

## Original Article

# miR-205 regulates A549 cells proliferation by targeting PTEN

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**Abstract:** miR-205 is an epithelial-specific miRNA and has been shown to orchestrate some cellular processes such as epithelial mesenchymal transition (EMT) and differentiation fate of stem cells in mammary gland. miR-205 play a part of a tumor suppressor in human cancers. However, the role of miR-205 in lung cancer is unclear. In this study, we detected the expression level of miR-205 in 46 cases clinical lung cancer specimens and adjacent normal tissues by stem-loop RT-PCR. We found that the expression of miR-205 was significantly increased in lung cancer specimens compared to adjacent normal tissues ( $P < 0.01$ ). Furthermore, we observed the expressions of PTEN protein and mRNA in lung cancer tissues and adjacent normal tissues by methods of western blot and Real time PCR respectively. We found that the expressions of PTEN protein and mRNA was significantly decreased in lung cancer specimens compared to adjacent normal tissues. And then, we found there is a negative relationship between the expression of miR-205 and PTEN mRNA in lung cancer by analyzed. To validate whether PTEN was direct targets of miR-205, a dual-luciferase reporter assay was employed, the result showed that PTEN is a target gene of MiR-205. In subsequent experiments, we examined the expressions of PTEN protein and mRNA after transfection of miR-205 mimics or inhibitor into A549 cells, and A549 cell proliferation was measured by CCK-8 tests. We found that the expression of PTEN protein and mRNA in A549 cells were significantly down-regulated or up-regulated after miR-205 mimics and miR-205 inhibitors transfected into, and miR-205 could inhibits A549 cells proliferation. These results indicate that miR-205 might inhibitor the proliferation of A549 cells by regulating the expression of PTEN.

**Keywords:** Lung cancer, miR-205, PTEN, proliferation

## Introduction

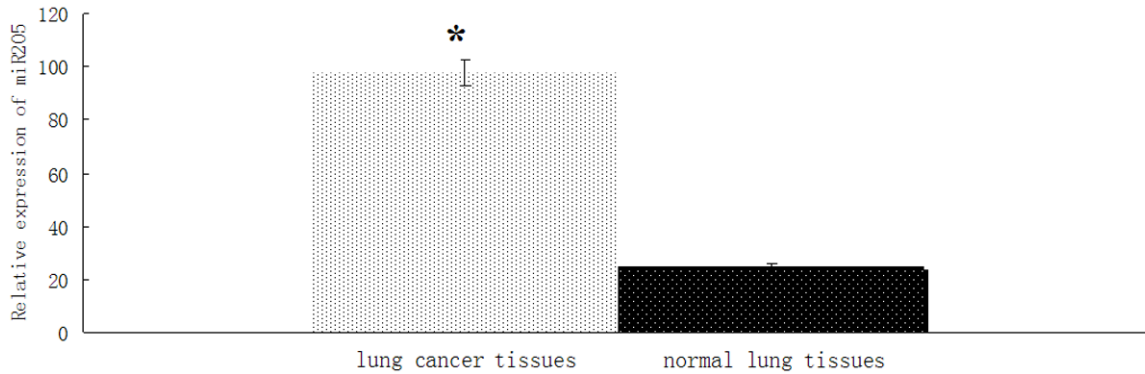
Lung cancer is a high incidence of cancer increasing year by year. With the development of science and technology, the treatment of lung cancer had penetrated the gene level. In recent years, miRNA has been paid more and more attention. MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression through base-pairing with their target messenger RNAs. miR-205 is a highly conserved microRNA and it has been proved that miR-205 shares a similar expression pattern with miR-200 family [1]. It has been proved that miR-205 might associated with the occurrence, development and prognosis of kinds tumors, and many studies suggest that expression levels of miR-205 were evidently different in different tumors. For example, the expressions of miR205 was down-regulated in Breast cancer [2], the former Prostate cancer [3], however, up-regulate in Endometrial carcinoma [4], non small cell lung

cancer [5], head and neck squamous cell carcinoma [6] and melanoma [7]. These results indicated that miR-205 might play a tumor suppressor in specific tissues. Therefore, further research is needed to understand the mechanism of action and function of miR-205 in different tumor tissues.

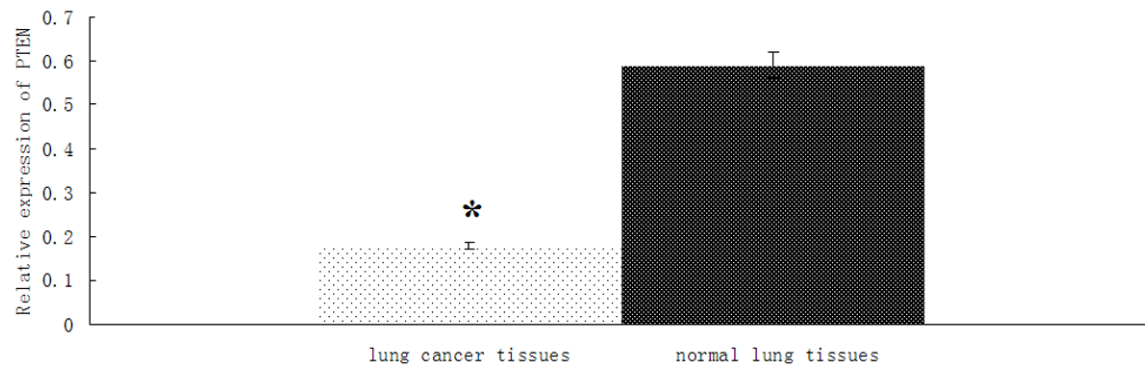
PTEN is a phosphatase that can affect the activity of Akt, a regulator involved in cell survival, which belongs to tumor suppressor gene family. Clinical studies have demonstrated that different degrees of PTEN gene mutation or loss exist in a variety of advanced tumor. Study found that the expression of PTEN in non small cell lung cancer (NSCLC) group was significantly lower than that of adjacent group, and irrelevant with gender, age and lymph node metastasis in patients [8]. There was low expression or no expression of PTEN in 74% regu/117) There was low expres [9]. The negative stained rate of PTEN inssion of PTEN in 74%regulator involved



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**Figure 1.** The expression of MiR-205 was up-regulated in lung cancer tissue. U6 RNA was used as an endogenous normalizer and the relative combined expression levels of miR-205 are shown.  $2^{-\Delta\Delta Ct}$  values  $\pm$  SEM,  $n = 46$ ,  $*P < 0.01$ .



**Figure 2.** The expression of PTEN is downregulated in lung cancer tissue. U6 RNA was used as an endogenous normalizer and the relative combined expression levels of miR-205 are shown.  $2^{-\Delta\Delta Ct}$  values  $\pm$  SEM,  $n = 46$ ,  $*P < 0.01$ .

negative control group, miR-205 mimics (miR-205), mimic NC, miR-205 inhibitor and inhibitor NC group [14]. Cells in logarithmic growth phase were transfected with negative control, miR-205 mimics or miR-205 inhibitor respectively according to Lipofectamin 2000 instructions.

### Luciferase reporter assay

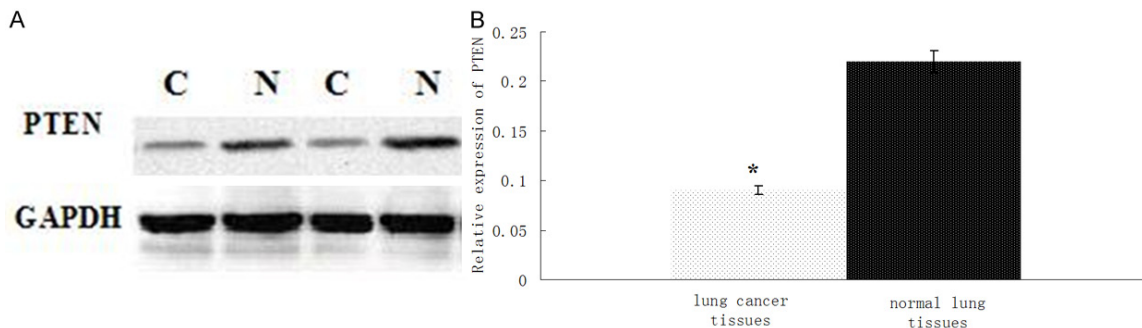
The target sequences of PTEN wild type 3'UTR were cloned into a luciferase vector which contained the *Renilla* luciferase gene. In addition, mutant 3'UTR was also cloned. HEK-293T cells were cotransfected with miR-NC or miR-205 mimics using Lipofectamine 2000 (Invitrogen). Cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), and luciferase activity values were normalized relative to that of the *Renilla* luciferase internal control.

### Cell Counting Kit-8

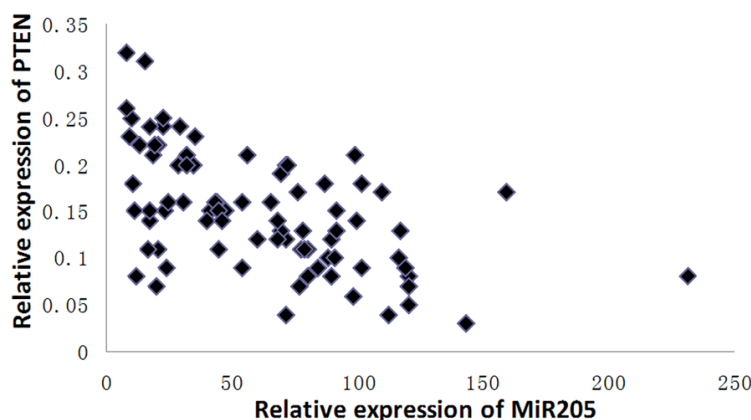
Cell proliferation analysis was performed using the Cell Counting Kit-8 (CCK-8) (Tongren, Shanghai, China). The optimum reaction time of CCK-8 was determined to be 2.5 h. When 80% confluent, A549 cells (100  $\mu$ L/well) were seeded into 96-well plates, and were left untransfected or transfected with miR-205-sense or miR-205-antisense, and further incubated for 12, 24, 48, and 72 hours using three replicates. Approximately 10  $\mu$ L of tetrazolium salt, WST-8, was added to each well for 1 hour. The optical density (OD), at 450 nm, of each well was determined by microplate reader.

### Statistical analysis

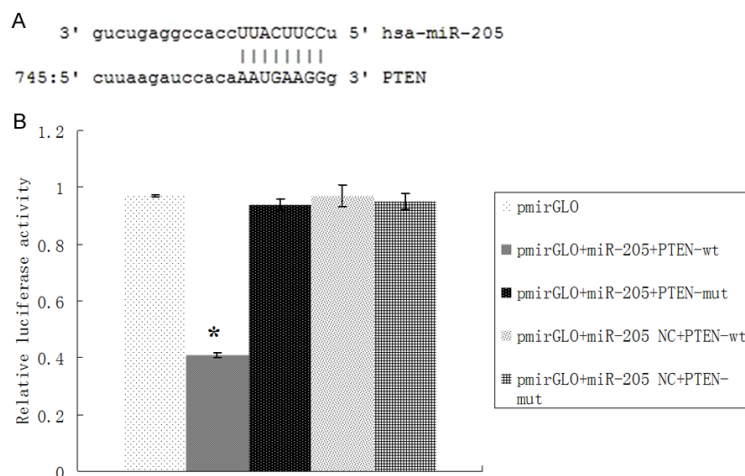
Statistical analysis of data was performed using SPSS19.0 software. Statistical evaluation was performed using one-way analysis of variance (ANOVA;  $P < 0.05$ ) and Student's t-test.



**Figure 3.** The expression of PTEN protein is downregulated in lung cancer tissue. (A) PTEN protein expression was determined by Western blot in lung cancer (C) and normal tissues (N). (B) Optical density values for combined PTEN protein relative to GAPDH in lung cancer tissues. N = 46. \* $P < 0.01$ .



**Figure 4.** The scatter diagram of the expression of miR-205 and PTEN.



**Figure 5.** PTEN as a target gene of miR205. A. Schematic diagram of the predicted miR-205 binding site in the PTEN 3'-UTR. B. Luciferase reporter assays in untreated HEK-293T cells, or cotransfected with wild type (wt) or mutant (mut) PTEN 3'-UTR, and miR-133b or miR-133b negative control (NC). \* $P < 0.01$ .

## Results

### *Expression of MiR-205 was upregulated in lung cancer tissues*

The expressions of miR-205 in 46 lung cancer specimens and their corresponding adjacent normal tissues (The distance of 5-10 cm from tumor) were detected by RT-PCR. We found that the expression of miR-205 was significantly higher in lung cancer specimens than in adjacent normal tissues ( $P < 0.01$ ; **Figure 1**). The results suggested that the up-regulation of miR-205 might be involved in human lung cancer disease processes.

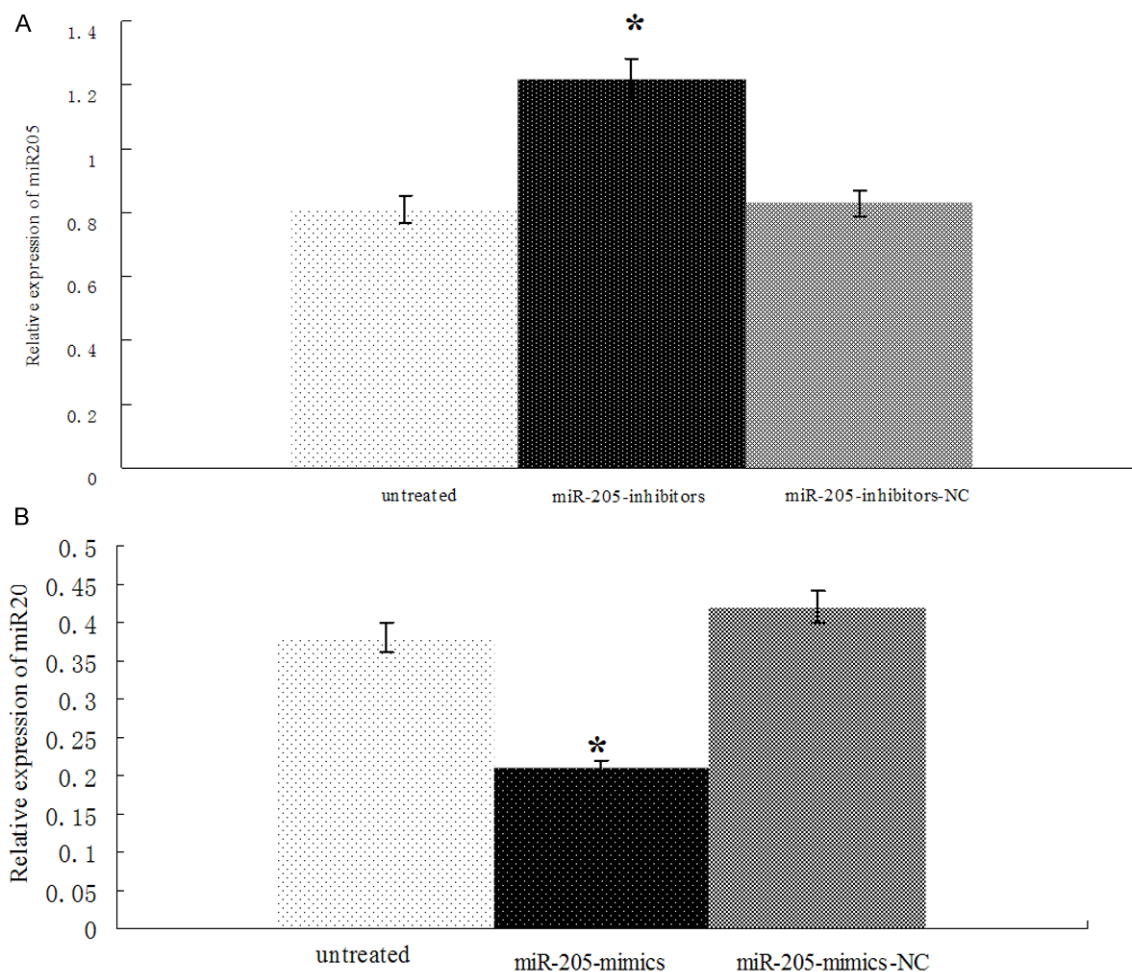
### *Expression of PTEN mRNA was downregulated in lung cancer tissues*

The expressions of PTEN mRNA in 46 lung cancer specimens and their corresponding adjacent normal tissues (The distance of 5-10 cm from tumor) were detected by RT-PCR. We found that the expression of PTEN mRNA was significantly lower in lung cancer specimens than in adjacent normal tissues ( $P < 0.01$ ; **Figure 2**). The results suggested that the down-regulation of PTEN mRNA might be

All data from experiments were expressed as mean  $\pm$  SD unless otherwise stated.

involved in human lung cancer disease processes.

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**Figure 6.** Determination of miR-205 mimics or miR-205 inhibitors transfection efficiency. A. The expression of miR-205 was significantly increased after miR-205 mimics' transfection into A549 cells. B. The expression of miR-205 was significantly decreased after miR-205 inhibitors transfection into A549 cells. \* $P < 0.01$ .

*Expression of PTEN protein was downregulated in lung cancer tissues*

The expression of PTEN in 46 lung cancer specimens and their corresponding adjacent normal tissues (The distance of 5-10 cm from tumor) was detected by western blot method. We found that the expression of PTEN was significantly decreased in lung cancer specimens compared to adjacent normal tissues ( $P < 0.01$ ; **Figure 3A, 3B**).

*A negative relationship between the expression of miR-205 and PTEN mRNA in lung cancer*

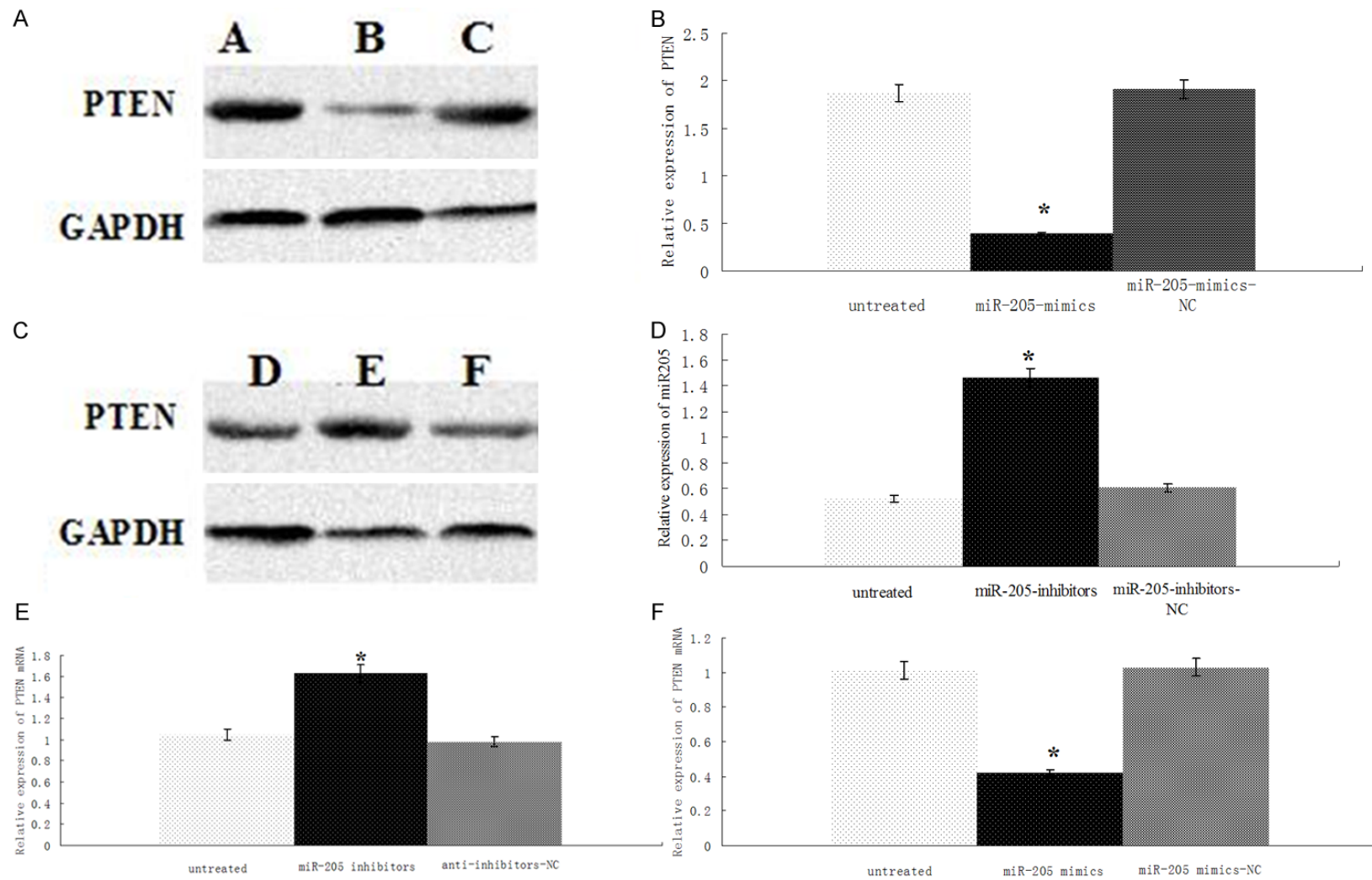
The scatter diagram was obtained according to experimental data, and Spearman correlation analysis showed that miR-205 level were negatively correlated with the expression of PTEN ( $r$

$= -0.594$ ,  $P < 0.01$ ) in lung cancer tissues (**Figure 4**).

*Luciferase reporter assay results*

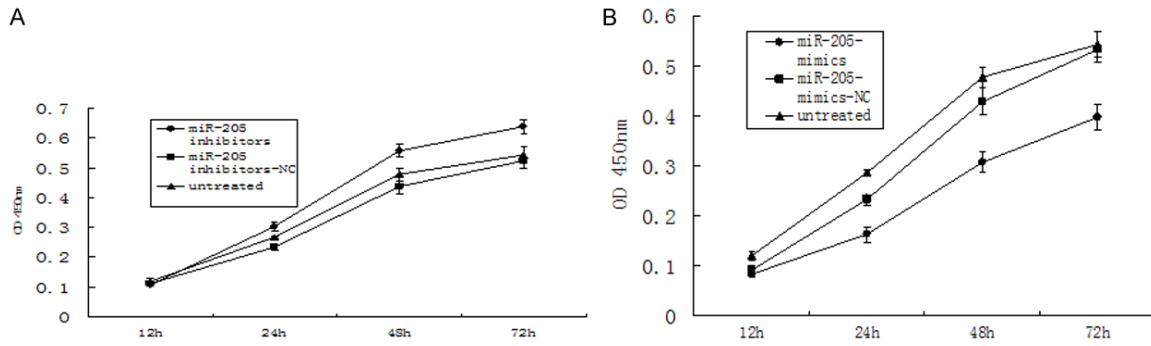
We employed the target gene prediction database to predict MiR 205 target genes, and we found that PTEN mRNA 3'UTR had multiple regions and 2~8 nucleotides of miR-205 Complete complementary (**Figure 5A**). Luciferase reporter assay results showed that the expression levels of fluorescent protein in PTEN 3'UTR and miR-205 mimics cotransfection group is lower than in PTEN 3'UTR and miR-205 control mimics cotransfection group ( $P < 0.01$ ); However, the expression levels of fluorescent protein in Mut PTEN 3'UTR and miR 205 mimics cotransfection group is higher than in PTEN 3'UTR and miR-205 mimics cotransfection group ( $P < 0.01$ ). These results

miR-205 and cell proliferation



**Figure 7.** Effect of miR-205 mimics and inhibitor on PTEN mRNA and protein levels in A549 cells. A549 cells were untreated (A, D), treated with miR-205 mimics (B), treated with miR-205 NC (C), treated with miR-205 inhibitors (E), treated with miR-205 inhibitors NC(F). (A, C) The expression of PTEN protein was determined by Western blot. (B, D) Optical density values for combined PTEN protein relative to GAPDH. (E) The expression of PTEN mRNA was determined by Realtime PCR, (F) Optical density values for combined PTEN mRNA relative to U6, n = 46. \*P < 0.01.

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**Figure 8.** Effect of miR-205 on proliferation of A549 cells. A. A549 cells were untreated or transfected with miR-205-NC or miR-205-mimics for up to 72 h. B. A549 cells were untreated or transfected with miR-205-inhibitor-NC or miR-205-inhibitor for up to 72 h. Cell proliferation was measured by CCK-8. \* $P < 0.05$ .

indicated that PTEN is a target gene of MiR205 (Figure 5B).

### Determination of miR-205 mimics transfection efficiency

The relative expression of miR-205 in A549 cells transfected by miR-205 mimics and normal control was determined by Real-time PCR. Compared with transfection of miR-205 normal control, the expression of miR-205 was increased significantly after transfection of miR-205 mimics ( $P < 0.01$ , Figure 6A), and decreased significantly after miR-205 mimics inhibitors ( $P < 0.01$ , Figure 6B).

### Effect of miR-205 on the expression of PTEN protein and mRNA in A549 cells

We employed western blot methods to study the effects of miR-205 mimics or inhibitor on the expression of PTEN in A549 cells. We found that the expression of PTEN in A549 cells was significantly down-regulated or up-regulated after miR-205 mimics (Figure 7A and 7B) or inhibitor (Figure 7C and 7D) was transfected into A549 cells respectively ( $P < 0.01$  for both). We also observed the expression of PTEN mRNA in A549 cells by Realtime PCR, and we found that the expression level of PTEN in A549 cells was significantly down-regulated or up-regulated after miR-205 mimics (Figure 7E) or inhibitor (Figure 7F) was transfected into A549 cells respectively ( $P < 0.01$  for both).

### MiR-205 inhibits cell proliferation of A549 cells

We employed Cell Counting Kit-8 (CCK-8) methods to study the effects of miR-205 mimics or

inhibitor on cell proliferation of A549 cells. Cell proliferation assays were performed at 12 h, 24 h, 48 h and 72 h after miR-205-mimics or miR-205-inhibitors transfection of A549 cells. Compared with untreated or miR-205-mimics-NC group, the cell proliferation of A549 cells in miR-205-mimics group was decreased significantly ( $P < 0.05$ ; Figure 8A); compared with untreated or miR-205-inhibitors-NC group, the cell proliferation of A549 cells in miR-205-inhibitors group was increased significantly ( $P < 0.05$ ; Figure 8B). These results indicate that miR-205 inhibits A549 cells proliferation.

### Discussion

miR-205 conducts conflicting functions in human cancers, where it may functions either as a tumor suppressor or an oncogene in a single tumor type depending on targeted mRNA [15-17]. Then, what would miR-205 be like in human lung cancer? We detected the expression of miR-205 in 46 lung cancer specimens and their corresponding adjacent normal tissues by stem-loop RT-PCR. We found the expression of miR-205 was significantly increased in lung cancer specimens compared to adjacent normal tissues. The result implied that the down-regulation of miR-205 might be involved in human lung cancer disease processes, which consistent with previous research [5].

PTEN was the first discovered tumor suppressor gene with dual specificity phosphatase activity, and its mutation, inactivation and decreased expression were closely related with the occurrence, development and prognosis of patients with lung cancer, playing an important

role in the tumor chemotherapy resistance [18-22]. We detected the expressions of PTEN mRNA and protein in 46 lung cancer specimens and their corresponding adjacent normal tissues, found the expressions of PTEN mRNA and protein was significantly decreased in lung cancer tissues than in adjacent normal tissues, which consistent with previous research [5]. However, it is a problem whether PTEN is a target of miR-205 in the pathogenesis of lung cancer.

To validate whether PTEN was direct targets of miR-205, at first, the statistical analyses of experimental data were done to draw a conclusion that miR-205 level were negatively correlated with the expression of PTEN in lung cancer tissues, and then we employed the target gene prediction database to predict miR-205 target genes, found that PTEN mRNA 3'UTR had multiple regions and 2~8 nucleotides of miR-205 complete complementary. At last, a dual-luciferase reporter assay was employed; the result showed that PTEN is a target gene of MiR205. But, it is a problem whether PTEN was regulated by miR-205 in lung cancer cells.

We designed miR-205 mimics and inhibitor for verifying the relationship between miR-205 and PTEN. We detected the expression change of PTEN mRNA and protein after miR-205 mimics or inhibitor were transfected into A549 cells. We found that the expression of PTEN protein and mRNA in A549 cells was down-regulated and up-regulated respectively after transfection of miR-205 mimics or inhibitor. These findings support the idea that PTEN might be a target of miR-205 in lung cancer. And then, CCK-8 tests were used to evaluate the effects of miR-205 on cell proliferation of A549 cells. We found that miR-205 overexpression caused A549 cell growth inhibition.

Thus, we hypothesized that miR-205 may function as a tumor suppressor, and the abnormal expression of miR-205 might be an important factor in lung cancer incidence.

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### Disclosure of conflict of interest

None.

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