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Original Article

MicroRNA-494 promotes tumor growth by targeting PTEN in non-small cell lung cancer

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Abstract: Lung cancer is the most common cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for 80% of all cases of lung cancer patients. MicroRNA-494 (miR-494) has been reported to play important roles in tumorigenesis of various human cancers. However, the roles and underlying molecular mechanism of miR-494 in NSCLC have not been totally elucidated. Therefore, the purpose of this study was to investigate the possible regulatory mechanisms of miR-494 in NSCLC. Herein, we demonstrated that miR-494 as oncogene MicroRNA in NSCLC progression was significantly up-regulated in NSCLC tissue samples and cell lines compared with normal lung tissues and cell line. The further research shows that down-regulation of miR-494 significantly delayed cell proliferation, increased apoptosis and pro-apoptotic related protein expression in NSCLC cell lines. Moreover, PTEN (phosphatase and tensin homolog), a unique tumor suppressor gene, was confirmed as a direct target of miR-494. We also found that the messenger RNA (mRNA) expression was decreased in NSCLC tissues and was inversely correlated with miR-494. Further study showed that knockdown of PTEN by siRNA could reverse the inhibitory effect of miR-494 inhibitor on proliferation and apoptosis of NSCLC cells. Thus, miR-494 may be a potential prognostic marker and of treatment relevance for NSCLC progression intervention.

Keywords: NSCLC, MicroRNA-494, PTEN, proliferation, apoptosis

Introduction

Lung cancer is the leading cause of cancer mortality worldwide [1]. Non-small cell lung cancer (NSCLC) represents the most frequent type of lung cancer including adenocarcinoma and squamous cell carcinoma [2]. Despite of great advances made in NSCLC treatment, the survival of this disease remains unsatisfactory. Thus, increased understanding of molecular mechanism involved in NSCLC formation and development is important. It is an urgent need to look for novel therapeutic targets and strategies for the treatment of human NSCLC.

MicroRNAs (miRNAs) are highly conserved, small, noncoding RNAs consisting of about 18-24 nucleotides in length, which mediate posttranscriptional regulation of target genes through their combination with 3'-untranslated regions (UTRs) [3]. Accumulating evidence has indicated that miRNAs played important roles in many biological events including cancer development in which these miRNAs were up-regulated or down-regulated and functioned as either oncogenes or tumor suppressors [4]. Recent research has showed that miRNAs such as miR-451, miR-145, miR-135b, miR-21, and miR-495 have been found to regulate the progress in NSCLC [5-9]. Additionally, miR-494 has been found to be regulate tumor progression in a variety of cancers, including gastric carcinoma [10], Breast cancer [11], pancreatic cancer [12], epithelial ovarian carcinoma [13], cervical cancer [14] and nasopharyngeal carcinoma [15]. However, the function of miR-494 in regulating NSCLC progression is poorly understood. Thus, identification of miR-494 would be critical to understand the characterization and underlying molecular mechanisms associated with NSCLC progression and development.

PTEN as a major tumor suppressor, negatively regulates tumor cell growth, cell cycle, apopto-

sis and metastasis by regulating multiple signal transduction pathways [16, 17]. MiR-494 has been proven to be involved in the carcinogenesis and development of various types of cancer by directly targeting PTEN [18]. However, whether miR-494 function as an oncogene by inhibiting PTEN in NSCLC remains unknown.

In the present study, we found that the expression level of miR-494 was up-regulated in NSCLC cell lines and clinical tissues samples. MiR-494 inhibitor could inhibit proliferation and promote apoptosis in NSCLC. Furthermore, we predicted PTEN to be the downstream target of miR-494 in NSCLC. Thus, our findings may reveal a novel strategy for the treatment of NSCLC.

Materials and method

Tissue samples

50 paired NSCLC tissues and normal lung tissues were obtained from 50 patients who underwent primary surgical resection of NSCLC between 2015 and 2016 at Yongchuan hospital of Chongqing medical university, China. NSCLC tissues and normal tissues were immediately snapped frozen in liquid nitrogen and stored at -80°C until total RNA was extracted. No patient had undergone chemotherapy, radiotherapy and adjuvant treatment before surgery. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Cell culture

Six NSCLC adenocarcinoma cell lines (A549, NCI-H358, NCI-H520, SPC-A1, NCI-H1299, NCI-H1703), a normal human bronchial epithelial cell line (16HBE) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). 16HBE, A549, NCI-H358, NCI-H520, NCI-H1299 and NCI-H1703 cells were cultured in RPMI 1640 (Gibco, USA) medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA), SPC-A1 cells were cultured in DMEM (Gibco, USA) medium supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, China). All cell lines were cultured at 37°C in 5% CO_2 and 95% humidified air atmosphere.

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

Total RNA containing miRNA and mRNA from tissue samples and cells was extracted by TRIzol reagent (Invitrogen, USA) for both miR-92a and PTEN mRNA analyses according to the manufacturer's instructions. RNA concentrations were measured using the NanoDrop2000 (Thermo Scientific). cDNA synthesis was performed using PrimeScript RT reagent Kit (Takara, China) according to the manufacturer's instructions. For detection of miR-494 and PTEN mRNA expression, qPCR was performed using the miScriptSYBR®green PCR Kit (Qiagen, USA) according to the manufacturer's protocol. The relative expression levels of interest gene were calculated by the $2^{-\Delta\Delta Ct}$ method. U6 and β-actin were used as internal controls for miRNAs and mRNAs. the primer for miR-494 were: 5'-UGA AAC AUA CAC GGG AAA CCU C-3' (sense) and 5'-ACG CAA ATT CGT GAA GCG TT-3' (antisense), and their reverse primer was the universal primer supported by the miScript-SYBR®green PCR Kit (Qiagen, USA). The PTEN mRNA forward primer was 5'-ACC AGT GGC ACT GTT GTT TCA C-3' and the reverse primer was 5'-TTC CTC TGG TCC TGG TAT GAA G-3'. the β-actin primer forward primer was 5'-GGC ACC ACA CCT TCT ACA ATG AG-3' and the reverse primer was 5'-GGA TAG CAC AGC CTG GAT AGC A-3'.

Cell transfection

miR-494 mimic (5'-UGA AAC AUA CAC GGG AAA CCU C-3'), mimic negative control (5'-UUC UCC GAA CGU GUC ACG UTT-3'), miR-494 inhibitor (5'-GAG GUU UCC CGU GUA UGU UUC A-3'), inhibitor negative control (5'-CAG UAC UUU UGU GUA GUA CAA-3') were designed and purchased from GenePharma (China). PTEN siRNA (5'-AAC CCA CCA CAG CUA GAA CUU-3') and scramble siRNA (5'-CCC AAC CAA CUC GGA AAU CU-3') were purchased from Santa Cruz Inc. (USA). These molecular productions were transiently transfected into A549 cells or H358 cells using Lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacturer's protocol.

Cell proliferation assay

Cell viability was measured using cell counting-kit-8 (CCK-8, Dojindo, Japan), according to the manufacturer's instruction. At 24 h post-trans-

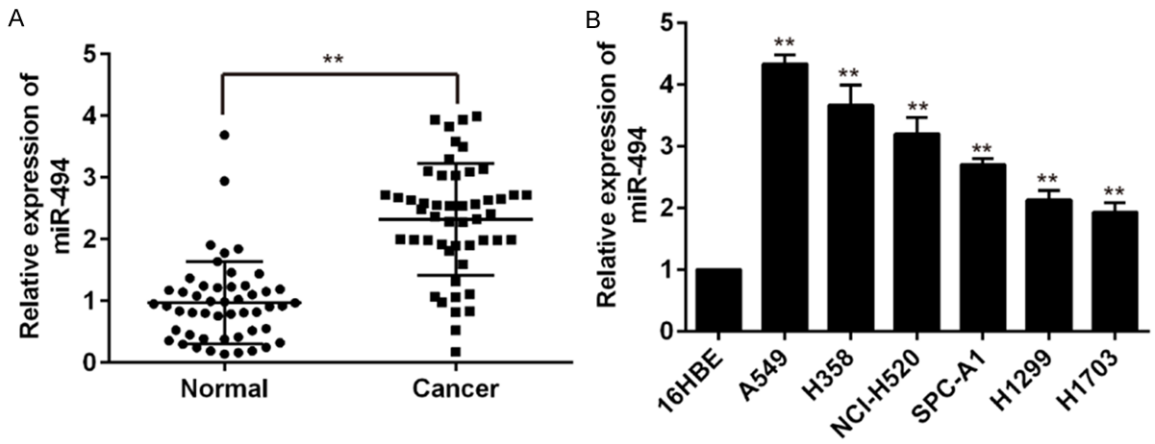

Figure 1. miR-494 was increased in both NSCLC tissues and cell lines. A. Expression of miR-494 in 50 pairs of NSCLC tissues and matched non-tumor tissues was detected by qRT-PCR, U6 was used as internal control. Data are showed as mean ± S.D. (n = 3). **P < 0.01. B. The expression levels of miR-494 in six human NSCLC cell lines (A549, NCI-H358, NCI-H520, SPC-A1, NCI-H1299, and NCI-H1703) and normal lung cell (16HBE) were detected by qRT-PCR. Data are showed as mean ± S.D. (n = 3). **P < 0.01.

fection with miR-494 inhibitor or inhibitor NC, A549 cells and H358 cells were seeded onto 96-well plates (2×10^3 cells/well), and cell proliferation was documented every 24 h for 5 days. The number of viable cells was assessed by measurement of the absorbance at 450 nm using a microplate reader (Tecan, Austria).

Flow cytometry analysis apoptosis

Cell apoptosis was examined by flow cytometry. Briefly, 48 h posttransfection, transfected cells were harvested for apoptosis assay. Then, A549 cells and H358 cells were trypsinized and washed twice with phosphate buffered saline (PBS). Cells (4×10^5/ml) were collected with l mL PBS and centrifuged at 1000 rpm for 10 min. The cells were suspended in 500 μL of Binding Buffer and mixed with 5 μL Annexin V (Bio-Science, China) and 5 μL PI (Bio-Science, China). After incubation at RT in the dark for 5 min, cell apoptosis assay was performed according to the manufacturer's protocol under a FACS Calibur flow cytometer (BD Biosciences, USA). The apoptosis ratio was calculated using CellQuest software (BD Biosciences, USA).

Western blot analysis

Whole-cell lysate was prepared using a lysis buffer (Beyotime, China) containing proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich, USA). The protein concentration was determined using a BCA protein assay kit (Thermo Scientific, USA). The proteins were separated on 10% SDS PAGE gel and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk for 1 hr and then incubated with primary antibodies overnight at 4°C. The primary antibodies used include Anti-PTEN (Cell Signal, USA), anti-cleaved caspase-3 (Cell Signal, USA), anti-cleaved PARP (Cell Signal, USA) and anti-β-actin (Abcam, USA). After three times washing, the membranes were incubated in horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, USA) for 1 hr at room temperature. Then, the protein band was visualized using the ECL Western blotting substrate (Promega, USA).

Vector construction and luciferase activity assay

For luciferase reporter experiments, luciferase reporter assays were performed in A549 cells. Cells were cotransfected with psiCheck-2 reporter plasmid (Promega, USA) containing the wild type or mutant type of PTEN 3'UTR, along with mimic negative control (NC), miR-494 mimic, inhibitor NC and miR-494 inhibitor by the Lipofectamine 2000 (Invitrogen, USA). The cells were harvested 24 h after transfection, and luciferase activity was measured with a dual luciferase reporter assay kit (Promega, USA) on a luminometer (Lumat LB9507, Germany).

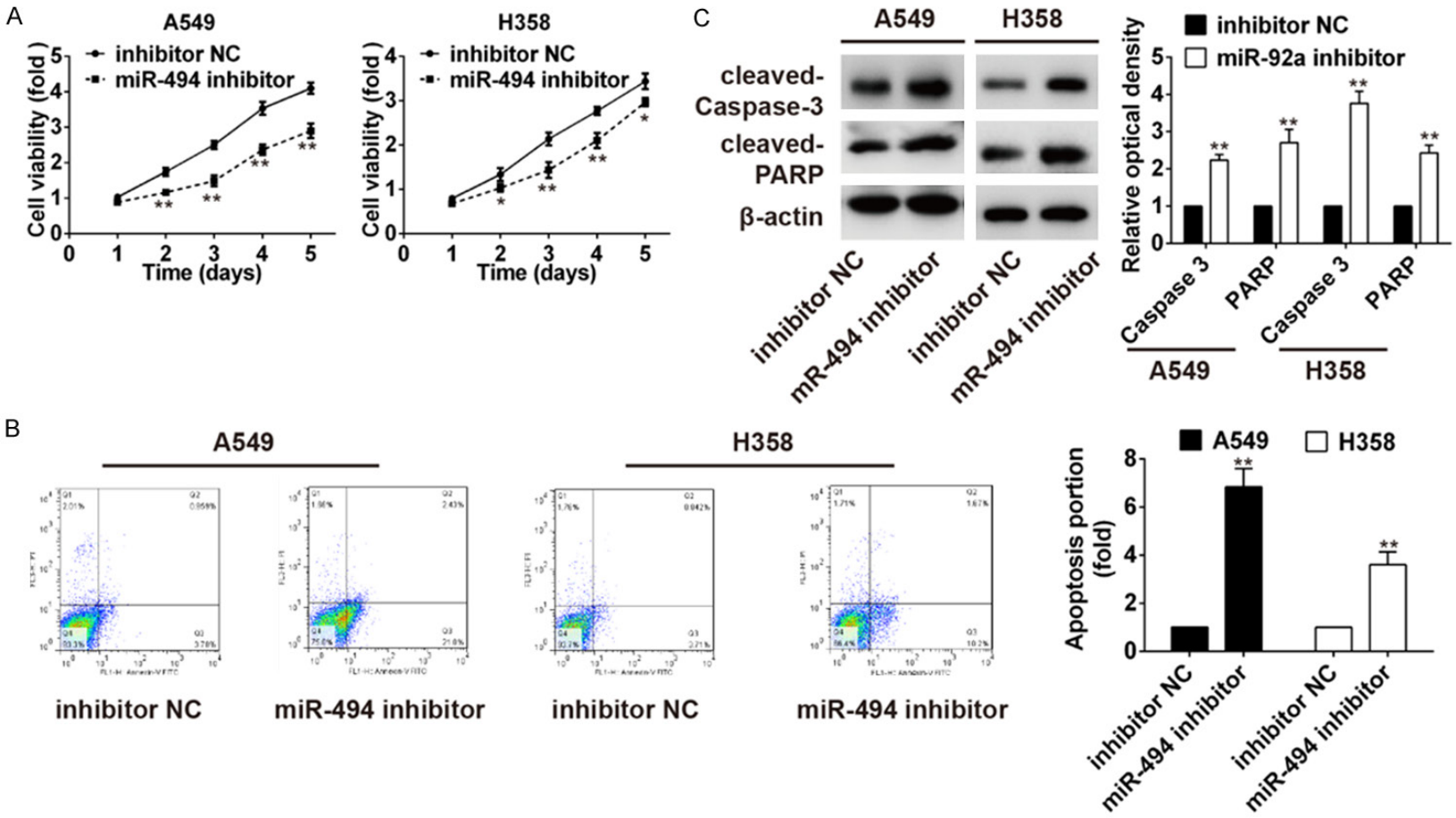

Figure 2. Down-regulated of miR-494 inhibits cell proliferation and increased cell apoptosis. A. The effect of miR-494 inhibitor on the proliferation of A549 and NCI-H358 cells. A549 and NCI-H358 cells were transfected with miR-494 inhibitor or inhibitor NC, the CCK8 assay was performed every 24 h for 5 days. B. Flow cytometry analysis of apoptosis in miR-494 inhibitor or inhibitor NC transfected A549 and NCI-H358 cells. C. Apoptotic related protein expressions were determined in miR-494 inhibitor or inhibitor NC transfected A549 and NCI-H358 cells by Western blot.

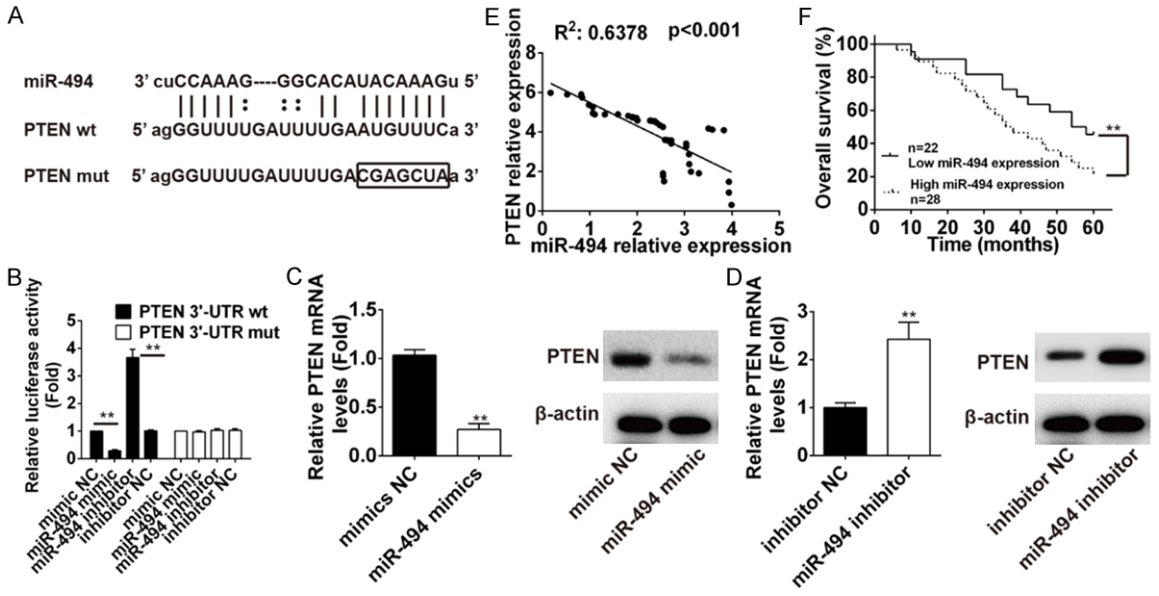

Figure 3. miR-494 directly targets PTEN in NSCLC cells. A. Predicted binding site (PTEN WT 3'UTR) and mutated binding site (PTEN MUT 3'UTR) of PTEN on miR-494. B. A luciferase reporter plasmid carrying wild PTEN 3'UTR or mutant PTEN 3'UTR was transfected into A549 cells alone, cotransfected with mimic NC or miR-494 mimic, or with inhibitor NC or miR-494 inhibitor and luciferase activity was measured. C. A549 cells were transfected with mimic NC and miR-494 mimic, mRNA and protein expression of PTEN was analyzed by qRT-PCR and Western blot. β-actin was used as an internal control. Data are showed as mean ± S.D. (n = 3). **P < 0.01. D. A549 cells were transfected with inhibitor NC and miR-494 inhibitor, mRNA and protein expression of PTEN was analyzed by qRT-PCR and Western blot. β-actin was used as an internal control. E. The reverse relationship between PTEN and miR-494 expression was explored in 50 NSCLC tissues by Spearman's correlation. F. Kaplan-Meier curves for overall survival and miR-494 expression in group of 50 NSCLC patients. Data are showed as mean ± S.D. (n = 5). **P < 0.01.

Statistical analysis

All statistical analyses were carried out using the GraphPad Prism 5.0 software and the SPSS 16.0 software. Experiments were repeated independently at least three times, and the results are expressed as mean ± SD (standard deviation). The differences between two experimental conditions were compared on a one-to-one basis using two-tailed Student's test. A value of $P < 0.05$ was considered statistically significant.

Results

miR-494 was up-regulated in NSCLC tissues and cell lines

To study the role of miR-494 in the pathogenesis of NSCLC, we analyzed miR-494 levels in 50 paired NSCLC tissues and normal lung tissues by qRT-PCR. As shown in **Figure 1A**, miR-494 was significantly up-regulated in NSCLC tissues samples versus non-tumor tissues (P < 0.01). The expression of miR-494 was further examined in various NSCLC cells, including A549, NCI-H358, NCI-H520, SPC-A1, NCI-H1299 and NCI-H1703. In parallel, the expression of miR-494 was also up-regulated in 6 NSCLC cell lines compared with 16HBE (normal human bronchial epithelial cell line) (**Figure 1B**, P < 0.01). These results suggest that miR-494 expression is frequently up-regulated in NSCLC and may be involved in the development of NSCLC.

miR-494 inhibitor delayed cell proliferation through inducing cell apoptosis

miR-494 is markedly up-regulated in NSCLC tissues and cell lines, it may thus function as a tumor promoter. Therefore, we tested whether miR-494 inhibitor in A549 and NCI-H358 cells affect cell growth. In a CCK-8 assay, both A549 cells and NCI-H358 cells transfected with miR-494 inhibitor all delayed cell proliferation than the inhibitor NC group (**Figure 2A**). The difference in proliferative activity indicates that miR-494 inhibitor inhibits the A549 cells and NCI-H358 cells growth activity. One of the hallmarks of cancer is its ability to evade apoptosis. Thus,

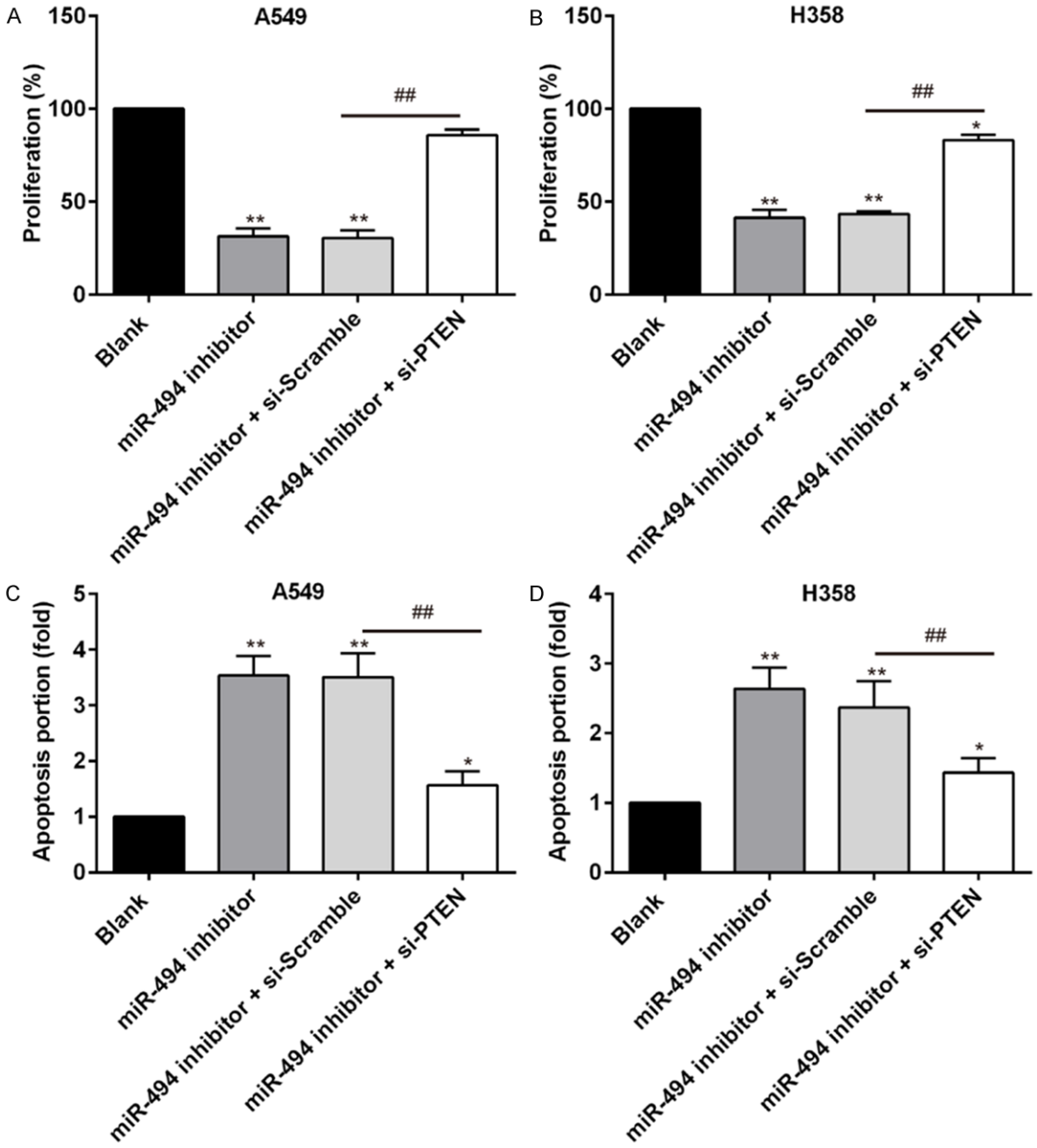

Figure 4. Down-regulated of PTEN could partially reverse miR-494 inhibitor induced anti-growth effects on NSCLC cells. A549 and H358 cells were co-transfected with miR-494 inhibitor and si-scramble or co-transfected with miR-494 inhibitor and si-PTEN. The CCK-8 assay and flow cytometry was used to detect cell proliferation and apoptosis. A. The cell proliferation was determined in A549 cells. B. The cell proliferation was determined in H358 cells. C. The cell apoptosis was determined in A549 cells. D. The cell apoptosis was determined in H358 cells. Data are showed as mean ± S.D. (n = 5). **P < 0.01.

we next examined the effect of miR-494 on apoptosis. Transfected with miR-494 inhibitor significantly increased apoptosis in A549 cells and NCI-H358 cells (**Figure 2B**). In addition, two apoptotic related protein, cleaved caspase-3 and cleaved PARP is selected for study the impact of miR-494 on apoptosis. Western blot was used to analysis the regulation of miR-494 inhibitor on cleaved caspase-3 and cleaved PARP protein levels in NSCLC cells. It was found that the protein levels of cleaved caspase-3 and cleaved PARP significantly increased in A549 cells and NCI-H358 cells transfected with miR-494 inhibitor when compared with trans-

fected with inhibitor NC (**Figure 2C**). These data indicate that miR-494 inhibitor may serve as a significant apoptotic factor in NSCLC.

PTEN is a direct target of miR-494 in NSCLC cells

Previous studies have indicated PTEN expression was negatively correlated with miR-494 expression in NSCLC tissues [19]. But whether PTEN was a direct target of miR-494 in NSCLC remains unknown. TargetScan and RNAhybrid algorithms assay demonstrated that PTEN target for miR-494 since there was a putative miR-494 binding sites within the 3'UTR of PTEN mRNA (**Figure 3A**). To experimentally demonstrate that PTEN is a direct target of miR-494, WT/Mut PTEN 3'UTR was subcloned into a luciferase reporter vector and transfected with miR-494 mimic or miR-494 inhibitor into the A549 cells. Luciferase reporter assays showed that transfection of miR-494 mimics in A549 cells with the wild-type 3'UTR significantly decreased the luciferase activity compared with transfection of miR-NC mimics ($P < 0.01$), but transfection of miR-494 inhibitor significantly increased the luciferase activity compared with transfection of inhibitor NC ($P < 0.01$). No luciferase activity change was observed when the cells were transfected with the Mut PTEN reporter plasmids (**Figure 3B**). QRT-PCR and western blotting showed that upregulation of miR-494 expression in A549 cells could decrease PTEN expression on mRNA level and protein level (**Figure 3C**). Whereas, down-regulation of miR-494 could increase PTEN expression on mRNA level and protein level in A549 cells (**Figure 3D**). In addition, we also examined expression of PTEN in NSCLC tissues by qRT-PCR. It was found that PTEN mRNA expression levels were negatively correlated with miR-494 expression levels (**Figure 3E**, $R^2 = 0.6378$, $P < 0.001$). In order to evaluate whether the survival time of NSCLC patients is associated with the mRNA expression level of miR-494. 50 NSCLC patients were divided into high miR-494 expression group and low miR-494 expression group. The result showed that NSCLC patients with higher expression of miR-494 have a shorter overall survival.

Knockdown of PTEN could partially rescue the suppression effects of miR-494 inhibitor on NSCLC cells

We investigated whether down-regulated of PTEN could rescue the anti-growth effect of miR-494 inhibitor. A549 and H358 cells were transfected with miR-494 inhibitor for 24 h and followed by transfection with si-PTEN or si-scramble. The cell proliferation was determined by a CCK-8 assay. We found that knockdown of PTEN by si-PTEN could rescue miR-494 inhibitor-induced inhibition of proliferation in A549 and H358 cells (**Figure 4A**, **4B**). In addition, the cell apoptosis were determined by flow cytometry, knockdown of PTEN by si-PTEN could reverse miR-494 inhibitor-induced apoptosis in A549 and H358 cells (**Figure 4C**, **4D**). From these results, we concluded that knockdown of PTEN by siRNA could at least partially rescue the anti-growth effects of miR-494 inhibitor on NSCLC cells. Therefore, miR-494 may functions as an oncogene by directly targeting PTEN on NSCLC.

Discussion

Lung cancer is the most commonly diagnosed cancer worldwide, the most frequent type of lung cancer is NSCLC. It has a high mortality rate due to lack early detection and efficient treatment [20]. Thus, it is urgent to find out a novel approach to understand the molecular mechanism involved in NSCLC formation and development. Recently, increasing evidence has suggested that aberrant expression of miRNAs plays important roles in NSCLC occurrence and development [21-27]. In the present study, we observed that miR-494 was significantly up-regulated in NSCLC tissue samples and cell lines compared with normal lung tissues and cell line. In addition, in miR-494 down-regulated NSCLC cells, cell proliferation rate was significantly reduced, conversely, cell apoptosis rate significantly increased as compared to control cells. We further identified PTEN as a target of miR-494 in NSCLC cells, and miR-494 expression was found to correlate inversely with PTEN expression in NSCLC. The functional experiments showed that knockdown of PTEN by si-PTEN directly reverse the tumor suppressive effect of miR-494 inhibitor on NSCLC cells. These data might provide new insights into the underlying molecular mechanisms of miR-494 in NSCLC, and reveal a novel strategy for the treatment of NSCLC.

MiRNAs play critical roles in human cancers in which these miRNAs were up-regulated or down-regulated and functioned as either oncogenes or tumor suppressors [5-7]. Some reports have demonstrated that miR-494

expression is down-regulated in nasopharyngeal carcinoma [15], epithelial ovarian carcinoma [13], cervical cancer [14], Esophageal Squamous Cell Carcinoma [28], pancreatic cancer [12], gastric carcinoma [10], Breast cancer [11] and oral cancer [29], suggesting that miR-494 may act as a tumor suppressor in these tumors. However, other studies found that miR-494 expression is up-regulated in several cancers and functions as oncogene and plays a critical role in cancer development. For example, Li et al. Results showed that the expression of miR-494 in human brain glioma tissues is up-regulated, miR-494 inhibitor could prevent migration, invasion, proliferation, and promote apoptosis in gliomas through PTEN/AKT pathway [30]. Lim et al. Results showed that miR-494 is overexpressed in human Hepatocellular carcinoma, miR-494 inhibition in human HCC cell lines decreases cellular transformation and anti-miR-494 treatment of primary MYC-driven liver tumor formation significantly diminishes tumor size [31]. Sun et al. Results showed that the expression of miR-494 in colorectal cancer tissues and cell lines was up-regulated, up-regulation of miR-494 more frequently occurred in tissue specimens with adverse clinical stage and the presence of distant metastasis, and promoted cell migration and invasion by targeting PTEN gene in colorectal cancer [32]. The dual roles of miR-494 could attribute to organ-specific actions, different cellular contexts, and its interaction target gene, which requires further investigation in detail tumor. Sun et al. study found that the expression of miR-494 was significantly higher in NSCLC than in normal lung tissues, the level of miR-494 expression was negatively correlated with PTEN expression [19]. Sun et al. study showed a consistent result with us. But, the detail function and underlying molecular mechanism of miR-494 in NSCLC have not been totally elucidated. In the present study, we analyzed miR-494 mRNA levels in 50 paired NSCLC tissues and six NSCLC cell lines, our results showed that miR-494 is mainly acting as an oncogenic effector in NSCLC, and up-regulated expression in NSCLC tissue samples and cell lines. MiR-494 inhibitor was acting as a tumor suppressor by inhibiting cell proliferation and promoting apoptosis. These results expanded the function of miR-494 in NSCLC and suggested that miR-494 may be served as a new treatment strategy for NSCLC.

PTEN as a well-established tumor suppressor is an essential regulator of cell proliferation, differentiation, growth, and apoptosis [16, 17]. PTEN deficiency is linked to cancer development and progression [33]. MiR-494 has been proven to be involved in the carcinogenesis and development of various types of cancer by directly targeting PTEN [18]. For example, Yang et al. results showed that miR-494 expression was significantly up-regulated in human cervical cancer cell lines and tissues. miR-494 up-regulation was significantly associated with PTEN down-regulation, adverse clinicopathological characteristics, poor overall and prognosis, inhibition of miR-494 suppressed cell proliferation and growth by directly targeting the 3'UTR of PTEN mRNA [34]. Sun et al. Results showed that low PTEN expression and high miR-494 expression are associated with high proliferation, low differentiation of tumor tissues, and high possibility of early invasion and metastasis. Additionally, their expression levels are closely related to postoperative 5 year survival of NSCLC patients [19]. However, the detail function and underlying molecular mechanism between miR-494 and PTEN in NSCLC have not been totally elucidated. In the present study, TargetScan and RNAhybrid algorithms assay demonstrated that PTEN target for miR-494, the luciferase reporter assay in cultured NSCLC cells also showed that PTEN was a target gene of miR-494. In addition, miR-494 expression was found to correlate inversely with PTEN expression in both NSCLC cell lines and clinical NSCLC tissues samples, the result is consistent with Sun et al. [19]. In order to further study the relationship of PTEN and miR-494, we investigated whether down-regulated of PTEN could rescue the effect of miR-494 inhibitor, result showed down-regulation of PTEN could show similar effects with miR-494 overexpression and partially reverse miR-494 inhibitor induced effects on NSCLC cells. These data indicated that miR-494 was possibly involved in the regulation of NSCLC by targeting gene PTEN.

In summary, the present study demonstrated that miR-494 was significantly up-regulated in NSCLC tissue samples and cell lines. Down-regulation of miR-494 inhibits NSCLC cells proliferation by inducing apoptosis. PTEN was a target gene of miR-494, and miR-494 expression was found to correlate inversely with PTEN

expression in NSCLC. Down-regulation of PTEN could partially reverse miR-494 inhibitor induced effects in NSCLC. These findings showed that expression levels of miR-494 and PTEN may serve as potential biomarkers of NSCLC, miR-494 may represent a potential therapeutic value in NSCLC therapy in the future.

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Disclosure of conflict of interest

None.

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