

Icariin inhibits the malignant progression of lung cancer by affecting the PI3K/Akt pathway through the miR-205-5p/PTEN axis

FENGJIE ZHU and ZHE REN

Department of Cardiothoracic Surgery, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310000, P.R. China

Received October 22, 2021; Accepted March 2, 2022

DOI: 10.3892/or.2022.8326

Abstract. Icariin (ICA) is one of the main bioactive monomer belonging to the flavonoid glycosides that has been widely studied in multiple diseases, including lung cancer. Although ICA has shown anticancer effects, its specific molecular mechanism of action remains to be elucidated. In the present study, the expression of microRNA (miR)-205-5p and Phosphatase and tensin homolog deleted on chromosome ten (PTEN) in human lung cancer and bronchial cells were analyzed. Cell viability, colony formation, migration, invasion, apoptosis and cell cycle distribution were investigated *in vitro*. In addition, the function of ICA on tumor growth was determined using a xenotransplantation model. The results showed that ICA decreased the viability of lung cancer cells. In addition, miR-205-5p was upregulated in lung cancer tissues but downregulated following ICA treatment, while PTEN showed a significantly lower expression in lung cancer cells. miR-205-5p could increase cancer cell proliferation, migration, invasion and cell cycle progression while suppressing cell apoptosis. Importantly, rescue experiment results showed that ICA could target the miR-205-5p/PTEN axis to affect the PI3K/Akt signaling, thereby suppressing the malignant cell phenotype of lung cancer. Finally, animal experiments confirmed that ICA could inhibit lung cancer growth *in vivo*. Taken together, our findings suggest that miR-205-5p is a key gene targeted by ICA to inhibit lung cancer progression.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, characterized by drug resistance and poor

prognosis (1). Of all lung cancer cases, lung adenocarcinoma (LUAD) accounts for >50% (2). Studies have made significant progress in LUAD targeted therapy, such as the various tyrosine kinase inhibitors used to effectively prolong the survival of patients with advanced LUAD (3). Therefore, it is of great significance to explore the intrinsic molecular mechanism of lung cancer progression.

Chinese medicine herb (CMH) is one of the alternative options for treating lung cancer. Among them, icariin (ICA) has been found to have lung-protective effects. However, the molecular mechanism of action remains to be elucidated (4,5). In addition, the dysregulation of micro (mi)RNAs expression levels is closely associated with the progression of lung cancer and some CMHs can exert antitumor effects by targeting aberrant miRNAs. For example, *Panax notoginseng* saponins significantly decreased the level of miR-222 regulated by Met and exhibited a therapeutic effect on lung cancer (6). It is also found that cinnamaldehyde can downregulate miR-1252 and increase the apoptosis of non-small cell lung cancer (NSCLC) cells (7). Additionally, a total of 162 miRNAs displayed abnormal expression in *Radix tetragyna hemsleyana* flavonoids-treated lung cancer cells (8). The above findings indicate that exploring aberrant miRNAs expression levels might be of clinical value for lung cancer treatment. Therefore, it is promising to explore the regulatory relationship between CMHs, such as ICA and abnormally-expressed miRNAs in lung cancer.

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is known to exhibit antitumor effects in various cancers. For instance, PTEN is abnormally downregulated in ovarian cancer and its overexpression can inhibit the malignant progression of ovarian cancer (9). In bladder cancer, PTEN is significantly downregulated and is regulated by circular (circ) SLC8A1 to inhibit the malignant behavior of cancer cells (10). PTEN is been shown to suppress NSCLC and may be used as a prognostic marker for those patients (11-13). Based on the above studies, it was hypothesized that it was important to decipher the level and regulation mechanism of PTEN in tumors, especially in lung cancer. However, most current studies focus on the regulatory relationship between PTEN and other genes and studies on the mechanism of PTEN in lung cancer based on CMHs are severely lacking.

Correspondence to: Dr Zhe Ren, Department of Cardiothoracic Surgery, The First Affiliated Hospital of Zhejiang Chinese Medical University, 54 Youdian Road, Hangzhou, Zhejiang 310000, P.R. China
E-mail: 2820355483@qq.com

Key words: lung cancer, icariin, microRNA-205-5p, phosphatase and tensin homolog deleted on chromosome ten, proliferation

In the present study, ICA was been found to downregulate the level of miR-205-5p and inhibit lung cancer cells proliferation. The downstream molecular mechanism of miR-205-5p was further explored in combination with bioinformatics prediction results. Taken together, the present study provided new insights into the use of ICA to target miR-205-5p as a potential therapeutic strategy for lung cancer.

Materials and methods

Cell culture. The human lung cancer cell line A549, NCI-H1975 and human bronchial epithelial cell line BEAS-2B were purchased from China General Microbiological Culture Collection Center. All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. 293T cells (American Type Culture Collection) were grown in Dulbecco's Modified Eagle Medium with 1% L-glutamine, 1% penicillin-streptomycin (Beyotime Institute of Biotechnology) and 10% FBS at 37°C with 5% CO₂. The cells used in the subsequent experiments were all passaged 3 times.

Cell transfection and drug treatment. miR-205-5p mimic (50 nM, 5'-UCCUUAUCCACCGGAGUCUG-3'), miRNA mimic negative control (mimic NC; 50 nM, 5'-UUCUCCGAA CGUGUCACGUTT-3'), miR-205-5p inhibitor (50 nM, 5'-CAG ACUCCGGUGGAAUGAAGGA-3'), miRNA inhibitor NC (inhibitor NC) (50 nM, 5'-CAGUACUUUUGUGUAGUA CAA-3'), small interfering (si)-PTEN (50 nM, 5'-GACGGG AAGACAAGUUAUTT-3') and siRNA NC (si-NC; 50 nM, 5'-GCACAGTTAACCGCATAAA-3') were all purchased from Guangzhou RiboBio Co., Ltd. Cell transfections were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), 0.5x10⁶ cells (NCI-H1975 or A549) were inoculated into 6-well plates and incubated at room temperature for 5 min. Following transfection, the cells were used for subsequent experiments 24 h later.

Lung cancer cells (NCI-H1975 or A549) in the logarithmic growth phase were treated with different concentrations of ICA (The concentration range was 5–40 µM and the concentrations of the four treatment groups were 5, 10, 20 and 40 µM, respectively) or control for 24 h at 37°C (14). ICA was obtained from Shanghai Tauto Biotech Co., Ltd.

Reverse transcription-quantitative PCR (RT-qPCR). The experimental steps were carried out according to the manufacturers' instructions. Total RNA in cells (NCI-H1975 or A549) at 80% confluence was extracted using TRIzol® (Thermo Fisher Scientific, Inc.). PrimeScript RT kit (Takara Biotechnology Co., Ltd.) and SYBR Select Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.) were used for reverse transcription and qPCR. The PCR conditions were as follows: Initial denaturation at 95°C for 2 min; followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 60 sec and elongation at 72°C for 30 sec and final extension at 72°C for 5 min. U6 and GAPDH were used for the standardization of miR-205-5p and PTEN. The sequences of primers were: miR-205-5p F, 5'-CTT GTCCTTCATTCCACCGGA-3' and R, 5'-TGCCGCTG AACTTCACTCC-3'; PTEN F, 5'-TGTGGGTGTGGTTGG

AAGTC-3' and R, 5'-CCTCAAGGTCAGACCCTTCC-3'; GAPDH F, 5'-GGAGCGAGATCCCTCCAAAAT-3' and R, 5'-GGCTGTTGTCATACTTCTCATGG-3'; and U6 F, 5'-CTC GCTTCGGCAGCACA-3' and R, 5'-AACGCTTCACGAATT TGCCT-3'. The quantitative analysis was performed using the 2^{-ΔΔC_q} method (15). All experiments were repeated 3 times.

Western blotting. The cells (NCI-H1975 or A549 at 80% confluence in 6-well plates) were lysed with RIPA buffer (Thermo Fisher Scientific, Inc.) and the protein concentration was determined using the bicinchoninic acid kit (Beyotime Institute of Biotechnology). Proteins (20 µg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (MilliporeSigma). Following blocking (with 5% nonfat milk in TBST at room temperature for 1 h), the membrane was incubated overnight at 4°C with the primary antibodies: PTEN (1:1,000; cat. no. ab267787), p-Akt (1:500; cat. no. ab131443), Akt (1:10,000; cat. no. ab179463), p-PI3K (1:500; cat. no. ab182651), PI3K (1:1,000; cat. no. ab191606) and GAPDH (1:1,000; cat. no. ab9485; all Abcam). Subsequently, PBST was used to rinse the membranes three times. Secondary antibody goat anti-rabbit IgG H&L (HRP; cat. no. ab6721; Abcam) was used to incubate the membranes at room temperature for 1 h. All protein bands were visualized using ECL chemiluminescence (Thermo Fisher Scientific, Inc.). Protein bands were visualized in a gel imaging system (MG8600, Bio-Rad). Images were using Image Pro Plus software (Version 7.0, Media Cybernetics).

Dual-luciferase assay. A luciferase vector pScheck (Sangon Biotech Co., Ltd.) for wild-type (WT) and mutated (MUT) PTEN 3'UTR was constructed. The binding sites between PTEN and miR-155-5p were predicted using miRbase (<https://mirbase.org>). miR-205-5p mimic/mimic NC and PTEN-pscheck WT/MUT were co-transfected into 293T cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h transfection, luciferase activity was measured using the dual-luciferase reporter gene assay kit (Shanghai Qcbio Science & Technologies Co., Ltd.). Luciferase activity was normalized to Renilla activity.

Colony formation assay. Cells (1x10³/well) were inoculated on Petri dishes and cultured until colonies were visible. Colonies were fixed with 4% formaldehyde (Thermo Fisher Scientific, Inc.) for 15 min at room temperature, stained with 0.1% crystal violet for 15 min (Thermo Fisher Scientific, Inc.) at room temperature and quantified under a light microscope (magnification, x40, three randomly selected fields). A colony was defined as an agglomeration of >50 cells.

Cell Counting Kit-8 (CCK-8) assay. After inoculating for 0, 24, 48, 72 and 96 h, CCK8 solution (10 µl/well) (Dojindo Molecular Technologies, Inc.) was added to the cells. After 4 h, the absorbance of cells at 450 nm was measured using a microplate reader (BioTek Instruments, Inc.).

Transwell assay. Cell suspension (200 µl) was added to the chamber. For induction, 5% FBS was added to the lower chamber. After 24 h, the upper cells were gently scraped off

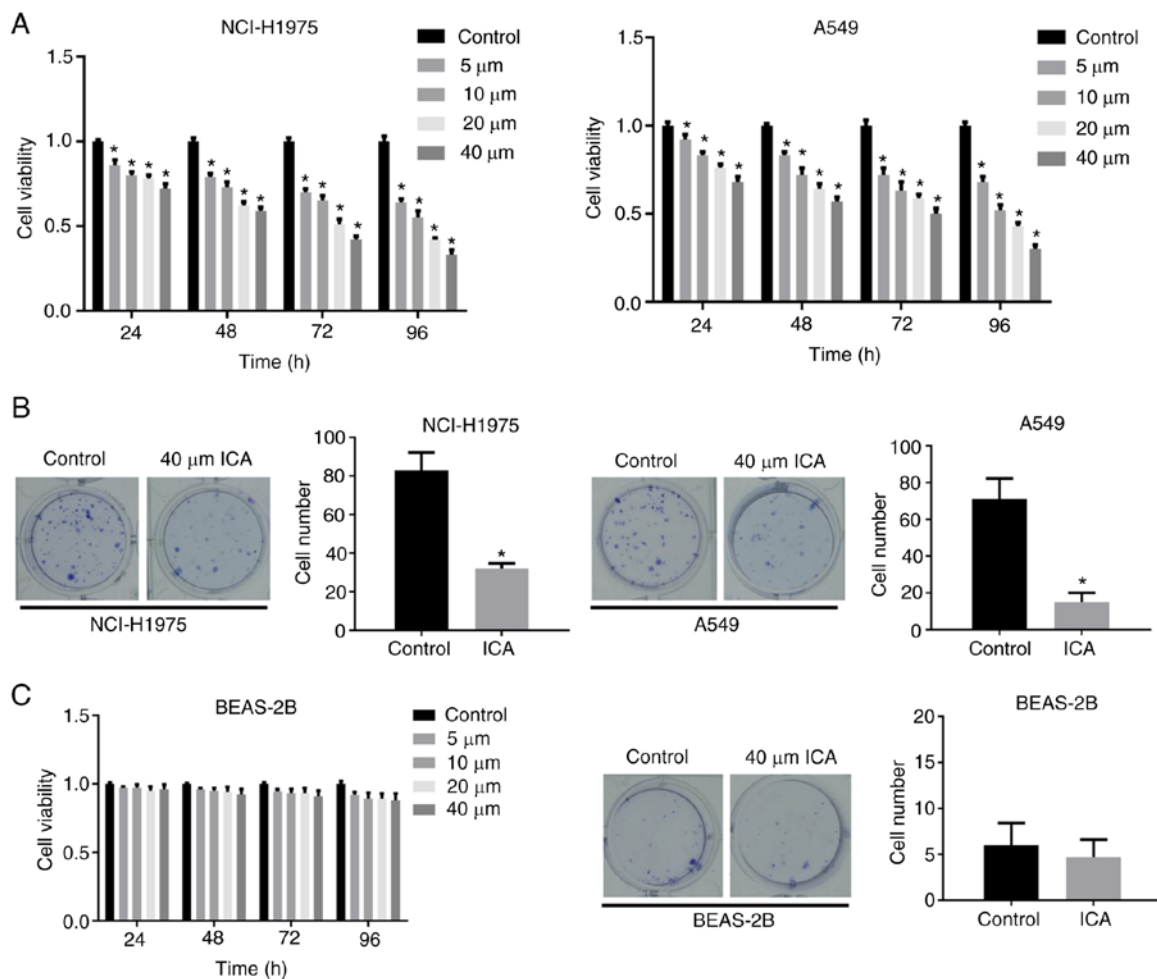


Figure 1. ICA suppresses the viability and colony formation ability of lung cancer cells but has little effect on lung epithelial cells. (A) CCK8 assays were performed to detect the proliferation ability of A549 cells and NCI-H1975 cells. (B) Colony formation assay was used to detect the colony formation ability of NCI-H1975 and A549 cells when treated with 40 μ m ICA. (C) CCK8 and colony formation assay analyzed the effect of ICA on BEAS-2B cells. Results are represented as the mean \pm SD of three experiments; * P <0.05 vs. control. ICA, icariin; CCK8, Cell Counting Kit-8.

and the migratory cells were stained with 0.1% crystal violet for 30 min at room temperature (Thermo Fisher Scientific, Inc.), observed and counted. The upper chamber was pre-coated with Matrigel (BD Biosciences) at 37°C for 30 min to detect the invasiveness of cells and the other operations were basically the same as the migration assay.

Flow cytometry (FCM). FCM was used to detect apoptosis, as previously described by Xu *et al* (16). In short, in the presence of 50 μ g/ml RNase A (MilliporeSigma), cells were stained with Annexin V/FITC and propidium (PI; Thermo Fisher Scientific, Inc.) and analyzed by flow cytometer (CytoFLEX; Beckman Coulter). The data were analyzed using FlowJo software (version 10.0.2, FlowJo LLC). Apoptosis rate=percentage of early + late apoptotic cells. For cell cycle detection, cells were treated with trypsin, fixed for 2 h at 4°C with 70% ethanol which was precooled in a 4°C refrigerator, then stained with PI for 15 min at 4°C. Cell cycle was analyzed by FCM.

Nude mouse transplantation tumor experiment. BALB/C nude mice (n=15, female, aged 6 weeks, weighed 16-18 g, purchased from Laboratory Animal Resources, Chinese Academy of Sciences) were raised in a 12-h light/dark cycle at a temperature

of 25°C \pm 1°C and relative humidity of 50 \pm 10%. All the mice were given standard diet and sterile water *ad libitum*. Mice were randomly divided into A, B and C groups with 5 mice in each group. Mice in group A were inoculated with A549 cells transfected with NC Agomir (Guangzhou RiboBio Co., Ltd.) and mice in groups B and C were inoculated with A549 cells transfected with miR-205-5p Agomir (Guangzhou RiboBio Co., Ltd.). Mice in group C were additionally injected with ICA (10 mg/kg) every 3 days (4).

Tumor diameter was measured every 7 days. After the experiment, the mice were sacrificed by inhalation of CO₂ (50% of the chamber volume/min) and the tumors were resected and weighed. The flow of CO₂ was maintained for at least 2 min after respiratory arrest. All animal experiments were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhejiang Chinese Medical University (approval No. 2021026) in accordance with the National Research Council Guide for Care and Use of Laboratory Animals.

Immunohistochemistry (IHC). IHC was performed using the principle of antigen and antibody-specific binding. The tumor tissue was fixed with 4% paraformaldehyde at room

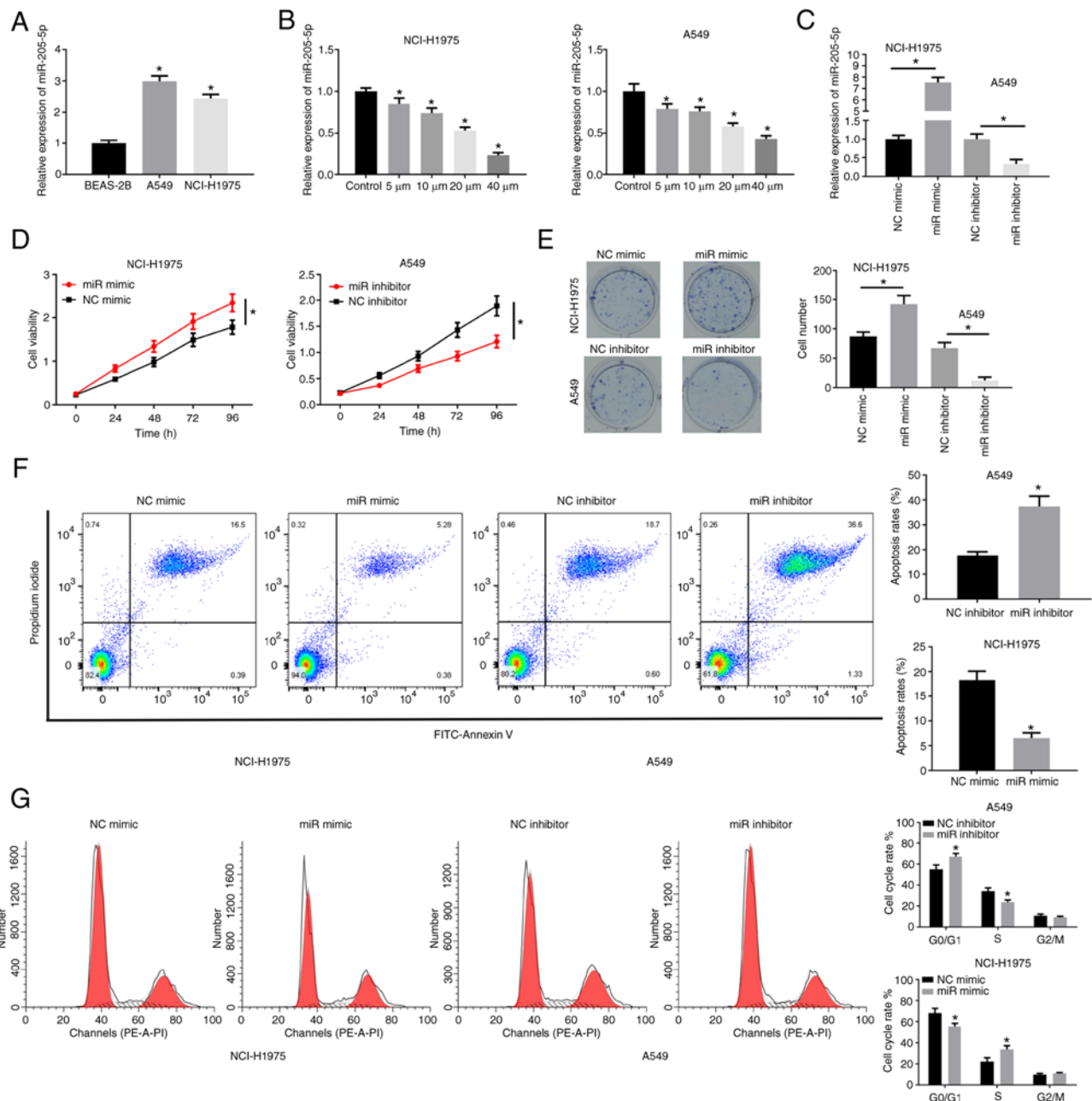


Figure 2. miR-205-5p is highly expressed in lung cancer and promotes cell proliferation. The expression of miR-205-5p in cells was detected by (A-C) reverse transcription-quantitative PCR, (D) CCK8, (E) colony formation assays and (F and G) flow cytometry. Data are represented as the mean \pm SD of three experiments; * P <0.05 vs. BEAS-2B, control, NC mimic or NC inhibitor. miR, microRNA; CCK8, Cell Counting Kit-8; NC, negative control.

temperature for 24 h, embedded with paraffin and sectioned. The paraffin sections were dewaxed with xylene, washed with 100% ethanol twice, 95% ethanol twice and dH₂O twice. The sections were then boiled in 10 mM sodium citrate buffer for 10 min. After 3 washes with dH₂O, the slices were incubated with 3% H₂O₂ for 10 min and washed for twice. The sections were blocked in 5% goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature. Primary antibody Ki-67 (cat. no. ab15580; Rabbit; Abcam) was added and incubated overnight in a 4°C refrigerator, followed by secondary antibody IgG (cat. no. ab6721; Goat Anti-Rabbit; Abcam) for half an hour at room temperature. Specific immunostaining was performed with a 3,3'-diaminobenzidine substrate (MilliporeSigma) at room temperature and

monitored under a light microscope, until the target tissue appeared brownish yellow when staining was stopped and counterstained with hematoxylin (~1-2 min) at room temperature. Finally, five fields were randomly selected using a light microscope (Olympus Corporation; magnification, x200).

Statistical analysis. At least three biological and technical duplications were independently performed. All data were expressed as Mean \pm Standard deviation (SD) using GraphPad Prism 6 software (La Jolla, USA). Differences between two or multiple groups were conducted using the Student t-test or one-way ANOVA followed by a post hoc Tukey's test. P <0.05 was considered to indicate a statistically significant difference.

Results

Icariin suppresses the viability and colony formation ability of lung cancer cells but had little effect on lung epithelial cells. Lung cancer cell lines, A549 and H1975, were exposed to different ICA concentrations. The results of the CCK-8 assay revealed that ICA could markedly reduce cell viability in a time and dose-dependent manner compared with the control group (Fig. 1A). In addition, ICA could significantly reduce the cell colony-forming ability of cancer cell lines compared with the control group (Fig. 1B). At the same time, the above experiments were also performed with BEAS-2B cells and the results showed that the viability and proliferation ability of BEAS-2B were almost unaffected by the increase in ICA treatment time and concentration (Fig. 1C). These results suggest that ICA inhibited the growth of lung cancer cells but had almost no toxic effects on normal cells.

miR-205-5p is highly expressed in lung cancer and promotes cell proliferation. miR-205-5p has been shown to be upregulated in various cancers (17,18). In the present study, miR-205-5p was highly expressed in NCI-H1975 and A549 cell lines (Fig. 2A). Subsequently, the cancer cells were treated with different concentrations of ICA and it was found that miR-205-5p decreased as the dose increased (Fig. 2B). NCI-H1975 and A549 were selected for follow-up research. miR-205-5p mimic (Fig. 2C) could markedly increase the viability and colony formation of lung cancer cells, while miR-205-5p inhibitor treatment had the opposite effect (Fig. 2D and E). FCM results revealed that miR-205-5p inhibited apoptosis but increased cell cycle progression (Fig. 2F and G). Together, these results further suggested the tumor-promoting effect of miR-205-5p in lung cancer cells.

miR-205-5p can promote the invasion and migration of lung cancer cells. Next, a Transwell migration and invasion assay was performed to determine if miR-205-5p could promote the migration and invasiveness of lung cancer cells. It was found that miR-205-5p overexpression could facilitate the invasion and migratory abilities of lung cancer cells (Fig. 3A and B), while inhibition of miR-205-5p could suppress these effects.

miR-205-5p translationally represses PTEN by targeting specific sequences in the 3'UTR of PTEN. To further explore the mechanism of action of miR-205-5p in lung cancer, the targeted binding sites of miR-205-5p and PTEN were predicted (Fig. 4A). PTEN mRNA expression was found to be significantly downregulated in lung cancer cell lines (Fig. 4B). Dual-luciferase reporter gene assay was applied to explore the binding relationship between miR-205-5p and PTEN (Fig. 4C). The expression of PTEN in different treatment groups was determined. PTEN was notably downregulated in cells overexpressing miR-205-5p, while cells depleted of miR-205-5p showed the opposite trend (Fig. 4D). The above results thus indicated that miR-205-5p could suppress PTEN in lung cancer.

ICA inhibits the proliferation of lung cancer cells through the miR-205-5p/PTEN axis. Having demonstrated that PTEN is a target gene of miR-205-5p, the present study next investigated

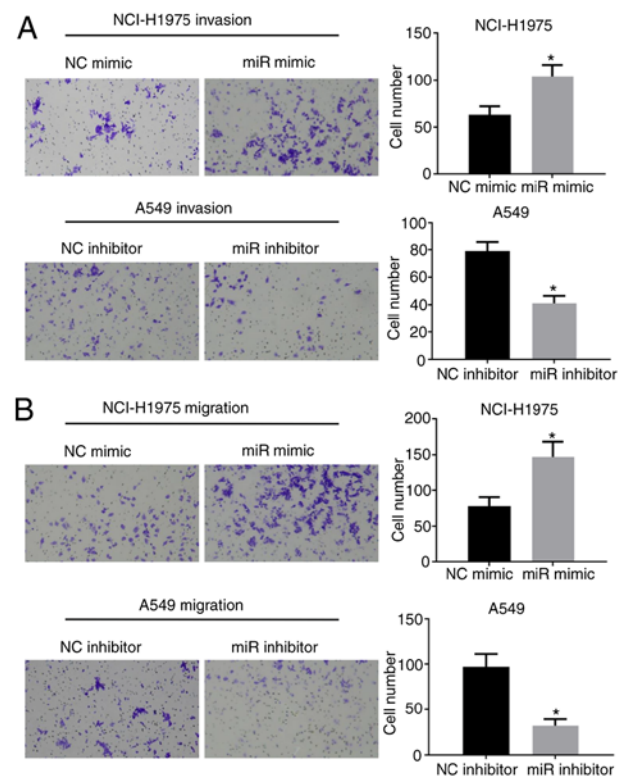


Figure 3. miR-205-5p can promote the invasion and migration of lung cancer cells. Transwell assays detected cellular (A) invasiveness and (B) migratory ability. Magnification, $\times 100$. Data are shown as the mean \pm SD of three experiments; * $P < 0.05$ vs. NC mimic or NC inhibitor. miR, microRNA; NC, negative control.

whether ICA could regulate the expression of PTEN by inhibiting miR-205-5p. In A549 cells, ICA treatment could upregulate the level of PTEN mRNA, while overexpression of miR-205-5p or downregulation of PTEN reversed that effect (Fig. 5A). In A549 cells, ICA treatment could suppress the cell vitality and colony formation effects of miR-mimic or si-PTEN transfection (Fig. 5B and C). Moreover, FCM results showed that ICA could promote cell apoptosis and induce cell cycle arrest, while miR-205-5p or inhibition of PTEN attenuated this effect (Fig. 5D and E).

ICA can affect the migration and invasion ability of cancer cells and regulate the PI3K/Akt signaling pathway through the miR-205-5p/PTEN axis. Next, the migratory and invasive abilities of A549 cells were detected. Transwell assay revealed that ICA could significantly decrease the migratory ability and invasiveness of cells, but following transfection with miR mimic or si-PTEN, the above trends were restored (Fig. 6A and B). To explore the regulatory effect of the axis identified in this study on the PI3K/Akt signaling pathway, the pathway-related proteins (Akt, PI3K and its corresponding phosphorylation level) were measured. It was found that PTEN protein expression was decreased and the PI3K/Akt pathway was activated when cells were transfected with miR mimic and si-PTEN. However, phosphorylation of Akt and PI3K decreased following ICA treatment, while it was markedly increased in the ICA + miR mimic or the ICA + si-PTEN group compared with the ICA treatment group (Fig. 6C). In short, ICA could inhibit the malignant phenotype of lung cancer cells through the miR-205-5p/PTEN axis.

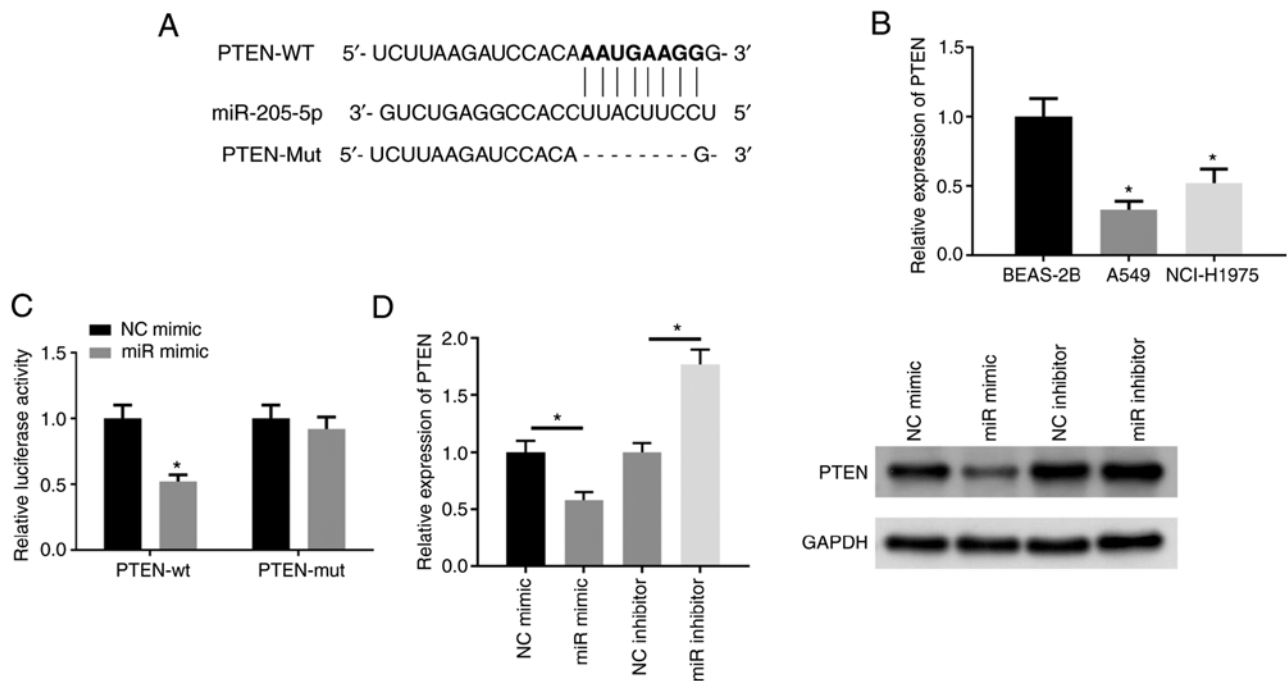


Figure 4. miR-205-5p translationally represses PTEN by targeting specific sequences in the 3'UTR of PTEN. (A) miR-205-5p and PTEN have targeted binding sites. (B) RT-qPCR detected the expression level of PTEN mRNA in cells. (C) Dual-luciferase assay verified the targeted binding of miR-205-5p and PTEN. (D) PTEN mRNA and protein expression levels were detected by RT-qPCR and western blotting, respectively. Data are represented as the mean \pm SD of three experiments; * P <0.05. miR, microRNA; PTEN, phosphatase and tensin homolog deleted on chromosome ten; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; wt, wild type; mut, mutant.

ICA impairs the promoting effect of exogenous miR-205-5p on tumor growth. In addition to *in vitro* studies, a xenograft model was also employed to explore the effect of ICA on miR-205-5p mediated cancer promotion. The tumor growth curve showed that tumor growth was faster in the miR agomir group compared with the NC group, with ICA administration significantly suppressing the tumor growth rate (Fig. 7A). Tumor weight was significantly increased in mice treated with miR agomir when compared with control mice. Following ICA administration, tumor weight was significantly reduced compared with the miR agomir group (Fig. 7B). IHC staining revealed that Ki67 was highly expressed in miR agomir xenograft tumor, while Ki67 was downregulated following ICA administration (Fig. 7C). Moreover, miR-205-5p was upregulated and PTEN mRNA was downregulated in the miR agomir group, while the above-mentioned phenomena were inhibited in the ICA + miR agomir group (Fig. 7D). The above findings revealed that miR-205-5p could promote lung cancer progression, while this promoting effect was reversed when ICA was added concurrently.

Discussion

At present, a number of CMHs have shown good efficacy in lung cancer treatment. For example, the xihuang pill can inhibit lung cancer by regulating the amino acid metabolism pathway in the Lewis lung cancer animal model (19). In addition, Rg3 is shown to regulate DNA damage in NSCLC cells, thereby inhibiting the development of cancer cells (20). Salidroside can also suppress the malignant phenotype of human lung cancer cells (21). However, the clinical application of CMHs

has been greatly limited because of their elusive mechanisms of action to prevent tumor progression.

As a flavonoid glycoside from genus *Epimedium* plants, ICA has been shown to promote the blood flow of cardiovascular and cerebrovascular vessels, hematopoiesis, immune function, anticancer effects (22,23). The present study found that ICA could suppress the viability and colony formation ability of lung cancer cells, which is similar to the results of a previous study (4). However, to further strengthen the theoretical basis of ICA to facilitate future clinical application, the present study also used BEAS-2B cells and found that ICA had almost no effect on the growth of BEAS-2B cells, signifying that ICA has little to no toxicity effect on normal cells. Several studies have previously shown that ICA can exert an important role via miRNAs or mRNAs in multiple diseases. For instance, in liver fibrosis, ICA can regulate miR-875-5p to attenuate the epithelial-mesenchymal transition process by targeting the hedgehog signaling pathway (24). ICA can also regulate the biological function of ovarian cancer cells by regulating miR-21 (25). In addition, a study found that ICA could target miR-625-3p and inhibit thyroid cancer cell growth (26). The results of previous studies suggest that exploring the mechanism of the anticancer activity of ICA might help to expand its clinical application. The present study revealed for the first time, to the best of the authors' knowledge, that miR-205-5p was upregulated in lung cancer cells and that ICA could inhibit its level, miR-205 is located at the 1q32.2 locus of the human genome and plays an important role in multiple diseases. miR-205-5p is reported to inhibit apoptosis and promote invasion of A549 cells (27). Furthermore, miR-205-5p can also inhibit LRP1 and regulate

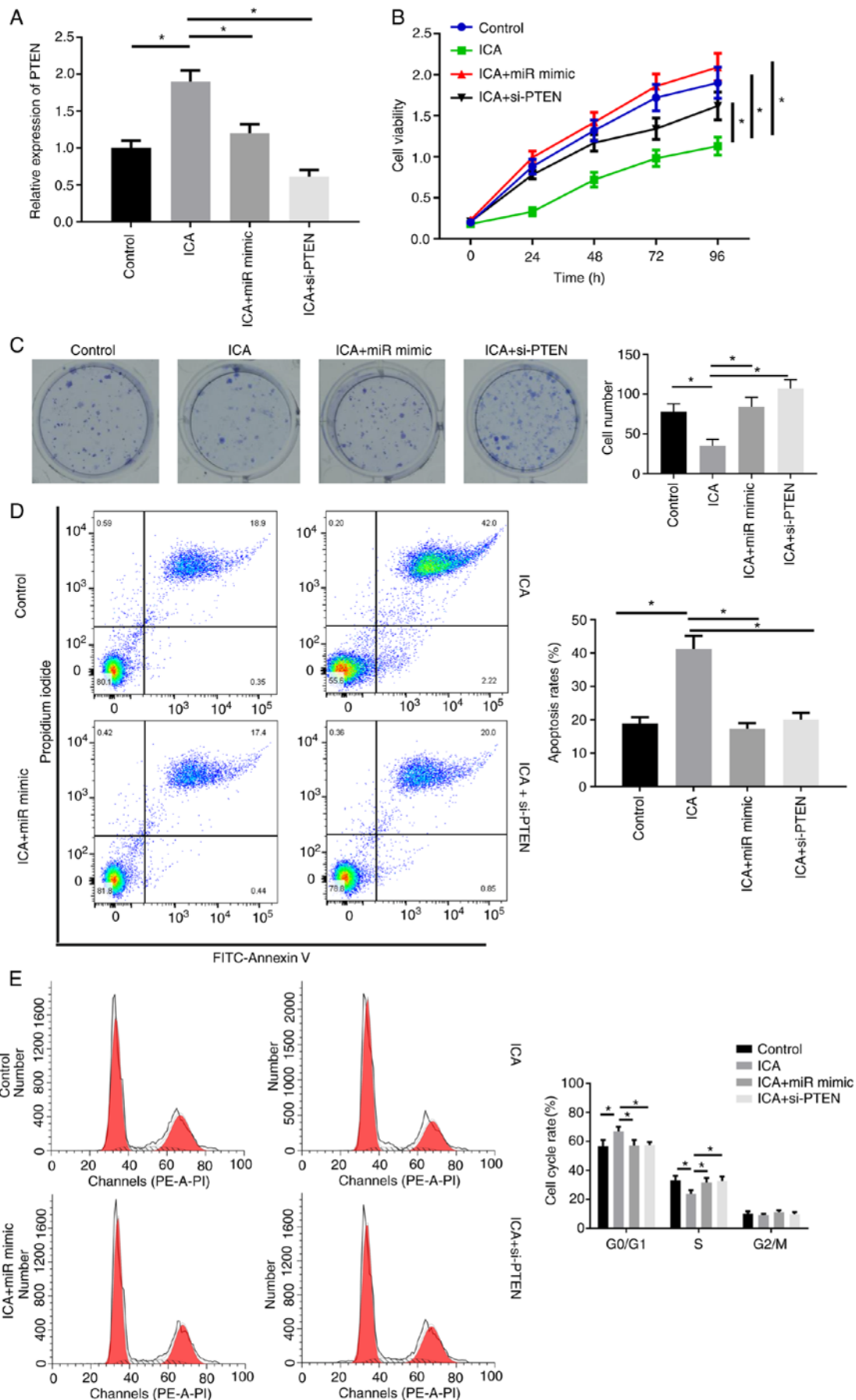


Figure 5. ICA inhibited the proliferation of lung cancer cells through the miR-205-5p/PTEN axis. The expression of PTEN mRNA was detected by (A) RT-qPCR, (B) CCK-8, (C) colony formation assays and (D and E) flow cytometry were used to detect proliferation, colony formation, apoptosis and cycle distribution of transfected cells in each group. Data are shown as the mean \pm SD of three experiments; * P <0.05. ICA, icariin; miR, microRNA; PTEN, Phosphatase and tensin homolog deleted on chromosome ten; RT-qPCR, reverse transcription-quantitative PCR; CCK8, Cell Counting Kit-8.

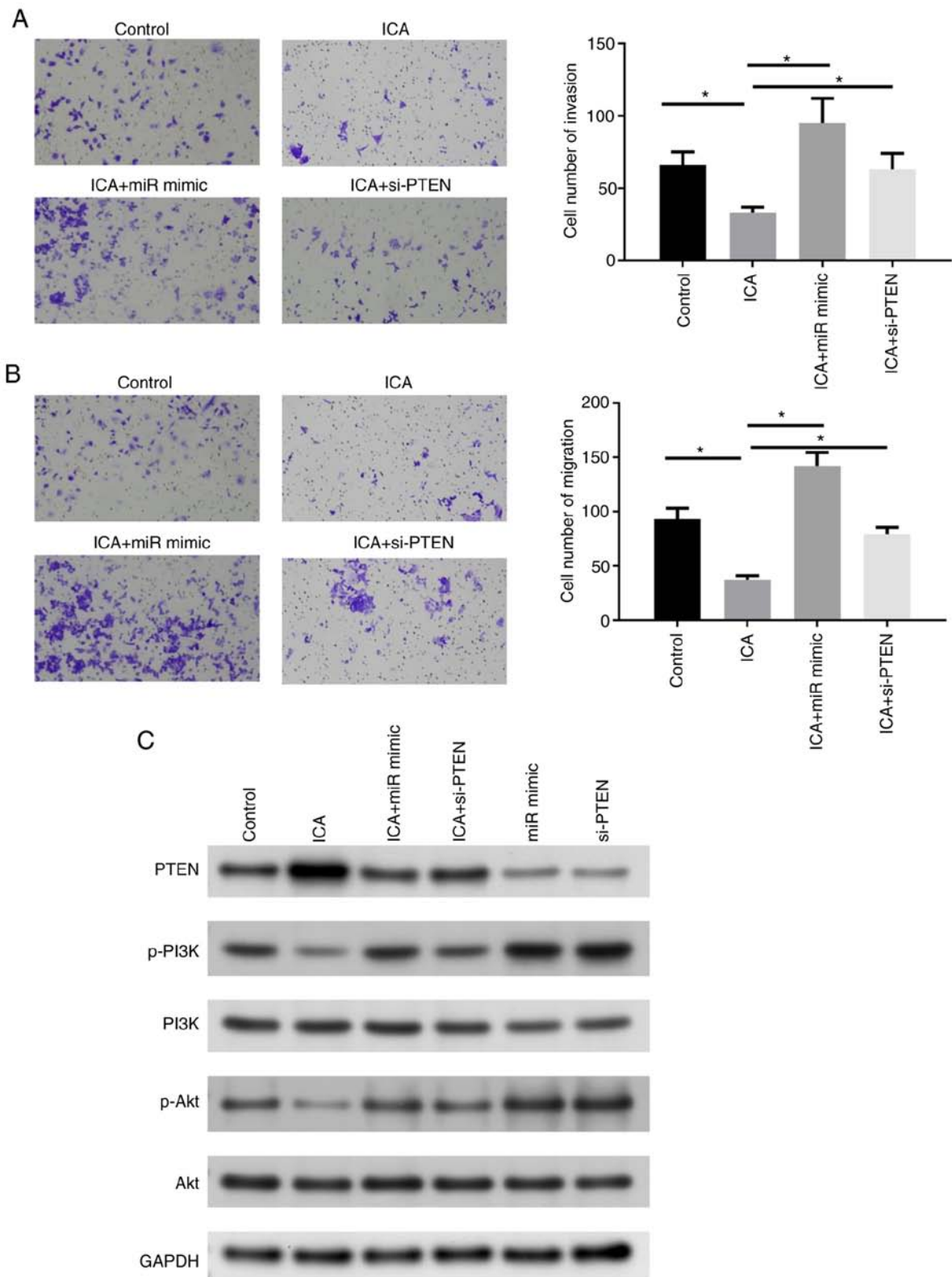


Figure 6. ICA can affect the migration and invasion ability of cancer cells and regulate the PI3K/Akt signaling pathway through the miR-205-5p/PTEN axis. (A) Invasive ability and (B) migratory ability of cells in each group were detected by Transwell assay; magnification, x100. (C) Western blotting detected the expression of PI3K/Akt pathway-related proteins in transfected cells. Data represented as the mean \pm SD of three experiments; * $P < 0.05$. ICA, icariin; miR, microRNA; PTEN, Phosphatase and tensin homolog deleted on chromosome ten; p-, phosphorylated.

NSCLC proliferation (28). *In vitro* functional studies in the present study showed that the overexpression of miR-205-5p could significantly enhance the progression of lung cancer cells, in line with previous reports.

The present study also predicted that miR-205-5p and PTEN had binding sites and a double luciferase assay further verified the relationship between the two. PTEN is considered a common tumor suppressor and expression loss often leads

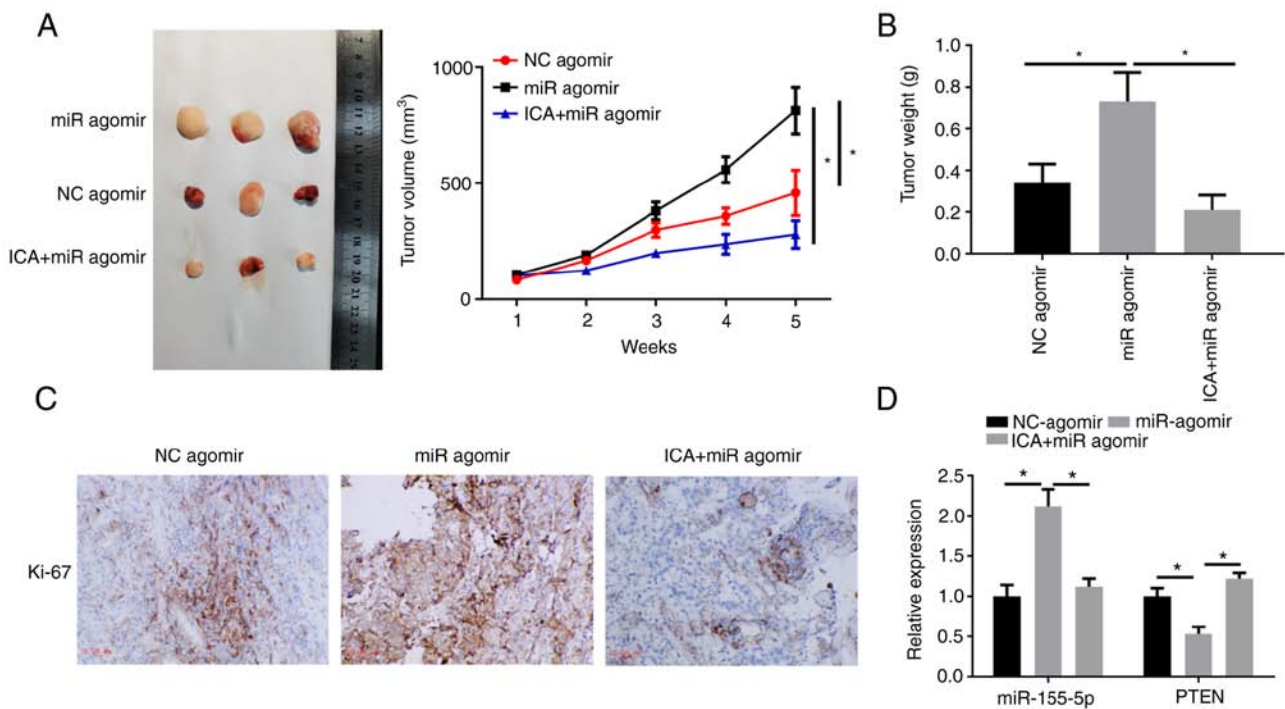


Figure 7. ICA impairs the promoting effect of exogenous miR-205-5p on tumor growth. (A) Tumor volume in each group. (B) Tumor quality in each group. (C) IHC detected the expression of Ki-67 in the tumor tissues of each group. (D) The expression of miR-205-5p and PTEN mRNA levels in each group were detected by RT-qPCR. Data are shown as the mean \pm SD of experiments (n=3), *P<0.05. ICA, icariin; miR, microRNA; IHC, Immunohistochemistry; PTEN, Phosphatase and tensin homolog deleted on chromosome ten; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.

to abnormal activation of the PI3K signaling pathway (29). In bladder cancer, long non-coding RNA LINC00641 is shown to target miR-197-3p/KLF10/PTEN/PI3K/Akt and inhibit bladder cancer (30). In NSCLC, the PTEN/PI3K/Akt signaling affects a variety of cellular functions (11). Aberrant activation of the PI3K/Akt axis is often one of the inducing factors for the development of a number of tumors. Therefore, targeting the PI3K/Akt signaling pathway can also be considered a potential strategy for treating tumors (31). The present study found that ICA could inhibit the progression of lung cancer cells through the PI3K/Akt pathway in a miR-205-5p/PTEN axis-dependent manner. Further corroboration was obtained through *in vivo* experiments. Moreover, it was also learned that there are a number of other targets (including SMAD4) downstream of miR-205-5p and a number of other miRNAs (including miR-382-5p) upstream of PTEN (32-34). Whether these pathways are the molecular mechanisms through which ICA regulates lung cancer progression warrants further exploration.

In conclusion, the present study confirmed that ICA could significantly slow lung cancer progression to a certain extent. Notably, it revealed that ICA could suppress lung cancer progression via the miR-205-5p/PTEN and PI3K/Akt pathways *in vivo* and *in vitro*. Together, the findings provided novel molecular targets and the foundation for the clinical treatment of lung cancer. Further research into other possible molecular mechanisms of ICA affecting the progression of lung cancer will further improve the assessment of the therapeutic potential of ICA for lung cancer.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The two authors designed the experiments, wrote the manuscript and acquired and analyzed the data. The two authors approved the manuscript and are responsible for confirming the authenticity of the raw data. The two authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments were approved by the Experimental Animal Ethics Committee of the First Affiliated Hospital of Zhejiang Chinese Medical University (Approval No. 2021026).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2019. *CA Cancer J Clin* 69: 7-34, 2019.
2. Jordan EJ, Kim HR, Arcila ME, Barron D, Chakravarty D, Gao J, Chang MT, Ni A, Kundra R, Jonsson P, *et al*: Prospective comprehensive molecular characterization of lung adenocarcinomas for efficient patient matching to approved and emerging therapies. *Cancer Discov* 7: 596-609, 2017.
3. Pakkala S and Ramalingam SS: Personalized therapy for lung cancer: Striking a moving target. *JCI Insight* 3: e120858, 2018.
4. Wu X, Kong W, Qi X, Wang S, Chen Y, Zhao Z, Wang W, Lin X, Lai J, Yu Z and Lai G: Icariin induces apoptosis of human lung adenocarcinoma cells by activating the mitochondrial apoptotic pathway. *Life Sci* 239: 116879, 2019.
5. Handoussa H, AbdAllah W and AbdelMohsen M: UPLC-ESI-PDA-MSⁿ Profiling Of phenolics involved in biological activities of the medicinal plant *halocnemum strobilaceum* (Pall.). *Iran J Pharm Res* 18: 422-429, 2019.
6. Yang Q, Wang P, Cui J, Wang W, Chen Y and Zhang T: *Panax notoginseng* saponins attenuate lung cancer growth in part through modulating the level of Met/miR-222 axis. *J Ethnopharmacol* 193: 255-265, 2016.
7. Tian F, Yu CT, Ye WD and Wang Q: Cinnamaldehyde induces cell apoptosis mediated by a novel circular RNA hsa_circ_0043256 in non-small cell lung cancer. *Biochem Biophys Res Commun* 493: 1260-1266, 2017.
8. Liu P, Yang X, Zhang H, Pu J and Wei K: Analysis of change in microRNA expression profiles of lung cancer A549 cells treated with *Radix tetragynis* flavonoids. *Onco Targets Ther* 11: 4283-4300, 2018.
9. Zhao W, Han T, Li B, Ma Q, Yang P and Li H: MiR-552 promotes ovarian cancer progression by regulating PTEN pathway. *J Ovarian Res* 12: 121, 2019.
10. Lu Q, Liu T, Feng H, Yang R, Zhao X, Chen W, Jiang B, Qin H, Guo X, Liu M, *et al*: Circular RNA circSLC8A1 acts as a sponge of miR-130b/miR-494 in suppressing bladder cancer progression via regulating PTEN. *Mol Cancer* 18: 111, 2019.
11. Perez-Ramirez C, Canadas-Garre M, Molina MA, Faus-Dader MJ and Calleja-Hernandez MA: PTEN and PI3K/AKT in non-small-cell lung cancer. *Pharmacogenomics* 16: 1843-1862, 2015.
12. Zang X, Gu J, Zhang J, Shi H, Hou S, Xu X, Chen Y, Zhang Y, Mao F, Qian H, *et al*: Exosome-transmitted lncRNA UFC1 promotes non-small-cell lung cancer progression by EZH2-mediated epigenetic silencing of PTEN expression. *Cell Death Dis* 11: 215, 2020.
13. Wang H, Ma Z, Liu X, Zhang C, Hu Y, Ding L, Qi P, Wang J, Lu S and Li Y: MiR-183-5p is required for non-small cell lung cancer progression by repressing PTEN. *Biomed Pharmacother* 111: 1103-1111, 2019.
14. Zhao J, Ohba S, Shinkai M, Chung UI and Nagamune T: Icariin induces osteogenic differentiation in vitro in a BMP- and Runx2-dependent manner. *Biochem Biophys Res Commun* 369: 444-448, 2008.
15. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
16. Xu F, Li Q, Wang Z and Cao X: Sinomenine inhibits proliferation, migration, invasion and promotes apoptosis of prostate cancer cells by regulation of miR-23a. *Biomed Pharmacother* 112: 108592, 2019.
17. Wu M, Duan Q, Liu X, Zhang P, Fu Y, Zhang Z, Liu L, Cheng J and Jiang H: MiR-155-5p promotes oral cancer progression by targeting chromatin remodeling gene ARID2. *Biomed Pharmacother* 122: 109696, 2020.
18. Li N, Cui T, Guo W, Wang D and Mao L: MiR-155-5p accelerates the metastasis of cervical cancer cell via targeting TP53INP1. *Onco Targets Ther* 12: 3181-3196, 2019.
19. Liu N, Ma M, Qu N, Wang R, Chen H, Hu F, Gao S and Shan F: Low-dose naltrexone inhibits the epithelial-mesenchymal transition of cervical cancer cells in vitro and effects indirectly on tumor-associated macrophages in vivo. *Int Immunopharmacol* 86: 106718, 2020.
20. Liu T, Zuo L, Guo D, Chai X, Xu J, Cui Z, Wang Z and Hou C: Ginsenoside Rg3 regulates DNA damage in non-small cell lung cancer cells by activating VRK1/P53BP1 pathway. *Biomed Pharmacother* 120: 109483, 2019.
21. Ren M, Xu W and Xu T: Salidroside represses proliferation, migration and invasion of human lung cancer cells through AKT and MEK/ERK signal pathway. *Artif Cells Nanomed Biotechnol* 47: 1014-1021, 2019.
22. Shen R and Wang JH: The effect of icariin on immunity and its potential application. *Am J Clin Exp Immunol* 7: 50-56, 2018.
23. Fang J and Zhang Y: Icariin, an Anti-atherosclerotic Drug from Chinese Medicinal Herb Horny Goat Weed. *Front Pharmacol* 8: 734, 2017.
24. Ye L, Yu Y and Zhao Y: Icariin-induced miR-875-5p attenuates epithelial-mesenchymal transition by targeting hedgehog signaling in liver fibrosis. *J Gastroenterol Hepatol* 35: 482-491, 2020.
25. Li J, Jiang K and Zhao F: Icariin regulates the proliferation and apoptosis of human ovarian cancer cells through microRNA-21 by targeting PTEN, RECK and Bcl-2. *Oncol Rep* 33: 2829-2836, 2015.
26. Fang L, Xu W and Kong D: Icariin inhibits cell proliferation, migration and invasion by down-regulation of microRNA-625-3p in thyroid cancer cells. *Biomed Pharmacother* 109: 2456-2463, 2019.
27. Jiang M, Zhong T, Zhang W, Xiao Z, Hu G, Zhou H and Kuang H: Reduced expression of miR2055p promotes apoptosis and inhibits proliferation and invasion in lung cancer A549 cells by upregulation of ZEB2 and downregulation of erbB3. *Mol Med Rep* 15: 3231-3238, 2017.
28. Wang P, Chen D, Ma H and Li Y: Long non-coding RNA MEG3 regulates proliferation and apoptosis in non-small cell lung cancer via the miR-205-5p/LRP1 pathway. *RSC Adv* 7: 49710-49719, 2017.
29. Wise HM, Hermida MA and Leslie NR: Prostate cancer, PI3K, PTEN and prognosis. *Clin Sci (Lond)* 131: 197-210, 2017.
30. Li Z, Hong S and Liu Z: LncRNA LINC00641 predicts prognosis and inhibits bladder cancer progression through miR-197-3p/KLF10/PTEN/PI3K/AKT cascade. *Biochem Biophys Res Commun* 503: 1825-1829, 2018.
31. Ebrahimi S, Hosseini M, Shahidales S, Maftouh M, Ferns GA, Ghayour-Mobarhan M, Hassanian SM and Avan A: Targeting the Akt/PI3K signaling pathway as a potential therapeutic strategy for the treatment of pancreatic cancer. *Curr Med Chem* 24: 1321-1331, 2017.
32. Chu P, Liang A, Jiang A and Zong L: MiR-205 regulates the proliferation and invasion of ovarian cancer cells via suppressing PTEN/SMAD4 expression. *Oncol Lett* 15: 7571-7578, 2018.
33. Liu D, Zhong L, Yuan Z, Yao J, Zhong P, Liu J, Yao S, Zhao Y, Liu L, Chen M, *et al*: MiR-382-5p modulates the ATRA-induced differentiation of acute promyelocytic leukemia by targeting tumor suppressor PTEN. *Cell Signal* 54: 1-9, 2019.
34. Zhao YS, Yang WC, Xin HW, Han JX and Ma SG: MiR-182-5p knockdown targeting PTEN inhibits cell proliferation and invasion of breast cancer cells. *Yonsei Med J* 60: 148-157, 2019.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.