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Dipotassium Glycyrrhizinate Inhibits Histamine-Induced Proliferation by Suppressing Cell Cycle and P38/FAK Pathway of Lung Cancer Cells In-Vitro

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Abstract: *Histamine is released from activated mast cells which have been found around tumor tissue area. The aim of this study was to examine the function of histamine on human lung cancer cell line A549 cell; to investigate the anti-histamine effects of dipotassium glycyrrhizinate (DG) on histamine mediated proliferation and migration of A549 cells and to explore the potential inhibitory mechanism of DG on lung cancer cell metastasis. The in-vitro histamine/A549 cells model was established, the cell proliferation, migration and cell cycle were examined, and the intracellular glutathione (GSH) level and signal proteins were detected. Data showed that histamine promote cell viability, proliferation and migration, while DG inhibited the histamine-induced cell proliferation and migration. DG decreased histamine-induced expressions of FAK, matrix metalloproteinases-2/9 (MMP-2/9), P38, PCNA, and Bcl-2, and DG up-regulated Bax, P53 and Becline-1 expressions against histamine's effects. DG altered the histamine-driven M-phase in cell cycle and decreased the histamine-elevated GSH content in A549 cells, leading to cell apoptosis. This study revealed that DG inhibited the histamine-induced cell proliferation and promoted cell apoptosis of A549 cells, implicating its potential therapeutic mechanisms on lung cancer cell proliferation, metastasis and development.*

Keywords: *lung cancer; histamine; dipotassium glycyrrhizinate; A549 cell*

1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide [1,2]. Metastasis is one of the most prominent features of malignant tumors; especially lung cancer metastasis is the main cause of treatment failure and patient death [3]. The proliferative activity of tumor cells is an important marker in cancer development [4]. Tumor metastasis involves a complex and multistage process termed the metastatic cascade, which requires tumor cells to detach from the primary site, intravasate, disseminate in the circulation, extravasate, adapt to the foreign microenvironment, and form organ-specific colonization [5]. The intracellular signal proteins P38, FAK and P53 play important role in regulating tumor growth and metastasis; Bax/Bcl-2 and Beclin-1 are involved in cell apoptosis and autophagy. Inhibition of cell proliferation and induction of apoptosis of cancer cells are the main therapeutic method.

Mast cell (MC) is a type of immune granulocyte, and mainly distributed in the connective tissue and mucous membrane. Mast cells have been found around tumors to shape tumor cells and the tumor microenvironment [6]. Direct cell-cell interaction or the production of mediators that either directly or indirectly promote proliferation and survival are two ways that MCs might boost the growth of cancer cell [7]. Activated MCs release the large quantities of histamine, chymase, tryptase, etc, affecting the different stages of angiogenesis, extracellular matrix (ECM) degradation, migration and proliferation of endothelial cells; involve in the generation and distribution of new blood vessels and boosting tumor growth. Histamine is one of the MC specific mediators that induces cell proliferation, increases cytokine secretion, involves in allergy and inflammation, and also induces airway mucus production, vascular permeability, and secretion of gastric acid. Histamine works through its specific receptors, such as H1R, H2R, H3R, H4R [8]. Histamine receptor links with G protein to further regulate

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intracellular signaling proteins.

Dipotassium glycyrrhizinate (DG) is a natural bioactive pentacyclic triterpene glycoside and a derivative of Glycyrrhizic acid. DG has anti-inflammatory, anti-biological oxidation effects, and is traditionally used to treat hepatitis [9,10]. Earlier studies reported that DG was able to induce nuclear and cell morphology changes in U87MG and T98G cells, leading to apoptosis caused by caspase-3 activation and DNA fragmentation [11]. It has been reported that human lung cancer cell line A549 cells express histamine receptors, H1R, H2R and H4R that medicate cancer cell growth [12], so that histamine/A549 cells model was established to study the effects of DG on lung cancer cell proliferation, migration and apoptosis, to investigate the intracellular signal pathway, and to explore the cellular mechanism and the therapeutic potential of DG.

2. Materials and methods

2.1. Histamine/A549 cells model and cell viability assay

A cell line A549, human adenocarcinoma alveolar basal epithelial cell was purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China).

As mentioned in previous study [13], cells were seeded (8×10^3 cells/well) in a 96-well plate in completed DMEM and incubated overnight at 37°C in 5% CO_2 till 75% confluence, then cells were treated with Histamine (1×10^{-3}, 1×10^{-2}, 0.1, 1mM), DG (0, 0.1, 1, 10, 25, 30, 50, 70, 100 mg/ml), the mixture of DG (25 mg/ml) with histamine (0.1 mM) respectively, in triplicates for 24 h in serum-free (SF) condition. The supernatants were collected for gelatin zymography assay; the cells were washed with PBS thrice and further incubated with MTT (5 mg/ml) for 4 h. Dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, and the OD values were read by Bio-Rad microplate reader at 570 nm. Microplate Manager6 software was used to analyze the relative growth rate (RGR%) of A549 cells in different treatment conditions relative to untreated cells (n = 3-6).

2.2. Gelatin zymography analysis

MMP-2/9 activity in cell supernatant was evaluated using gelatin zymography analysis as previously described [14]. The images were recorded by the camera (Gel Doc XR+, Bio-Rad). The intensities of the bands in gels were determined and semi-quantified using the Image Lab software (version 7.0, Bio-Rad Laboratories).

2.3. Cell cycle analysis

As mentioned in previous study [14], the cells were seeded in a 24-well plate and cultured overnight. Cells were treated when 80% confluence with Histamine (0.1 mM), DG (25 mg/mL) or the mixture of Histamine (0.1 mM) with DG (25 mg/mL) for 24h. The cells were digested with Trypsin (without EDTA), collected and washed with PBS, then stain with pyridine iodide (PI) (50 μg/mL) and RNAS A (100 mg/mL) at 4°C for 30 min in the dark. Flow cytometer (BD FACSCalibur) and ModFit LT 3.1 software package were used for cell cycle analysis.

2.4. Western blot assay

As mentioned in previous study [13], the cell lysates were made using lysis buffer containing 1 mmol/L phenylmethylsulfonyl fluoride protease inhibitor (Beyotime Biotechnology, Shanghai, China), and three cycles of the freethaw method were applied for protein extraction. Total protein content in cell lysis was determined using the BCA kit (Sigma-Aldrich). Equal proteins were loaded for separation by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 2% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20 (TPBS) at room temperature for 1 h and incubated with the corresponding antibodies (P38, P53, Bax, Bcl-2, Becline-1, PCNA, GAPDH) for 90 min. The membranes were then washed with TPBS and incubated with horseradish peroxidase-linked secondary antibody for 45 min. Enhanced chemiluminescence (PerkinElmer) was used to develop the signals. The images were captured using a ChemiDoc CRS + Molecular Imager (Bio-Rad Laboratories, USA) and quantified by Image Lab software (version 7.0, Bio-Rad Laboratories).

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2.5. Immunocytochemistry (ICC)

As mentioned in previous study [15], cells were seeded onto sterile glass coverslips (4×10^3 cells/ piece) in 24-well plate and cultured in different conditions. After 24 h, cells on coverslips were fixed with 100% chilled methanol for 30 min at 4°C; then incubated with 0.05% Triton X-100 aqueous solution for 15 min at 37°C and blocked with 3% BSA for 1 h at room temperature. The anti-FAK antibody (1:200 in PBS) were added and incubated at room temperature for 2 h; and subsequently incubated with FICT-labelled secondary antibodies (1:200) for 30 min at room temperature. After three washes with PBS, PI nucleus staining (100 μg/ml) was applied for 10 min. The coverslips were mounted with Fluorescent Mounting Medium onto glass slides and examined under a fluorescent microscope (Carl Zeiss, Germany); ZEN3.0 software was used for image analysis.

2.6. Cell migration assay

As described in previous study [15], cells were plated into 24-well plates and cultured overnight. The cell gaps were made with fine pipette tips after cell monolayer confluence, and then washed with PBS to remove the debris. The cell monolayers with gaps were treated with histamine (0.1 mM), DG (25 mg/ml), DG mixed with Histamine (25 mg/ml with 0.1 mM) in SF condition (n = 3-6). The widths of cell gaps were measured before treatment (0 h) and after treatments at time points (8, 24, 48 h); the cell-gap closure rate (%) relative to the gap at 0 h was calculated = [1 - (width of treated cell gaps at 8, 24, 48 h) / (width of cell gaps at 0 h)] × 100%. The photos were taken at each time point under an inverted microscope (× 40) (Carl Zeiss, Germany).

2.7. The levels of GSH in A549 cells

As described in previous study [16], cells were seeded in a 96-well plate in completed DMEM and incubated overnight at 37°C in 5% CO_2. untreated, His (0.1 mM), DG (25 mg/mL), His and DG (0.1 mg/mL and 25 mM). After 24 hours, the cells were collected in a 96-well plate, and the content of glutathione in the cells was detected by the glutathione fluorescence method as described previously.

3. Statistical analyses

GraphPad Prism 8.0 software was used for statistical analysis and graph. Data have been presented as the means ± standard errors of the mean (SEM). Statistically significant differences were analyzed using a two tailed Student's t-test; *$P < 0.05$ was taken as significant; **$P < 0.01$ very significant; ***$P < 0.001$ very very significant.

4. Results

4.1 DG inhibited the histamine-induced cell proliferation and MMP-2/9 activity of A549 cells

The results demonstrated that histamine increased the viability and growth of A549 cells dose-dependently (Figure 1A), and DG decreased the histamine-induced viability and growth of A549 cells (Figure 1B). After calculation by Prism, the IC_{50} of DG on A549 cells is 15 mg/mL. When A549 cells were co-incubated with the mixture of DG and histamine, the anti-histamine effects of DG can be seen in which DG inhibited the histamine-induced excessive cell proliferation (Figure 1C). The gelatin zymogram analysis illustrated the changes of MMP-2/9 activity in the supernatant of cells under various conditions. Compared with untreated cells, DG inhibited the histamine-induced expression/activity of MMP-2/9 (Figure 1D).

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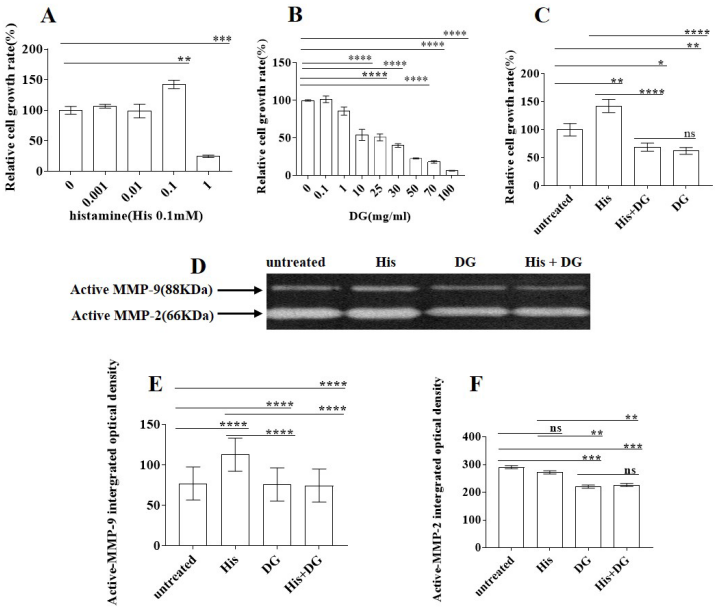

*Figure 1: Histamine (His) or dipotassium glycyrrhizinate (DG) influenced cell viability and proliferation of A549 cells. The relative growth rates of the cells treated with: (A) a range of concentrations of His; (B) a range of concentrations of DG; (C) His (0.1 mM), DG (25 mg/mL), the mixture of His with DG (His + DG, 0.1 mM and 25 mg/mL). MMP-2/9 in the supernatant of cell cultures: (D) gelatin zymography images; (E) band intensity semi-quantization of MMP-9; (F) band intensity semi-quantization of MMP-2. Data are shown as means ± SEM. *P ＜ 0.05; **P ＜ 0.01; ***P ＜ 0.001; ****P < 0.0001 (n = 3-6)*

4.2. DG reduced the histamine-upregulated cell populations at S and M phases of cell cycle

Cell cycle analysis (Figure 2) show that compared to the untreated cells, histamine decreased the cell population at G0/G1 phase by 19.58%, and increased the S phase and G2/M phase by 17.02% and by 2.55%. The cells treated with DG, the cell population at G0/G1 phase was decreased by 4.18%, S phase increased by 4.75%, G2/M phase decreased by 0.57% respectively. Compared to the histamine only treated cell, the cells after co-treatments of DG and histamine, the cell population at G0/G1 phase was increased by 10.95% leading cell arresting; the S phase and G2/M phase were both decreased by 9.84% and 1.1% resulting in the inhibition on cell cycle and proliferation (Figure 2A-E)**.**

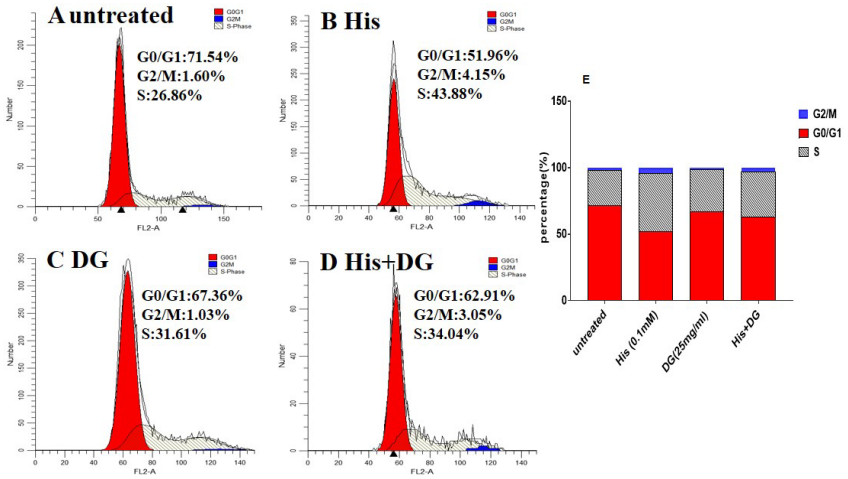

Figure 2: Anti-histamine effects of DG on cell cycle. The periodogram of A549 cells treated with (A) untreated; (B) His (0.1mM); (C) DG (25mg/mL) and (D) the mixture of His with DG (His + DG, 0.1 mM and 25 mg/mL) for 24 h; PI staining and Flow-cytometry analysis were carried out. ModFit LT 3.1 software package were used to examine the cell cycle. (E) The summary of the changes in percentage of the cells at three phases (G0/G1, S, G2/M) of cell cycle in different treatment conditions.

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4.3. Dipotassium glycyrrhizinate regulated the expressions of P38, P53, Bax, Bcl-2, Becline 1, PCNA in A549 cells against histamine's effects

The data from Western blotting (Figure 3) showed that compared to the untreated cells, histamine increased the expressions of P38, Bcl-2, PCNA and decreased P53, Bax, Becline-1 in A549 cells; while DG decreased the protein expression of P38, Bcl-2, PCNA, P53 and increased Bax, Becline-1 of A549 cells. When cells were treated with the mixture of histamine and DG, DG reduced the histamine-induced effects on cells.

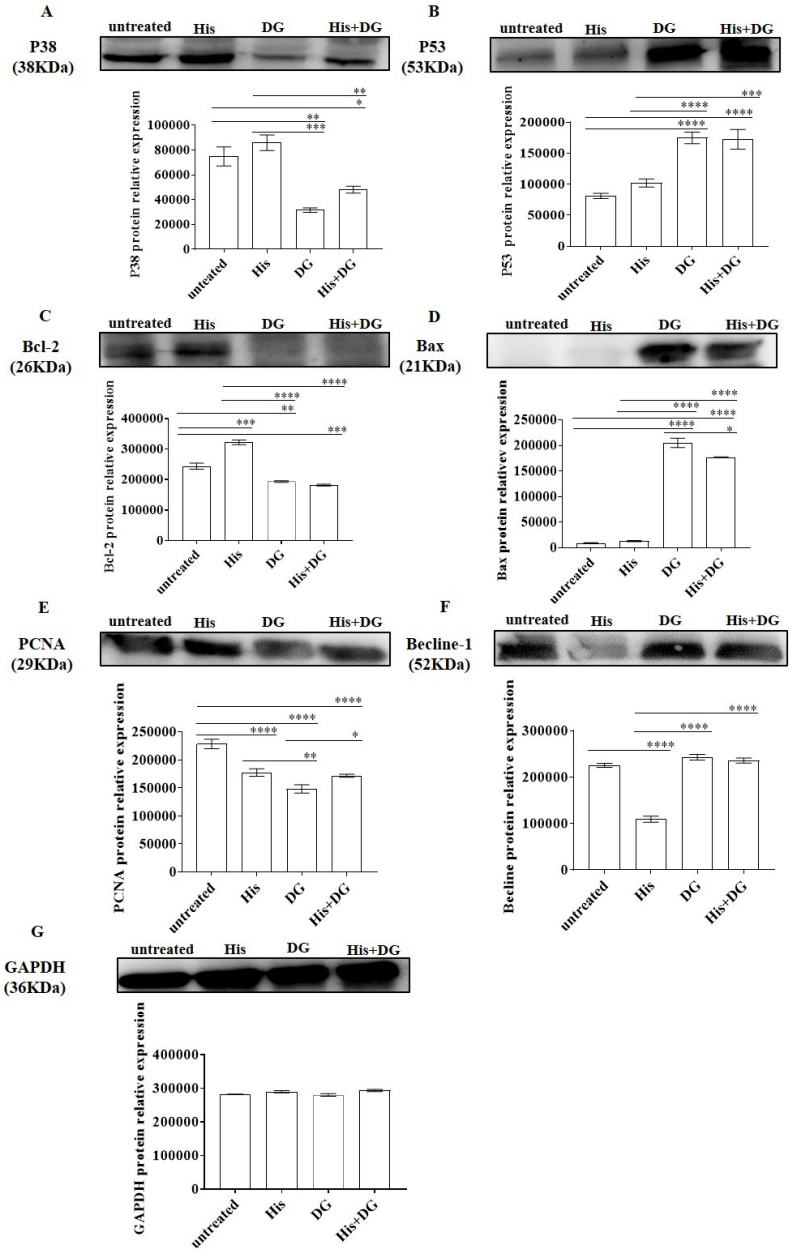

*Figure 3: Anti-histamine effects of DG on cell signaling protein expressions. Protein expressions in cell lysis samples collected from cells cultured with His (0.1 mM), DG (25 mg/ml), DG mixed with His (DG + His, 25 mg/ml and 0.1 mM) for 24 h: (A) P38, (B) P53, (C) Bcl-2, (D) Bax, (E) PCNA, (F) Becline-1 (G) GAPDH. The images were taken by Bio-Rad Laboratories and quantified by Image Lab software (Bio-Rad). Data are shown as means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 (n = 3-6)*

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4.4 Dipotassium glycyrrhizinate down-regulated the focal adhesion kinase (FAK) expression in A549 cells against histamine's effect

The data from immunocytochemistry (Figure 4) illustrated that after histamine treatment, the FAK expression of A549 cells were increased. Compared with the untreated cells, histamine treatment up-regulated and DG treatment down-regulated FAK expression. Compared to the cells treated with histamine, the cells treated with the mixture of histamine with DG, the FAK expression was reduced. However, the anti-histamine effect on FAK expression was observed in A549 cells.

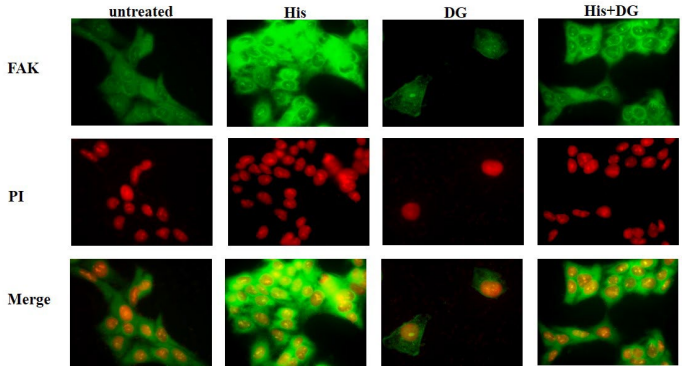

Figure 4: Histamine up-regulated and DG down-regulated focal adhesion kinase (FAK) expression of A549 cells. The A549 cells treated with His (0.1 mM), DG (25 mg/mL) and the mixture of His with DG (His + DG, 0.1 mM and 25 mg/mL) for 24h. The green fluorescence is for target protein FAK and the red fluorescence is cell nucleus staining with propidium iodide (PI). Images were taken and combined using fluorescent microscope (Carl Zeiss, ZEN-3- 0-blue-Hotfix-4, X100).

4.5 Dipotassium glycyrrhizinate inhibited the histamine-induced cell migration of A549 cells

The data from cell migration assay (Figure 5) demonstrated the promotion effect of histamine and the inhibitory effect of DG on cell migration rates respectively. DG significantly inhibited the histamine-induced migration ability of A549 cells, especially at 24 h after treatments.

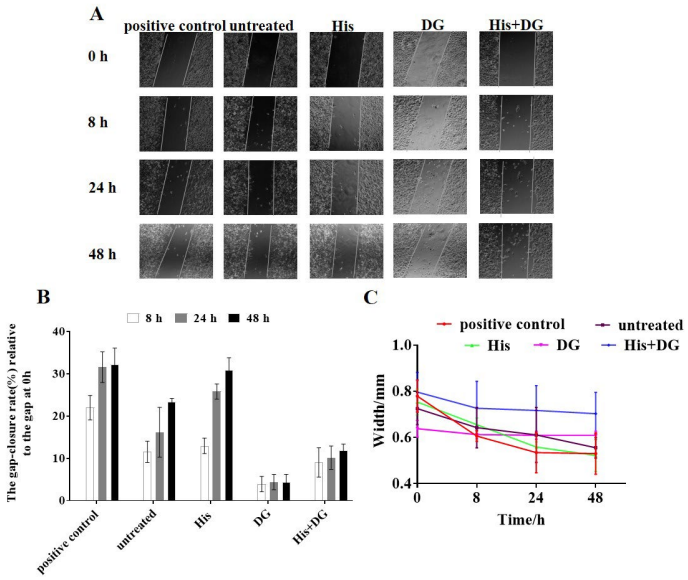

Figure 5: DG inhibited the Histamine-induced cell migration of A549 cells. A scratch-line on the cell monolayers and treated with His (0.1 mM), DG (25 mg/ml), DG mixed with His (DG + His, 25 mg/ml and 0.1 mM) in SF condition, the completed DMEM with 10% fetal calf serum was used as the positive control. At the time points: 0 h (before treatment) and after treatments for 8, 24, 48h, the widths of cell gaps were measured (n = 6), and the photos were taken with Carl Zeiss (Germany). (A) Pictures of A549 cells during the cell-gap healing period. The migration rate (%) relative to the gap width at 0 h (before treatment) was calculated. (B) the gap closure rate (%) relative to the gap at 0 h; (C) over the culture period, the changes in the width of cell gaps.

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4.6. Dipotassium glycyrrhizinate decreased the histamine-induced glutathione (GSH) content in A549 cells

The date from GSH assay (Figure 6) showed that histamine increased GSH level and that DG decreased the histamine-increased GSH level in A549 cells.

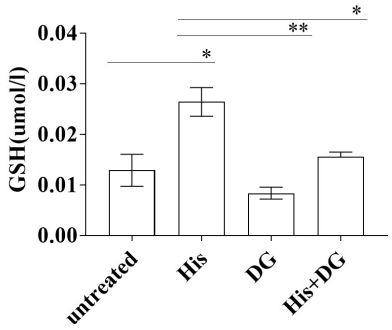

*Figure 6: DG decreased the histamine-induced GSH content in A549 cells. A549 cells were treated by His (0.1 mM), DG (25 mg/mL), the mixture of His with DG (His + DG, 0.1 mM and 25 mg/mL), after 24 hours, GSH content was measured (n = 3) and shown as means ± SEM. *P < 0.05; **P < 0.01 (n = 3-6).*

5. Discussion

The therapeutic concepts for cancer study have been considered that the cancer cell proliferation and migration are the pharmaceutical targets and that how to induced cancer cell apoptosis for cancer therapy. The present study using an histamine/A549 cell model demonstrated the simulative effects of histamine on cells proliferation, migration and cell cycle (Figure 1A, 4 and 2), which are associated with the up-regulation of MMP-2/9, P38, Bcl-2, PCNA and FAK expressions (Figure 1D, 3A, 3C, 3E and 4) and increased level of GSH (Figure 6); and the anti-histamine effects of DG were clear observed in comparison with histamine on cell proliferation, migration, cell cycle and intracellular protein expressions. Especially DG up-regulated P53, Bax and Becline-1 expressions, leading to cell apoptosis and autophagy (Figure 3B, 3D, 3F).

Almost all stimulated cellular activities, such as cellular proliferation, differentiation, and stress response, are regulated by a family of signaling proteins called mitogen-activated protein kinases (MAPKs). Many pathologies, including cancer, inflammation, developmental disorders, and neurological diseases, are associated with dysregulation of these kinases [17]. P38 is a key cellular regulator for gene expression, cytoskeleton remodeling, cell cycle and apoptosis. P53 protein is a transcription factor called "genome guardian", which plays a key role in maintaining genome integrity and cell cycle. Many of these mutant P53 proteins have oncogenic characteristics, and therefore modulate the ability of cancer cells to proliferate, escape apoptosis, invade and metastasize [18]. Proliferating Cell Nuclear Antigen（PCNA）exists in the nucleus and expressed in proliferative stage cells or tumor cells [19], its main functions are DNA replication, DNA excision repair, cell cycle control, chromatin assembly and RNA transcription [20,21]. Histamine up-regulated these factors to promote A549 cell growth and migration, and the anti-histamine effects of DG were involved in the down-regulation of P38, PCNA and up-regulation P53 expressions to inhibit cell proliferation and cell cycle progress, indicating that DG has an ability to target lung cancer cells for treatment.

Bcl-2 controls whether cell die or survive by controlling mitochondrial apoptosis pathway [22]. Bax is a pro-apoptotic protein, controlling apoptosis [23]. The over expression of Bcl-2 and the inhibition of Bax expression will lead to the imbalance of cell homeostasis, and then lead to cancer. DG depressed Bcl-2 and upregulated Bax which increased that ratio of Bax/Bcl-2, ultimately induced A549 cell apoptosis; also, DG depressed the histamine-induced cell growth leading A549 cell death. Autophagy is a molecular phenomenon used to eliminate damaged organelle and protein aggregates, which is characterized by the formation of autophagosomes and interaction with the lysosome [24]. Beclin-1 is an essential autophagy protein with a role in tumor suppression. DG up-regulated Becline-1 expression in A549 cells, indicating that the part of DG function on A549 is making tumor cell death including cell autophagy.

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MMP-2 and MMP-9 are enzymes and collectively referred to as gelatinases from MMPs family, which are associated with collagen degradation and involve in ECM remodeling by hydrolyzing ECM components [25]. MMPs play a multifaceted role in cancer cell invasion, they not only help the degradation of the surrounding ECM barrier, but also release active growth factors to promote tumor invasion, metastasis and angiogenesis [26]. The expression of MMP-2 is regulated by P38, also MMP-2 is a regulator for cell activation and proliferation. DG inhibited the histamine-induced cell proliferation and migration can be associated with inhibiting histamine-induced MMP-2/9 activity of A549.

FAK is a cytoplasmic non-receptor protein tyrosine kinase mediating cell adhesion, migration. Studies have reported that FAK-related integrin and growth factor receptors mediated cell survival and cell motility through focal adhesion complex dynamics, and that FAK affects cancer cell survival and proliferation through kinase-independent function of protein scaffolding [27]. DG significantly reduced FAK expression and inhibited histamine-induced migration of lung cancer cells are associated with depressed FAK expression, suggesting that DG function on A549 by down-regulating FAK signal which is important finding of its therapeutic mechanism.

Glutathione (GSH) is a tripeptide formed by glutamic acid, cysteine, and glycine. GSH has two forms: reduced (G-SH) and oxidized (G-S-S-G), under physiological conditions, the majority is the reduced glutathione. GSH plays an important role in the intracellular redox, which not only participates in antioxidant defense system, but also in many metabolic processes. GSH relates to the maintenance of reduced glutaredoxin or thioredoxin in DNA synthesis. In many tumor cells, the increased GSH levels are associated with cell proliferation and cell cycle progression. Excessive GSH promotes tumor progression [28,29]. Our study revealed that histamine can elevate intracellular GSH level to promote A549 proliferation. DG can decrease the histamine-induced effect on GSH in A549 cells, exhausting GSH to induce apoptosis, and possibly ferroptosis.

6. Conclusion

In summary, this study emphasizes the cellular mechanism that DG involves in A549 cells responses to histamine. The new findings in present study on suppressed P38, FAK, Bcl-2/Bax, GSH and cell cycle by DG indicate that DG might be able to impair the interaction between mast cells and lung cancer cells by inhibiting histamine effects. This study implicates the potential therapeutic mechanism of DG against lung cancer development and metastasis.

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