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Original Research

Esculetin inhibits the proliferation of human lung cancer cells by targeting epithelial-to-mesenchymal transition of the cells

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Abstract: Lung cancer is characterized by high mortality and it is a serious threat to human health. At present, strategies used for treatment of lung cancer are not effective. Hence, there is need for new drugs that can effectively treat the metastatic stage of the cancer. The present study investigated the effect of esculetin on proliferation and epithelial-mesenchymal transition (EMT) of human lung cancer (A549) cells, and the underlying mechanism.Cell proliferation was assessed using MTT assay. Real-time quantitative polymerase chain reaction (qRT-PCR) and Western blotting were employed for the determination of changes in the expression levels of vimentin, Snail and E-cadherin mRNAs. Cell invasion and migration were determined using Transwell assay. The results showed that esculetin significantly and time- and dose-dependently inhibited the proliferation of A549 cells ($p < 0.05$). It also significantly and dose-dependently reduced their invasive ability ($p < 0.05$). The levels of expression of vimentin and Snail mRNAs were significantly and dose-dependently down-regulated in esculetin-treated A549 cells, when compared with the control cells ($p < 0.05$). Esculetin treatment significantly and dose-dependently upregulated the expression of E-cadherin mRNA ($p < 0.05$). These results demonstrate that esculetin effectively inhibits the proliferation of A549 cells, and regulates EMT of the cells via the down-regulation of vimentin and Snail, and up-regulation of E-cadherin expressions.

Key words: Esculetin; Lung cancer; Epithelial-mesenchymal transition; Proliferation; Expression.

Introduction

Lung cancer is one of the most common malignant tumors with high incidence of mortality in the world (1). In the last 50 years, the incidence of lung cancer has significantly increased, and the disease is more common in males than in females (2, 3). Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for about 85 % of reported cases (4). Cancer metastasis is one of the leading causes of treatment failure and death in patients with lung cancer. Chemotherapy remains the only available method for treating cancer metastasis. However, chemotherapy cannot satisfactorily improve the quality of life of sufferers, hence the need for new treatment strategies. Traditional Chinese Medicine (TCM) which employs different herbal preparations and techniques, has shown potential in the treatment of diverse diseases, (5, 6).

Qinpi is the dry branch or stem bark of osmanthus plants such as *Fraxinus rhynchop hylla* Hance, *Fraxinus chinensis* Roxb., *Fraxinus aboana* Lingelsh, and *Fraxinus stylosa* Lingelsh (7). Osmanthus is indigenous to several provinces of China such as Shaanxi, Hebei, Henan, Liaoning and Jilin. Esculetin (6, 7-dihydroxycoumarin, Fig.1), the active principle in *qinpi,* possesses varied pharmacological effects such as anti-inflammatory (8), antioxidant (9), antidiarrheal (10), antibacterial (11), and antitumor properties (12). Studies have shown that esculetin exerts significant inhibitory effect on human leukemia and liver cancer cells *in vitro* (13, 14). However, reports on the effect of esculetin on human lung cancer cells are scanty.

Epithelial-mesenchymal transition (EMT) refers to the biological process by which epithelial cells are transformed into mesenchymal phenotype cells by a specific procedure which makes epithelial-derived malignant cells acquire migratory and invasive abilities. It is a key step in the invasion and metastasis of malignant tumors (15). Studies have shown that EMT is closely associated with distant metastasis of lung cancer (16). The inhibitory effect of esculetin on the proliferation and migration of lung cancer cells has been reported, but the specific mechanism has not been fully elucidated (17). The present study investigated the effect of esculetin on proliferation and EMT of A549 cells, and the underlying mechanism.

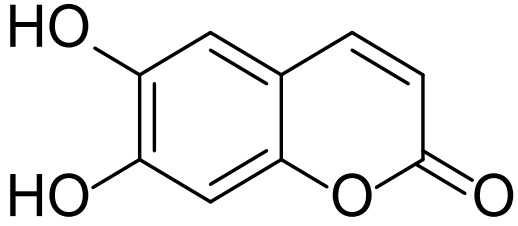

Figure 1. Chemical structure of esculetin.

Materials and Methods

Drugs and reagents

Esculetin was obtained from Chengdu Manster Biotechnology Co., Ltd., and dimethyl sulfoxide (DMSO) was purchased from Selleckchem (USA). Gli-1 primers and probes, Gli-2 primers and probes, E-cadherin primers and probes, vimentin primers and probes, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probe were products of Life Technologies (USA). Epithelial cadherin (E-cadherin), mouse anti-human monoclonal antibody, and vimentin rabbit anti-human monoclonal antibody were obtained from Abcam (USA), while GAPDH mouse anti-human monoclonal antibody was purchased from Santa Cruz (USA). Bicinchoninic acid (BCA) protein assay kit, M-PER cultured cell, and total protein extraction reagent were products of Thermo Fisher Scientific (USA). Protease inhibitor was purchased from Roche (Germany), while total RNA extraction kit was a product of Qiagen (Germany). Taqman gene expression master mix was a product of Applied Biosystems (USA). Mini-PROTEAN TGX Preform, Tris/Glycine/Sewing Buffer, Tris/Glycine Buffer and cDNA synthesis kit were obtained from Bio-Rad (USA).

Cell culture

Human lung cancer (A549) cells purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) were cultured in RPMI-1640 medium containing 10 % FBS and streptomycin (100 U/mL) at 37 ˚C in a humidified atmosphere containing 5 % CO_2 and 95 % air. Cells in logarithmic growth phase were selected and used for this study.

MTT assay

This was performed to determine the proliferative ability of the cells and their viability in the presence of esculetin. After trypsinization with 0.25 % trypsin, the cells (2×10^5 cells/well) were seeded into 96-well plates and cultured in DMEM for 24 h. Then, esculetin (0 - 40 µM) was added to the cells and incubated for 24, 36 and 48 h at 37 °C. At the end of the second day, 20 µL of 5.0 g/L MTT solution was added to the wells, followed by incubation for another 4 h. The medium was finally replaced with 150 mL of 0.1 % DMSO solution, agitated at 50 oscillations/min for 10 min, and absorbance of the sample was read in a microplate reader at 570 nm. The control cell cultures were incubated in medium containing DMSO alone. Cell proliferation was calculated as shown in Equation 1:

$$Cell\ proliferation\ (\%) = \frac{Absorbance\ of\ the\ experimental\ group \times 100}{Absorbance\ of\ the\ control\ group} \quad (1)$$

Cell invasion assay

The invasive ability of A549 cells was determined using Transwell assay. The cells (2 x 10^5 cells/mL) were placed in Transwell chamber coated with Matrigel (1:2 dilution) and cultured in 500 µL FBS serum-free RPMI-1640 medium. Then, they were incubated for 48 h with varied doses of esculetin (0 - 40 µM). Medium containing 10 % FBS was added to the lower chamber. After 24 h, the cells that passed through the matrix gel membrane were stained with crystal violet after fixation, photographed and counted using an inverted microscope. The number of transmembrane cells was counted thrice from the top, bottom, left, right and middle views of each membrane, and the mean was taken.

qRT-PCR

Real-time fluorescent quantitative PCR was used to determine the effect of esculetin on the expression levels of E-cadherin, vimentin, and Snail. Esculetin (5 and 20 µM) was added to A549 cells with 70 to 80 % fusion growth in 12-well plates and incubated for 24 h, after which the cells were washed with phosphate buffered saline (PBS). Trizol RNA extraction reagent was used to extract total RNA from A549 cells after 48 h of incubation with esculetin. Trizol reagent (1 mL) was added to the cells, followed by chloroform treatment. The resultant cell suspension was then centrifuged at 12,000 rpm for 20 min at 4 ˚C to obtain a supernatant which was treated with isopropyl alcohol and subsequently centrifuged at 12,000 rpm for 15 min at 4 ˚C. The resultant RNA pellet was dissolved in ethyl alcohol, and centrifuged as before. The products obtained were suspended in aqueous solution of diethyl pyrocarbonate. The concentration of RNA was measured using a UV-spectrophotometer. The 5X All-In-One RT MasterMix system was used for the reverse transcription of RNA into cDNA. The reactions were carried out at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. The RNA samples were maintained at a temperature of -20 ˚C. Taqman gene expression premix was used for the measurement of the levels of expression of E-cadherin, vimentin and Snail. The cycling process consisted of 95 °C for 10 sec, 60 °C for 10 sec, 72 °C for 10 sec, over a total of 40 cycles. The average threshold value for each cycle was normalized to the expression of GAPDH. Data were analysed using the 2-ΔΔCt method.

Western blotting

The expressions of EMT-related marker proteins in esculetin-treated A549 and control cells were determined using Western blotting. The cells at a density of 5 x 10^8cells/L, were incubated with 5 and 20 µM esculetin for 48 h. The cells were then washed twice with PBS, and then lysed with ice-cold radio-immunoprecipitation assay buffer (RIPA) containing protease inhibitor. The resultant lysate was centrifuged at 12, 000 rpm for 20 min at 4 °C, and the protein concentration of the supernatant was determined using BCA assay kit. A portion of total cell protein (10 µg) from each sample was separated on a 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min. Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 °C with primary antibodies of rabbit polyclonal anti-E-cadherin, Snail, vimentin and GAPDH, each at a dilution of 1 to 1000. Thereafter, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was

performed using Enhanced chemiluminescence (ECL). The respective protein expression levels were normalized to that of GAPDH which was used as a standard.

Statistical analysis

Data are expressed as mean ± SD, and the statistical analysis was performed using GraphPad Prism (7.0). Groups were compared using Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Effect of esculetin on the proliferation of A549 cells

As shown in Figure 2, esculetin significantly and time- and dose-dependently inhibited the proliferation of A549 cells ($p < 0.05$).

Effect of esculetin on A549 cell invasion

Treatment of A549 cells with esculetin significantly and dose-dependently reduced their invasive ability ($p < 0.05$; Figure 3).

Effect of esculetin on E-cadherin, vimentin and Snail mRNAs expressions

The expressions of vimentin and Snail mRNAs were significantly and dose-dependently down-regulated in esculetin-treated A549 cells, when compared with the control cells ($p < 0.05$). In contrast, esculetin treatment significantly and dose-dependently upregulated the expression of E-cadherin mRNA ($p < 0.05$). These results are shown in Figure 4.

Expression of E-cadherin protein in EMT

The results of Western blotting showed that the expression of E-cadherin protein in EMT was significantly and dose-dependently increased in esculetin-treated cells relative to the control cells ($p < 0.05$). Esculetin treatment significantly and dose-dependently down-regulated the expressions of vimentin and Snail ($p < 0.05$; Figure 5).

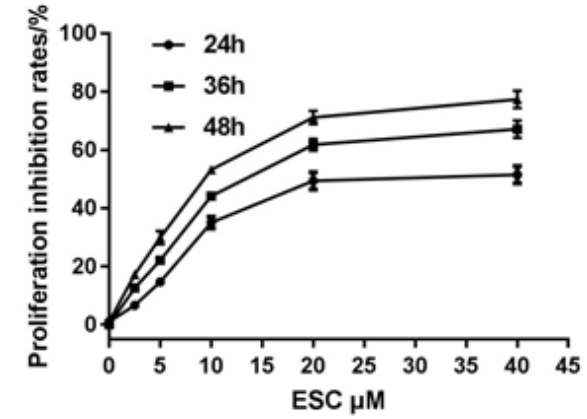

Figure 2. Inhibitory effect of esculetin on A549 cell proliferation .

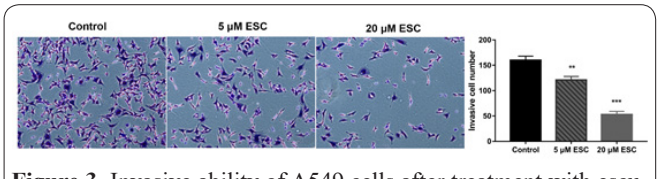

Figure 3. Invasive ability of A549 cells after treatment with esculetin. $^{**}p < 0.01$, $^{***}p < 0.001$, when compared with control cells.

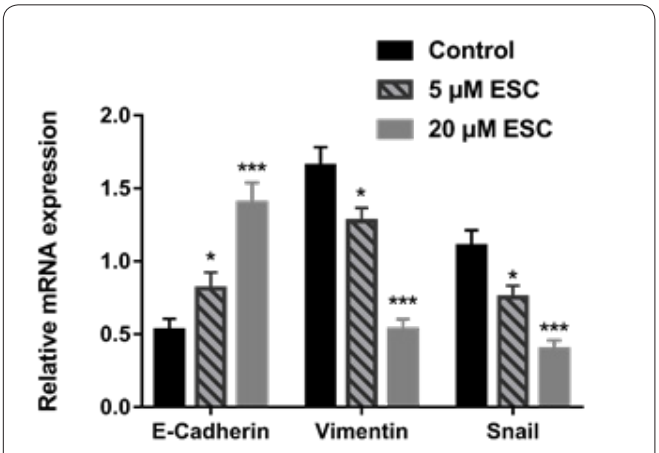

Figure 4. Expression levels of E-cadherin, vimentin and Snail mRNAs in esculetin-treated A549 and control cells. $^{*}p < 0.05$, $^{***}p < 0.001$, when compared with control cells.

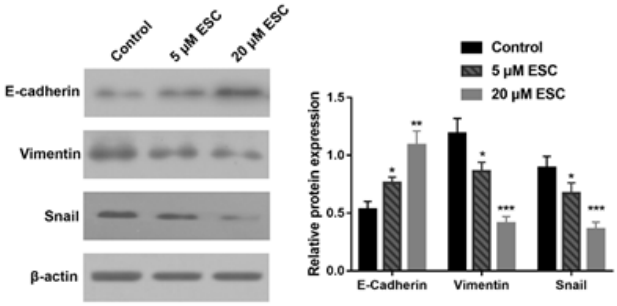

Figure 5. Effect of esculetin on the expressions of E-cadherin, vimentin and snail proteins in A549 cells. $^{*}p < 0.05$, $^{**}p < 0.01$ & $^{***}p < 0.001$, when compared with control cells.

Discussion

Lung cancer, also known as lung carcinoma, is a malignant lung tumor characterized by uncontrolled cell growth in tissues of the lung. It is one of the fastest growing malignancies in China. About 80 to 90 % of patients with lung cancer die from metastasis. Cancer metastasis is a major medical problem which has profound effect on effectiveness of chemotherapy, quality of life and prognosis of patients. Antitumor agents used in TCM have received huge attention due to their many beneficial health effects. Esculetin, the active principle in *qinpi* possesses antibacterial, anti-inflammatory, analgesic, antitumor and other pharmacological effects (18). In recent times, several attempts have been made to unravel the antitumor mechanism of this compound. The present study investigated the effect of esculetin on proliferation and EMT of A549 cells, and the underlying mechanism. The results suggest that esculetin may effectively inhibit the proliferation of A549 cells, and reduce their migratory and invasive properties.

Epithelial-mesenchymal transition (EMT), a biological process in which epithelial cells are transformed into mesenchymal cells through specific cellular processes, has received huge attention in antitumor research. Studies have shown that EMT participates in the development of tumors, and that it is closely associated with malignant behaviors of tumors such as invasion and metastasis (19). One of the EMT-specific markers is E-cadherin, and its down-regulation leads to decrease in tumor cells adhesion and enhancement of their migra-

tory ability, thereby resulting in metastasis (20). Vimentin and Snail, important markers of health of interstitial cells, change the spatial structure of cell adhesion spots, thereby affecting the adhesion and migration ability of tumor cells. Down-regulation of the expression of vimentin and snail reduces tumor cell adhesion and migratory ability (21).

The results of this study suggest that esculetin down-regulates the expressions of vimentin and Snail mRNAs, upregulates the expression of E-cadherin mRNA, and regulates EMT in lung cancer cells. Thus, it is likely that esculetin reduces the invasiveness of lung cancer cells.

Esculetin effectively inhibits the proliferation of A549 cells, and regulates EMT of the cells via the down-regulation of vimentin and Snail, and up-regulation of E-cadherin expressions.

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Conflict of Interest
There are no conflict of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Zhong Han; Hua Li, Qi Wang, Yunli Wang, Ze Xu and Zhong Han collected and analysed the data; Hua Li wrote the text and all authors have read and approved the text prior to publication.

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