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Original Article

MiR-34c-3p suppresses the proliferation and invasion of non-small cell lung cancer (NSCLC) by inhibiting PAC1/MAPK pathway

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Abstract: MicroRNAs have become recognized as key players in the development of malignancy. They are a family of small non-coding RNAs (22 nt~30 nt) that can negatively regulate the expression of cancer-related genes by sequence selective targeting of mRNAs, leading to either mRNA translational repression or degradation. Lung cancer is the leading cause of cancer-related death worldwide with a substantially low survival rate. In this study, we analyzed the expression profile of miR-34c-3p in non-small cell lung cancer (NSCLC) tissues and cell lines, as its participation in some other types of cancer has been shown by previous reports. We found that miR-34c-3p was downregulated both in NSCLC tissues and cell lines. Overexpression of miR-34c-3p suppressed cell proliferation and colony formation and also limited migration and invasion in A549 cells. Furthermore, our results also shown miR-34c-3p reduction was associated with increased PAC1 expression levels in which miR-34c-3p downregulated PAC1 expression by recognizing and binding to specific binding sites in PAC1 3'-UTR. Taken together, our study implicates important roles of miR-34c-3p in NSCLC pathogenesis and implicates its potential application in cancer therapy.

Keywords: miRNA-34c-3p, invasion, PAC1/MAPK pathway, NSCLC

Introduction

Lung cancer remains the leading cause of cancer related death worldwide. Non-small cell lung cancer (NSCLC) comprises approximately 80% of all lung cancers [1]. NSCLC population has grown quickly over the past five years in China [2]. Although surveillance and clinical treatment strategies have been improved, the 5-year survival of NSCLC patients after curative resection is reported to be only 30-60% [3]. Therefore, elucidating the potential mechanism that mediate the initiation and progression of NSCLC is urgent and of great interest. Although significant progress had been made in the discovery of lung cancer-related genes, the pathophysiological functions and mechanisms underlying these aberrant genes remain unclear [4, 5].

The rationale of miRNA therapy in lung cancer is based on two criteria: one is that miRNAs play an essential role in lung development and their expression levels are deregulated in lung cancer compared to normal tissues and also in the blood of patients, and the second one is that several studies demonstrated that modulation of miRNA expression, both *in vitro* and *in vivo*, can modify the cancer phenotype [6-8]. MiRNAs silence their target genes by binding to the 3' untranslated region (3'-UTR) of target messenger RNAs (mRNAs), causing either degradation or inhibition of translation [9, 10]. Many studies have shown that miRNAs play important roles in numerous developmental processes, including cell proliferation, apoptosis, and tumorigenesis [11-13]. Numerous studies have suggested that miRNAs undergo aberrant regulation during tumorigenesis [14]. In lung cancer, it has been shown that the miRNA expression profiles and specific miRNAs in the lung tissue correlate with disease prognoses and clinical outcomes [15]. miRNA analysis provides a more comprehensive understanding of the pathogenesis of lung cancer [11].

Table 1. Clinico-pathological variables and miR-34c-3p in NSCLC tissues

Characteristic	N	miR-34c-3p expression		*P*-value[a]
		High	Low	
Gender				0.517
Male	13	7	6	
Female	7	3	4	
Age (y)				0.211
≥55	12	5	7	
<55	8	5	3	
Tumor size				0.436
≥3 cm	9	4	5	
<3 cm	11	6	5	
Histological grade				0.312
I	6	3	3	
II	8	5	3	
III	6	2	4	
Histology				0.189
Adenocarcinoma	14	8	6	
Squamous cell cancer	6	2	4	
Lymph node status				0.017[b]
No metastasis	13	9	4	
Metastasis	7	1	6	

[a]The x^2 test. [b]Indicates *P* value <0.05.

miR-34c is significantly down-regulated in several cancers, including breast cancer, malignant peripheral nerve sheath tumors, glioma, and lung cancer [16, 17]. As the down-regulation of miR-34c is related to a number of cancers, it has been hypothesized that miR-34c may play an important role in tumorigenesis and tumor development [18]. However, the function of miR-34c especially in NSCLC remains unclear. In this study, we addressed miR-34c-3p as a negative regulator of growth, migration, and invasion of NSCLC lung cancer cell lines, A549 cell lines, and identified the mechanism of miR-34c-3p inhibiting on growth, migration, and invasion of NSCLC might be through targeting PAC1/MAPK pathway.

Materials and methods

Cell culture

The human NSCLC cell lines A549 were purchased from American Type Culture collection (ATCC, Manassas, VA, USA). A549 cells were cultured in Ham's F12K media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), HEK293 cells were purchased from ATCC and grown were cultured in DMEM media containing 10% FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Specimens

In this study, 20 paired NSCLC and adjacent non-tumor specimens were collected from the Department of Respiration and Oncology, Jinan Central Hospital (Jinan, Shandong, China). All tissue samples were flash-frozen in liquid nitrogen immediately after collection and stored at -80°C until use. The study protocol was approved by Jinan central hospital Ethical Committee. Informed consent was obtained from all patients. All clinic pathologic and biological data were available for those patients. Both tumor and non-tumor samples were confirmed by pathological examination. No patients received chemotherapy or radiotherapy prior to surgery. The demographic and clinical features of the NSCLC tissues and the corresponding normal tissues are presented in **Table 1**.

Prediction of miRNAs targeting PAC1

miRNA target predicting algorithms miRDB (http://mirdb.org/miRDB/), TargetScan (http://www.targetscan.org/), and PicTar (http://pictar.mdcberlin.de/) were used to predict miRNAs targeting PAC1 and the binding regions.

Quantitative RT-PCR

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), using the standard method. cDNA synthesis was performed with 2 μg of total RNA, using the miScript II RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real-time PCR was performed on the ABI 7500 cycler (Applied Biosystems, CA, USA), using the miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Hsa-RNU6B and glyceraldehyde-3-phosphate dehydrogenase (GAP DH) were used as endogenous controls for miRNAs and mRNAs expression, respectively.

Luciferase reporter assay

3'-UTR of PAC1 and a mutation sequence were amplified by PCR using the primers that includ-

ed a Bgl II restriction site on the 5' and 3' strands. The PCR products were inserted into the Bgl II sites of the pGL3-control vector (Promega, Madison, WI, USA) and identified by DNA sequencing. The wild-type plasmid was created containing the 3'-UTR of PAC1 with complementary sequence of miR-34c-3p (pGL3-PAC1 3'-UTR wild), and a mutant plasmid with the mutation sequence without complementary sequence of miR-34c-3p (pGL3-PAC1 3'-UTR mut). For the luciferase reporter assay, the A549 cells were seeded on 24-well plates and co-transfected using Lipofectamine 2000 (Invitrogen) with 100 ng per well of the resulting luciferase UTR-report vector, 2 ng per well of pRLCMV vector (internal control, Promega) and 20 ng per well of miR-34c-3p precursor molecules or control precursor (Applied Biosystems) following the manufacturer's instructions. After 24 h the cells were lysed and the relative luciferase activity was assessed with the Dual-Luciferase Assay Reporter System (Promega).

Transfection

miR-34c-3p mimics/inhibitors, obtained from Lifetechnologies (Grand Island, NY, USA), were transfected into A549 cells with the FuGENE 6 transfection reagent (Promega, Madison, USA) at a final concentration of 50/100 nM. siRictor and siRaptor duplexes were synthesized by GenePharma (Invitrogen, Carlsbad, CA, USA), and a non-silencing siRNA was used as a negative control. The transfection protocol for siRNA was the same as that for miR-34c-3p mimics. Briefly, cells in wells of 96-well plates were grow to 80~90% confluence. Cells were incubated with small RNA complexes for 8 h before the medium was changed.

Immunocytochemical assay

After transfection A549 cells cultured in 24-well plates were washed with PBS and fixed with 4% paraformaldehyde for 15 min. 0.5% Trixon-100 was used for permeabilization. The cells were then blocked in 2% goat serum (diluted in PBS). After blocking, the cells were incubated with the anti-myosin primary antibody at 37°C for 1-2 h, and then the fluorescent secondary antibodies at 37°C for 1 h. The nuclei were stained with DAPI (Lifetechnologies) for 10 min. Images were captured using fluorescence microscope (Olympus, Tokyo, Japan).

In vitro proliferation assay

Cell viability was assayed by a colorimetric procedure, using the Cell Counting Kit-8 (Lifetechnologies, NY, USA) according to the manufacturer's protocol. The absorbance at 460 nm was determined with a microplate reader. To detect the exact proliferation rates of A549 cells, an EDU (5-ethynyl-20-deoxyuridine) incorporation assay was executed with the Cell-Light™ EdU In Vitro Imaging Kit (Lifetechnologies, NY, USA) according to the producer's instructions. Briefly, cells at 70-80% confluence were treated with 50 nM EDU in ECM medium and incubated for 2 h before fixation in 4% paraformaldehyde. After EDU staining, cell nuclei were stained with Hoechst 33342 and observed with an inverted fluorescent microscope (Olympus, Tokyo, Japan).

Wound healing assay

The A549 cells wound healing assay was performed as described with minor modifications. Briefly, A549 cells were first transfected with miR-34c-3p mimics, inhibitors or negative control (NC) RNAs in a 6-well plate and were allowed to grow overnight until confluent. Next, miRNA was added to each well, except for the control one, at a final concentration of 100 ng/ml as a 24 h pre-treatment, and miRNA treatment continued until the end of the test. At the 0 h time point, the cell monolayer in each well was scraped with a sterile 200 μl pipette tip 3 times to form parallel lines, followed by 1 wash with ECM. The same wound areas were examined and photographed with a Nikon Eclipse TS100 Microscope (Nikon, Japan) at the 0 h and 24 h post-injury time points. The areas of the cells that migrated into the wound fields were measured with Adobe Photoshop software.

Cell cycle analysis

A549 cells in wells of 6-well plate were transfected with miR-34c-3p mimics, inhibitors or negative control (NC) for 48 h. Then, the cells were harvested for Flow Cytometry analysis. Briefly, Cells were fixed in 80% ethanol, suspended in PBS, and incubated with RNase A

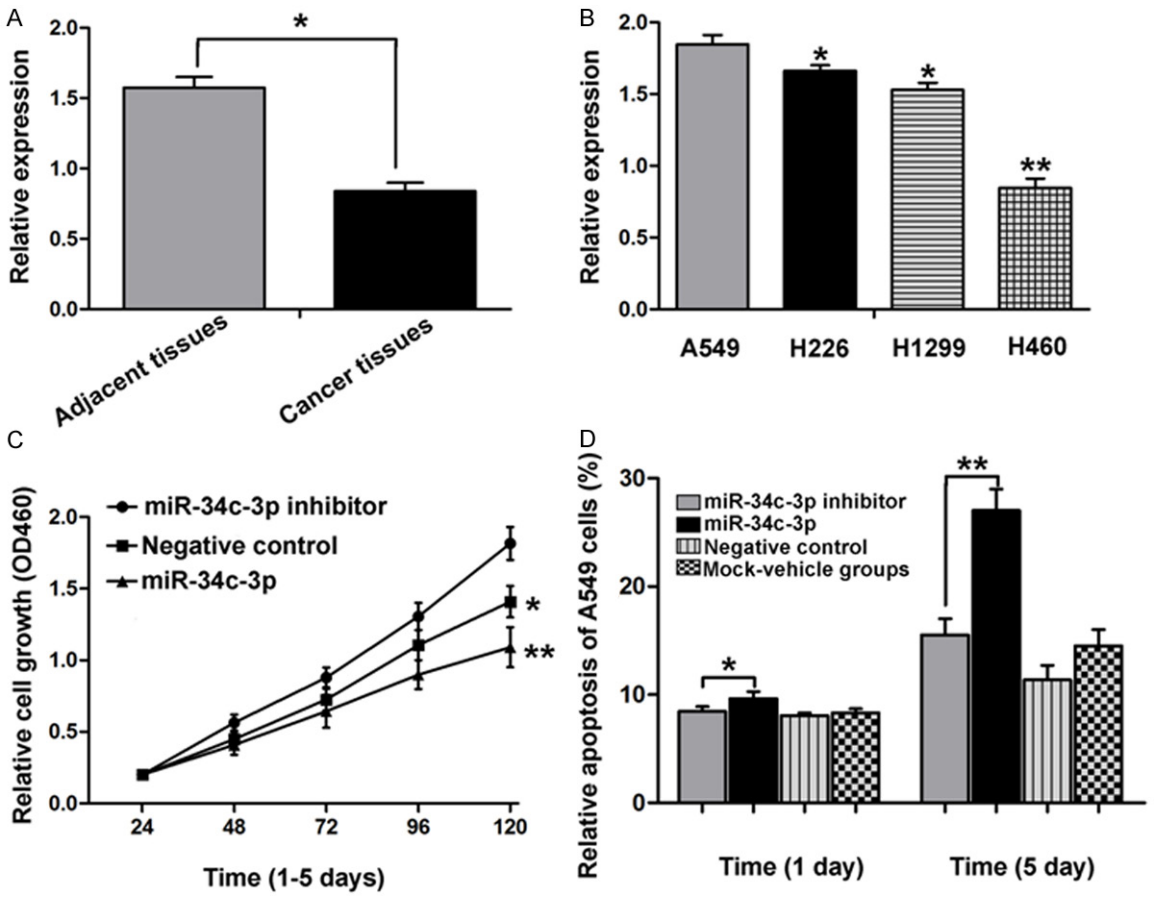

Figure 1. microRNA-34c-3p was decreased in NSCLC tissues and cell lines, also suppressed the proliferation of NSCLC cells. A. The expression levels of miR-34c-3p in 20 pairs of NSCLC tissues and their matched normal tissues were measured by quantitative real-time PCR. U6 was used as an internal control. B. The expression levels of miR-34c-3p in four NSCLC cell lines (A549, H226, H1299 and H460). C. MTT assay were performed to detect cell viability. D. Cell apoptosis was detected using flow cytometry.*P<0.05, **P<0.01.

(0.5 μg/μl) for 30 min at 37°C. Then the cells were stained with propidium iodide on ice for 1 h and subsequently measured with FACScan Flow Cytometry (Becton Dickinson, San Jose, USA). The percentage of cells in the G1, S, and G2 phases were analyzed by using the Cell cycle analysis Software.

Migration assay

A549 cells migration were determined by using a modified two chamber migration assay with a pore size of 8 μm (Transwell chamber, Corning, NY, USA). For migration assay, 1×10^5 cells were seeded in serum-free medium in the upper chamber. After 12 h incubation at 37°C, cells in the upper chamber were carefully removed with a cotton swab and the cells that had traversed the membrane were fixed in methanol, stained with Giemsa and photographed in five independent ×100 fields for each well.

Western blot

Cells in a 6-well plate were scraped in RIPA lysis buffer that was supplemented with 1 mM PMSF. Proteins (20 μg) were separated on 10% or 12% (for RhoB protein assay) SDS-polyacrylamide gels and were electro-transferred to polyvinylidene difluoride (PVDF) membranes (Lifetechnologies, NY, USA). After a blocking incubation with 5% milk-TBST, the membranes were incubated overnight in primary antibodies at appro- priate dilutions, followed by 1 h incubations in a secondary antibody that was conjugated to horseradish peroxidase (1:10000 dilution). After incubations in an enhanced chemiluminescence reagent (Life-

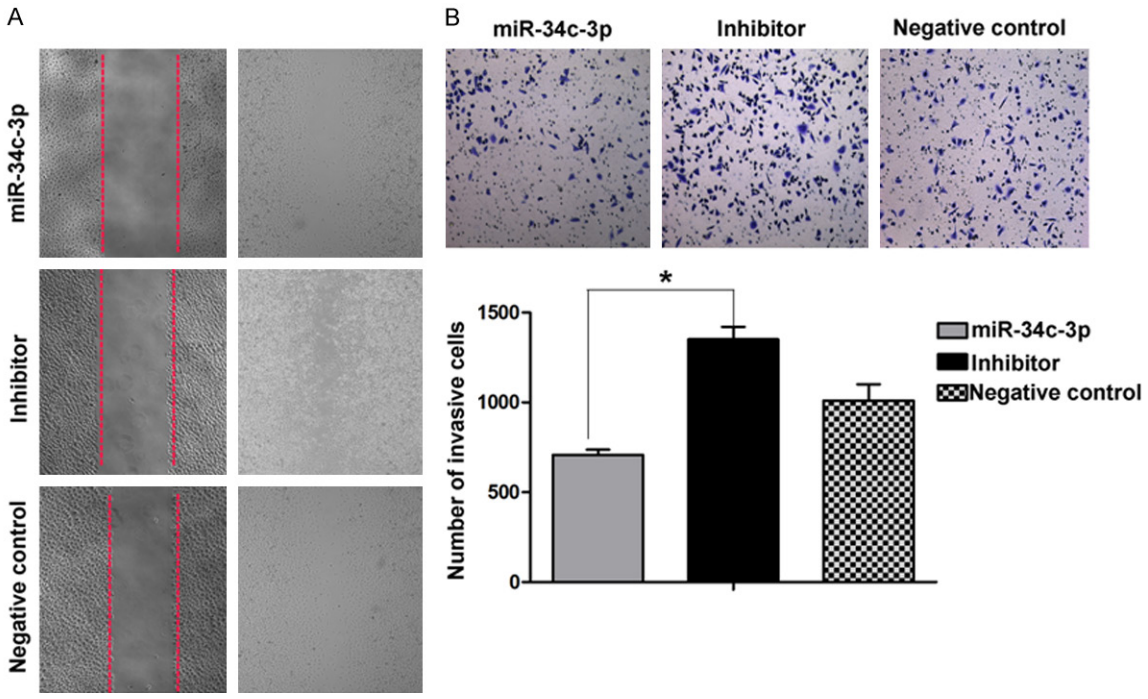

Figure 2. miR-34c-3p suppressed the migration and invasion of non-small-cell lung cancer (NSCLC) cells. A. A549 cells were transfected with miR-34c-3p and inhibitor or negative control mimics, and in vitro migration was assessed by wound healing assay. B. In vitro invasion assay. *P<0.05.

technologies, NY, USA), the images were captured on the image reader LAS-4000 system (Olympus, Tokyo, Japan).

Statistical analysis

All experiments were performed at least three times. The relative quantification of gene expression detected by real-time PCR was analysed by Student's t-test. Linear or rank correlation analysis was performed to determine the correlation between the gene expression levels. Data were presented as the means ± SEM. Analyses were conducted with SPSS 19.0 software, using the unpaired Student's t-test for comparisons between two groups or one-way ANOVA for multiple comparisons. *P* values <0.05 were considered statistically significant.

Results

miR-34c-3p is downregulated in NSCLC tissues and cell lines

The expression level of miR-34c-3p was examined in NSCLC specimens and the corresponding normal tissues. The average expression level of miR-34c-3p was significantly lower in NSCLC specimens compared with adjacent normal tissues ($P<0.01$) (**Figure 1A**). We also determined the expression level of miR-34c-3p in different human NSCLC cell lines by quantitative RT-PCR. Compared with the normal intestinal epithelial cell line, the expression level of miR-34c-3p was lower in the six examined NSCLC cell lines (**Figure 1B**).

MiR-34c-3p inhibited NSCLC cells proliferation and regulated cell cycle of NSCLC cell

To test the function of miR-34c-3p in NSCLC cells, stable cell lines A549 cells expressing miR-34c-3p and negative control (NC) were established by Lipo-2000 transduction. Then we examined the effect of miR-34c-3p on the proliferation of A549 cells from 0h to 96 h ($P<0.05$). Further, we observed that over expression of miR-34c-3p dramatically suppressed the proliferation of A549 cells. In contrast to use a miR-34c-3p inhibitor promoted the proliferation of A549 cell ($P<0.05$) (**Figure 1C**). To determine whether cell cycle was a contributing factor to cell growth inhibition, we analyzed the effect of miR-34c-3p expression on apoptosis of NSCLC cells. As shown in **Figure 1D**, the apoptotic rate of miR-34c-3p-transfected cells was significantly increased by

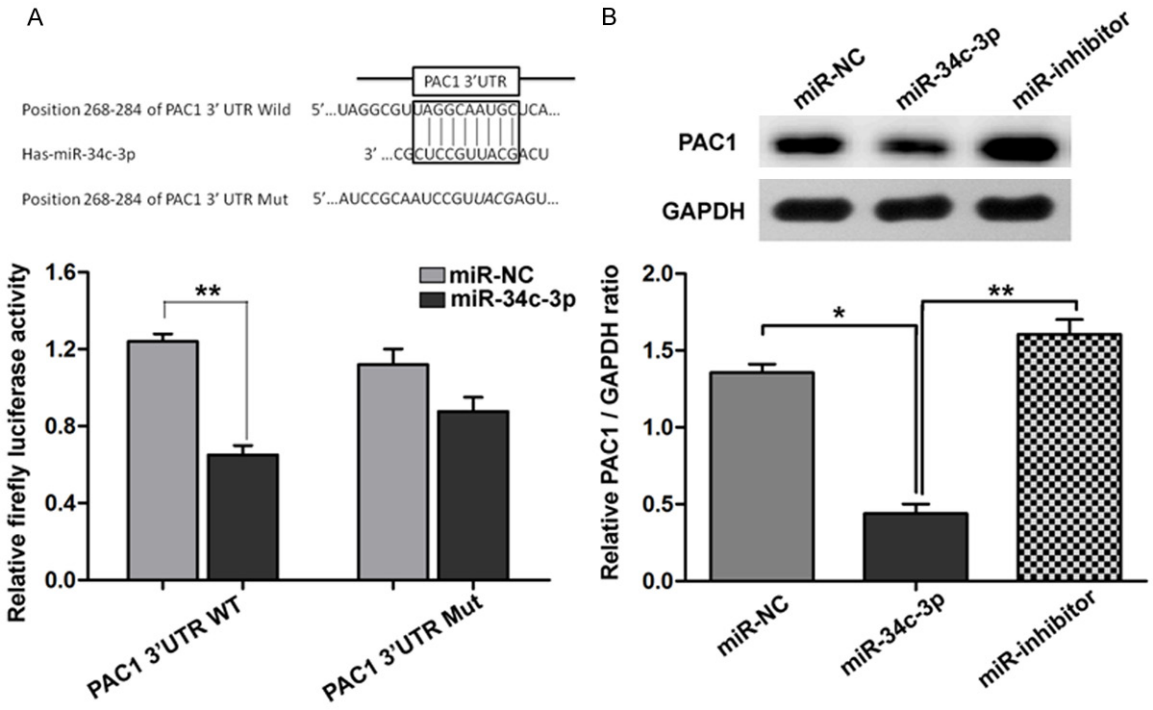

Figure 3. PAC1 was a direct target of miR-34c-3p. A. Schematic representation of wild-type (WT) and mutated (Mut) putative miR-34c-3p-binding sites in the 3'-UTR of PAC1. A549 cells were cotransfected with PAC1 3'-UTR, pGL-3 control and either miR-34c-3p or negative control mimics. Relative firefly luciferase activity was normalized to Renilla luciferase activity. B. Protein levels were detected by western blot analysis in A549 cells transfected with miR-34c-3p or control mimic and in A549 cells transfected with miR-34c-3p or control inhibitor. GAPDH was used as an internal control. *P<0.05, **P<0.01.

10.21±0.82 % compared with miR-NC-transfected cells (P<0.01). Together, these results indicated that the inhibition of cell growth by miR-34c-3p was associated with increased apoptosis.

MiR-34c-3p inhibited NSCLC cell migration and invasion

To determine the function of miR-34c-3p in NSCLC cells, the cell migration and invasion were determined. Overexpression of miR-34c-3p in NSCLC cells significantly suppressed cell migration (P<0.05) and cell invasion (P<0.01), whereas loss of its expression promoted NSCLC cellular migration (P<0.01) (**Figure 2A**) and cellular invasion (P<0.01) (**Figure 2B**). These observations suggest that miR-34c-3p plays an important role in inhibiting migration and invasion of NSCLC A549 cells.

PAC1 is the direct target of miR-34c-3p

We searched the sequence in the TargetScan and found that the 3'-UTR of the PAC1 gene contains one separate miR-34c-3p-binding seed sequences that are conserved through evolution. To demonstrate the direct interaction between miR-34c-3p and PAC1 mRNA, we then constructed the luciferase reporter system containing the binding site (PAC1-3'-UTR-wt) or mutated site (PAC1-3'-UTR-mut) to downstream of the pRLCMV luciferase reporter gene. This vector was co-transfected into 293T cells with miR-34c-3p mimic or negative controls. Luciferase activity in miR-34c-3p group was decreased markedly compared with negative controls by 32.6% (**Figure 3**). MiR-34c-3p mimic did not affect the luciferase activity in the pGL3-PAC1-mut vector (**Figure 3A**). When blocking the expression of miR-34c-3p with miR-34c-3p inhibitor, we get increased luciferase intensity in 293T cells (**Figure 3B**). These results support the bioinformatics prediction indicating the 3'-UTR of PAC1 mRNA as a target for miR-34c-3p.

PAC1 expression is inversely correlated with miR-34c-3p expression in NSCLC patients

We further compared expression levels of PAC1 and miR-34c-3p in NSCLC patient tissues and found that PAC1 protein level was also upregu-

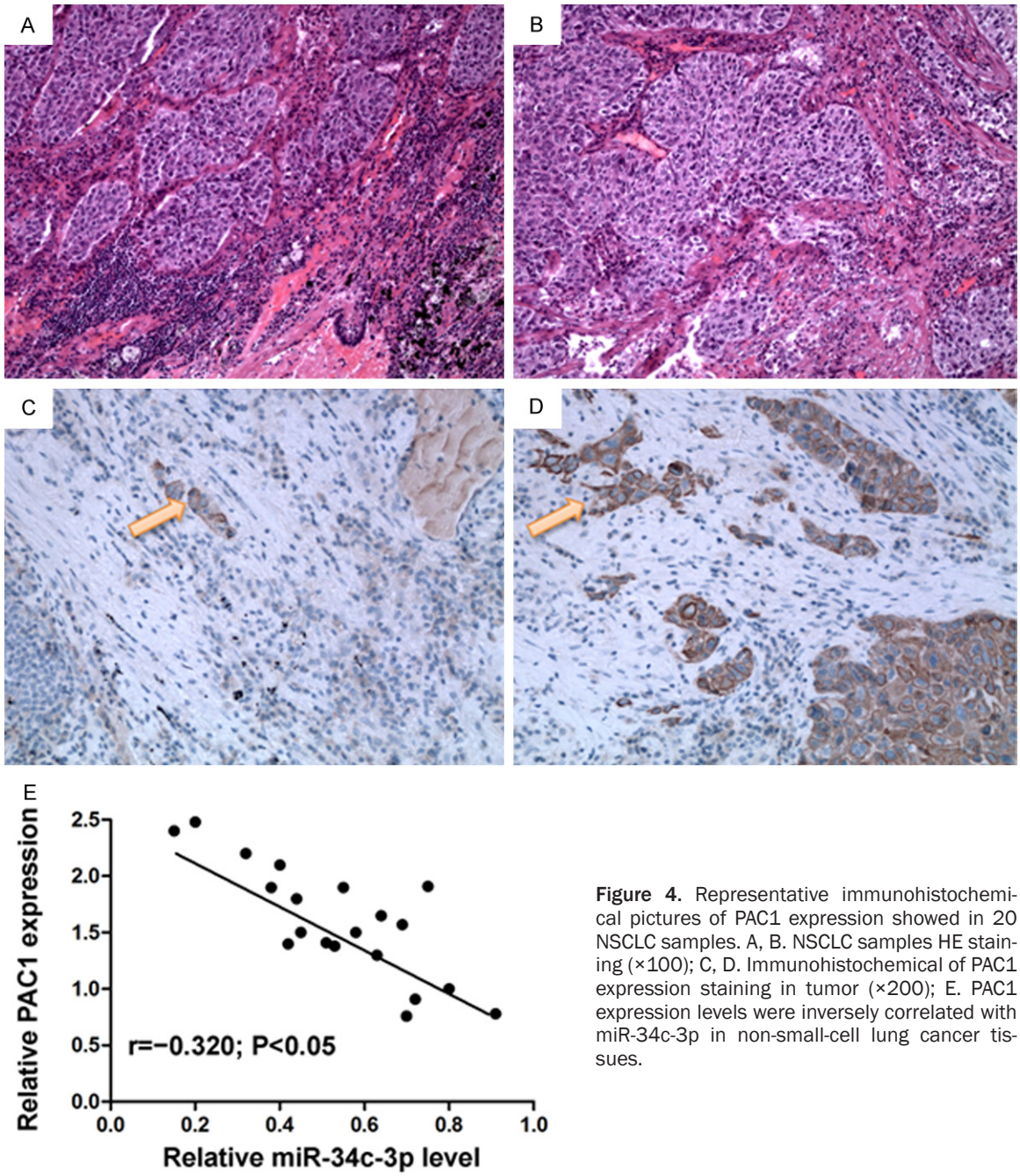

Figure 4. Representative immunohistochemical pictures of PAC1 expression showed in 20 NSCLC samples. A, B. NSCLC samples HE staining (×100); C, D. Immunohistochemical of PAC1 expression staining in tumor (×200); E. PAC1 expression levels were inversely correlated with miR-34c-3p in non-small-cell lung cancer tissues.

lated in miR-34c-3p downregulated NSCLC tissues confirming that endogenously expressed PAC1 is regulated by miR-34c-3p expression. Bivariate correlation analysis revealed a significant inverse correlation between expression of PAC1 and miR-34c-3p (r=-0.320; $P<0.05$; **Figure 4**). Thus, these data further support that downregulation of miR-34c-3p was inversely correlated with upregulation of PAC1 in NSCLC tissues. Taken together, these data demonstrate that PAC1 is a direct target of miR-34c-3p and further suggest that miR-34c-3p may exert its apoptosis promoting effect through inhibition of PAC1 expression.

MiR-34c-3p overexpression regulate p38 MAPK relevant pathway expression

Since MAPK pathway play a crucial role in cell proliferation and tumorigenesis, we then carried out the western blot and immunocytochemistry assay to compare the activity of P38

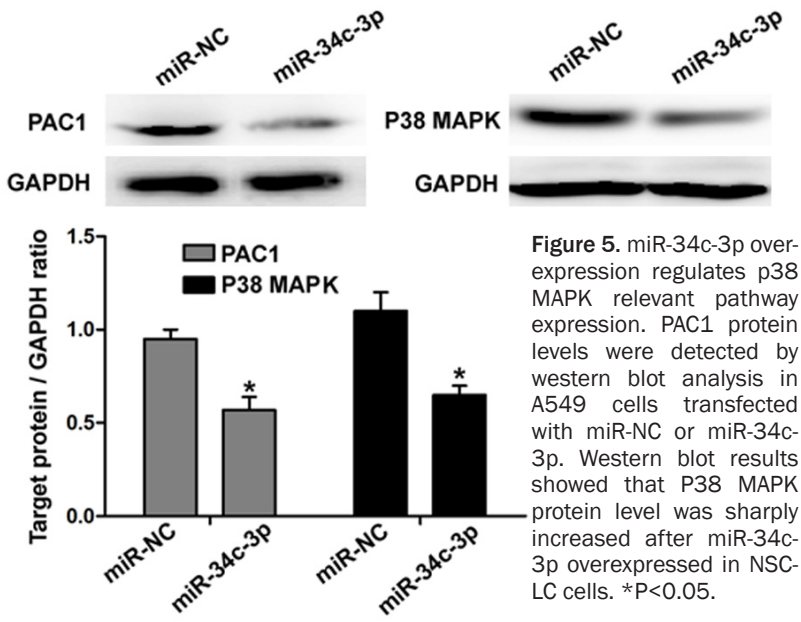

Figure 5. miR-34c-3p overexpression regulates p38 MAPK relevant pathway expression. PAC1 protein levels were detected by western blot analysis in A549 cells transfected with miR-NC or miR-34c-3p. Western blot results showed that P38 MAPK protein level was sharply increased after miR-34c-3p overexpressed in NSCLC cells. *P<0.05.

MAPK pathway in miR-34c-3p-overexpressing and control cells. As shown in **Figure 5**, P38 MAPK protein expression was dramatically increased in miR-34c-3p overexpressing cells compared with the control cells, respectively. Then, we performed Western blot to examine the P38 MAPK expression in NSCLC cells. Western blot results showed that P38 MAPK protein level was sharply increased after miR-34c-3p overexpressed in NSCLC cells (**Figure 5**).

Discussion

Takamizawa *et al* were the first to relate microRNA expression to lung cancer [19]. Since then there have been large number of studies relating microRNA expression with lung cancer [20]. The miRNAs have been reported to play essential roles in carcinogenesis and tumor progression [12, 21]. In addition, acting as either tumor suppressors or oncogenes, miRNAs are involved in several aspects of cancer biology including cell proliferation, apoptosis, migration and invasion [22]. Deregulation of miRNAs such as miR-221, miR-222, miR-449a, miR-21, miR-205, miR-10b, miR-143 and miR-181a in NSCLC is a key factor potential tumorigenesis [23]. These findings prompted us to investigate the regulation of miR-34c-3p in NSCLC H1299 and A549 cells. Recent studies showed that miR-34c-3p regulated growth of glioma cells, down-regulation of miR-34c-3p maintained self-renewal and inhibited apoptosis in glioma tumor-initiating cells, miR-34c-3p regulated Notch2 expression during cellular senescence [24]. However, the role of miR-34c-3p in cancers especially in NSCLC is not very much known. Understanding the relationship between NSCLC and dysregulated miRNAs may help find a biomarker and further develop treatment approaches to improve the cure and survival rates of this cancer. In this study, we confirmed that oncogene PAC1 was directly targeted by miR-34c-3p in NSCLC cells. Downregulation of miR-34c-3p and up-regulation of PAC1 protein levels were also found in NSCLC tissues compared to adjacent non-tumor tissues.

It is well-documented that the mature miRNA-34 family, as tumor suppressors, shows a global decrease in expression in many different human cancers, including laryngeal carcinoma, prostate cancer and cervical carcinoma [16, 17, 25]. In this study, we demonstrated that miR-34c-3p is downregulated in NSCLC tissues as well as in NSCLC cell lines. Therefore, miR-34c-3p was suggested to function as a tumor suppressor gene in NSCLC, which is consistent with its roles in glioblastomas, pituitary adenomas, and lymphocytic leukemia [26, 27]. To verify this hypothesis, we validated the tumor suppressive roles of miR-34c-3p in NSCLC cell line A549 cells. Restoration of miR-34c-3p expression could dramatically inhibit growth, suppress cell migration, and invasion ability and promotes apoptosis of NSCLC cell lines in vitro. However, in chronic lymphocytic leukemia and breast cancer, miR-34c-3p expression is reported to be elevated in these malignancies [28]. Hence, deregulation of miR-34c-3p may be different in different types of cancer, and the roles of miR-34c-3p deregulated in cancer development remain to be further investigated.

Many investigators have demonstrated that expression of the miR-34 family resulted in G0/G1 cell cycle arrest in diverse cellular contexts

[29]. The typical sub-G0 population would occur as well as cell apoptosis. The results delineate a novel regulatory network employing miR-34c-3p and PAC1 to fine-tune cell proliferation, invasion and apoptosis. Interestingly, a recent study has shown that miR-34c-3p can suppress the metastasis of human malignant glioma by downregulating the c-Met and Notch signaling pathway [24]. These studies, combined with ours, reveal the importance of miR-34c-3p targeting PAC1/MAPK as a novel regulatory pathway in lung cancer progression.

Recent studies showed that PAC1 activates the Rap1 to MAPK pathway from intracellular organelles as does TrkA, and because of the possibility of trans-activation of TrkA by PAC1 in intracellular organelles, one might predict a common early endosomal intermediate for these two receptors [30]. In the present study, we also predicted that PAC1 was the precise intracellular target of miR-34c-3p by using miRanda, TargetScan and PICTAR databases. PAC1/MAPK signaling is key in the regulation of multiple tumor cell proliferation [31, 32]. The MAPK signaling is mediated by p38 MAPK, ERK1/2 and JNK, which are important in the control of cell proliferation, differentiation and apoptosis [33]. Accumulating evidences reveal that the roles of MAPKs in these functions are controversial and complicated, that depend on the stimuli, intensity, and duration, as well as cell types [34]. JNK plays opposite roles in carcinogenesis, which is involved in induction of apoptosis, but also implicated in promotion of cell survival and proliferation [35]. In addition, under stimulation of wound healing, p38 and ERK1/2 coordinate the dynamics of the processes through inducing migration by p38 and enhancing proliferation by ERK1/2 activation in corneal epithelial cells [36]. Recently, the dual role of MAPK in response to different stimulations has been demonstrated in fibroblast cells [37]. Exposure to anisomycin causes cellular stress and induces strong and persistent MAPK activation, leading to cell cycle arrest [38]. In contrast, mitogenic stimulation by serum results in weaker and transient MAPK activation to induce cyclin D1 and inactivate Rb by phosphorylation, leading to enhancing cancer cell proliferation [39, 40].

To the best of our knowledge, this study provided the first evidence of miR-34c-3p values in NSCLC patients' tissues. Transfection with target miRNA inhibited the cell proliferation, cell cycle changes, apoptosis and cell invasion. Moreover, we identified PAC1 as a direct target of miR-34c-3p and found that overexpression of miR-34c-3p downregulated both mRNA and protein levels of PAC1. We demonstrated that miR-34c-3p inhibited PAC1 expression by directly targeting its 3'-UTR. Although further studies are needed to validate the other targets and determine the expression of miR-34c-3p in lung cancer tissues or other cell lines, our experimental data suggest an important role of miR-34c-3p in inhibiting the growth, migration, and invasion of NSCLC and implicates restoration of miR-34c-3p may be a potential therapeutic strategy for NSCLC. Our findings prompt important roles of miR-34c-3p in NSCLC etiology and provide potential candidates for treating NSCLC patients. More experiments are required to further validate the effect of miR-34c-3p on the PAC1/MAPK pathway.

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Disclosure of conflict of interest

None.

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