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Original Article

MiR-21/PTEN signaling modulates the chemo-sensitivity to 5-fluorouracil in human lung adenocarcinoma A549 cells

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Abstract: An aberrant expression of microRNA-21 (miR-21) has been found in multiple human cancers, including lung carcinoma. Our work aims at investigating the role of miR-21 in human lung adenocarcinoma A549 cells and cells treated with 5-fluorouracil and their potential molecular mechanisms. A549 cells were transfected with an miR-21 mimic, an miR-21 inhibitor, and their respective negative controls using Lipofectamine 2000. Real-time quantitative PCRs (qRT-PCRs) was applied to evaluate the cells' miR-21 expression levels. EdU incorporation and a cell viability assay were used to confirm the cell proliferation. Flow cytometry was performed to analyze the effects of miR-21 on the A549 cell cycle determination. Using flow cytometry and western blot analysis, we measured the A549 cell apoptosis and necrosis and the potential mechanism. Our findings demonstrated that the overexpression of miR-21 decreased 5-fluorouracil-induced apoptosis and necrosis, and the opposite effects were obtained by the suppression of miR-21. Further, we found that the phosphatase and tensin homologue (PTEN) was regulated by the alteration of miR-21 in A549 cells treated with 5-fluorouracil. Finally, we co-transfected an miR-21 mimic or/and PTEN into A549 cells and found that the anti-apoptotic effects of the miR-21 mimic on the A549 cells could be reversed by overexpressing PTEN. Our present work indicated the involvement of the miR-21/PTEN axis in the 5-fluorouracil-induced cell apoptosis of NSCLC. Therefore, the inhibition of the miRNA-21/PTEN pathway may be a novel therapeutic target to block 5-fluorouracil-induced chemotherapy resistance in NSCLC.

Keywords: miR-21/PTEN, 5-fluorouracil, cell apoptosis, A549, chemotherapy resistance

Introduction

Lung carcinoma is a leading cause of morbidity and mortality in the world and leads to approximately 1.6 million deaths every year [1]. Of the most frequent pathologic types of lung cancer, non-small cell lung cancer (NSCLC), accounts for approximately 85% of all lung cancer cases and is associated with a poor, 5-year overall survival rate of less than 15% [2]. Although molecular biology has developed rapidly in recent years and treatments for adenocarcinoma have improved, the treatments remain unsatisfactory, and the mortality rate of patients with lung cancer remains poor [3, 4]. Thus, the identification of novel treatment approaches is urgently needed for NSCLC therapy.

MicroRNAs (miRNAs), a class of small non-coding RNAs of 19~22 nucleotides in length, act as endogenous inhibitors of gene expression and post-transcriptionally modulate their targeted genes, primarily by binding to the 3'-untranslated region (3'-UTR) of target mRNAs that leads to mRNA down-regulation and/or translational inhibition [5, 6]. To date, approximately 1000 miRNAs have been identified and each miRNA can regulate and control hundreds of gene expressions [7]. And it has been reported that more than 60% of cellular protein coding genes are readjusted by miRNAs [8]. Accordingly, miRNAs are closely interconnected in a wide range of cell functions, including cell division, differentiation, proliferation and apoptosis [9]. More importantly, increasing evidence has demonstrated that aberrant expressions of miRNAs are closely associated with the chemotherapy resistance of NSCLC. MiR-181c contributes to cisplatin resistance in non-small cell lung cancer cells by targeting Wnt inhibition factor 1

[10]. MiR-513a-3p sensitizes human lung adenocarcinoma cells to chemotherapy by targeting GSTP1 [11]. MiR-638 is a new biomarker for the outcome prediction of non-small cell lung cancer patients receiving chemotherapy [12]. MicroRNA-130b targets PTEN to mediate chemoresistance to cisplatin in lung cancer cells by regulating the Wnt/β-catenin pathway [13]. Studies have demonstrated that miR-21 is the only upregulated miRNA in all human cancers [14]. In addition, miR-21 can decrease the PDCD4 expression level and regulate PI3K/AKT/mTOR signaling, thereby modulating the radiosensitivity of NSCLC cells [15]. The MiR-21/PTEN signaling pathway regulates gefitinib resistance in NSCLC. However, the roles of miR-21 in the chemosensitivity of NSCLC cells to 5-fluorouracil still remains to be elucidated.

The function of miR-21 on PTEN expression was confirmed in the NSCLC cell lines and in the NSCLC tumor tissue samples [16]. MiR-21 was overexpressed concomitantly to the depression of PTEN in the PC-9 gefitinib resistant cell lines in comparison with the PC-9 cells [17]. Therefore, we postulated that miR-21 regulated PTEN as one of several target genes of miR-21 in NSCLC. Our present work was undertaken to illustrate the function of miR-21 in NSCLC and to identify the modulation of PTEN by miR-21 and confirm the mechanisms of this role. We first demonstrate that miR-21 does not promote A549 proliferation, cell cycle progression, or apoptosis. However, it enhances cellular apoptosis and necrosis and represses PTEN expression with 5-fluorouracil treatment in A549 cells.

Materials and methods

Cell culture and transfection

The human lung adenocarcinoma A549 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and routinely cultivated in an RPMI 1640 medium and maintained at 37°C with 5% CO_2 supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, USA) and 1% penicillin/streptomycin sulfate (Invitrogen, Carlsbad, USA). The MiR-21 mimic, inhibitor and their negative controls were purchased from RiboBio (Guangzhou, China). The non-small lung cancer cell line A549 cells were transfected with the miR-21 mimic (50 nM), inhibitor (100 nM) and their negative controls (NC) for 48 h using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocols.

Cell viability assay and EdU incorporation

A549 cells were seeded into 96-well plates and were transfected with an miR-21 mimic (50 nM), an inhibitor (100 nM) or their negative controls using Lipofectamine 2000 (Invitrogen). After 48 hours, the cells were harvested for cell proliferation assays using Cell Counting Kit-8 (CCK-8) (Dojindo, Japan), according to the manufacturer's manuals. Absorbance was then performed at 450 nm using a microplate reader (Bio-Rad). Next, A549 cells were planted in 24-well plates. After transfection with the miR-21 mimics, inhibitors, and their negative controls, the cells were incubated with EdU for 8 hours before they were quantified. The actively proliferating cells were then evaluated using a Cell-Light™ EdU Cell Proliferation Detection kit (RiboBio, China) following the manufacturer's instructions.

Cell cycle analysis

The A549 cells were seeded into 6-well plates and were then transfected with an miR-21 mimic (50 nM), an miR-21 inhibitor (100 nM) or their negative controls for 48 h before determination. Then A549 cells were separated using 0.025% trypsin, cleaned once with PBS, and fixed in 70% ethanol at 4°C overnight. The cellular DNA content was stained using propidium iodide (PI) (Sigma, USA) and evaluated using a MoFlo XDP Cell Sorter (Beckman Coulter). The number of cells in each phase of the cell cycle was confirmed using FlowJo software (Treestar Inc., USA).

Flow cytometry determination of cell apoptosis and necrosis

The MiR-21 mimic, inhibitor, or respective controls were transfected to the A549 cells for 48 h, and the cell apoptosis and necrosis were assessed using Annexin V-FITC and propidium iodide (PI) kits (Dojindo, Japan) according to the manufacturer's manuals, followed by flow cytometry detection (Beckman Coulter, USA).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent following the manufacturer's manuals.

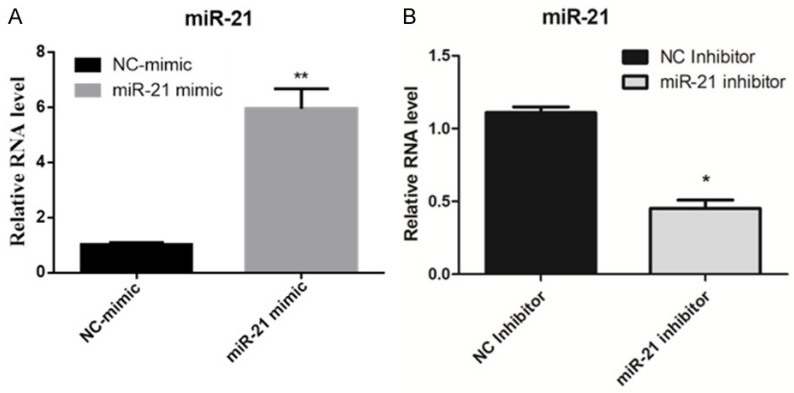


Figure 1. Quantitative reverse transcription polymerase chain reactions (qRT-PCRs) confirm that miR-21 mimics and inhibitors take effect in A549 cells. A. miR-21 mimics significantly up-regulate miR-21 levels in A549 cells. **P<0.01. B. miR-21 inhibitors successfully down-regulate miR-21 expression levels in A549 cells. *P<0.05.

For miR-21 qRT-PCR, total RNA was reverse transcribed using a miRNA-specific primer and the miScript Reverse Transcription kit (Qiagen, Germany) by a stem-loop RT primer using a PrimeScript RT Reagent Kit (Takara). Relative expression was determined by the comparative Ct method and normalized to the expression level of U6 small nuclear RNA.

Western blot analysis

A549 cells were lysed with a RIPA buffer (KeyGen, China) including 1% phenylmethanesulfonyl fluoride (PMSF). Total proteins were isolated with 10% SDS-PAGE gels, and then they were transferred onto PVDF membranes (Millipore, Bedford, MA, USA) for western blotting analysis and were probed with anti-PTEN Ab (1:1000; Epitomics, ab154812), anti-Bcl-2 ab (1:1000; Abclonal, A0208), anti-Bax ab (1:1000; Abclonal, A2211). The enhanced chemiluminescence ECL System was used to analyze the protein bands with a ChemiDocTM XRS Plus luminescent image analyzer (Bio-Rad, USA).

Luciferase assays

The 3'-UTR of PTEN, with putative binding sites for miR-21, was inserted into the 3'-end of the firefly luciferase gene of the dual-luciferase miRNA target expression vector luciferase reporter vector (pGL3) (Promega corporation, WI, USA). For the luciferase reporter assay, A549 cells were co-transfected with the luciferase reporter vectors or/and miR-21 mimics, miR-21 inhibitor using Lipofectamine 2000. Luciferase activity was standardized to Renilla luciferase. After 48 h transfection, Firefly luciferase activity was evaluated using the Dual-Luciferase Reporter Assay system (Promega Corporation, USA) following the manufacturer's manuals.

Statistical analysis

Results are presented as the mean ± SEM. An independent-samples *t*-test or one-way ANOVA was performed with Bonferroni's post-hoc test. A *P*-value, less than 0.05 was regarded as statistically significant. All data in the study were analyzed using IBM SPSS 20.0 for Windows.

Results

MiR-21 does not induce A549 cells' cell viability and proliferation

To examine whether miR-21 could modulate the cell viability of A549 cells, a transfection of the miR-21 mimic, inhibitor, or their negative controls were conducted. The transfection effect of the mimics, inhibitors, or their negative controls has been previously indicated [18]. As confirmed by qRT-PCR, we determined that the miR-21 expression level was successfully increased by miR-21 mimic, but it was decreased by miR-21 inhibitor, indicating that the miR-21 mimic and inhibitor significantly modulated miR-21 the expression level in the A549 cells (**Figure 1**). The proliferation of the A549 cells was evaluated using CCK-8 cell viability and EdU incorporation assays, and the miR-21 mimic did not significantly increase cell viability, and the miR-21 inhibitor did not decrease their viability (**Figure 2A**). The MiR-21 mimic did not significantly increase cell proliferation, but miR-21 inhibitor did not decrease their proliferation (**Figure 2B**). These data demonstrate that miRNA-21 does not contribute to A549 tumor features by regulating cell growth.

MiR-21 does not regulate A549 cells' cell cycle and apoptosis

To further elucidate the biological effect of miR-21 on A549 cells' progression, flow cytometry

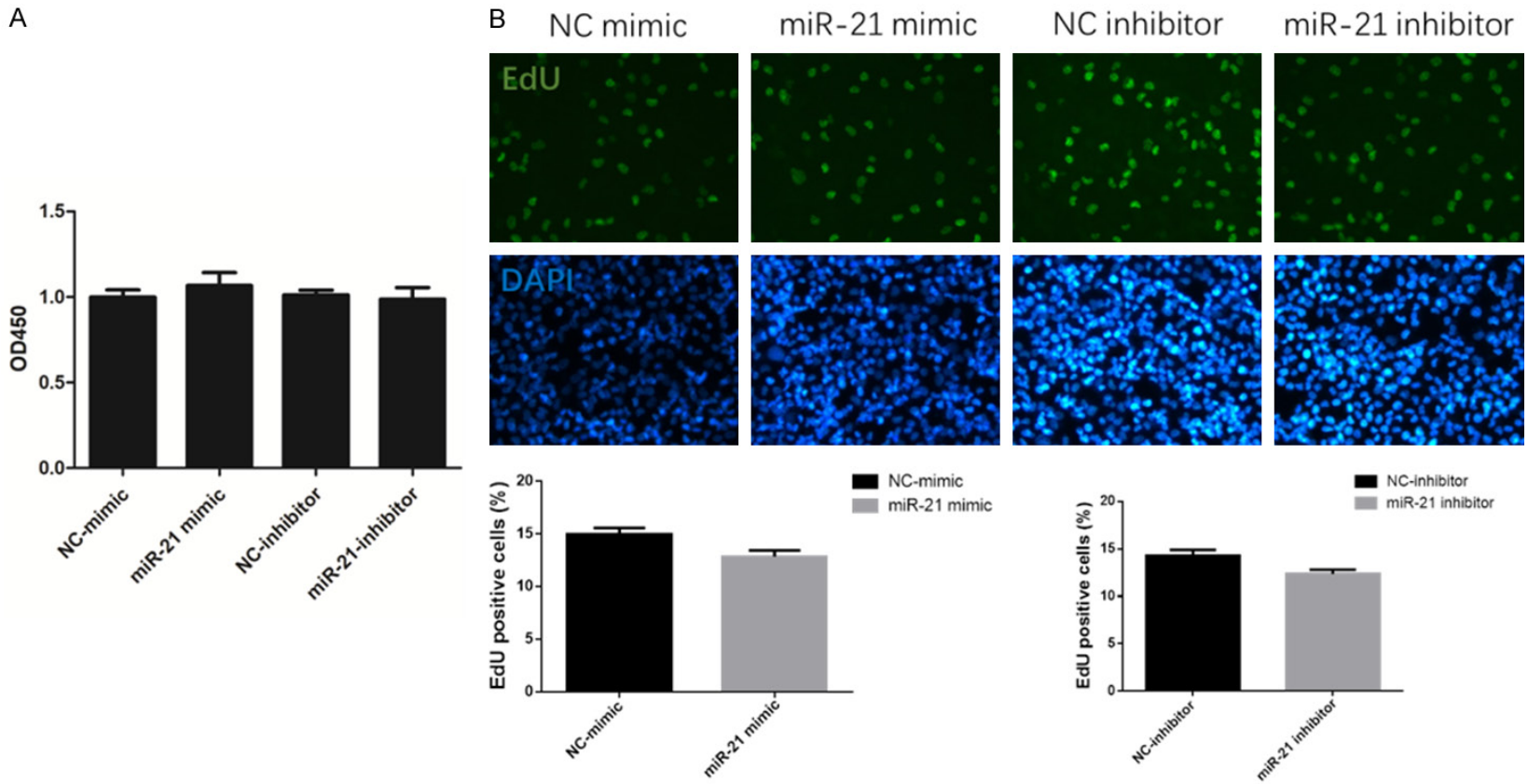


Figure 2. miR-21 does not regulate the cell viability and cell proliferation of A549 cells. Cell Counting Kit-8 assays indicate that miR-21 mimics do not increase the cell viability of A549, while the miR-21 inhibitors do not decrease the cell viability of A549 (A). 5-Ethynyl-2'-deoxyuridine (EdU) staining indicates that miR-21 mimics do not increase the proliferation of A549 cells (A), while miR-21 inhibitors do not decrease the proliferation of A549 cells (B).

was performed to evaluate the effects of miR-21 on the progression of the A549 cell cycle and cell apoptosis. The percentage of A549 cells was the same in both the S and G2 phases with the miR-21 mimic (**Figure 3A**), which paralleled with a zero difference by the miR-21 inhibitor (**Figure 3B**). As enhanced cell proliferation is closely related to altered cell apoptosis, flow cytometry indicated that the miR-21 mimic or inhibitor do not regulate the A549 cells' progression by increasing or decreasing apoptosis and necrosis (**Figure 4A**, **4B**). These results showed that miR-21 can't alter cell cycle progression via an increase or decrease in the population of A549 cells.

MiR-21 regulates 5-fluorouracil-induced cell apoptosis in A549 cells

To examine the functional roles of miR-21 in A549 cells, transfection of the miR-21 mimic, inhibitor, or their miR-NC, were conducted. The MiR-21 mimic was determined to be sufficient to increase the relative miR-21 level, but the miR-21 inhibitor had the opposite effect, validating that the miR-21 mimic and inhibitor successfully had its effect in the A549 cells (**Figure 5A**). Flow cytometry revealed that the miR-21 mimic reduced the A549 cells, but the miR-21 inhibitor aggravated that (**Figure 5B**). Simultaneously, the overexpression of miR-21 led to the increased expression of Bcl-2, decreased the expression of the Bax ratio at the protein levels, but the inhibition of miR-21 had the opposite effects (**Figure 5C**). These results indicate the anti-apoptotic effect of miR-21 against 5-fluorouracil-induced cell apoptosis in A549 cells.

PTEN is a target gene of mir-21 modulating 5-fluorouracil-induced cell apoptosis in A549 cells

We determined how miR-21 controls 5-fluorouracil-induced cell apoptosis in A549 cells. PTEN is a proverbial downstream target gene of miR-21 [17, 19-21]. In our present work, a western blot analysis showed that PTEN was negatively regulated by miR-21 in the A549 cells (**Figure 6A**). In addition, up-regulating miR-21 alone led to decreased apoptosis in 5-fluorouracil-induced cell apoptosis in the A549 cells, but overexpressing PTEN alone resulted in a significantly increased apoptosis in the A549 cells. Moreover, the co-transfection of the overexpression of PTEN and the miR-21 mimic could abolish the anti-apoptotic effects of the miR-21 mimic on cell apoptosis in the A549 cells treated with 5-fluorouracil (**Figure 6B**), demonstrating that miR-21 regulated 5-fluorouracil-induced cell apoptosis, at least in part, by targeting PTEN.

Discussion

In recent years, investigators report that miRNAs play critical regulatory roles in the occurrence and development of human cancers and involve in pathogenesis of a variety of diseases such as cardiovascular diseases and cancers [22, 23], and have critical roles in the regulation of various vital processes containing tumor formation, growth, invasion, metastasis, differentiation and chemotherapy resistance [24-29], with the molecular mechanisms underlying the roles of the miRs still unclear.

MiR-21 is one of the most common dysfunctional miRNAs in human tumors and has been described in a variety of types of human cancers. Several studies have illustrated that miR-21 is increased in breast [30], ovarian [31], colorectal [32], prostate [33], pancreatic [34], and thyroid cancer [35], as well as in lung carcinoma [36, 37]. MiR-21 acts as a prognostic [38, 39], diagnostic [40], and predictive [41] biomarker and as a therapeutic target in lung cancer. The overexpression of miR-21 was found to weaken sensitivity to gefitinib in PC9 cells by inhibiting PTEN and activating the Akt and ERK pathways. In addition, miR-21/PTEN expression in the regulation of TKI sensitivity in NSCLC promotes cell invasive ability and induces cell apoptosis [17]. Wang, et al. found that miR-21 could modulate adriamycin (ADR) resistance of breast cancer cells, at least in part, by targeting the tumor suppressor gene PTEN [42]. However, the associations of miR-21 expression with human lung adenocarcinoma cell line A549, especially in chemotherapy resistance, are still poorly elucidated. Here, we found that miR-21 did not markedly regulate lung adenocarcinoma cell line A549 growth but reduced A549 cells' cell apoptosis treated with 5-fluorouracil, which prompted us to further explore the underlying mechanisms.

PTEN is a proverbial target gene of miR-21, which mainly modulates cancer cell growth and proliferation [16, 32, 43, 44]. Meanwhile, the

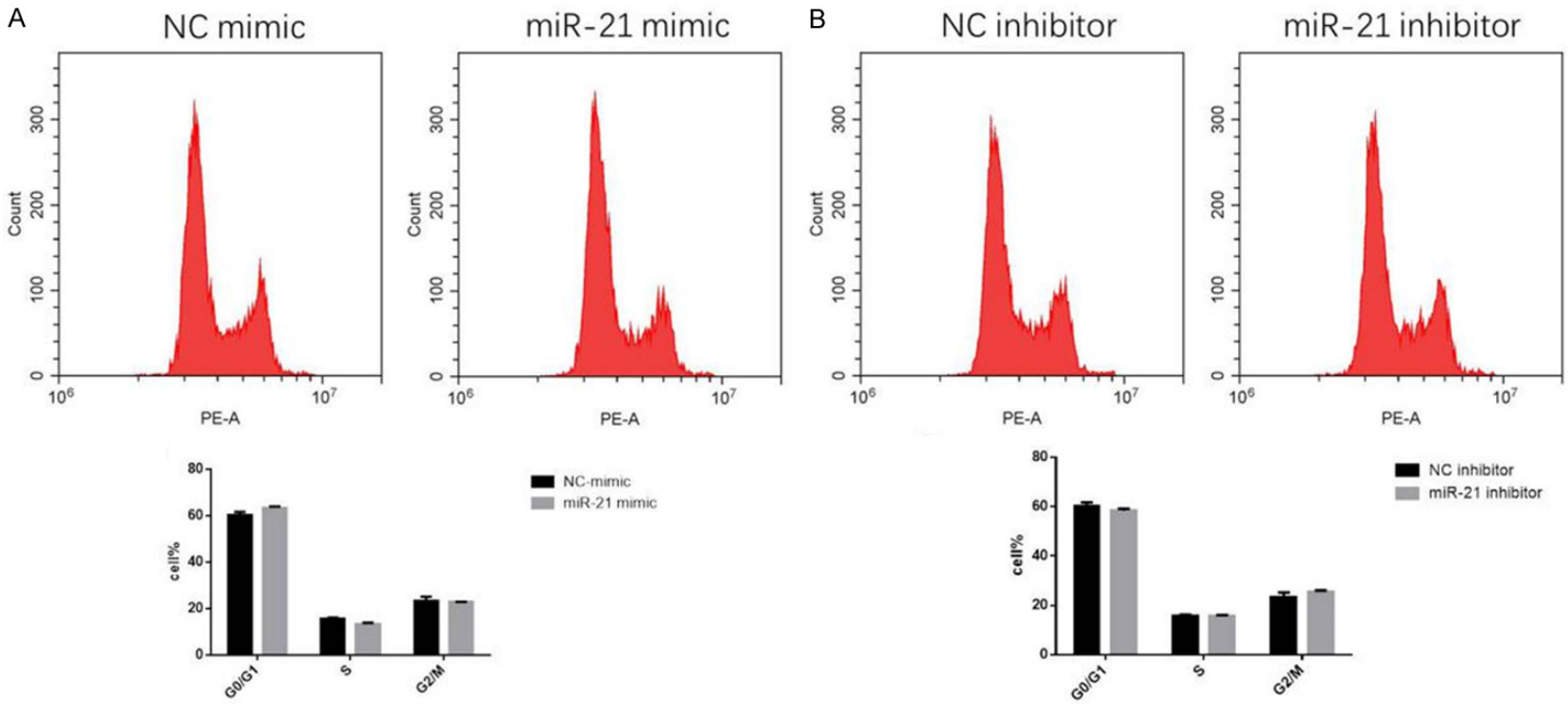


Figure 3. MiR-21 does not reduce A549 cell cycle progression. (A) MiR-21 mimics do not increase the percentage of A549 cells in cell phases. (B) MiR-21 inhibitors do not decrease the percentage of A549 cells in cell phases (A, B).

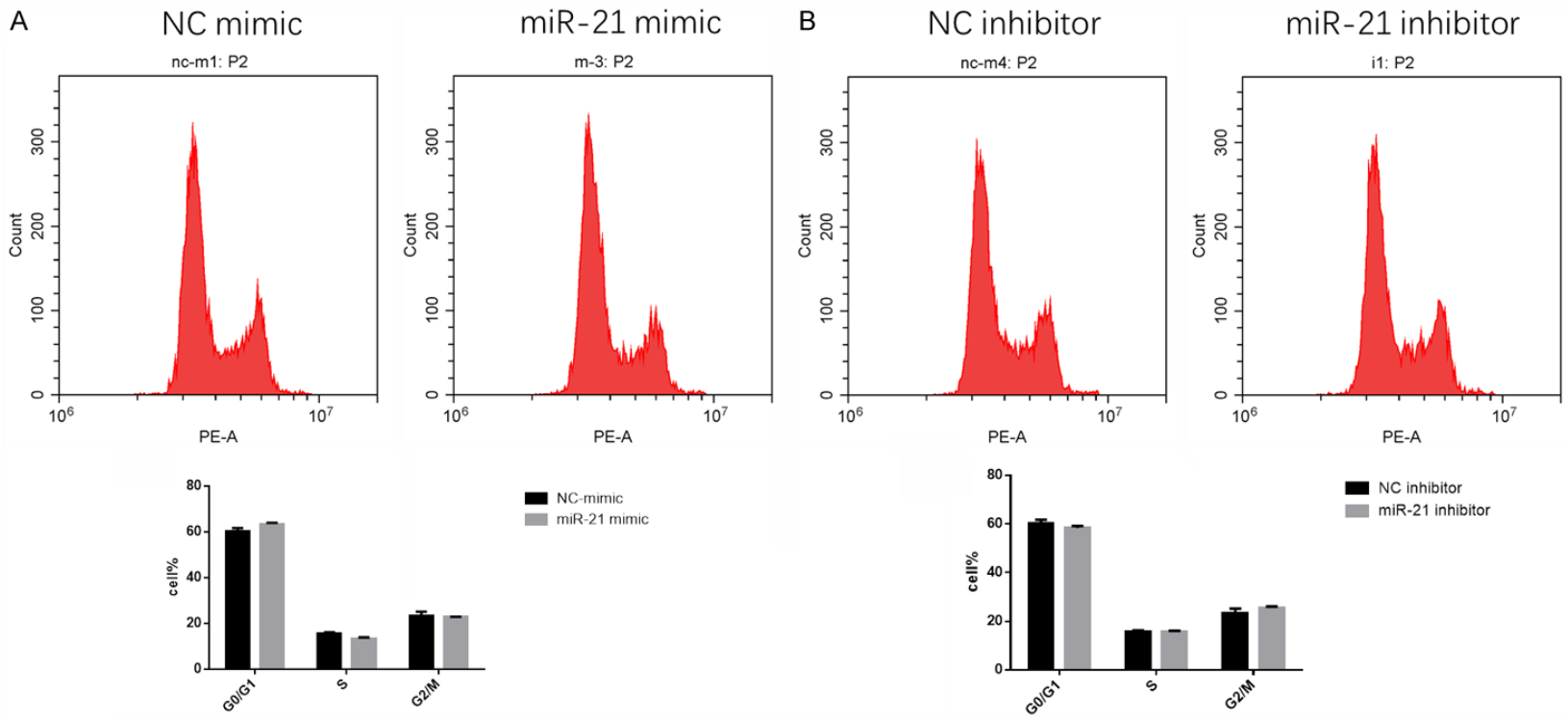
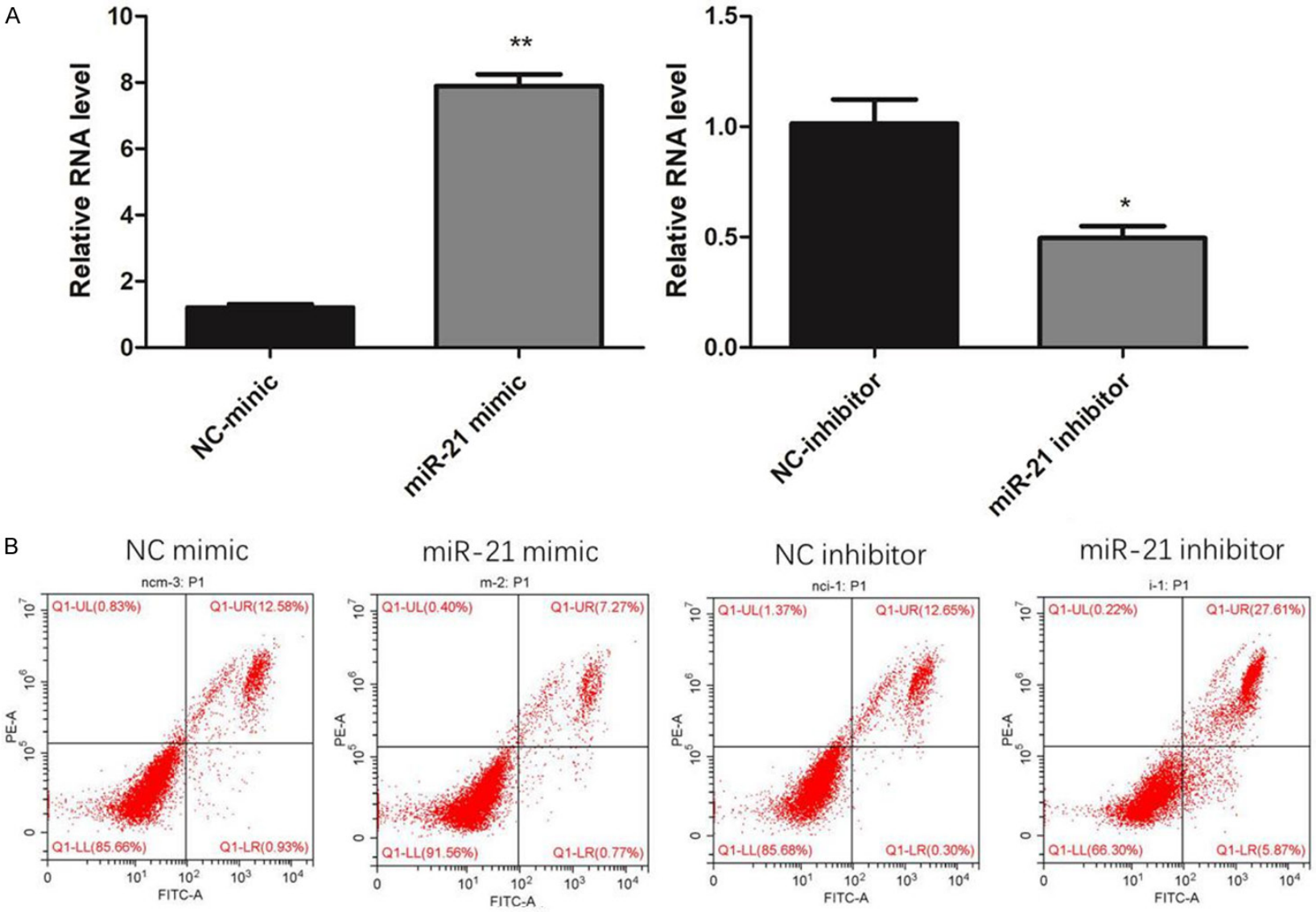


Figure 4. MiR-21 does not control A549 cells' cell apoptosis and necrosis. Flow cytometry indicates that miR-21 mimics or inhibitors do not facilitate A549 cells' progression by increasing or decreasing apoptosis and necrosis (A, B). *P<0.05.


A
Relative RNA level
10
8
6
4
2
0
**
NC-minic
miR-21 mimic
Relative RNA level
1.5
1.0
0.5
0.0
*
NC-inhibitor
miR-21 inhibitor
B
NC mimic
ncm-3: P1
Q1-UL(0.83%)
Q1-UR(12.58%)
Q1-LL(85.66%)
Q1-LR(0.93%)
miR-21 mimic
m-2: P1
Q1-UL(0.40%)
Q1-UR(7.27%)
Q1-LL(91.56%)
Q1-LR(0.77%)
NC inhibitor
nci-1: P1
Q1-UL(1.37%)
Q1-UR(12.65%)
Q1-LL(85.68%)
Q1-LR(0.30%)
miR-21 inhibitor
i-1: P1
Q1-UL(0.22%)
Q1-UR(27.61%)
Q1-LL(66.30%)
Q1-LR(5.87%)
PE-A
FITC-A

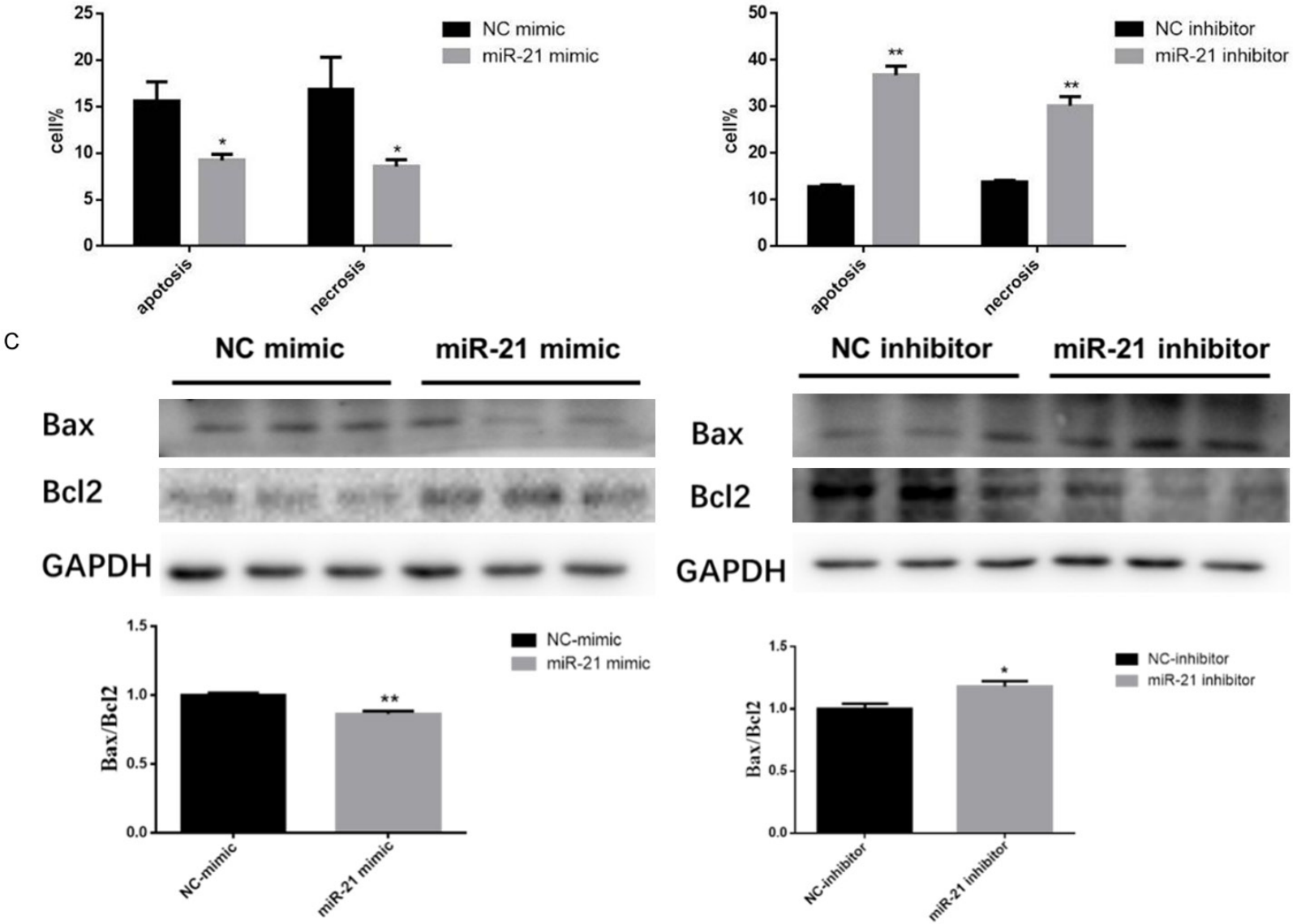
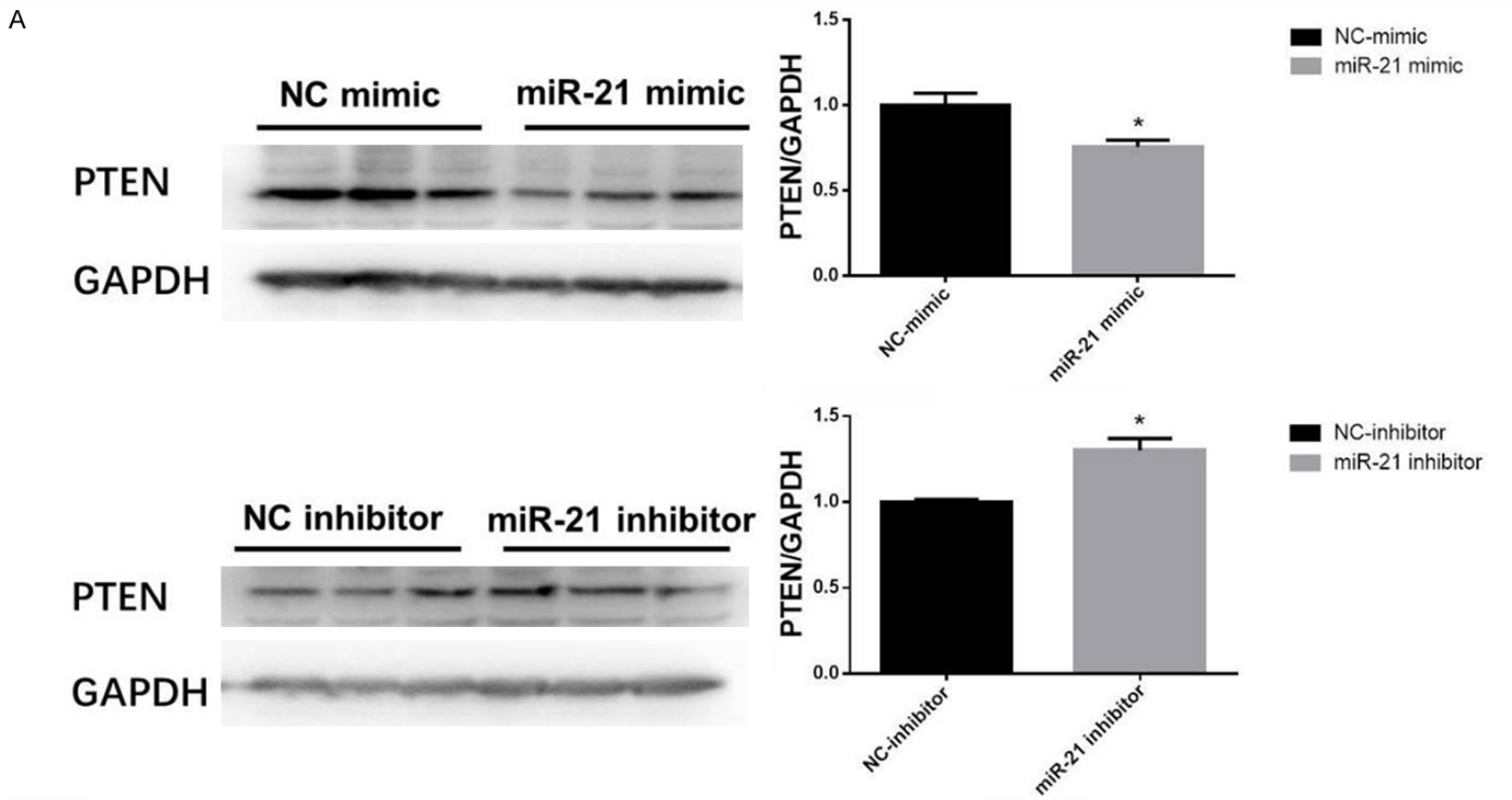
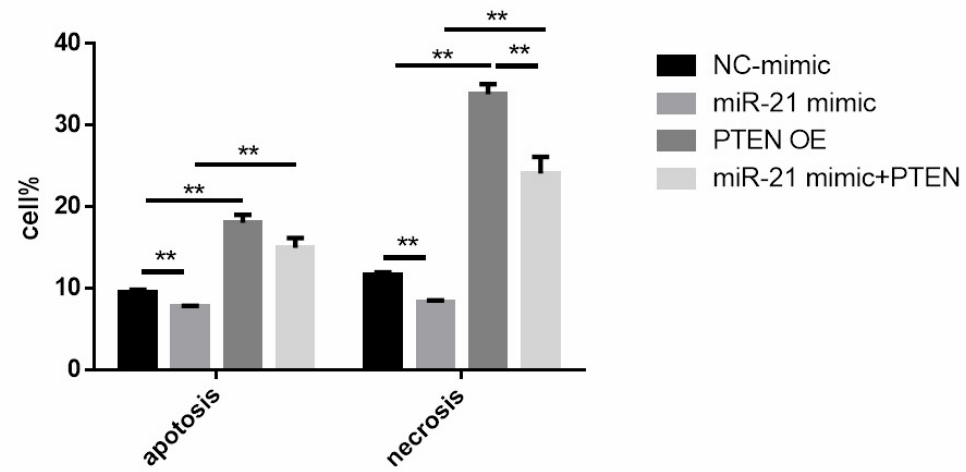
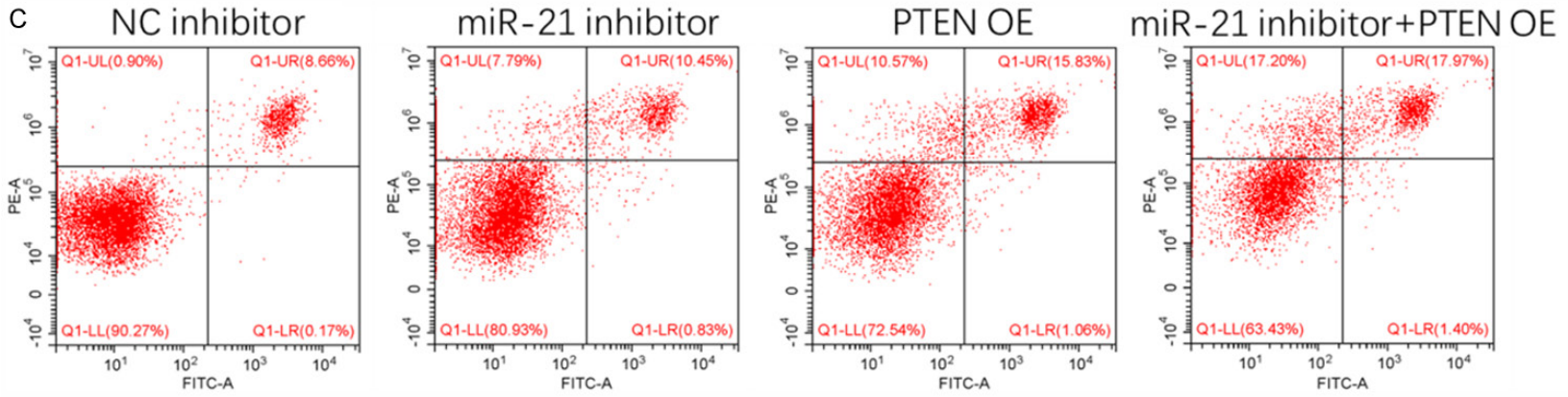


Figure 5. MiR-21 regulates 5-fluorouracil-induced cell apoptosis in A549 cells. A. miR-21 mimic and inhibitor successfully took effect in A549 cells treated with 5-fluorouracil. B. Flow cytometry reveals that the miR-21 mimic reduced A549 cells' cell apoptosis with 5-fluorouracil treatment, while miR-21 inhibitor had the opposite effect. C. Accordingly, Immunoblot analysis for Bcl-2/Bax ratio with miR-21 overexpression or inhibition in A549 cells treated with 5-fluorouracil. *P<0.05, **P<0.01.


A
NC mimic
miR-21 mimic
PTEN
GAPDH
PTEN/GAPDH
NC-mimic
miR-21 mimic
NC inhibitor
miR-21 inhibitor
NC-inhibitor
miR-21 inhibitor
B
NC mimic
miR-21 mimic
PTEN OE
miR-21 mimic+PTEN OE
Q1-UL(2.73%)
Q1-UR(9.48%)
Q1-LL(87.57%)
Q1-LR(0.22%)
Q1-UL(0.77%)
Q1-UR(7.76%)
Q1-LL(91.32%)
Q1-LR(0.15%)
Q1-UL(16.60%)
Q1-UR(17.99%)
Q1-LL(64.23%)
Q1-LR(1.17%)
Q1-UL(9.78%)
Q1-UR(14.99%)
Q1-LL(74.35%)
Q1-LR(0.89%)
PE-A
FITC-A





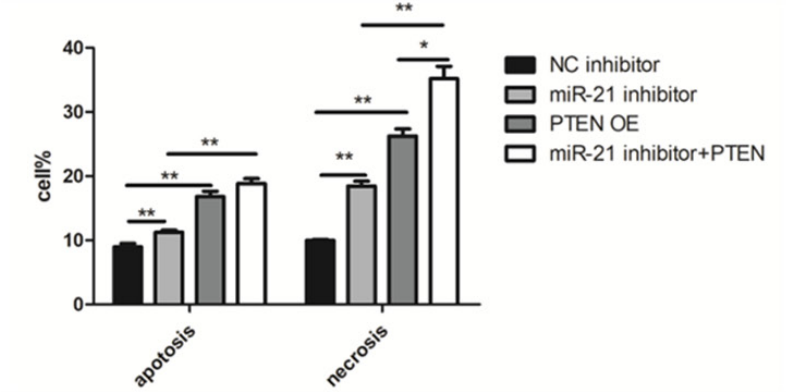


Figure 6. PTEN is a downstream target gene of miR-21 in regulating 5-fluorouracil-induced A549 cell apoptosis. A. Western blot assay showed that PTEN was negatively regulated by miR-21 in A549 cells treated with 5-fluorouracil. B. Flow cytometry analysis for apoptosis and necrosis of 5-fluorouracil-treated A549 cells with the overexpression of miR-21 and/or PTEN overexpression. C. Estimates for apoptosis and necrosis of 5-fluorouracil-treated A549 cells with miR-21 inhibition and/or PTEN overexpression using flow cytometry. *P<0.05, **P<0.01.

overexpression of PTEN has been shown to enforce cellular apoptosis [45, 46]. Based on that, we investigated whether PTEN could be a downstream regulator of miR-21, mediating its effect in 5-fluorouracil-induced A549 cells' apoptosis. As intended, PTEN was reverse-regulated by miR-21 at the protein level in the A549 cells treatment with 5-fluorouracil. Importantly, overexpressing PTEN could reverse the anti-apoptotic effect of the miR-21 mimic in A549 cells treated with 5-fluorouracil. These results demonstrate that PTEN is a downstream effector of miR-21 controlling A549 cells' apoptosis induced by 5-fluorouracil.

In conclusion, our work indicates that miR-21 regulates 5-fluorouracil-induced human lung adenocarcinoma cell line A549 cells' apoptosis by targeting PTEN. Our findings may provide new evidence for understanding the potential roles of miR-21 in the pathogenesis of NSCLC and become a potential therapeutic target of the resistance of NSCLC to chemo-resistance.

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Disclosure of conflict of interest

None.

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