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Original Article

MiR-98 inhibits cell proliferation and invasion of non-small cell carcinoma lung cancer by targeting PAK1

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Abstract: A family of small non-coding RNAs, ~22 nt in length, known as microRNAs (miRNAs), regulating ~30% of all human gene expression, have been reported to be involved in the pathogenesis of a number of types of cancers, including non-small cell carcinoma lung cancer (NSCLC). P21-activated protein kinase 1 (PAK1) is a clinical biomarker of Non-small carcinoma lung cancer. Here, we found that miR-98 is down-regulated, whereas PAK1 is highly expressed in NSCLC tissues and cells. We demonstrated that miR-98 directly targets the 3'UTR of PAK1 and down-regulates its expression at the mRNA and protein level. Also, miR-98 inhibited and PAK1 stimulated proliferation, migration, invasion and apoptosis of NSCLC cells. In agreement, PAK1 over-expression counteracted the inhibitory effect of miR-98. This current study suggests that exogenous miR-98 may serve as novel potential maker for NSCLC therapy.

Keywords: miRNA, miR-98, PAK1, non-small cell carcinoma lung cancer

Introduction

Lung cancer is the leading cause of cancer mortality worldwide, accounting for more deaths than any other cancer [1, 2]. Diagnosis of lung cancer normally occurs in late stages of the disease, thus limiting the options for treatment. The most common type of lung cancer (~85%) is non-small cell lung cancer (NSCLC), which has three main types: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma [3, 4]. Although the detection and surgical techniques continue to advance, the 5-year survival rate for NSCLC patients remains poor [5, 6]. Previously unknown biomarkers such as noncoding RNA gene products may also be associated with NSCLC. Therefore, a better understanding of the novel molecular mechanisms involved in NSCLC is urgently needed to guide the diagnosis, prognosis, and treatment of this disease.

In this several years, a class of novel non-coding RNAs called MicroRNAs (miRNAs) had been discovered in plants and animals. MicroRNAs (miRNAs) include 18-26 nucleotides, which post-transcriptionally regulate gene expression in multicellular organisms by affecting both the stability and translation of miRNAs. In the process of tumor formation, the abnormal expression or the loss of the dynamic balance between oncogenes and tumor suppressor genes, leads tumorigenesis and development of cancer. MiRNA, as an important regulation factor of gene expression, also was involved in tumor formation and progression. Considerable evidence showed critical functions for miRNAs in diverse biological processes, as proliferation [7-9], apoptosis [10-13], angiogenesis [14-16], cell differentiation [17-19], adhesion and metastasis [20] of tumor cells. A high expression of miR-155 and a low expression of let-7 correlate with a poor survival in the two most common forms of NSCLC (adenocarcinoma and squamous cell carcinoma) [21, 22]. Overexpression of let-7 miRNA family members has been shown to suppress tumor development in mouse models of lung cancer [23]. Therefore, down-regulation or up-regulation of certain miRNA expression may cause a variety of imbalance, including many kinds of cancers.

P21-activated protein kinase 1 (PAK1), a member of group I PAKs, is a main downstream

effector of the small Rho GTPases. The Rho family of GTPases is a family of small (~21 kDa) signaling G proteins, and is a subfamily of the Ras superfamily. The members of the Rho GTPase family have been shown to regulate many aspects of intracellular actin dynamics, and are found in all eukaryotic organisms including yeasts and some plants. Three members of the family have been studied a great deal: Cdc42, Rac1, and RhoA. All G proteins are "molecular switches", and Rho proteins play a role in organelle development, cytoskeletal dynamics, cell movement, and other common cellular functions [24, 25]. The p21-associated kinases (PAKs) were first discovered in a screen for proteins that interact with the small G-proteins Rac1 and Cdc42 in 1994 [26]. As a set of evolutionarily conserved serine/threonine protein kinase, PAKs influence actin polymerization by regulating cofilin and actin depolymerizing factor (ADF), which are actin binding proteins that regulate cytoskeletal dynamics by severing and depolymerizing actin filaments [27, 28]. Based on domain structure, there are six members in the PAK family subdivided into two groups: group I (contains PAK 1-3) and group II (contains PAK 4-6). PAKs serve as important mediators of Rac and Cdc42 GTPase function as well as pathways required for Ras-driven tumorigenesis.

As noted above, the incidence of lung cancer involves a number of oncogenes and tumor suppressor gene expression changes. Using quantitative Real-time PCR, we found that microRNA-98 (miR-98) was significantly downregulated in human NSCLC tissue samples. In this study, we focused on the effects of miR-98 on the phenotypes of NSCLC cells as well as the identification of its direct target genes PAK1, in order to illuminate the molecular mechanisms of miR-98 in the initiation and progression of NSCLC.

Materials and methods

Bioinformatics analysis

MicroRNA target prediction was performed using TargetScan v6.2, Microcosm v5.0 and miRanda, followed by expression correlation between miRNA-mRNA pairs. Matinspector (http://www.genomatix.de) and GeneGo (http://www.genego.com/metacore.php) were used for searching the transcription factor binding sites as well as analyzing the miRNA-mRNA network, respectively. MatInspector is a software tool that utilizes a large library of matrix descriptions for transcription factor binding sites (TFBS) to locate matches in DNA sequences. By introducing a matrix family concept, optimized thresholds, and comparative analysis, the program produces concise results that avoid redundant and false-positive matches. It assigns a quality rating to matches and thus allows quality-based filtering and selection of matches. GeneGo's MetaCore™ is an integrated "knowledge-based" platform for pathway analysis of OMICs data and gene lists. MetaCore™ is based on a proprietary manually curated database of human protein-protein, protein-DNA, and protein compound interactions, as well as metabolic and signaling pathways for human, mouse, and rat, supported by proprietary ontologies and controlled vocabulary.

Cell culture and transfection

A549 and H1299 cells, were cultured in DMEM (GIBCO) and 10% heat-inactivated fetal bovine serum, 100 IU penicillin/ml, 0.1 mg streptomycin/ml in a humidified 5% (v/v) atmosphere of CO2 at 37°C. SPC-A1 cells were cultured in RPMI1640 (GIBCO) and 10% heat-inactivated fetal bovine serum, 100 IU penicillin/ml, 0.1 mg streptomycin/ml in a humidified 5% (v/v) atmosphere of CO_2 at 37°C. Transfected with Lipofectamine 2000 Reagent (Invitrogen), followed the manufacturer's protocol.

Western blotting

Cultured cells were lysed by RIPA (0.1% SDS, 1% Triton X-100, 1 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) in 4°C for 25 min. Collected the lysates and cleared by centrifugation, and protein concentration was determined. Total cell lysates (50 μg) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electro-transferred onto nitrocellulose membranes. Nonspecific binding sites of membranes were saturated with 5% skim milk in TBST solution (100 mmol/L Tris-Cl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20) and incubated for 2 hours with antibodies at room temperature. The following antibody were used: anti-PAK1 and anti-GAPDH. After 4 washes with TBST, the filters were incubated with goat anti-mouse peroxidase-conjugated secondary anti-

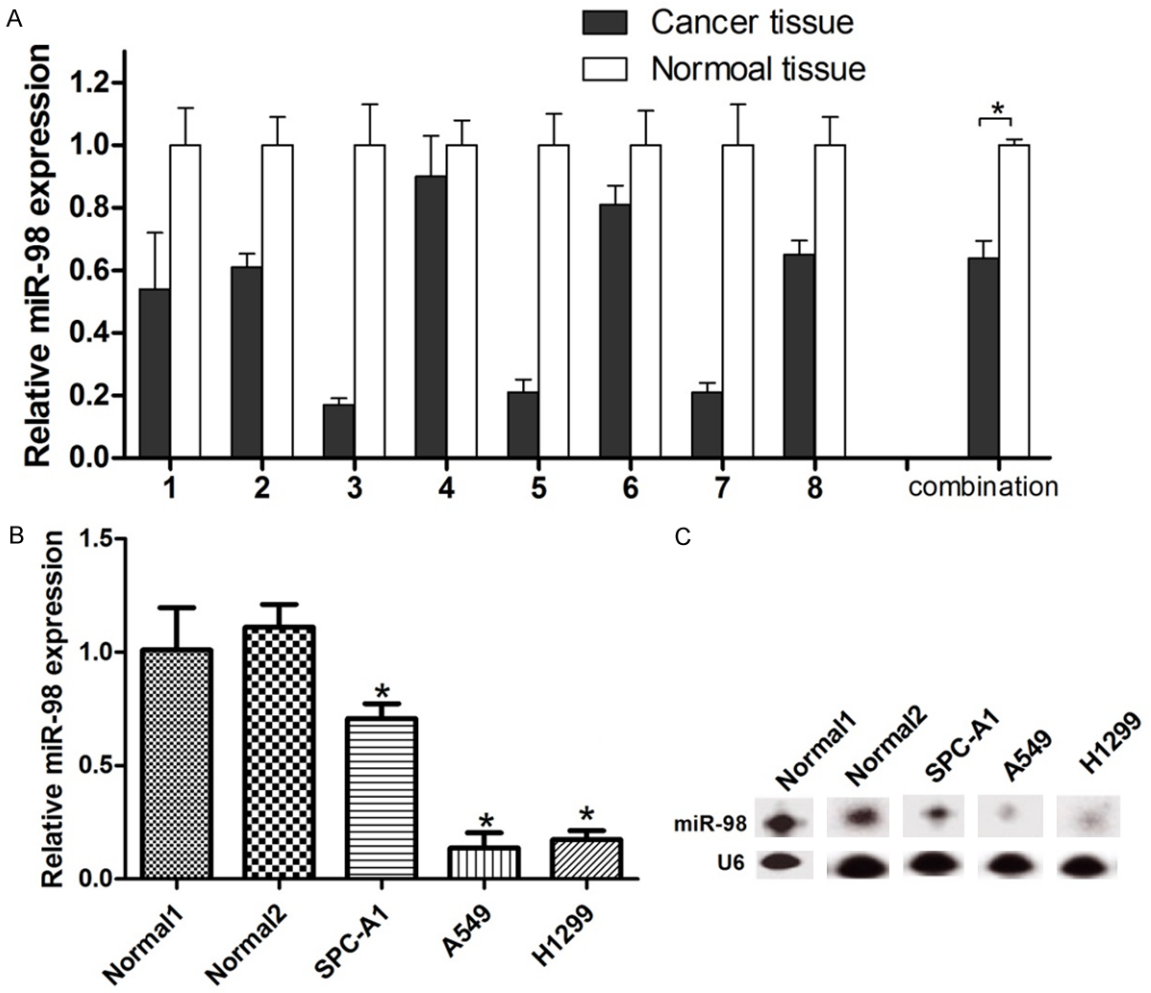

Figure 1. Identification of differential expression of miR-98 in human NSCLC tissues and cells. A. We use quantitative Real-time PCR to detect miR-98 differential expression level in 8 pairs of human NSCLC tissues and the adjacent normal tissues. U6 snRNA was regarded as an endogenous normalizer and the relative miR-98 expression level of the 8 pairs of human NSCLC tissues (means ± SD) is shown. Error bars indicate the S.D. of at least three independent analyses ($^{*}P < 0.05$). B. We use Real-time PCR to detect miR-98 differential expression level in 3 kinds of human NSCLC cells (SPC-A1, A549, and H1299) and 2 kinds of normal cells ($^{*}P < 0.05$). C. The northern blot result shows the same tendency with Real-time PCR result.

body (Sigma) in 5% skim milk in TBST solution for 1 hour at room temperature; reactions were developed using enhanced chemoluminiscence (Perkin Elmer, USA).

Cell proliferation assay

A549 and H1299 cells were seeded in 96-well plate at 6,000, 7,000 and 8,000 cells per well the day before transfection. The cells were transfected with miR-98, or control vector 0.2 μg per well. MTT assay was used to measure the viable, proliferating cells at 48 h after transfection. The absorbance at 570 nm was measured using a μQuant Universal Microplate Spectrophotometer (Bio-tek Instruments).

Colony formation assay

After transfection, A549 and H1299 cells were counted and seeded in 6-well plates (in triplicate) at 50, 60 and 75 cells per well. Fresh culture medium was replaced every 3 days. Colonies were counted only if they contained more than 50 cells, and the number of colonies was counted from the 6th day after seeding and then the cells were stained using crystal violet.

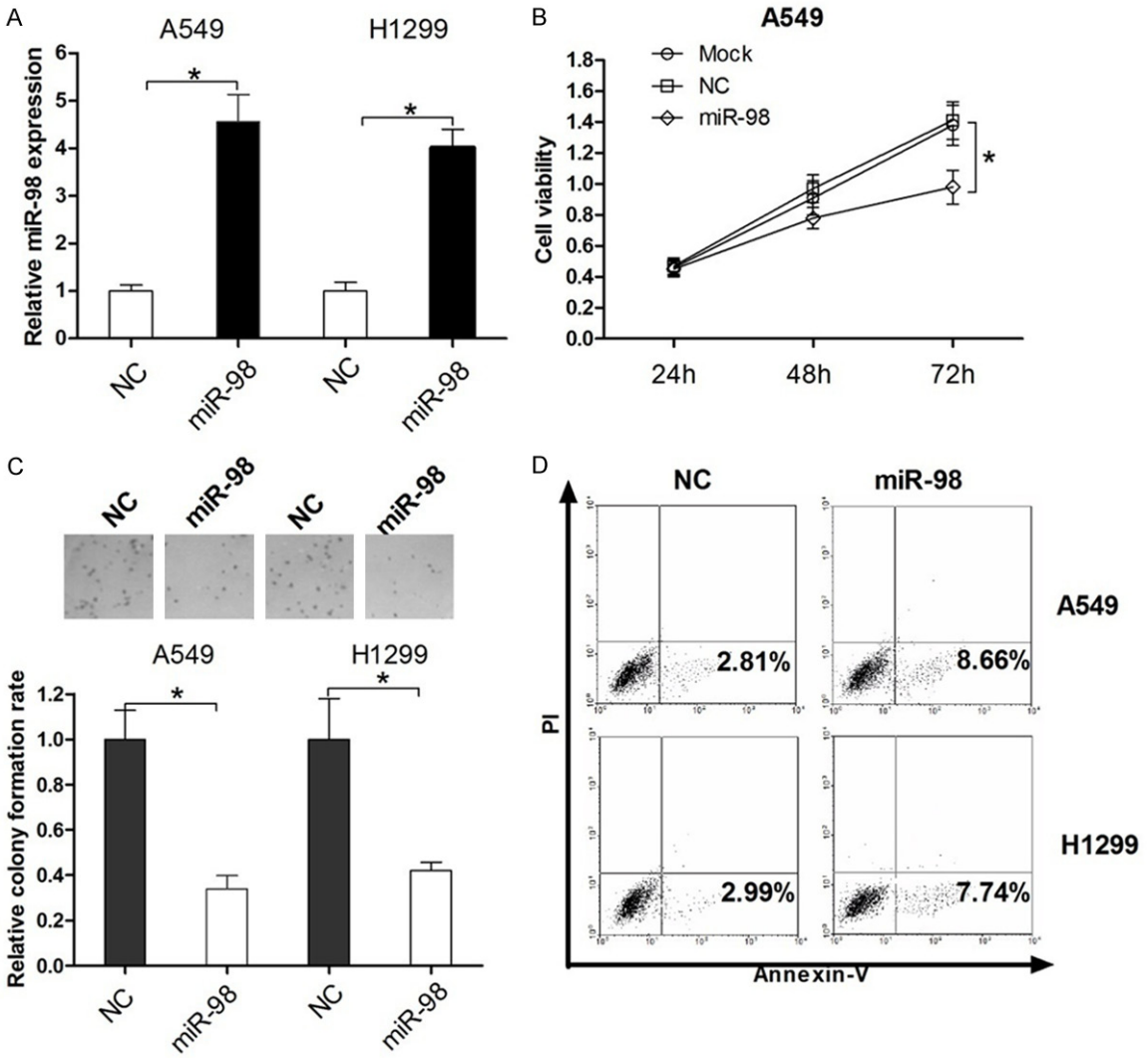

Figure 2. Overexpression of miR-98 suppresses ovarian cancer cells proliferation and promotes the cell apoptosis. A. The relative level of miR-98 expressed in NSCLC cells after the transfection with miR-98 or control vector. B. NSCLC cells were transfected with miR-98 or control vector. Cell growth activity was determined at 12 h, 24 h and 48 h post-transfection by MTT assay. Values are means ± SD of three duplications and the relative cell growth activity is shown. Error bars indicate the S.D. of at least three independent analyses ($^*P < 0.05$). C. The cell independent growth activity in vitro was assessed by colony formation assay. NSCLC cells were transfected with miR-98 or control vector. The colony formation assay was shown. Colonies were counted only if they contained more than 50 cells, and the number of colonies was counted from the 6th day after seeding. The number of colonies was counted from the 6th day after seeding. The colony formation rate was calculated and was shown. Error bars indicate the S.D. of at least three independent analyses ($^*P < 0.05$). D. The effect of miR-98 on apoptosis was examined by FCM analysis. NSCLC cells were transfected with miR-98 mimics or control, and then the medium was replaced with serum-free DMEM for 48 h. OC cells after transfected were analyzed for apoptotic rate after staining with Annexin V-FITC and PI. Data represent means ± S.D. from four independent experiments ($^*P < 0.05$).

Flow cytometry analysis

After 48 hours transfection as earlier described, the cells were harvested and washed twice with PBS. Washed cells were resuspended in 0.6 mL PBS, and fixed by the addition of 1.4 mL 100% ethanol at 4°C overnight. The fixed cells were rinsed twice with PBS, and resuspended in propidium iodine (PI) solution, including 50 mg/mL PI and 50 mg/mL RNase A (Sigma) in PBS without calcium and magnesium, and incubated at 37°C for 30 minutes in the dark. Stained cells were passed through a nylon mesh sieve to remove cell clumps and analyzed

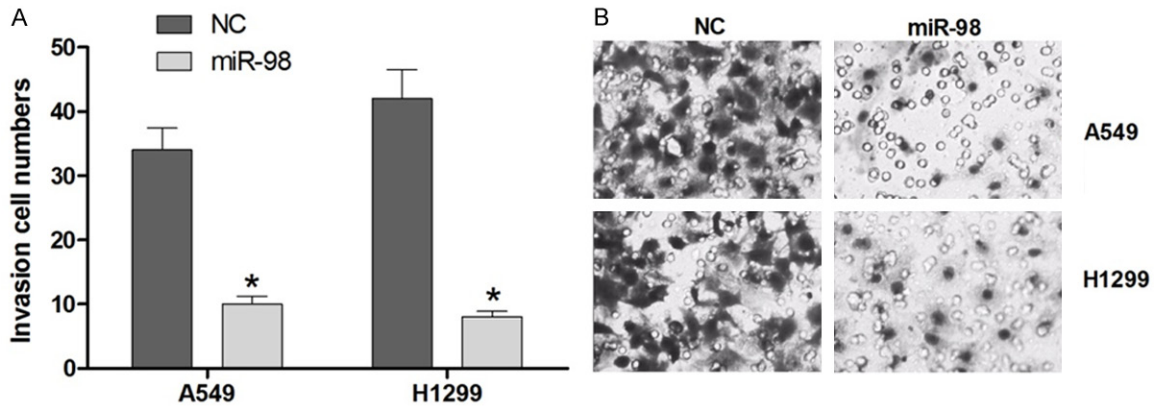

Figure 3. Over expression of miR-98 inhibits cell migration and invasion in NSCLC cells. A. Transwell analysis of NSCLC cells after treatment with miR-98 mimics or control. The relative ratio of invasive cells per field is shown below. Error bars indicate the S.D. of at least three independent analyses ($^{*}P < 0.05$). B. Transwell analysis of NSCLC cells after treatment with miR-98 mimics or control.

by a FACScan flow cytometer and Cell Quest analysis software (Becton Dickinson, San Jose, CA, USA). Flow cytometry analysis was repeated 3 times.

Transwell assay

NSCLC cells (2×10^5) were transiently transfected with or without miR-23b. Post transfection 48 h, 1.5×10^5 cells were resuspended in 300 μL serum free medium and seeded to the transwell of uncoated polycarbonate membranes with 8.0 μm pores (BD Bioscience) with the bottom supplemented with 800 μL complete medium. After 20 h incubation, cell migrated to the other side of transwell were stained with 0.005% crystal violet. Ten photographs were taken randomly and the cell number was counted.

Statistical analysis

Data are expressed as means ± standard deviation (SD), and $P < 0.05$ is considered as statistically significant by Students-Newman-Keuls test.

Results

MiR-98 expression level in NSCLC cells and tissues

We used quantitative real-time PCR to detect miR-98 differential expression level in 8 pairs of human NSCLC tissue and corresponding adjacent normal tissue. The results showed that miR-98 in human NSCLC tissue was significantly lower than their corresponding adjacent normal tissues (**Figure 1A**). We also used quantitative real-time PCR to detect miR-98 differential expression level in 3 kinds of human NSCLC cells, SPC-A1, A549, H1299. The results showed that, the expression level of miR-98 in human NSCLC cells was significantly lower than the normal cells (**Figure 1B**, **1C**). These data suggested that alterations of miR-98 could be involved in ovarian cancer progression.

MiR-98 inhibits the proliferation of human NSCLC cells in vitro and potentiates apoptosis in human NSCLC cells

Before identifying the effect of miR-98 on the proliferation of human NSCLC cells, the efficiency of miR-98 in A549 and H1299 cells was validated using qRT-PCR. The results revealed that miR-98 expression level was significantly higher than the control group (**Figure 2A**). To test the effects of miR-98 on NSCLC cells proliferation, we investigated cell growth by MTT assay and colony formation assay. We performed MTT assay to confirm the effects of miR-98 on cell proliferation. We found that miR-98 could obviously suppressed A549 cells growth (**Figure 2B**). The colony formation rate of A549 and H1299 cells transfected with miR-98 were significantly lower than the control group (**Figure 2C**). These two experiments showed that miR-98 played a role in suppressing cell growth and proliferation in NSCLC cells.

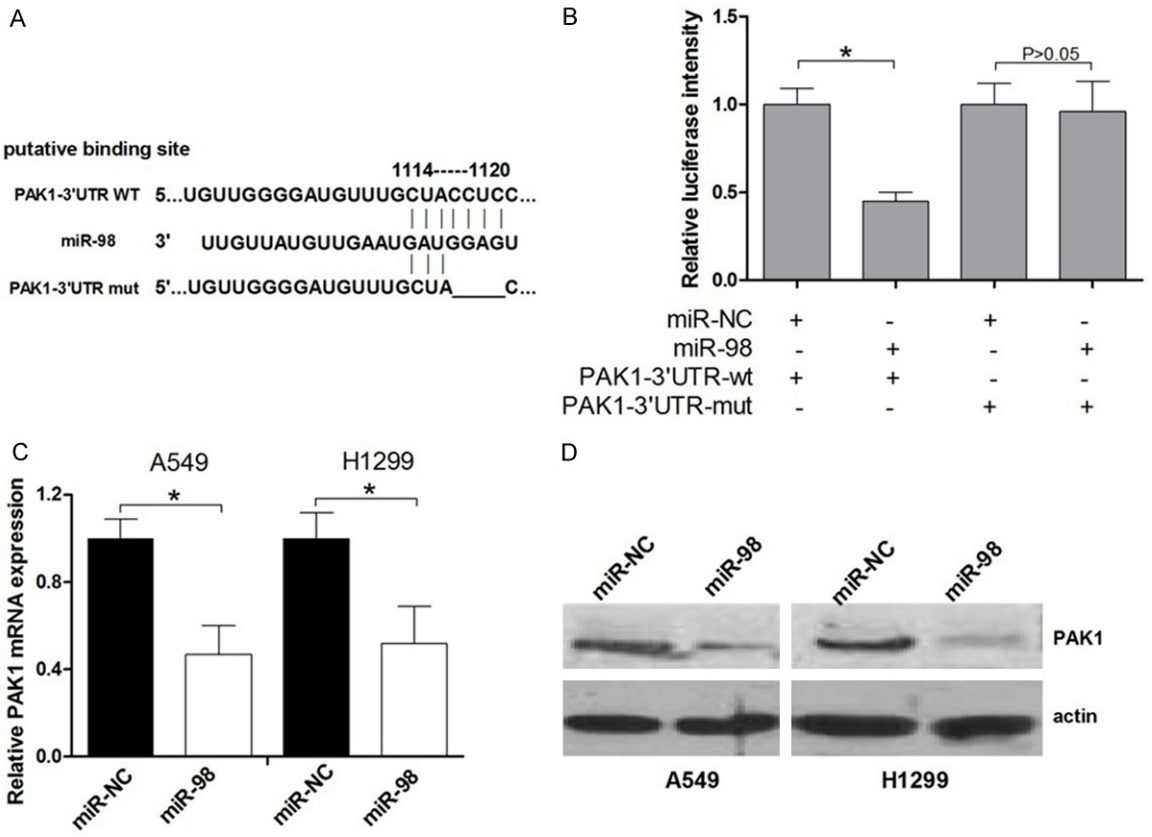

Figure 4. PAK1 is a directly target gene of miR-98. A. The predicted binding sites of miR-98 on PAK1 mRNA is shown. The mutant UTR with a 4 base pair for site-directed mutagenesis in the complementary seed sequences. B. NSCLC cells were transfected with the wild type of PAK1 reporter vector as well as miR-98 or control vector. MiR-98 suppressed the EGFP fluorescence intensity of PAK1-3'UTR-wt, while the group transfected with PAK1-3'UTR-mut was not significant different to the group. Error bars indicate the S.D. of at least three independent analyses ($^*P < 0.05$). C. NSCLC cells were transfected with miR-98 and control vector, the expression of PAK1 mRNA expression level were measured by quantitative RT-PCR. β-actin mRNA was regarded as an endogenous normalizer and the relative PAK1 mRNA expression level is shown. Error bars indicate the S.D. of at least three independent analyses ($^*P < 0.05$). D. NSCLC cells were transfected with miR-98 and control vector, the expression of PAK1 protein expression level were measured by Western blot. GAPDH protein was regarded as endogenous normalizer and the relative PAK1 protein quantity is shown ($^*P < 0.05$).

Up-regulating the miR-98, cell viability and proliferation were significantly inhibited.

To validate whether miR-98 is able to influence the cell apoptosis, Flow cytometry assay was performed. The results indicated that the significant increase in the apoptosis was observed in the A549 and H1299 cells transfected with miR-98 (**Figure 2D**). These results strongly suggested that introduction of miR-98 could inhibit human ovarian cancer cells growth by promoting early apoptosis of cancer cells.

MiR-98 inhibits cell migration and invasion in human NSCLC cells

To test whether miR-98 affects the ability of cancer cell migration and invasion, transwell assay were performed. Transwell assay demonstrated that over expression of miR-98 significantly reduced the migration and invasion capacity of NSCLC cells (**Figure 3A**, **3B**). These data demonstrated that overexpression of miR-98 suppressed migration and invasion in NSCLC cell lines.

MiR-98 directly inhibits expression of PAK1 via its 3'UTR

We used bioinformatics methods to predict miR-98 potential target genes. The 3'UTR region of PAK1 mRNA, contains miR-98 complementary binding sites (**Figure 4A**). Luciferase reporter assay has been widely used in verification of miRNA target genes. We performed lucif-

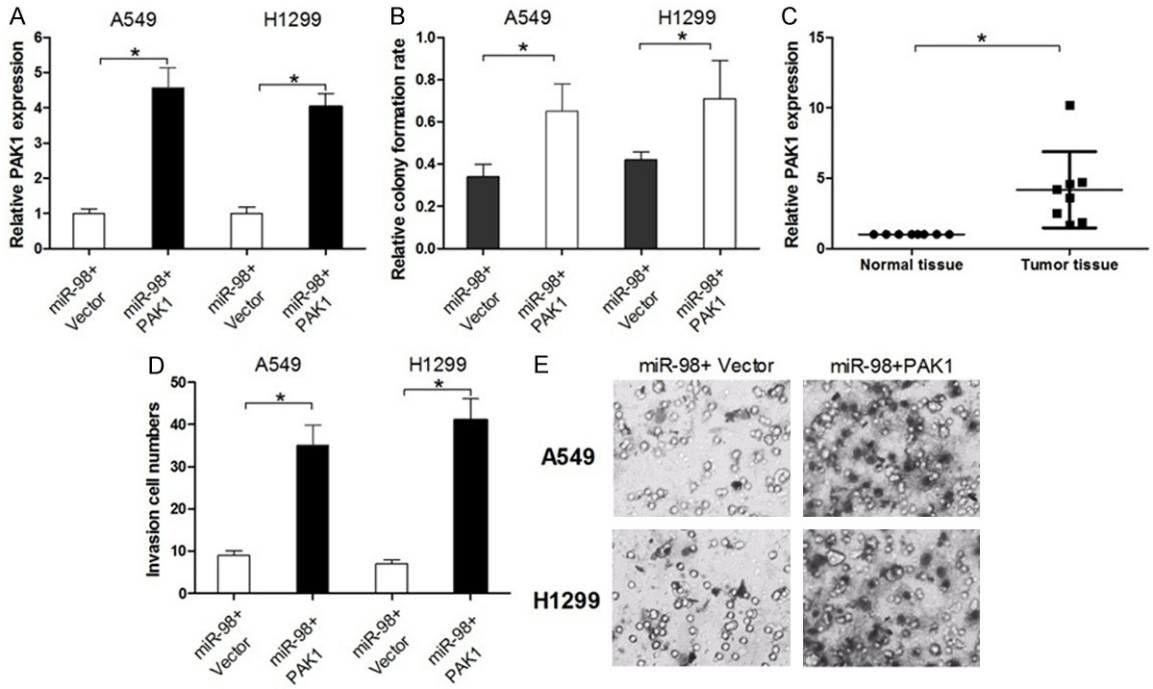

Figure 5. The ectopic expression of PAK1 counteracts the inhibition of the aggressive malignance induced by miR-98. A. NSCLC cells were cotransfected with miR-98 and PAK1 without its 3'UTR or the control vector and then western bolt assay was used to test the restoration of PAK1 protein by PAK1 in the presence of miR-98. The quantification of the bars are shown. B. The transfected cells were submitted to colony formation assays to test the proliferation of NSCLC cells. C. The relative level of PAK1 protein expressed in 8 pairs of NSCLC tissues and the adjacent normal ones. D. The effect of co-transfected with miR-98 and PAK1 on apoptosis was examined by FCM analysis. NSCLC cells after transfected were analyzed for apoptotic rate after staining with Annexin V-FITC and PI. Data represent means ± S.D. from four independent experiments ($^*P < 0.05$). E. Transwell migration/invasion assays and three-dimensional Matrigel culture to test the cells' abilities to migrate and invade.

erase reportor assay, engineering luciferase reportors, that have either the wild-type 3'UTR of PAK1, or the mutant UTR with a 4-base pair for site-directed mutagenesis in the complementary seed sequence to investigate whether PAK1 can be directly targeted by miR-98 (**Figure 4A**). First, A549 cells were transfected with PAK1-3'UTR-wt, miR-98 and control mimics. The results showed that, compared with the control group, co-transfected with miR-98, the fluorescent EGFP expression were significantly lower (**Figure 4B**), indicating that overexpression of miR-98 enhanced miR-98 binding to its target gene PAK1 mRNA 3'UTR, so that luciferase activities were decreased. In contrast, mutant reporters were not repressed by miR-98 (**Figure 4B**). After a 4-base pair mutant of miR-98, the luciferase activities were not decreased as well (**Figure 4B**). These all results suggested that, miR-98 could combine with the specific PAK1 mRNA 3'UTR binding sites and play a role in inhibiting the expression of PAK1 gene.

MiR-98 plays a negative regulatory role at PAK1 post-transcriptional level

MiRNAs regulate the target genes at the post-transcriptional level by binding their target genes 3'UTR to silence the gene function. We transfected A549 and H1299 cells with miR-98, in order to examine whether miR-98 depress endogenous PAK1 through translational repression, the expression of PAK1 protein was examined by Western blot. The results showed that overexpression of miR-98 made the expression level of PAK1 protein decreased (**Figure 4D**), suggesting that miR-98 negatively regulates endogenous PAK1 protein expression through translational repression mechanism. Meanwhile, high expression level of miR-98a in A549 and H1299 cells could also decrease the endogenous PAK1 mRNA level (**Figure 4C**).

Furthermore, in the 8 pairs of human NSCLC tissues, we found the expression level of PAK1 was higher than the normal tissue, which had a negative correlation with miR-98 expression (**Figure 5C**). All these data suggest that miR-98 negatively regulates the expression of PAK1 through mRNA cleavage mechanism at the post-transcriptional level.

The ectopic expression of PAK1 counteracts the inhibition of the aggressive malignance induced by miR-98

The above observations indicate that miR-98 and PAK1 have opposite effects on the aggressive phenotypes of NSCLC cells and that miR-98 down-regulates PAK1 expression. Based on these results, we questioned whether the effect of miR-98 on NSCLC cells was mediated by the miR-98 down-regulatory effect on PAK1 expression. We performed a rescue assay to address this issue. First, we co-transfected miR-98 with the PAK1 expression plasmid without the 3'UTR and confirmed the over-expression of PAK1 could rescue the decrease in PAK1 protein levels caused by miR-98 (**Figure 5A**). We then performed different functional rescue experiments. As expected, the restoration of PAK1 expression mostly blocked the inhibitory influence of miR-98 on the colony formation rate (**Figure 5B**). In addition, the ectopic expression of PAK1 counteracts the inhibition of migration/invasion induced by miR-98 in NSCLC cells (**Figure 5D**, **5E**).

Discussion

Transformation process of malignant tumors is regulated by the synergy of multiple genes, including overexpression of oncogenes and low expression or even loss of function of tumor suppressor genes. Recent research uncovered that the regulation of oncogenes and tumor suppressor genes was not only in the transcriptional level, but also in the post-transcriptional level, which was more important and accurate. MicroRNAs (miRNA), as important regulation factors, participate in gene expression in human carcinogenesis. Aberrant expression patterns of miRNAs were revealed in various cancers and dysregulation of miRNAs were found highly associated with the progression of different cancers [29, 30]. NSCLC is considered to be a heterogeneous neoplasm, which involves aberrant expression patterns of miRNA and mRNA. Abundant results about the expression of different miRNAs and their function in NSCLC patients were reported. In 2004, Takamizawa et al. [31], reported for the first time reduced expression of the let-7 microRNA in human lung cancers and the potential clinical and biological effects of such a microRNA alteration. The miR-1280 expression was significantly higher in the NSCLC tissues than distal normal tissues [32]. MiR-512-3p could inhibit tumor cell adhesion, migration, and invasion by regulating the RAC1 activity via DOCK3 in NSCLC A549 and H1299 cell lines [33]. MiR-1271 promotes non-small-cell lung cancer cell proliferation and invasion via targeting HOXA5 [34]. MiR-99a is downregulated and promotes proliferation, migration and invasion in non-small cell lung cancer A549 and H1299 cells [35]. MiR-342-3p targets RAP2B to suppress proliferation and invasion of non-small cell lung cancer cells [36]. Recent studies demonstrated that miRNAs could be good potential candidates for the development of therapeutic targets and novel biomarkers. The miR-98, a member of let-7 family, is thought to be associated with various cancers [37-39]. In the present study, we tried to identify miR-98 which regulates the expression of PAK1, and evaluate their effects on NSCLC cell phenotype. Initially, we used real-time PCR to find that miR-98 was significantly down-regulated in human NSCLC tissue/cells, compared with the normal tissues/cells. The results suggested that alterations of miR-98 could be involved in NSCLC progression. Therefore, we hypothesized that miR-98 was a negative factor of carcinogenesis in human NSCLC cells due to the low expression levels in NSCLC. We calculated the cell growth viability through the MTT and colony formation assay to detect the relationship between miR-98 and the growth capacity of NSCLC cell lineA549 and H1299. The cell growth viability of NSCLC cells transfected with the miR-98 was significantly decreased when compared to control group and lead to the early apoptosis. We also found that over expression of miR-98 significantly reduced the migration and invasion capacity of NSCLC cells.

Secondly, bioinformatics analyses predicted a miR-98 binding site on the PAK1 transcript. Experimental evidence indicated that PAK1 was indeed a target of miR-98. On one hand, the ability of miR-98 to regulate PAK1 expres-

sion was likely direct because it bound the 3'UTR of PAK1 mRNA complementarily to the miR-98 seed region. The EGFP fluorescence intensity of PAK1-3'UTR-wt was specifically responsive to miR-98 overexpression. Furthermore, mutation of the miR-98 binding site abolished the effect of miR-98 on the regulation of EGFP fluorescence intensity. On the other hand, the endogenous PAK1 mRNA and protein expression was decreased in NSCLC cells transfected with miR-98. These results suggested that miR-98 regulated PAK1 protein expression at the post transcription level.

PAK1 is regulated by miR-98 and promotes the development of NSCLC. We proposed that miR-98 may inhibit the aggressive phenotypes by decreasing PAK1 expression. Consistent with our expectations, miR-98 inhibited the proliferation, migration and invasion in NSCLC cells. Importantly, the ectopic expression of PAK1 without the 3'UTR mostly rescued the inhibitory effect of miR-98 on the aggressive phenotypes. These results prove that miR-98 inhibits the development of NSCLC and may partially (if not completely) through down-regulating PAK1 expression.

The PAK family members are important signaling molecules that frequently overexpressed in many cancerous tissues. Many human malignancies are associated with aberrant levels and over-activities of PAK, in general, and PAK1, in particular. Studies from other malignancies found that overexpression of PAK1 was associated with poor prognosis in colorectal cancer, ovarian cancer, and breast cancer [40-42]. Jagadeeshan et al. reported that PAK1 levels are significantly upregulated in pancreatic ductal adenocarcinoma samples compared with adjacent normal samples [43]. Yeo et al. showed that the natural product glaucarubinone reduced pancreatic cancer cell growth, at least in part via inhibition of pathways involving PAK1 and PAK4 [44]. Han et al. identified PAK1 was a novel prognostic marker for pathologically confirmed human pancreatic cancer [45]. Reduced expression of PAK1 correlates with poor histological differentiation in pancreatic cancer. So, discovering new compounds that inhibit or modulate the activity and the signal transduction pathways of the overactive and/or overexpressed serine/threonine kinases, particularly PAK1, may be useful in treating hyperproliferative and neoplastic diseases.

In conclusion, our results demonstrate that miR-98 inhibits the proliferation, migration, invasion and apoptosis through the down-regulation of PAK1 expression. These findings may provide new insights into the mechanisms of NSCLC development and present potentially diagnostic or therapeutic strategies for NSCLC.

Disclosure of conflict of interest

None.

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