Original Paper

Emodin: One Main Ingredient of Shufeng Jiedu Capsule Reverses Chemoresistance of Lung Cancer Cells Through Inhibition of EMT

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Key Words

Lung cancer • Emodin • EMT • Proliferation • Apoptosis

Abstract

Background: Chemoresistance has become a an important worldwide problem to cancer treatment. Understanding the mechanism of drug resistance is the key to solve this problem and improve the survival of the patient. Doxorubicin and its analogues are widely used as antitumor drugs but many doxorubicin resistant cases have been identified in recent years. Doxorubicin (Dox) resistance is a very serious phenomenon in lung cancer treatment. As we could show previously, Shufeng Jiedu Capsule (SFJDC) can effectively reverse H69AR cells resistance to Dox, thus, the present study was designed to explore the mechanism underlying the effects of the main ingredient Emodin on chemosensitivity of H69AR cells to Dox. **Methods:** First, the growth inhibition rate of lung cancer cells and normal bronchial epithelial cells (BECs) was determined by MTT. Then, the resistance-induced epithelial-mesenchymal transition (EMT) of H69AR cells was examined by western blot and the effect of Emodin on Twist, Snail or Slug was assayed by Real-time PCR and Western blot. The activation of NF-kappa B was assayed by Western blot. Proliferation, apoptosis, migration and invasion of H69AR cells induced by Twist, Snail and Slug were also assayed by flow cytometry and transwell chamber. **Results:** The results showed that after administration of Dox (10μM) with different concentrations of Emodin, the cells exhibited a dose-dependent inhibition action to H69AR cells at 48 hours. H69AR induced the expression of Twist, Snail, and Slug when compared with Dox-sensitive H69 cells. The expression of Twist, Snail, and Slug can be effectively inhibited by combination of Dox and Emodin. The reversal of resistance was associated with the inhibition of NF-kappa B. Twist, Snail and Slug promoted proliferation, migration and invasion and inhibited apoptosis. **Conclusion:** Our data suggest that Emodin can effectively reverse the resistance of H69AR to Dox, an effect paralleled by inhibition of EMT, cell proliferation, apoptosis, migration and invasion.

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Introduction

Lung cancer is one of the most common malignant tumor in all over the world and is the leading cause of death from cancer in China [1]. Over the past 20 years, the mortality of lung cancer in all malignant tumors rises to the top. In all cases of lung cancer, small cell lung cancer (SCLC) constitutes 15% while non-small cell lung cancer (NSCLC) constitutes 85%, with adenocarcinoma comprising the major histological type. Two-thirds of patients diagnosed with lung cancer of stages III~IV have lost the timing of the radical excision. Therefore chemotherapy plays a quite important role in treatment of cancer [2]. Doxorubicin (Dox) is widely used for the treatment of SCLC as chemotherapeutic agent. However, acquired resistance develops during treatment, leading to tumor recurrence and further progression. Therefore, understand the underlying mechanism of drug resistance and increasing the sensitivity to therapeutic drugs are key steps to improve the treatment of lung cancer.

At the same time, a lot of Chinese medicine monomers and compounds have been confirmed to show advantages in prolonging the survival period and reversing lung cancer drug resistance [3-5]. Shufeng Jiedu Capsule (SFJDC) was used in experiment and found that it reverses chemoresistance of H69AR. It consists of giant knotweed, fructus forsythiae, radix isatidis, radix bupleuri, Herba Patriniae, verbena, reed root, glycyrrhiza. Previously it was mainly used for treatment of acute upper respiratory tract infection. Further, HPLC-Q-TOF-MS/MS method was used to determine 94 compounds in SFJDC, among them emodin is the main ingredient which effectively reverse H69AR cells resistance to Dox. Emodin exerts anti-tumor activity against various human cancers. Emodin induces cell cycle arrest and apoptosis in cancer cells. According to previous reports [6, 7], Emodin has been proved to have good effects in preventing tumors, however, the effects and mechanism of Emodin on the chemoresistance have not reported.

Studies have shown that drug-resistant tumor cells and epithelial-mesenchymal transition (EMT) has a direct association. EMT is biological processes in which epithelial cells lose their apico-basolateral polarity and cell-cell adhesion, resulting in the formation of migratory mesenchymal stem cells with invasive properties. The main characteristics involves loss of cell adhesion molecules (such as E-cadherin) and gain morphologically mesenchymal markers such as vimentin, Snail and Slug [8]. Thus, EMT induction in cancer cells results in the acquisition of invasive and metastatic properties in several types of cancer progression, including lung cancer, therefore, suppression of EMT could significantly inhibit metastasis and growth of lung cancer [9, 10].

H69 cell line is a drug sensitive SCLC cell line, and H69AR cell line has a multidrug resistant (MDR) phenotype by treatment of H69 cells with increasing concentration of Dox [11]. In present study, we administrated Emodin combined with Dox and observed the reversal effects of Emodin on Dox-resistant lung cancer cell line H69AR by detecting EMT-related molecules. It was found that Emodin combined with Dox has the more effective anti-cancer effects than alone on Dox-resistant small cell lung cancer cells.

Material and Methods

Cell culture

H69 cells (ATCC, Manassas, VA, USA) were grown in fetal bovine serum to a final concentration of 10%. H69AR cells were induced by increasing concentration of Dox from 1 μ M to 50 μ M and grown in RPMI 1640 medium in our lab; supplemented with 2 mM L-glutamine, 10 nM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 20% fetal bovine serum (FBS) at 37°C in 5% CO₂ and saturated humidity. Normal human bronchial epithelial cells (BECs) was obtained from Saibaikang Ltd., Shanghai, China.

MTT assay

The MTT assay is a colorimetric assay for assessing cell metabolic activity. MTT assay was used to examine cell viability. H69, H69AR and normal BECs cell lines were respectively seeded into 96-well

plates (1×10^5 cells/well) and incubated at 37°C in 95% air and 5% CO_2 . Dox (molecular weight: 579.99) was purchased from Sigma Aldrich, USA with a purity $\geq 98\%$ and prepared in a stock solution of 5mM in de-ionized water according to manufacturer's instruction. Dox was added to cells so that the final concentrations were 1, 10, 20 and $50\mu\text{M}$. Emodin (molecular weight: 270.24) were obtained from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China) with a purity of 95%-99%. Emodin solution in MeOH were added to cells to obtain the final concentrations of 1, 10, 20 and $50\mu\text{M}$. 48 h later, the cell viability was assayed by MTT. $20\mu\text{l}$ of MTT solution (5 mg/mL; Sigma, MO, USA) reacted with $100\mu\text{l}$ cultured cells for 4 h at 37°C . After removing the culture medium, $150\mu\text{L}$ DMSO was added to each well and the plates were shaken for 10 min. Then the absorbent (OD) value was measured at 490 nm. Three replicates of each sample were analyzed in each assay. The growth inhibition rate (%) was calculated as: Growth inhibition (%) = $(1 - \text{OD}_{490}) / (\text{OD}_{490} \text{ at } 0\mu\text{M}) \times 100$.

Real-time PCR

RNA was isolated from lung cancer cells by using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and cleaned by using RNeasy® MinElute™ Cleanup Kit (Qiagen, Suzhou, China). After first Strand cDNA Synthesis by using SuperScript III Reverse Transcriptase (Invitrogen, USA), real-Time PCR was performed by SYBR Green real-time PCR Kit (Takara, Dalian, China) at 95°C , 5 min, then 95°C , 15 s, 60°C , 1 min for 35 amplification cycles. The primers used in the study are 5'-TTAGGTTTAAGTTTCTTTGTCCCG-3' and

5'-CTCAGCTACGCCTTCTCGGTCT-3' for Twist-1; 5'-GTTCTACGTGTAGGCTTCGG-3' and

5'-CCCTCGTCCTTCTCCTCTACTTCAGT-3' for Snail; 5'-AAGAAAGGGTCACTAATAAAGGGG-3' and

5'-ACGGTCGTAATACACCTAAATAGCCC-3' for Slug. The ΔCt (average Ct-average Ct of Homo-18SrRNA) and $\Delta\Delta\text{Ct}$ [$\Delta\text{Ct} = \Delta\text{Ct} (\text{experimental}) - \Delta\text{Ct} (\text{control})$] were calculated. The relative expression was calculated from experimental to control as $2^{-\Delta\Delta\text{Ct}}$ [12].

Western blot analysis

Lysates of lung cancer cells (75 μg) after different treatments were quantified by spectrophotometry (Eppendorf, Hamburg, Germany) and separated on 10% SDS-PAGE and then transferred onto PVDF membrane (Dingguo, China). After membranes were blocked with 5% skim milk for 15 min, they were incubated with monoclonal mouse anti-human E-cadherin, N-cadherin, Snail, Slug and Twist-1 antibodies (R&D Biotechnology, Shanghai, China) and polyclone rabbit anti-phospho-NF- κB p65 antibody (R&D Biotechnology, Shanghai, China) at 37°C for 4 hours. Followed by three times of wash, membranes were incubated with peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:2,000; San Cruz, USA) for 1 h at room temperature. The results were then detected using an ECL chemiluminescent detection system (Dingguo, China). β -actin was used as an internal control. Developed bands were scanned and band densitometry results were calculated using AlphaEase software version 2200. All bands were run under identical conditions with the same batch of reagents and densitometry results were standardized to β -actin.

Transfection with Twist-1, Snail and Slug Chimera RNAi

Chimera RNAi for Twist-1 (H00007291-R01), Snail (H00006615-R01) and Slug (H00006591-R01) and control RNAi were purchased from Novus Canada. Cells were seeded into 24-well plates at 10^5 cells per well. 30% confluent monolayer cultures were incubated with final concentration of 5 nM Chimera RNA or Negative control using Lipofectamine RNAiMAX (QIAGEN, China) for 24 h.

Measurement of cell cycle by flow cytometry

After various treatments, lung cancer cells were fixed in cold 70% ethanol and stored at -20°C overnight. After twice washes with PBS, the fixed cells were stained in a propidium iodide solution (PI, 50 $\mu\text{g}/\text{ml}$, Sigma-Aldrich, St. Louis, MO, USA) for 1 hour, and treated with a ribonuclease A solution (20 $\mu\text{g}/\text{ml}$, Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes. Flow cytometry (BD Pharmingen, San Jose, CA, USA) was then using to examine cell cycle. Cells were labeled with $150\mu\text{l}$ of hoechst33342 and $75\mu\text{l}$ of PI for 10 min in darkness and at room temperature for apoptosis assay.

Analysis of cell migration or invasion assay

To monitor cell migration/invasion in real time, we used the transwell chambers with 24-well inserts (pore size, 8 μm ; Corning Costar, Corning, NY, USA). 10^5 cells pretreated with Twist, Snail or Slug RNAi for

8 hours were plated in the top chamber with or without Matrigel (BD Biosciences, San Jose, CA, USA) and incubated for 24 h. The cells in the top chamber were plated in medium without serum or growth factors, and medium supplemented with serum was used as a chemoattractant in the lower chamber. After 24 h of incubation, migrated or invaded cells through the pores were counted under a light microscope after crystal violet staining.

Statistical analysis

Data are expressed as means $\pm$ standard errors of the means. Analysis was performed using SPSS 15.0 for windows. Statistical significance were tested using either one-factor analysis of variance if the data consisted of a normal distribution or a rank test if the data were not consistent with a normal distribution. A *P* value of <0.05 was considered statistically significant.

Results

Emodin reverses the resistance of H69 AR cells to Dox

The chemical structure of Emodin was listed in Fig. 1A. The growth rate of H69 or H69 AR was evaluated by using MTT assay after administration of different concentrations of Dox or Emodin. The growth rate of H69 decreased by approximately 50% when treated with 1 μ M Dox and 99% with 50 μ M Dox for 48h, while growth rate of H69AR showed no inhibition and difference at different concentrations of Dox (1, 10, 20 and 50 μ M) (Fig. 1B). At the same time after the administration of different concentrations of Emodin on H69 or H69 AR, the cells exhibited a dose-dependent inhibition action. The proliferation rate decreased by 40% on H69 cells and 35 % on H69 AR cells with 50 μ M of Emodin at 48 hours (Fig. 1C). However, after administration of Dox (10 μ M) with different concentrations of Emodin, the cells exhibited a dose-dependent inhibition action to 90% at 48 hours, indicating Emodin reverses the resistance of H69 AR cells to Dox (Fig. 1D).

Resistance is related with EMT

Under microscope, H69AR cells showed obvious mesenchymal phenotype with vigorous growth when compared with its parental H69 cells (Fig. 2A). EMT is mainly characterized by the loss of epithelial markers such as E-cadherin and the acquisition of mesenchymal

Fig. 1. Emodin reverses the resistance of H69AR cells to Dox (n=4). (A) The chemical structure of Emodin. (B) The effect of Dox on H69 or H69AR cells. (C) The effect of Emodin on H69 or H69AR cells. (D) The effect of combined Emodin and Dox. Emodin exhibited a dose-dependent inhibition on H69 or H69AR cells and reversed the resistance of H69AR cells to Dox. Statistical significance was tested using one-factor analysis of variance. **P* < 0.05 and ***P* < 0.01 vs. H69 cells.

Fig. 2. EMT was assayed by western blot and real-time PCR ($n=3$, $** P < 0.01$ vs H69 cells). (A) The morphology of H69 and H69AR cells. H69AR cells showed obvious metastatic growth. (B) EMT was assayed by western blot and real-time PCR. H69AR cells showed enhanced EMT.

Fig. 3. Effects of Dox or Emodin on Twist, Snail, Slug or NF- κ B were assayed by real-time PCR (A) and Western blot (B, C) ($n=4$, $++ P < 0.01$ vs. H69 cells; $** P < 0.01$ vs. H69AR and $## P < 0.01$ vs. H69 AR+E). Emodin can effectively inhibit the expression of Twist, Snail, Slug or NF- κ B. Combination of Emodin and Dox can further inhibit the expression of Twist. Statistical significance was tested using one-factor analysis of variance.

markers such as vimentin and N-cadherin. Proteins were collected from H69 or H69AR cells and evaluated by Western blot for the expression of E-cadherin and N-cadherin. The results showed that the expression of E-cadherin was decreased and N-cadherin expression was enhanced in H69 AR cells when compared with H69 cells, indicating chemoresistance is related with EMT between H69 and H69AR (Fig. 2B).

Emodin inhibits Twist, Snail, Slug and NF- κ B

Further, we used real-time PCR to determine the effects of Emodin or Dox on the EMT-related genes and the results showed that Emodin can effectively inhibit the mRNA expression of Twist, Snail and Slug, while Dox had no influence on the expression of these molecules. Moreover, after administration of Dox (10 μ M) with Emodin (20 μ M), the mRNA expression of Twist decreased obviously at 24 hours (Fig. 3A). Results of western blot had the same tendency (Fig. 3B). Inflammation was recognized as an important trigger of EMT [13]. NF- κ B signaling is closely related with inflammation. The results of western showed

Fig. 4. Effects of Emodin on Twist, Snail and Slug in A549 and H446 cells were assayed by real-time PCR (A) and Western blot (B). Emodin can effectively inhibit the expression of Twist, Snail and Slug. Statistical significance was tested using one-factor analysis of variance. n=4, ** P < 0.01 vs. A549 or H446.

Fig. 5. Effects of Emodin on normal BECs (n=4). (A) The effect of Emodin on normal BECs. Emodin exhibited a dose-dependent inhibition on BECs. (B) Emodin inhibited the activation of NF-kappa B. Statistical significance was tested using one-factor analysis of variance. *P<0.01 versus concentration at 0 μM.

that Emodin can significantly decrease NF-kB expression, indicating Emodin inhibited EMT via inhibition of inflammatory signaling pathway (Fig. 3C).

Emodin has similar inhibitory effects on NSCLC

In order to verify the inhibitory effects of Emodin on Twist, Snail and Slug on other lung cancer strain, we used real-time PCR and western blot to determine the effects of Emodin on the Twist, Snail, and Slug in A549 cells and H446 cells. The results showed that Emodin can effectively inhibit the mRNA expression of Twist, Snail, and slug (Fig. 4A). Results of western blot had the same tendency (Fig. 4B).

Lung cancer cells is more sensitive to Emodin than normal BECs

After the administration of different concentrations of Emodin, the normal BECs also exhibited a dose-dependent cytotoxicity action. The proliferation rate decreased by 10.07 % with 20 μM of Emodin at 48 hours (Fig. 5A) while the proliferation rate decreased by 20.14 % in H69 AR cells and 31.66 % in H69 cells with 20 μM of Emodin at 48 hours (Fig. 1C), indicating that lung cancer cells are more sensitive to Emodin. By using western blot, Emodin also obviously inhibited the activation of NF-kappa B in normal BECs (P < 0.01, Fig. 5 B).

Twist, Snail and Slug promotes the metastatic phenotype of lung cancer

With the application of PI staining to detect the changes of cell cycle with or without Twist, Snail or Slug RNAi administration, we observed that the number of H69 AR cells in G0/G1 phase increased gradually with the administration of Twist, Snail or Slug RNAi, while

Fig. 6. Effects of Twist, Snail and Slug on proliferation, apoptosis, migration and invasion of H69AR cells (n=4, * $P < 0.05$ and ** $P < 0.01$ vs. Control RNAi). Twist-1, Snail or Slug promoted proliferation, migration and invasion and inhibited apoptosis. Statistical significance was tested using one-factor analysis of variance.

the number of cells in S phase decreased. This prompted tumor cells arrest in G0/G1 phase (Fig. 6).

Flow cytometry detected the apoptosis of cells as shown in Fig. 5, after pretreatment of Twist-1, Snail or Slug RNAi, the apoptosis of H69 AR showed a significant increase at 24 hours when compared with H69AR (Fig. 6A).

Migration and invasion abilities are two essential respects for cancer cell metastasis and drug resistance. To obtain further details, cell migration and invasion were measured in real time. The results showed that Twist-1, Snail or Slug RNAi inhibited the migration at 24h (Fig. 6B). In consistent with migration, the cell invasion ability also reduced at 24 h after administration of Twist-1, Snail or Slug RNAi in H69AR cells (Fig. 6B). Taken together, these data suggest that Twist, Snail and Slug may play a metastatic role in lung cancer metastasis.

Discussion

Chemotherapy represents the backbone of treatment for many cancers at different stages of the disease. However, Rapid resistance to chemotherapy and the possibility of relapse remain the main obstacle in the treatment of lung cancer. To address these limitations, many efforts are being done such as continuous development of new drugs and forms of chemotherapy [14]. Recently, natural extracts are being more interesting and important efforts as an adjuvant to chemotherapeutic drugs, because they can relieve the side effects and enhance the effects of both radiotherapy and chemotherapy [15]. Natural extracts are substance made by extracting a part of a natural raw material, often by using a solvent such as ethanol or water. Among many natural extracts, Emodin is one of compounds which is

recognized as a drug candidate for cancer therapy. Previous reports have shown that Emodin inhibits cell growth in several types of cancer cells by regulating genes related to apoptosis, oncogenesis, proliferation and cancer cell invasion and metastasis [16, 17]. In this study, we examined Emodin and Dox effects to lung cancer. The result shown that Emodin inhibited cell viability of H69AR cells in a dose-dependent manner and lung cancer cells were more sensitive to Emodin than normal BECs and also Dox below 50 μ M had no effects on H69AR cells, however, the combination of Emodin with Dox can effectively reverse the resistance.

Epithelial to mesenchymal transitions (EMT) are transdifferentiation programs that are required for tissue morphogenesis during embryonic development. The EMT process can be regulated by a diverse array of cell markers, cytokines and growth factors, such as E-cadherin, Vimentin, and TGF- β whose activities are dysregulated during malignant tumor progression. Thus, EMT induction in cancer cells results in the acquisition of invasive and metastatic properties. Recent evidence now indicates that EMT of tumor cells not only causes increased metastasis but also contributes to drug resistance and drug resistance reflect changes in the differentiation state of the tumor, which is likely to reflect EMT. For example, oxaliplatin-resistant colorectal cancer, paclitaxel-resistant ovarian cancer and gefitinib-resistant lung cancer cells are all linked with down-regulation of epithelial cells marker such as E-cadherin and up-regulation of mesenchymal cell markers such as Vimentin, fibronectin, N-cadherin, alpha-smooth muscle actin (α -SMA) [18-20]. Therefore, many reports suggested that targeting EMT is a promising therapeutic strategy, for example, Kitamura reported that inhibition of the EMT process reversed TGF- β 1-induced resistance to gefitinib in lung cancer cells [10]; Wu reported that downregulation of EMT reversed resistance in gemcitabine-resistant hepatocellular cancer cells [21]; and Haslehurst reported that knockdown of Snail or Slug reversed cisplatin resistance in ovarian cancer [22], indicating that regulation of EMT is a new way to overcome drug resistance in cancer cells and improve cancer treatment.

Many transcription factors can repress E-cadherin directly or indirectly. For example, Snail and Slug can bind to E-cadherin promoter and repress its transcription, whereas Twist repress E-cadherin indirectly [23]. In present study, we examined chemoresistance was associated with the enhanced EMT in H69AR cells. The expression levels of Twist, Snail and Slug were enhanced in H69AR. Emodin administration decreased the expression of Twist, Slug and Snail. We also observed that combination of Emodin with Dox inhibited the expression and activation of Twist-1, indicating that the inhibition of Emodin on Twist was specific association with reversal of Dox-resistance lung cancer. In order to verify the inhibitory effects of Emodin on Twist, Slug and Snail in other lung cancer cells, we used real-time PCR to determine the effects of Emodin on Twist, Snail and Slug in NSCLC A549 and H446 cells. The results showed that Emodin can effectively inhibit the mRNA expression of Twist, Snail and Slug, indicating Emodin can effectively inhibit enhanced EMT in H69AR cells.

Inflammation was recognized as an important trigger of EMT [24]. Homeobox gene, which target DNA sequence and modulate gene expression, also plays a very important regulatory role in EMT [25]. NF- κ B signaling was recognized as the important inflammatory signaling pathway. When NF- κ B is activated, the combined form of p50 and p65 enters the nucleus and then bind with DNA thereby causing the expression of various inflammatory cytokines such as IL-4, IFN- γ and IL-17 [26]. Emodin can significantly decrease NF- κ B expression, indicating Emodin inhibited EMT through inhibition of inflammatory signaling pathway.

Cell proliferation, apoptosis, migration and invasion are important processes of tumors formation. Research has proved that blocked cell apoptosis and unlimited proliferation of malignant cells are one of the important mechanisms of many diseases especially tumors. In the present study, we preliminarily examined the effects of Twist-1, Slug or Snail on proliferation, apoptosis, migration and invasion and confirmed that Twist, Slug or Snail can promote the cell proliferation, migration and invasion and inhibited cell apoptosis. Emodin may inhibit proliferation, migration and invasion and promoted apoptosis through inhibition of Twist, Slug, and Snail. However, a lot of work need to be done on the resistant mechanism of Twist, Slug, Snail and the inhibitory effects of Emodin on them.

Although the mechanisms underlying Emodin activities are still not clear, we can conclude from the present study that, in SCLC lines, i) Emodin can effectively reverse the resistance of H69AR to Dox; ii) Emodin can effectively inhibit the expression of Twist, Snail or Slug and the combination of Emodin with Dox synergistically inhibits expression of Twist-1; iii) Emodin can effectively inhibit the activation of NF- κ B.

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Disclosure Statement

The authors have declared that no competing interests exist.

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