Effect of Icaritin on non-small cell lung cancer NCI-H1299 cells

Lixia Hou1,2, Xinru Feng1,2, Bo Xiao1,2, Dong Yao3,4,5,*

1Key Laboratory of Respiratory Diseases, Affiliated Hospital of Guilin Medical University, Guilin, 541004, China
2Key Laboratory of Basic Research on Respiratory Diseases, Guangxi Health Commission, Affiliated Hospital of Guilin Medical University, Guilin, 541004, China
3Department of Respiratory and Critical Care Medicine, The Second Affiliated Hospital of Guilin Medical University, Guilin, 541199, China
4Guangxi Clinical Research Center for Diabetes and Metabolic Diseases, Guangxi Health Commission Key Laboratory of Glucose and Lipid Metabolism Disorders, The Second Affiliated Hospital of Guilin Medical University, Guilin, 541199, China
5Guangxi Key Laboratory of Metabolic Reprogramming and Intelligent Medical Engineering for Chronic Diseases, The Key Laboratory of Respiratory Diseases, Education Department of Guangxi Zhuang Autonomous Region, Guilin Medical University, Guilin, 541000, China

*Corresponding author

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Abstract: Chinese herbal medicine has indeed been used for centuries in China as a treatment for various ailments, including lung cancer. The use of Chinese herbal medicine in cancer treatment is often based on traditional Chinese medicine (TCM) principles, which emphasize restoring balance and harmony within the body. Icaritin is a natural compound extracted from plants of the Epimedium genus, the anticancer activity of which has been extensively studied. However, the anti-tumor effect of icaritin on the non-small cell lung cancer cell line NCI-H1299, which lacks the P53 gene, has not been reported. In this study, we evaluated the anticancer effect of icaritin on NCI-H1299 cells by treating these cells with different concentrations of icaritin. We find that icaritin significantly induces apoptosis and autophagy and inhibits proliferation and migration, icaritin induced autophagy in H1299 cells. These results indicate that icaritin is a potential therapeutic drug for patients with lung cancer with P53 gene deletion.

1. Introduction

Lung cancer is the most common cause of cancer-related deaths worldwide[1]. Fewer than 7% of patients survive 10 years following diagnosis across all stages of lung cancer. Diagnosis at a late disease stage and lack of effective and personalized medicine reflect the need for a better
understanding of the mechanisms that underlie lung cancer progression[2]. Therefore, the discovery of effective therapeutics is an urgent need for patients with non-small cell lung cancer.

Icaritin is the hydrolytic form of icariin, which may be isolated from plants of the genus Epimedium; this compound exerts an anti-tumor effect in a variety of tumor types including lung cancer[3], breast cancer[4], prostate cancer[5], acute myeloid leukemia, and liver cancer[6,7]. The anti-tumor mechanism of icaritin primarily involves promoting tumor cell apoptosis and necrosis[8, 9], inducing cell cycle arrest[10], inhibiting tumor angiogenesis[11,12], and interfering with tumor-related signaling pathways, such as the MAPK/ERK/JNK and PTEN/Akt/HIF-1α pathways[13, 14].

In this article, we evaluated the proliferation, apoptosis, migration, and autophagy in human lung cancer NCI-H1299 cells after icaritin treatment. These results highlight a potential strategy for the treatment of lung cancers in which the P53 gene is deleted.

2. Materials and Methods

2.1 Reagents

Icaritin with purity exceeding 99% was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. Cell Counting Kit-8 (CCK8) was purchased from Dojindo Chemical Technology Co., Ltd. Propidium iodide (PI), BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488, and the BCA Protein Assay Kit were purchased from Beyotime Biotechnology. The FITC Annexin V Apoptosis Detection Kit was purchased from BD Biosciences (USA). Primary antibodies against β-actin and cyclin E were purchased from Santa Cruz Biotechnology, whereas those against GAPDH, LC3 and cleaved caspase-3 were obtained from Cell Signaling Technology (Boston, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary anti-rabbit and anti-mouse antibodies were obtained from Zhongshan Golden Bridge Biotechnology (Beijing).

2.2 Cell culture

The NSCLC cell line NCI-H1299 was purchased from the Chinese Academy of Sciences Shanghai Cell Bank. Cells were cultured in RPMI-1640 complete culture medium (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (Gemini, USA) in a humidified atmosphere of 5% CO₂ at 37°C.

2.3 CCK8 assay

A CCK8 assay was used to detect cell proliferation. Cells were seeded in 96-well plates at a density of 5 × 10³ cells per well, and after 24 h, different concentrations of icaritin (5, 10, 20, 40, 60, 80, and 100 μM) were added to the cells, which were cultured for 24, 48, or 72 h. Then, 10 μL of CCK8 solution was added to the cultures, which were further incubated at 37°C for 2 h. The absorbance was measured at 450 nm using a standard instrument. Each group was tested in triplicate.

2.4 EdU staining assay

EdU was used to detect cell proliferation. Cells were seeded on sterile coverslips in 24-well plates at a density of 1 × 10⁴ cells per well and were incubated with different concentrations of icaritin (5, 10, 20, 40, 60, 80, and 100 μM) for 24, 48, or 72 h. Then, a cell proliferation analysis
was performed using a BeyoClick™ EdU-488 Cell Proliferation Detection Kit (C0071S, Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. First, the cells were incubated with 10-μM EdU working solution for 2 h for labeling. Second, the cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. Third, the cells were permeabilized via incubation in 0.3% TritonX-100 for 15 min; after which, the click reaction solution was prepared sequentially and added to the cells, which were then incubated at room temperature for 30 min in the dark. The cells were darkened with Hoechst 33342 for 10 min to stain the nucleus, and the EdU+ cells were determined using fluorescence microscopy (Olympus BX53, Tokyo, Japan).

2.5 Cell cycle analysis

H1299 cells were seeded in 6-well plates at a density of 3 × 10⁵ cells per well and cultured for 24 h at 37°C with 5% CO₂. The cells were then treated with icaritin at different concentrations for 48 h. Next, the cells were collected and washed in ice-cold PBS and then were resuspended in PBS; 70% precooled ethanol was rapidly added and mixed by pipetting; after which, the suspension was incubated overnight at 4°C. The PI staining solution containing RNase A was incubated with the cells for 15 min at 37°C. The cell cycle of H1299 cells was detected using flow cytometry (FACSCalibur™; BD Biosciences, Franklin Lakes, NJ, USA), and the data were analyzed with FlowJo V10 software.

2.6 Apoptosis assay

Apoptosis was analyzed using a FITC Annexin V Apoptosis Detection Kit I (BD Biosciences). According to the manufacturer's protocol, collection of cells in each tube were co-stained with 5 μL FITC Annexin V and 5 μL PI for 15 min in the dark. Then, 200 μL 1× binding buffer was added, and flow cytometry was used to detect the stained cells. The data were analyzed using FlowJo V10.

2.7 Wound healing assay

H1299 cells were grown to confluence in 6-well plates for 24 h and then wounded using a sterile 200-μL pipette tip in the middle of each well. After wounding, cells were washed three times with serum-free media before the addition of icaritin diluted in serum-free media. Wounds were then imaged at 0, 6, 12, and 24 h. The data were analyzed using ImageJ.

2.8 Immunofluorescence

After the cells were treated with icaritin, they were fixed in 4% PFA at room temperature for 30 min. The slides were then washed in PBS and permeabilized with 0.2% TritonX-100 at room temperature for 20 min. After three washes in PBS, nonspecific proteins were blocked with 1% BSA at room temperature for 1 h; after which, the slides were incubated with the primary antibody overnight at 4°C. Subsequently, the slides were washed and incubated with the secondary antibody for 1 h in the dark at room temperature. Last, the slides were washed and mounted with anti-fluorescence quenching mounting tablets and observed under a fluorescence microscope.

2.9 Western blot assay

The cells were lysed in RIPA lysis buffer (Beyotime Biotechnology) and incubated on ice for 30 min. Each sample tube was centrifuged at 12,000 rpm for 30 min at 4°C. The supernatants were
collected and quantified using a BCA Protein Assay Kit (Beyotime Biotechnology). Then, the supernatants were mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and boiled at 99°C for 5 min. Each protein was separated by 8%–12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk powder, the membranes were incubated with specific primary antibodies against β-actin, cyclin E, LC3, and cleaved caspase-3 overnight at 4°C. This was followed by incubation with HRP-conjugated secondary antibodies for 1 h at room temperature. All signals were visualized via electrochemiluminescence.

2.10 Statistical analysis

All data are represented as the mean ± standard deviation. SPSS 22.0 software was used for the statistical analysis. Measurement data were expressed as x ± s. One-way analysis of variance was used for comparisons of multiple groups. Further pairwise comparison was performed using the LSD-t method. Values of *P < 0.05 were considered statistically significant.

3. Results

3.1 Icaritin inhibited NCI-H1299 cell proliferation

(A) OD value of NCI-H1299 cells treated with icaritin (0, 5, 10, 20, 40, 60, 80, and 100 μM) for 24, 48, or 72 h. (B) EdU staining was performed after treatment with different concentrations of icaritin for 24, 48, or 72 h. (C) The proportion of proliferating cells was calculated in (B) *P < 0.05, **P < 0.01 compared with 0 μM icaritin.

Figure 1: Icaritin inhibits the proliferation of H1299 cells
The NSCLC cell line H1299 was used to evaluate the potential therapeutic effect of icaritin on NSCLC. The proliferation of H1299 cells treated with different concentrations of icaritin was detected using CCK8 assay. As shown in Figure 1A, icaritin inhibited the proliferation of H1299 cells in a dose-dependent manner (*P < 0.05, **P < 0.01). The inhibition rate was over 40% after treatment with 20-μM icaritin for 48 h. To further confirm the inhibitory effect of icaritin on the proliferation of H1299 cells, proliferating cells were detected via EdU staining. As shown in Figure 1B and 1C, after treatment for 48 h with doses of 20, 40, and 80 μM, the number of proliferating cells decreased by 26.7%, 36.6%, and 54.5%, respectively (**P < 0.01). These results indicated that icaritin inhibited H1299 cell proliferation. Because a high concentration of icaritin (≥100 μM) may induce cytotoxicity, we primarily analyzed four concentrations of icaritin in media.

3.2 Icaritin interrupted the cell cycle of NCI-H1299 cells

The cell cycle status was determined using PI staining and flow cytometry. As shown in Figure 2, after treatment with icaritin at doses of 0, 10, 20, 40, and 80 μM for 48 h, the percentages of cells in the S phase were 9.19%, 9.75%, 13.9%, 18.6%, and 20.5%, respectively. After treatment with 40-μM icaritin for 48 h, the percentage of cells in the S phase increased from 9.2% to 18.6% (*P < 0.05), whereas the percentage of cells in the G0/G1 phase decreased from 68.2% to 56.7% (**P < 0.01). These results indicated that icaritin caused cell cycle arrest in the S phase and revealed certain concentration dependence.

(A) Cells were treated with icaritin (0, 10, 20, 40, and 80 μM) for 48 h, and the cell cycle distributions were analyzed using flow cytometry. *P < 0.05, **P < 0.01 compared with 0 μM icaritin. (B) Cell cycle ratio of H1299 cells after icaritin treatment. *P < 0.05, **P < 0.01 compared with 0 μM icaritin.

Figure 2: Icaritin blocks S phase of the cell cycle in H1299 cells.
3.3 Icaritin induced apoptosis in NCI-H1299 cells

(A) Cells were treated with different concentrations of icaritin for 48 h, and apoptosis was analyzed using flow cytometry after staining with FITC and PI. (B) The apoptosis ratio of H1299 cells after icaritin treatment. *P < 0.05, **P < 0.01 compared with 0 μM icaritin.

Figure 3: Apoptosis of H1299 cells after icaritin treatment.

To evaluate whether icaritin affects apoptosis in H1299 cells, apoptosis was detected using flow cytometry after FITC Annexin V/PI double staining. As shown in Figure 3, icaritin induced apoptosis in H1299 cells in a dose-dependent manner. (*P < 0.05, **P < 0.01). Compared with that of the control group, the apoptosis rate was 4.27%, 4.31%, 5.05%, 8.02%, and 27.68% after treatment with icaritin at doses of 0, 10, 20, 40, and 80 μM, respectively. When treated with icaritin at concentrations of 40 and 80 μM, the apoptosis rate was significantly increased, and the difference was statistically significant.

3.4 Icaritin suppressed the migration of NCI-H1299 cells

(A) Cells were treated with different concentrations of icaritin for 6, 12, or 24 h, and cell migration was measured using scratch assay. (B) The cell migration ratio of H1299 cells after icaritin treatment. *P < 0.05, **P < 0.01 compared with 0 μM icaritin.

Figure 4: Icaritin inhibits the migration of H1299 cells.
A cell scratch test was used to detect cell migration ability. As shown in Figure 4, icaritin inhibited the migration of H1299 cells in a dose-dependent manner (*P < 0.05, **P < 0.01). The migration of H1299 cells was inhibited by approximately 40% after treatment with 20 μM icaritin for 24 h. The results showed that icaritin inhibited H1299 cell migration and that this inhibition was concentration dependent.

3.5 Icaritin altered the expression of proliferation-, apoptosis-, and autophagy-related proteins

Cells were treated with different concentrations of icaritin for 48 h, and autophagy was detected using immunofluorescence.

Figure 5: Icaritin promotes autophagy of H1299 cells.

(A) The expression of LC3 II, cleaved caspase-3, and cyclin E proteins was analyzed using western blot. (B) Graphic representation of LC3 II, cleaved caspase-3 and cyclin E proteins expression in five groups as indicated. **P < 0.01 compared with 0 μM icaritin.

Figure 6: Effects of icaritin on the expression levels of proteins involved in proliferation, apoptosis, and autophagy. Cells were treated with different concentrations of icaritin for 48 h.
Autophagy is a conserved evolutionary process that is involved in many other important physiological processes in cells, such as apoptosis and proliferation. Autophagy exerts a dual effect on apoptosis. We detected the effect of icaritin on H1299 cell autophagy. After treatment of H1299 cells with icaritin for 48 h, the autophagy marker LC3 II was detected via fluorescence microscopy, and we found that the fluorescence intensity of LC3 II increased as the icaritin concentration increased (Figure 5). Furthermore, western blot analysis showed that the protein expression level of LC3 II increased after icaritin treatment in a dose-dependent manner (Figure 6). As cyclin E is involved in the S phase cell cycle progression and cleaved caspase-3 functions as a pro-apoptotic protein, we examined the expression of these two proteins. As shown in Figure 6, the expression level of cyclin E was significantly reduced, whereas that of cleaved caspase-3 was significantly increased in a dose-dependent manner after icaritin treatment.

3.6 Icaritin promoted autophagy flux

As the expression of LC3 II protein increased significantly as the icaritin concentration increased to 20, 40, and 80 μM compared with that of the control group, Bafilomycin A1 was added to detect whether the autophagy flux was normal. Bafilomycin A1 was added at a final concentration of 100 nM 4 h before cell harvest. We found that the LC3 II expression in the icaritin + Bafilomycin A1 group was increased compared with that in the icaritin-alone group (Figure 7). This result indicated that the autophagy flux in H1299 cells was normal after treatment with icaritin, and the increase in LC3 II protein indicated that icaritin promoted the formation of autophagosomes in H1299 cells.

Cells were treated with different concentrations of icaritin for 48 h. The autophagy inhibitor Bafilomycin A1 at a final concentration of 100 nM was added 4 h before harvest. (A) The expression of LC3 II was analyzed using western blot. (B) Graphic representation of LC3 II proteins expression in eight groups as indicated.

Figure 7: Effects of icaritin on autophagy flux.

4. Discussion

Chinese medicine, as a natural anti-tumor therapy, has a long history in China, and its role in inhibiting the growth of tumor cells is receiving increasing attention. Chinese medicines contain a variety of anti-tumor components, such as polysaccharides, alkaloids, flavonoids, and so on, which can inhibit the DNA synthesis of tumor cells, block the cell cycle, and reduce the growth rate of tumor cells. Flavonoids are a class of components with anti-tumor effects in traditional Chinese medicine, which are widely found in plants and have a variety of biological activities such as antioxidant, anti-inflammatory and anti-tumor. The flavonoid icaritin studied in this paper is a
monomer compound obtained by extraction, isolation, purification and enzymolysis from the natural medicinal plant epimedium. Icaritin, a natural anti-tumor compound, exerts an anti-tumor effect by inhibiting tumor cell proliferation and promoting apoptosis.

In recent years, many natural compounds have attracted substantial attention for the prevention and treatment of cancer, especially lung cancer. Icaritin has been shown to inhibit proliferation and induce apoptosis in many types of cancer cells[15]. In the current study, we first examined the effect of icaritin on H1299 cell proliferation using CCK8 and EdU assays. We found that icaritin inhibits H1299 cell proliferation dose dependently and time dependently. Flow cytometry analysis revealed that icaritin significantly induces cell cycle arrest at the S phase and promotes apoptosis in H1299 cells after 48 h of icaritin treatment. In addition, a cell wound scratch assay showed that icaritin inhibits the migration of H1299 cells in a dose-dependent and time-dependent manner. These results imply that icaritin may be beneficial in the treatment of lung cancer.

Autophagy is an important intracellular homeostatic mechanism due to its degradation of cytoplasmic waste components via acidic vesicles[16]. However, excessive activation of autophagy may induce autophagic death of tumor cells[17]. Furthermore, studies on cells with multidrug resistance have shown that anticancer drugs that induce autophagy may also activate apoptotic signaling pathways[18]. In this study, expression of the autophagy marker protein LC3-II was detected via immunofluorescence staining and western blot. We found that icaritin promotes LC3-II expression and that after addition of the autophagy flux inhibitor Bafilomycin A1, LC3-II protein expression was significantly increased. These results indicate that icaritin promotes autophagy in H1299 cells. However, the way in which autophagy influences the anticancer effects of icaritin as well as the relationship between autophagy and apoptosis in H1299 cells remains to be further elucidated.

In this study, we first demonstrated that icaritin inhibits proliferation and migration, while induces apoptosis and autophagy in NCI-H1299 cells. Therefore, icaritin may be an effective drug for the treatment of NSCLC.

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References


