

Review Article

Ursolic acid induces apoptosis of lung cancer cells by regulating miR-21/KLF6 axis

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Abstract: Background: Ursolic acid (UA) is considered a potential drug for the treatment of carcinoma due to its ability to inhibit the growth of carcinoma cells. However, the antitumor mechanism of UA has not been clarified. Objective: To understand the effects of UA on the biological behavior of lung cancer (LC) cells and its related mechanisms. Methods: Human LC cells were placed in a culture medium containing multiple incubation concentrations of UA at 0, 5, 10, 20 and 40 μ M, and miRNA-21 (miR-21) and Kruppel-like factor 6 (KLF6) over-expression or inhibition vectors were transfected into LC cells. CCK-8, Transwell and flow cytometry were used to detect the changes in cell behavior after treatment. The relationship of miR-21 with KLF6 was analyzed by dual luciferase reports. Results: UA could inhibit the growth and invasion of LC cells and promote apoptosis. In addition, UA could also reduce miR-21 in LC cells. Down-regulation of miR-21 or increase of KLF6 could inhibit the proliferation, invasion and promote apoptosis of LC cells. miR-21 could negatively regulate KLF6. Both increasing miR-21 and inhibiting KLF6 could reduce the influence of UA on the biological behavior of LC cells. Conclusion: UA plays an anti-cancer role in LCs by regulating the miR-21/KLF6 axis.

Keywords: Ursolic acid, lung cancer, miR-21, KLF6

Introduction

LC is one of the major malignant tumor types leading to carcinoma related death in patients [1]. At present, surgery is the only way to cure LC. Due to the absence of obvious specific clinical manifestations in the early stages of LC and the characteristics of high invasion and metastasis of LC, about 78% of patients are in the middle and late stages when they are diagnosed, thus being past the best point for surgery, therefore resulting in high mortality rates of patients [2-4]. As such, it is urgent to find potential therapeutic drugs and early molecular targets for improving the prognosis of patients.

Ursolic acid (UA) is a pentacyclic triterpene compound, which widely exists in plants such as rosemary and holy basil [5]. UA not only has anti-inflammatory, antioxidant and anti-angiogenic effects, but it also has anti-cancer effects [6]. Previous studies have found that UA can effectively inhibit the growth of carcinoma cells

such as in LC, breast cancer and liver cancer [7-9]. Therefore, UA is considered a potential drug for the treatment of carcinoma. However, the mechanism by which UA regulates the biological behavior of LC cells has not been clarified. According to previous data, UA can play an anti-cancer role in tumors by regulating miRNA (miR). For example, UA can inhibit the growth of cancer cells by up-regulating miR-5400 expression in colorectal cancer [10]. In gastric cancer, UA can suppress the growth and metastasis of cancer cells by up-regulating miR-133a [11]. miR is a kind of endogenous non-coding small RNA molecule, and it can regulate the expression of human genes by suppressing mRNA translation or inducing mRNA degradation [12, 13]. Abnormal expression of miR has been found in a variety of human cancers, and the abnormal expression of miR is often closely associated with tumor growth [14, 15]. miR-21 is an important member of the miR family. Previous studies have found that miR-21 is up-regulated in LC and exists as a tumor promoter

[16]. Therefore, we have suspected that UA can play an anti-cancer role in LC by regulating miR-21.

This research was mainly designed to evaluate the effect of UA on the biological behavior of LC cells and explore whether UA plays its role by regulating miR-21.

Materials and methods

Cell processing

UA treatment: The human LC cell line A549 was purchased from ATCC cell bank and was placed in a DMEM medium containing 10% PBS and cultured at 37°C and 5% CO₂. The cells were adjusted to 2×10^5 /well and inoculated into 6-well plates overnight in an incubator at 37°C. The cell density was adjusted to 1×10^5 cells/mL. Next, 100 µL of cell suspension was inoculated into 96-well plates and cultured for 24 hours. UA (Sigma, USA) with concentrations of 0, 5 µM, 10 µM, 20 µM and 40 µM was added respectively and cultured for 24 hours. **Cell grouping:** A549 cells in a logarithmic growth phase were divided into a miR-21-inhibitor group, miR-NC, sh-KLF6, sh-NC, UA+miR-21-mimics, UA+miR-NC, UA+si-KLF6 and UA+sh-NC groups. pcDNA 3.1 plasmid was used as vector to establish miR-21 and KLF6 over-expression or inhibition plasmids respectively, and Lipofectamine™ 2000 kit (Thermo Scientific, USA) was used to transfect cells. UA+miR-21-mimics, UA+miR-NC, UA+si-KLF6 and UA+sh-NC groups were transfected for 24 hours, and then 20 µM was added for further culture.

Detection of qRT-PCR (qPCR)

Total RNA was obtained from cells in accordance with the instructions of the TRIzol kit (Invitrogen, USA). Next, the purity, concentration and integrity of total RNA were determined by UV spectrophotometer and agarose gel electrophoresis, respectively. The qualified total RNA was obtained and the reverse transcription kit (Invitrogen, USA) was used for reverse transcription. The amplification was performed with SYBR_Premix ExTaq II (Takara, China). Amplification system was as follows: SYBR Premix Ex Taq II (2X) 10 µL, cDNA 2 µL, each 0.8 µL of upstream and downstream primers, and then Sterile purified water was supplemented to 20 µL. Amplification conditions are

as follow: pre-degeneration at 95°C for 30 s, degeneration at 95°C for 5 s, annealing and extension at 60°C for 30 s, with a total of 40 cycles. U6 was used as the internal reference. 2-ΔΔct method was used to analyze the data [17].

Detection of Western blot

Total proteins were extracted from cells by RIPA lysis. BCA was used to test protein concentration. Next, it was separated by SDS-PAGE electrophoresis, and then transferred to a PVDF membrane. Then, the membrane was stained with ponceau working solution, soaked in PBST for 5 min, rinsed and sealed with 5% skim milk powder solution for 2 h. Then, KLF6 (1:1000) and β-catenin (1:1000) primary antibody (Abcam, USA) were added and placed in a refrigerator at 4°C to bind overnight. The membrane was washed with PBST for 3 times. Then goat anti-rabbit second antibody (1:20000) (Abcam, USA) was added, cultivated at room temperature for 1 h, and washed 3 times with PBST. It was developed in a darkroom. ECL was used to illuminate and develop the bands. The protein bands were scanned. The protein expression was measured and calculated using Quantity One.

CCK-8 proliferation detection

Cell proliferation was tested in accordance with the CCK-8 kit instructions (Beyotime Institute of Biotechnology, China). The details are as follows: After transfection for 24 hours, cells were cultured in 96-well plates at a cell density of 2.5×10^3 per well. CCK-8 (10 µl) was added to the wells at 24 h, 48 h, 72 h and 96 h respectively. The plates were incubated at room temperature for 2 hours, the absorbance of each well was detected at 490 nm by using enzyme-labeling instrument (USA) and the corresponding growth curve was drawn.

Detection of cell invasion

Transwell kit (Gibco, USA) was used for invasion detection. Cells in each group were adjusted to 5×10^4 cells, inoculated in a 6-well plate, washed with PBS and inoculated in the upper chamber. Next, 200 µL of 500 ml of DMEM without and with 20% FBS, were put in the upper chamber and the lower chamber respectively, and cultured at 37°C for 48 h. Cells and

substrates that did not pass through the membrane surface in the upper chamber were wiped away, washed with PBS 3 times, fixed with paraformaldehyde for 10 min, washed, dried and stained with 0.5% crystal violet. Cell invasion was analyzed with a microscope.

Apoptosis test

Transfected cells were collected and digested with 0.25% trypsin. After digestion, the cells were prepared into 1×10^6 /mL suspension. Then, Annexin-FITC and PI (Shanghai Yisheng Biotechnology Co., Ltd.) were added in sequence. Then, the cells were cultivated at room temperature for 5 minutes in the dark. FC-500MCL flow cytometry system was used for detection.

Double fluorescein report

The miR-21 target gene was predicted by using gene prediction online Targetscan7.2. KLF6-3' UTR wild type (Wt) and KLF6-3' UTR Mutant (mut) vectors were established by Lipofectamine™ 2000 kit and transferred to the downstream of luciferase reporter gene to sequence and identify the constructed plasmid, and the plasmid with correct sequencing was co-transfected into A549 cells with miR-21-inhibitor or miR-NC, respectively. The luciferase activity was tested using the double luciferase reporter gene detection kit (Solarbio, Beijing, China).

Statistical analysis

In this study, SPSS 18.0 was used to make statistical analysis on the collected data, and GraphPad 7 was used to illustrate the figures. Independent sample t test was used for comparison between groups. One-way ANOVA was applied for comparison among multiple groups. LSD-t test was used for pairwise comparison afterwards. Repetitive measurement and analysis of variance was used for expression at multiple time points. Bonferroni was used for post test. There was a statistical difference indicated with $P < 0.05$.

Results

UA inhibited the malignant growth of LC cells and reduced miR-21 expression

In order to evaluate the anti-cancer effect of UA in LC cells, different concentrations of UA were

cultured with LC cells. Then, CCK-8, Transwell and flow cytometry were used to observe the changes of cell behavior. The results showed that UA intervention could effectively inhibit the proliferation and invasion of carcinoma cells and promote cell apoptosis. With the increase of UA concentrations, the inhibitory and apoptosis-promoting effects were gradually increased. In order to evaluate the relationship between UA and miR-21, we detected miR-21 level changes in LC cells treated with different concentrations by qPCR. It was found that miR-21 decreased with the increase of UA concentration. This suggested that UA might play an anti-cancer role by reducing miR-21 (**Figure 1**).

miR-21 promoted the progression of LC

In order to understand the function of miR-21 in LC, miR-21 inhibition was carried out on LC cells. CCK-8 showed that the proliferation of LC cells was inhibited after transfection of miR-21-inhibitor. Transwell experiment showed that invasion ability of LC cells was also inhibited after transfection of miR-21-inhibitor. Finally, flow cytometry was used to detect the apoptosis of LC cells after transfecting with miR-21-inhibitor, and it was found that miR-21-inhibitor could improve the apoptosis rate of LC cells. These results showed that miR-21 could promote the progression of LC (**Figure 2**).

miR-21 negatively regulated KLF6

Further understanding of the mechanism of miR-21 regulating biological events in LC cells, resulted from findings of complementary nucleotide sequences between miR-21 and KLF6 through miR target gene prediction website Targetscan7.2. The KLF6 in LC cells transfected with miR-21-inhibitor was increased. Subsequently, the targeted relationship between them was revealed by detection of double luciferase activity. The results were expected in that transfection of miR-21-inhibitor enhanced KLF6-3' UTR Wt luciferase activity and had no effect on UCA12-3' UTR Mut luciferase activity. This indicated that miR-21 could negatively regulate KLF6 (**Figure 3**).

KLF6 inhibited the progression of LC

We showed that miR-21 could regulate the expression of KLF6 in LC cells, and therefore were curious if miR-21 could promote the pro-

Exploration of the effects of ursolic acid on the biological behavior of lung cancer cells

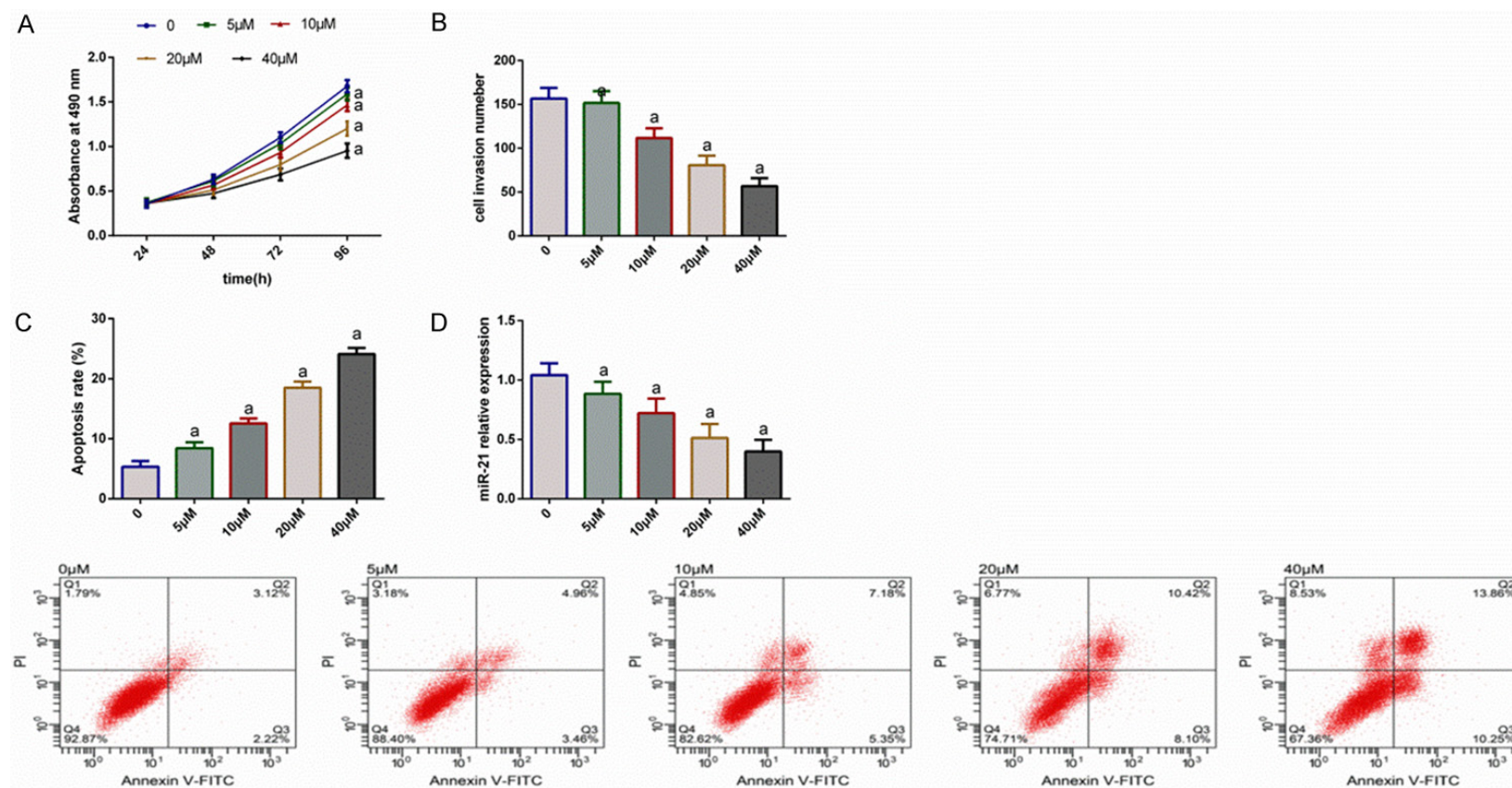


Figure 1. UA inhibited the malignant growth of cancer cells and reduced miR-21. A. UA intervention effectively inhibited the proliferation ability of LC cells. B. UA intervention effectively inhibited the invasion ability of LC cells. C. UA intervention effectively promoted apoptosis of LC cells. D. UA intervention effectively inhibited miR-21 changes in LC cells. Note: Compared with the concentration of 0, a represents $P < 0.05$.

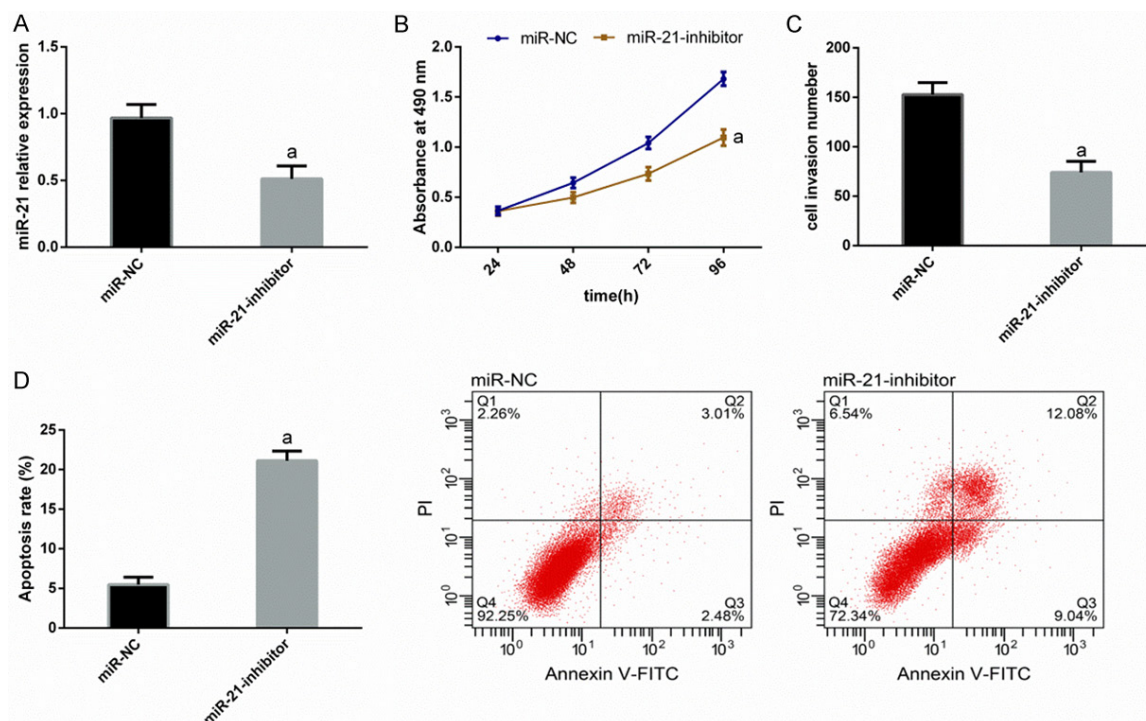


Figure 2. miR-21 promoted the progression of LC. A. Compared with cells transfected with miR-NC, miR-21 was down-regulated in cells transfected with miR-21-inhibitor. B. Compared with cells transfected with miR-NC, the proliferation of cells transfected with miR-21-inhibitor was decreased. C. Compared with cells transfected with miR-NC, the invasion of cells transfected with miR-21-inhibitor was decreased. D. Compared with cells transfected with miR-NC, the apoptosis rate of cells transfected with miR-21-inhibitor was increased. Note: Compared with miR-NC, a represents $P < 0.05$.

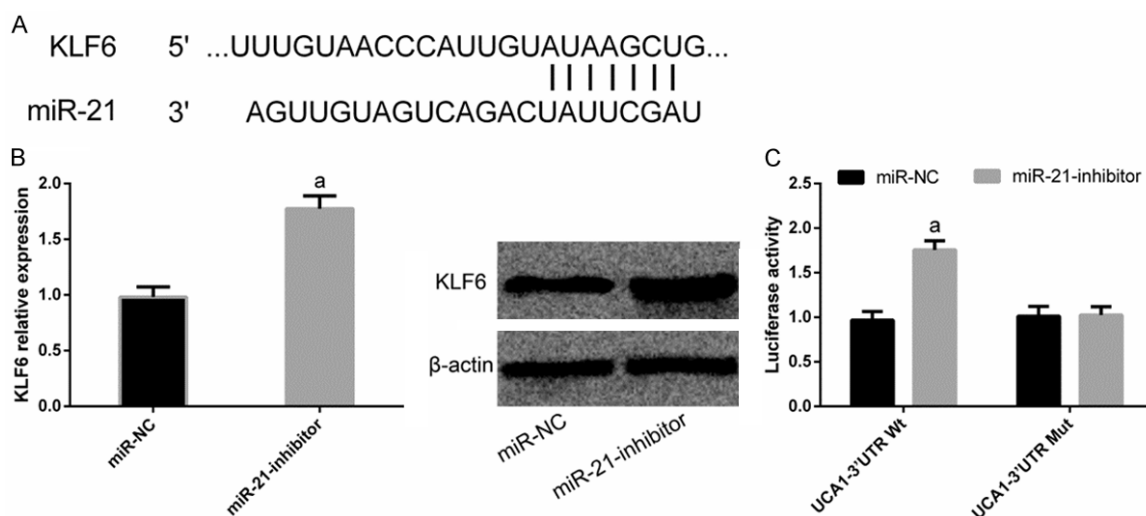


Figure 3. miR-21 negatively regulated KLF6. A. There were complementary nucleotide sequences between miR-21 and KLF6. B. Compared with cells transfected with miR-NC, KLF6 was increased in cells transfected with miR-21-inhibitor. C. Transfection of miR-21-inhibitor enhanced KLF6-3' UTR Wt luciferase activity and had no effect on UCA12-3' UTR Mut luciferase activity. Note: Compared with miR-NC, a represents $P < 0.05$.

gression of LC through the targeted regulation of KLF6. To prove this conjecture, it was necessary to understand the role of KLF6 in LC. LC

cells were transfected with sh-KLF6 and sh-NC respectively, and the biological behavior of the transfected cells was analyzed. It was found

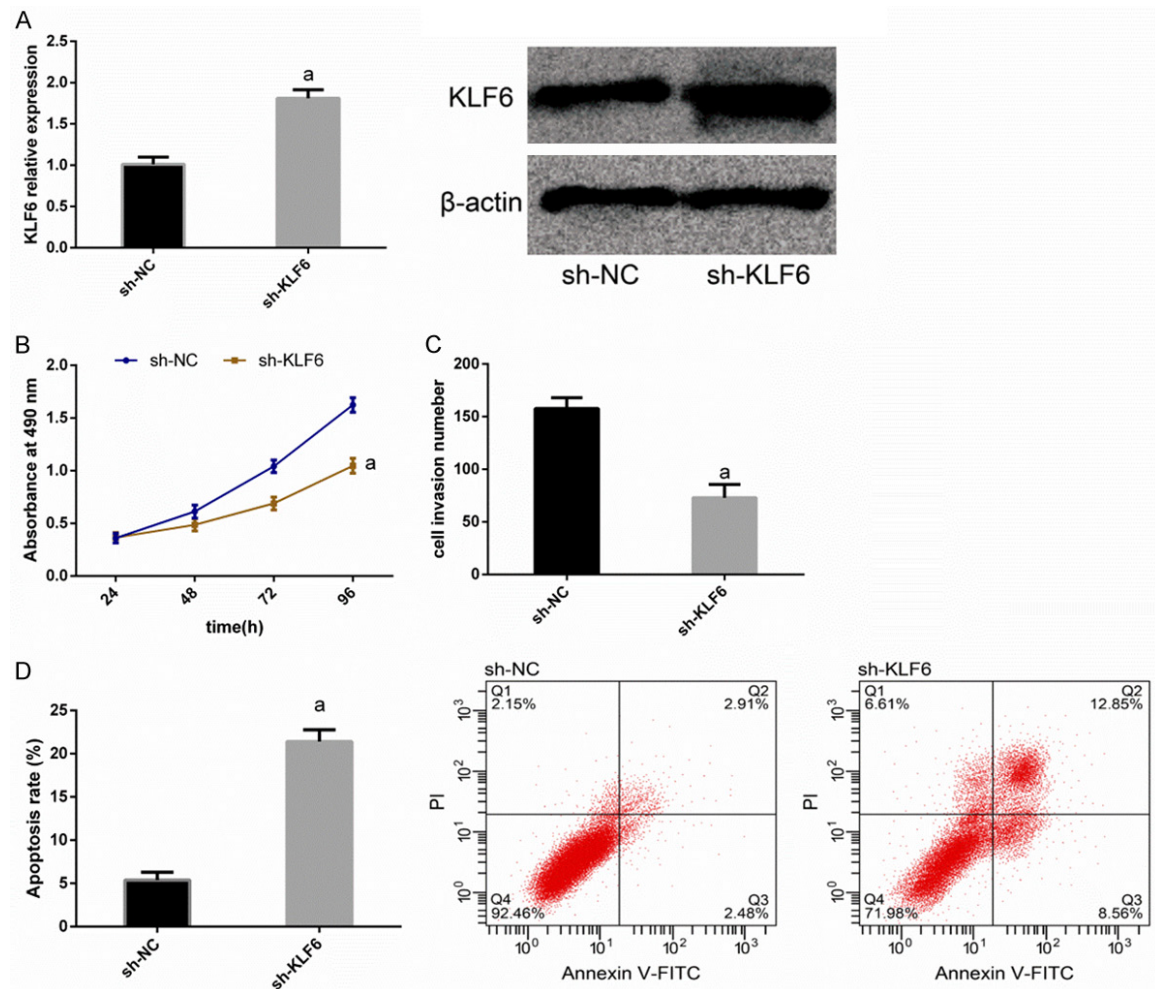


Figure 4. KLF6 inhibited the progression of LC. A. Compared with cells transfected with sh-NC, KLF6 was increased in cells transfected with sh-KLF6. B. Compared with cells transfected with sh-NC, the proliferation ability of cells transfected with sh-KLF6 was reduced. C. Compared with cells transfected with sh-NC, the invasion ability of cells transfected with sh-KLF6 was reduced. D. Compared with cells transfected with sh-NC, the apoptosis rate of cells transfected with sh-KLF6 was increased. Note: Compared with sh-NC, a represents $P < 0.05$.

that compared with cells transfected with sh-NC, KLF6 was enhanced in cells transfected with sh-KLF6, and cell proliferation and invasion ability were reduced, while cell apoptosis rates were enhanced. The effect of KLF6 was similar to that of miR-21-inhibitor, which indicated that KLF6 and miR-21 had opposite effects (Figure 4).

Increase of miR-21 or inhibition of KLF6 could reduce the anticancer effects of UA

Based on the above results, we could preliminarily speculated that the role of UA in anti-LC progression was related to the miR-21/KLF6 axis. For the purpose of exploring the relationship between UA and the miR-21/KLF6 axis

more deeply, 20 μM UA was used to intervene in LC cells transfected with miR-21-inhibitor or sh-KLF6, and the changes in cell behavior were observed after treatment. The results showed that both increasing miR-21 and inhibiting KLF6 could reduce the influence of UA on the behavior of LC cells (Figure 5).

Discussion

At present, there are many treatment options for patients with LC, such as chemotherapy, radiotherapy and surgery, but the overall prognosis of patients with LC is still unsatisfactory [18]. Therefore, it is urgent to find new potential therapeutic drugs and targets. UA has great clinical application prospects due to its anti-

Exploration of the effects of ursolic acid on the biological behavior of lung cancer cells

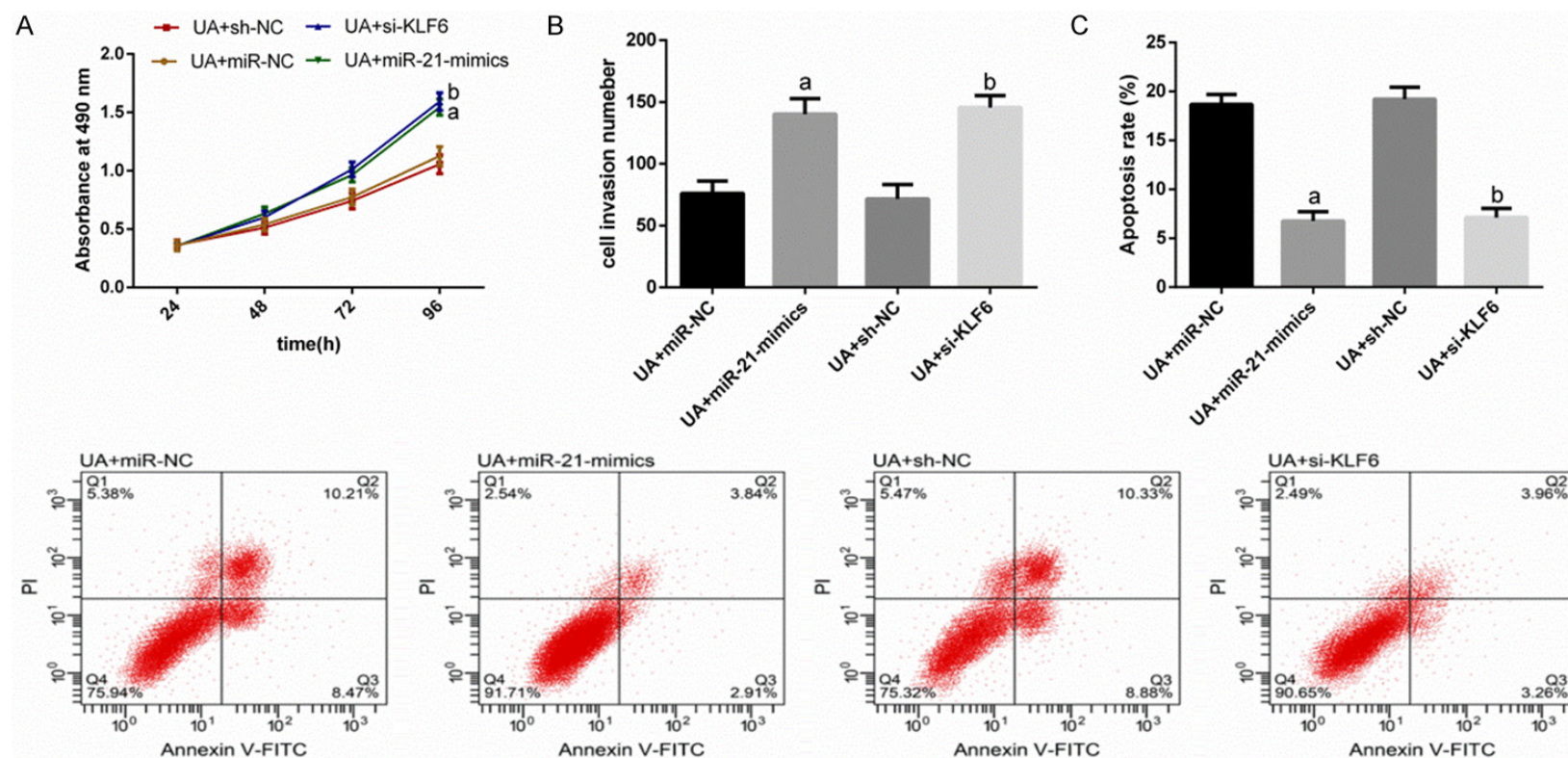


Figure 5. Increase of miR-21 or inhibition of KLF6 reduced the anticancer effect of UA. A. Increase of miR-21 or inhibition of KLF6 reversed the effect of UA on proliferation of LC cells. B. Increase of miR-21 or inhibition of KLF6 reversed the effect of UA on invasion ability of LC cells. C. Increase of miR-21 or inhibition of KLF6 reversed the effect of UA on apoptosis of LC cells.

tumor effects [7-9]. However, the anticancer mechanism of UA has not been clarified.

Abnormal expression of miR in the human body is considered an important reason for the development and progression of diseases [19]. In tumors, miR can play two distinct roles in promoting cancer or inhibiting cancer to participate in tumor progression [20-22]. Therefore, miR is widely studied as an important target for tumor therapy. Previous studies have shown that UA can play an anti-cancer role by regulating miR [10, 11]. miR-21 is one of miRs that has been deeply studied. Numerous evidence has revealed that it plays an anti-cancer role in tumors [23-25]. Previous studies have revealed that UA inhibits the proliferation and fibrosis of cardiac fibroblasts by down-regulating the expression of miR-21 [26]. Therefore, we believed that UA might also play an anti-cancer role by inhibiting miR-21. This study revealed that UA and inhibition of miR-21 could inhibit the proliferation and invasion of LC cells and promote apoptosis, while UA could reduce miR-21 in LC cells. In addition, we also found that the anti-cancer effect of UA was weakened after transfection of miR-21-inhibitor into LC cells intervened by UA. This showed that our conjecture was correct, and UA also played an anti-cancer role by inhibiting miR-21.

It is well known that miR can promote the degradation of transcripts by binding to the 3' UTR of mRNA sequences, thus participating in a variety of basic biological behaviors [27]. We found that there were complementary nucleotide sequences between miR-21 and KLF6. KLF6 is one of the KLF family members, which can regulate cell function, such as cell proliferation, apoptosis and differentiation [28]. Previous studies have found that KLF6 plays an anti-cancer role in renal cell carcinoma, glioblastoma, liver cancer and other tumors [29-31]. In this paper, we found that the proliferation and invasion activity of LC cells transfected with sh-KLF6 were reduced, while apoptosis rate was increased, indicating that KLF6 was also a cancer suppressor in LC. Subsequently, through the detection of double luciferase activity, we found that KLF6 could be targeted and regulated by miR-21. Furthermore, inhibition of KLF6 could weaken the effect of UA on the biological behavior of LC cells. Combined with all the results of this paper, we concluded

that UA plays an anti-LC role through the miR-21/KLF6 axis.

Although this article has revealed for the first time that UA can play an anti-LC role through the miR-21/KLF6 axis, there are still some deficiencies. First of all, tumor formation in nude mice was not conducted, and the influences of UA/miR-21/KLF6 on tumorigenesis effects are not known. Secondly, it was not investigated whether miR-21 can regulate U6 to exert an anti-LC role through other target genes besides KLF6. These deficiencies are expected to be improved in future research.

In summary, UA can inhibit the proliferation and invasion of LC cells and promote cell apoptosis, which is related to the regulation of the miR-21/KLF6 axis.

Disclosure of conflict of interest

None.

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