaluate the phosphorylation of AKT, PI3K and mTOR in A549 or H1975 cells treated with 25, 50 and 100 μM AGL. ns, non-significant. $**P < 0.01$, $***p < 0.001$

DISCUSSION

Lung cancer is the leading cause of cancer-related death, and developing novel molecular therapies are necessary for lung cancer treatment. Studies have shown that AGL has antibacterial and non-steroidal anti-inflammatory properties [9], and it also induces the apoptosis and regulates ROS-modulated STAT3/JAK2 pathway in gastric cancer cells [10]. AGL treatment promotes the apoptosis of cervical cancer cells and inhibits cell migration through the regulation of the PTEN/PI3K/AKT pathway [11]. Moreover, Apigenin inhibits tumor angiogenesis and growth in non-small cell lung carcinoma [1]. A similar study was performed, and the cell viability and EdU incorporation analysis showed that AGL treatment represses the proliferation and the acceleration of apoptosis of A549 and H1975 cells, which is consistent with those previous findings. Thus, it is reasonable to conclude that AGL repressed proliferation and promoted the apoptosis of lung cancer cells.

Transwell assay revealed that AGL functioned in suppressing the migration and invasion of A549 and H1975 cells. It was also reported that the AGL treatment accelerated the apoptosis of cervical cancer cells and inhibited cell migration, invasion and tumor development [11]. Apigenin attenuated cell migration and diminished focal adhesion kinase (FAK) activities to prevent melanoma metastasis. It also effectively inhibited the migration of HeLa and C33A cells via the inactivation of FAK signaling pathways [12]. These studies suggested that Apigenin blocked the migration and invasion of cancer cells. It

makes sense that AGL inhibits the migration and invasion of lung cancer cells.

Multiple studies have clarified that ROS directly activates the mitochondrial permeability transition and releases cytochrome C [2,5,6], initiating the PI3K/Akt pathway [7]. Furthermore, the pro-oxidant and antioxidant effects of Apigenin in tumors effectively explains the relationship between its ROS-modulating function and its anti-cancer potential [13]. A clarification on how AGL affects ROS generation would also facilitate the discovery of the mechanism of which AGL utilizes ROS repressing proliferation. In this work, Cytochrome C expression was analyzed to indicate the accumulation of ROS. In addition, previous studies had reported that Apigenin increased the production of intracellular GSH and superoxide dismutase (SOD), enhancing the endogenous defense against oxidative stress [14]. Furthermore, the AGL treatment resulted in an increase of MDA, and a reduction of the GSH expression in injured lung cells [15]. In this section, the concentration of MDA and GSH was evaluate to indicate the ROS level [16], and DCFH-DA staining was used to confirm the generation of ROS. These results are consistent with the conclusions reported previously. Thus, it is convincing to conclude that AGL induces the accumulation of ROS in lung cancer cells.

It has been reported that apigenin attenuated PI3K/AKT signaling (PI3K, AKT, and mTOR) to promote mitochondrial-mediated apoptosis, arrest cell cycle at G2/M-phase, and inhibit migration in cervical cancer cells [14]. Furthermore, Apigenin represses proliferation and migratory properties by targeting the PI3K/Akt/mTOR pathway in Barrett esophageal adenocarcinoma cells [17]. Inflammation in infantile pneumonia was alleviated by Chrysoeriol through the inhibition of the PI3K/AKT/mTOR signaling pathway [18]. For these reasons, the phosphorylation of PI3K, AKT and mTOR was evaluated in this study, and consistently, the data revealed that AGL significantly reduced the phosphorylation of PI3K, AKT, and mTOR.

CONCLUSION

The findings of this work show that AGL inhibits proliferation and promotes apoptosis of lung cancer cells. It also suppresses migration and invasion, and induces ROS accumulation in lung cancer cells by suppressing PI3K/Akt/mTOR pathway. These findings indicate the potential of AGL as a target for drug development in lung cancer treatment.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Chen Chen and Qi Wang designed the study and carried them out; Chen Chen, Sheng Zhong, Hua Wu, Jintong Ge supervised the data collection, analyzed the data, interpreted the data, and Chen Chen and Qi Wang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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