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ORIGINAL ARTICLE

Downregulation of microRNA-21 expression restrains non-small cell lung cancer cell proliferation and migration through upregulation of programmed cell death 4

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Preliminary studies showed that miR-21 is overexpressed in some human cancers. However, the role of miR-21 in cancer is still unclear and even controversial. Our purpose was to investigate the biological effects of miR-21 on A549 non-small cell lung cancer (NSCLC) cells and the underlying mechanisms of those effects. The expression of miR-21 was quantified in serum samples from patients with NSCLC. A549 cells were transfected with miR-NC-sponge or miR-21-sponge only, or with miR-21-sponge plus PDCD4 small-interfering RNA (siRNA). The expression of miR-21 and *PDCD4* mRNA in transfected cells was quantified by real-time polymerase chain reaction and the expression of PDCD4 protein by Western blot. Cell proliferation, apoptosis, migration, and invasion assays were performed to determine the biological effects of miR-21 expression and PDCD4 inhibition. miR-21 was overexpressed in serum from patients with NSCLC. Reduced miR-21 expression was observed following transfection with miR-21-sponge in A549 NSCLC cells. Co-transfection of miR-21-sponge with PDCD4 siRNA upregulated miR-21 expression in these cells. *PDCD4* mRNA and protein levels were increased 2.14-fold and 2.16-fold, respectively, following inhibition of miR-21 expression. Inhibition of miR-21 expression following transfection of miR-21-sponge reduced cell proliferation, migration, and invasion of A549 cells. Depletion of PDCD4 by siRNA transfection reversed the reduction of cell proliferation, migration, and invasion induced by inhibition of miR-21 in A549 cells. It indicates that miR-21 is highly expressed in patients with NSCLC and inhibition of miR-21 expression reduces proliferation, migration, and invasion of A549 cells by upregulating PDCD4 expression. Modulation of miR-21 or PDCD4 expression may provide a potentially novel therapeutic approach for NSCLC.

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INTRODUCTION

Lung cancer is the most common cause of cancer-related death in men and women worldwide, and non-small cell lung cancer (NSCLC) accounts for 80% of all cases.[1,2] Surgical resection remains the main treatment for NSCLC, but the majority of cases are diagnosed at a locally advanced or metastatic stage that rules out curative resection. For these patients, conventional cytotoxic chemotherapy is the best available treatment, but their prognosis is poor, with a 5-year survival rate of only 13%.[3,4] Therefore, a novel therapeutic strategy is needed to improve the prognosis and outcomes.

MicroRNAs (miRNAs) are evolutionarily conserved, endogenous, non-protein-coding, 20- to 23-nucleotide single-stranded RNAs. These molecules regulate gene expression by binding to 3′-untranslated regions, the coding sequences of target messenger RNAs (mRNAs), and influence many biological processes.[5–7] miRNAs are involved in virtually all biological processes, including growth, differentiation, apoptosis, motility, and malignant transformation.[8] Several miRNAs have been functionally classified as proto-oncogenes or tumor suppressors, and aberrant miRNA expression is involved in the initiation of many diseases, including cancer.[9,10] Altered serum levels of miRNAs are associated with cancer development and progression and with clinical outcomes.[9,11]

Among these miRNAs, miR-21 has been verified to be overexpressed in various human cancers and affects their biological behavior by negatively regulating downstream genes.[12,13] However, the role of miR-21 in cancer is still unclear and even controversial. For instance, in contradiction to the findings of other studies,[13–15] one study found that miR-21 was not differently expressed in carcinomas and matched normal tissues and had no biological effects in prostate cancer. These researchers speculated that the oncogenic properties of miR-21 could be cell and tissue dependent and that the potential role of a given miRNA as a therapeutic target should be contextualized with respect to the disease.[16]

Programmed cell death 4 (PDCD4), known as a tumor suppressor, reduces neoplastic transformation and tumorigenesis, inhibits cellular invasion, and promotes cell apoptosis.[17] PDCD4 may influence transcription and translation as well as to modulate signal transduction pathways depending on cell type and gene regulation.[17] However, the precise role and effect of PDCD4 and its regulation by miR-21 on specific biological activities (e.g., apoptosis, proliferation, migration, and invasion) in NSCLC has, to our knowledge, not been studied.

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In the present study, we investigated the role and potential mechanisms of miR-21 in serum samples from NSCLC patients and in A549 NSCLC cells. Our purpose was to identify antitumor pathways for therapeutic intervention through understanding of the biological effects of miR-21 and the potential mechanisms for those effects.

MATERIALS AND METHODS

Patient samples

Patients with NSCLC ($n = 17$) before the surgery and chemotherapy were recruited for a one-time blood draw, and matched healthy volunteers ($n = 16$) were used as the controls. This study was approved by the Institutional Ethics Review Board of the First Affiliated Hospital of Wenzhou Medical University. Written informed consent was obtained from all study participants. Blood from the 17 NSCLC patients and 16 healthy age-matched volunteers was collected into serum separator tubes and processed within 6 h as previously described.[18] Briefly, the tubes were rotated end-over-end at room temperature for 30 min and subjected to centrifugation at 795 *g* at 4 °C for 20 min. Serum from each tube was moved to a new 1.5-ml RNase-free Eppendorf tube and subjected to re-centrifugation at 15 000 *g* for 10 min to remove cellular contaminants. Serum was aliquoted into RNase-free Eppendorf tubes and immediately cryopreserved at − 80 °C for analysis.

Cell culture

A549 human NSCLC cells were maintained in RPMI 1640 medium (Gibco, Karlsruhe, Germany) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Merck Millipore, Darmstadt, Germany) in a 5% CO_2 incubator at 37 °C. Cells were harvested using 0.25% trypsin with 0.02% ethylene-diamine-tetraacetic acid (EDTA) (Beyotime, Jiangsu, China) and passaged every 2-3 days. Cultures were maintained at 80% confluence for use in experiments.

Cell transfection and derivation of stable cell clones

We constructed a plasmid vector (GenePharma, Shanghai, China) that expressed three copies of the bulged miR-21 binding site in the 3′ end of CMV promoter-driven green fluorescent protein (*GFP*) mRNA (Additional file 1: Supplementary Figure S1). Expression of this constructed plasmid vector in the tumor cells served as a 'sponge' that quenched endogenous miR-21.[19] To exclude the non-specific toxicity of the plasmid, a constructed plasmid vector (GenePharma, Shanghai, China) that included the non-specific miR-NC (5′-AAATGTACTGCGCGTGGAGAC-3′) was used as a negative control. Another plasmid vector (GenePharma) labeled with red fluorescent protein and including *PDCD4* binding sites (5′-ACATTCTTCTACAAACCGCTCC-3′), which could silence PDCD4 expression, was also constructed. To dilute out off-targets, 4 different SiRNAs against PDCD4 were designed, named as PDCD4-Si465, PDCD4-Si620, PDCD4-Si1052, and PDCD4-Si1645 respectively. The interference against PDCD4 was identified by Western Blot (Supplementary Figure S2). The constructed vectors were named miR-21-sponge, miR-NC-sponge, and PDCD4 small-interfering RNA (siRNA), respectively.

A549 cells seeded in 6-well plates were transfected with 1.6 μg plasmid DNA (miR-21-sponge or miR-NC-sponge), with the addition of 4 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to optimize the transfection efficiency. For co-transfections, A549 cells seeded in 6-well plates were transfected with 1.6 μg miR-21-sponge and 1.6 μg PDCD4 siRNA with the addition of 8 μl Lipofectamine 2000. Stably transfected cells were selected after 14 days incubation in the medium containing 500 μg/ml of G418 (Invitrogen). The cells were then expanded and maintained in medium containing 300 μg/ml of G418. The stable cell clones were identified by Western blot analysis and successful clones were pooled for further analysis (Supplementary Figure S3).

TaqMan miRNA assay

The PrimeScript RT reagent kit (Takara, Dalian, China) and miRNA-specific stem-loop primers (Applied Biosystems, Foster City, CA, USA) were used for miRNA reverse transcription (RT) from patient serum samples. Each reaction consisted of 2 μl serum with the addition of 6 μl of a reaction mixture containing 2 μl 5× PrimeScript Buffer, 3 μl RNase-free dH2O, and 1 μl 5× TaqMan miRNA RT primer. After the mixture was incubated at 70 °C for 10 min and on ice for 2 min, 0.5 μl PrimeScript RT Enzyme Mix I and 1.5 μl RNase-free dH2O were added into the mixture. RT was carried out in a SensoQuest LabCycler (Göttingen, Niedersachsen, Germany) at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. A no-RT negative control was included in each experiment to ensure that polymerase chain reaction (PCR) products were not due to contamination by genomic DNA.

miRNAs were quantified by PCR (qPCR) with TaqMan HotStart PCR Mastmixture (Bio-Serve, Beltsville, MD, USA) and individual miRNA primers and hydrolysis probes (Applied Biosystems) according to the manufacturer's instructions. Mixtures of 2 μl RT product with 7.3 μl nuclease-free H_2O, 0.4 μl ROX I (50×), 10 μl TaqMan PCR Mixture (2×), and 0.3 μl TaqMan MiRNA Assay (20×) primer were prepared. qPCR was performed on the StepOnePlus Real Time PCR instrument (Applied Biosystems) as follows: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s and 60 °C for 1 min. The relative expression of miR-21 in serum was normalized using the $2^{-\Delta\Delta C(t)}$ method relative to a spiked control (ath-miR-173). All TaqMan PCR analyses were performed in triplicate.

Quantitative real-time RT-qPCR

Total RNA was extracted from A549 cells using Trizol (Invitrogen) for both miR-21 and *PDCD4* mRNA analyses. cDNA was synthesized with the PrimeScript RT reagent kit according to the manufacturer's instructions. Total RNA was subjected to qRT-PCR using the QuantiTect SYBR Green PCR kit (Qiagen, Milan, Italy). Reactions were performed on the StepOnePlus Real Time PCR instrument. Cycling parameters were set at 95 °C for 5 min, then 40 cycles of 95 °C for 10 s and annealing/extending at 60 °C for 30 s. The relative expression of miR-21 and *PDCD4* mRNA was normalized using the $2^{-\Delta\Delta C(t)}$ method relative to U6 and β-actin, respectively. The primers for miR-21 and U6 were synthesized by RiboBio (Guangzhou, China). The primers for *PDCD4* were synthesized by Takara as follows: forward 5′-GAAGGTTGCTGGATAGGC-3′, reverse 5′-ATAAACACAGTTCTCCTGGTCA TCA-3′. The primers for β-actin were also synthesized by Takara: forward 5′-ACCCACACTGTGCCCATCTAC-3′, reverse 5′-TCGGTGAGGATCTTCATGAG GTA-3′. All qRT-PCR analyses were performed in triplicate.

Western blot analysis

Cells were washed with phosphate-buffered saline solution and subjected to lysis in RIPA Lysis Buffer (Beyotime, Jiangsu, China). Protein concentrations were determined by the bicinchoninic acid Protein Assay Kit (Beyotime). Aliquots (50 μg) were separated on a 10% polyacrylamide gel by electrophoresis in sodium dodecyl sulfate and transferred to nitrocellulose membrane (0.45 μm) (Beyotime) by electrophoretic transfer. The membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated at 4 °C overnight with the following primary antibodies: rabbit anti-PDCD4 and rabbit anti-GAPDH (1:2000 dilution; Epitomics, Burlingame, CA, USA). The blots were then incubated with anti-rabbit immunoglobulin G horseradish peroxidase–conjugated secondary antibody (1:5000 dilution; Epitomics) for 1 h at room temperature. After five washings with TBST, the blots were analyzed by an ECL plus western blotting detection system (Beyotime).

Cell proliferation and cell apoptosis analysis

Cell proliferation was assayed by the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). This assay is based on bioreduction of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H tetrazolium sodium (WST-8) to orange-colored formazan to measure cell viability. Briefly, cells were seeded in 96-well plates in triplicate at 1500 cells per well and incubated at 37 °C in a 5% CO_2 incubator. At the same time every day over a period of 7 days, the culture medium was replaced with fresh medium containing 10% CCK-8 reagent, then incubated 1 h at 37 °C. The absorbance was measured at 450 nm on a Multimode Microplate Reader-Varioskan Flash (Thermo Scientific, Waltham, MA, USA). Absorbance values were corrected by subtracting the values from the control wells containing no cells.

Apoptosis was quantified by the Annexin V–FITC Cell Apoptosis Analysis kit (KeyGen Biotech, Nanjing, China) or Caspase-3 Activity Assay kit (Beyotime) according to the manufacturer's protocol. Briefly, for Annexin V–fluorescein isothiocyanate (FITC) analysis, cells were first seeded into 12-well tissue plates (Corning, NY, USA). When cells were grown to 80% confluence, cells were harvested and stained with FITC and propidium iodide for 15 min and analyzed by a flow cytometer.

In vitro migration assay

Migration and invasion are the prominent characteristics of tumor cells. To determine the role of miR-21 on the migration capacity of A549 cells, we performed a wound-healing assay that mimics cell migration and proliferation.[20] After the cells reached 80% confluence in 6-well tissue culture plates, an artificial homogeneous wound was created on the cell monolayer with a sterile plastic 20-μl micropipette tip. The cells were then incubated in growth medium containing 2% serum. Wound width was determined every 24 h over a period of 72 h at a magnification of 100× under a Nikon Eclipse TS100 microscope. Data are expressed as the percent wound closure, which was calculated as follows: percent wound closure = $1 - (\text{width}_t/\text{width}_0) \times 100\%$.

Invasion assay

To determine the role of miR-21 on the invasion capacity of A549 NSCLC cells, a Transwell invasion assay was conducted. Twenty-four-well Transwell plates with a polycarbonate membrane with an 8-μm pore size were obtained from Corning. The transfected cells were starved for 24 h by culturing in serum-free RPMI 1640 medium. After that, 5×10^4 cells were plated on Transwell chambers precoated with Matrigel (BD Biosciences, Bedfird, MA, USA). Medium containing 15% fetal bovine serum in the lower chamber served as a chemoattractant. After incubation at 37 °C for 24 h, non-invading cells were removed with cotton swabs, and invaded cells on the bottom of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. The number of invaded cells on the membrane was then counted at a magnification of 100 x under a Nikon Eclipse TS100 microscope.

Statistical analysis

Statistical analysis of data was performed using SPSS 17.0 (SPSS, Chicago, Illinois, USA). Data are expressed as the mean ± standard error of the mean. Statistical significance was determined by analysis of variance or unpaired two-tailed Student *t*-test. *P* value < 0.05 was considered statistically significant. All of the experiments were repeated at least three times.

RESULTS

miR-21 was upregulated in the serum of patients with NSCLC

Demographic and clinical data on the 17 NSCLC patients are summarized in Table 1. TaqMan PCR revealed that miR-21 was highly expressed in the serum of patients with NSCLC compared with the serum from age-matched healthy volunteers ($P < 0.01$; Figure 1).

Table 1. Demographic and clinical characteristics of 17 NSCLC patients

Factors	
Age (y)	64.8 ± 9.0
Sex (male/female)	11/6
Tumor	
Differentiation (poor/moderate-well/Tis) ($n = 13$) [a]	5/6/2
Histology (squamous/adenocarcinoma/other)	7/6/4
Lymph node metastasis (negative/positive)	15/2
Distal metastasis (negative/positive)	15/2
TNM stage (0/I–II/III–IV)	2/11/4

[a]No differentiation data were available for four of the patients.

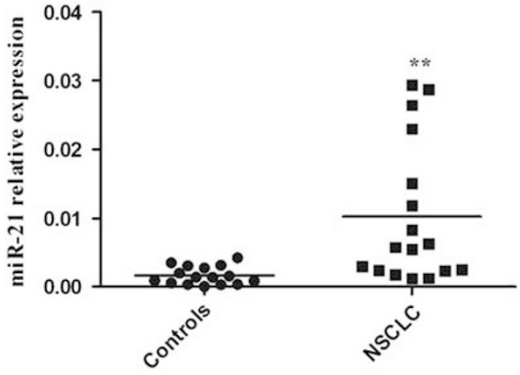

Figure 1. Serum levels of miR-21 in patients with NSCLC. Overexpression of miR-21 in the serum of NSCLC patients was detected by TaqMan PCR. **$P < 0.01$ vs controls.

Expression and regulation of miR-21 and PDCD4 in A549 cells

The transfection efficiencies of miR-NC-sponge and miR-21-sponge without or with co-transfection of PDCD4 siRNA in A549 cells were approximately 50–60%, as shown in Figure 2. This result conformed consistently to Western blot results and was used in subsequent experiments (Figure 2).

Expression of miR-21 in cells transfected with miR-NC-sponge was similar to that in untreated cells. miR-21 expression in cells transfected with miR-21-sponge was on average 44.3% lower than that in cells transfected with the miR-NC-sponge control ($P < 0.05$; Figure 3a). However, co-transfection of miR-21-sponge with PDCD4 siRNA reversed this inhibitory effect on miR-21 expression (Figure 3a). Transfection of miR-21-sponge into A549 cells resulted in increased expression of *PDCD4* mRNA ($P < 0.01$), while co-transfection of miR-21-sponge with PDCD4 siRNA attenuated this effect ($P < 0.05$; Figure 3b). These findings were confirmed by Western blot (Figure 3c and d).

Inhibition of miR-21 expression reduced A549 cell proliferation

The proliferation of cells transfected with miR-NC-sponge was similar to that of untreated cells. Cells transfected with miR-21-sponge showed a significant reduction in cell proliferation from day 5 compared with cells transfected with miR-NC-sponge ($P < 0.05$ to 0.01). However, A549 cells co-transfected with miR-21-sponge and PDCD4 siRNA had a higher rate of cell proliferation than cells transfected with miR-21-sponge only ($P < 0.05$; Figure 4). miR-21-sponge and/or PDCD4 siRNA had no effect on apoptosis of A549 cells (data not shown).

Inhibition of miR-21 expression restrained migration and invasion of A549 cells

As shown in Figure 5a and b and Table 2, the migration of miR-NC-sponge-transfected cells was similar to that of untreated cells. miR-21-sponge transfection inhibited the migration of A549 cells dramatically from 24–72 h compared with that of miR-NC-sponge-transfected cells ($P < 0.01$). Interestingly, co-transfection of miR-21-sponge with PDCD4 siRNA reversed this inhibitory effect on cell migration and facilitated wound closure compared with that of cells transfected with miR-21-sponge only ($P < 0.01$).

The invasion capacity of cells transfected with miR-NC-sponge was similar to that of untreated cells. The number of invaded cells was significantly lower among the cells transfected with miR-21-sponge than that among those transfected with miR-NC-sponge (40.45 ± 3.01 vs 126.38 ± 17.32, $P < 0.001$; Figure 6a and b). However, co-transfection of miR-21-sponge with PDCD4 siRNA reversed this effect compared to cells transfected with only miR-21-sponge (135.05 ± 11.56 vs 40.45 ± 3.01, $P < 0.001$; Figure 6a and b).

DISCUSSION

NSCLC is resistant to available modalities of treatment, perhaps because of its genetic heterogeneity and invasiveness.[11] Molecular

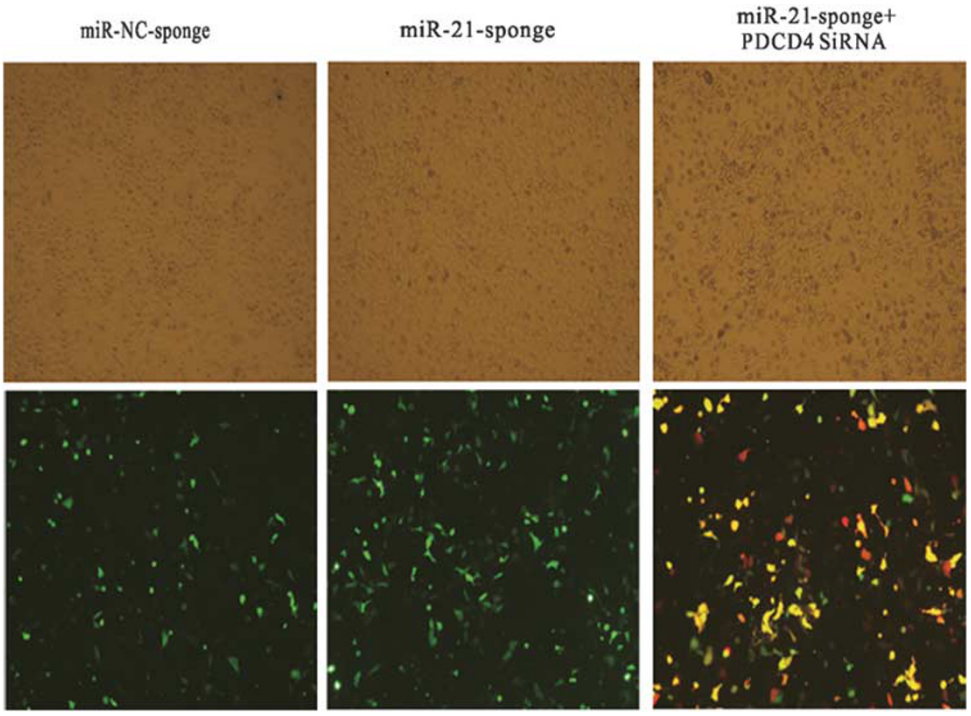

Figure 2. Transfection efficiency in A549 cells. The transfection efficiencies of miR-NC-sponge, miR-21-sponge, or PDCD4 siRNA plus miR-21 co-transfection in A549 cells were approximately 50–60% as detected by a fluorescence microscope (×100 magnification). miR-21-sponge and miR-NC-sponge were labeled with GFP and PDCD4 siRNA was labeled with red fluorescent protein.

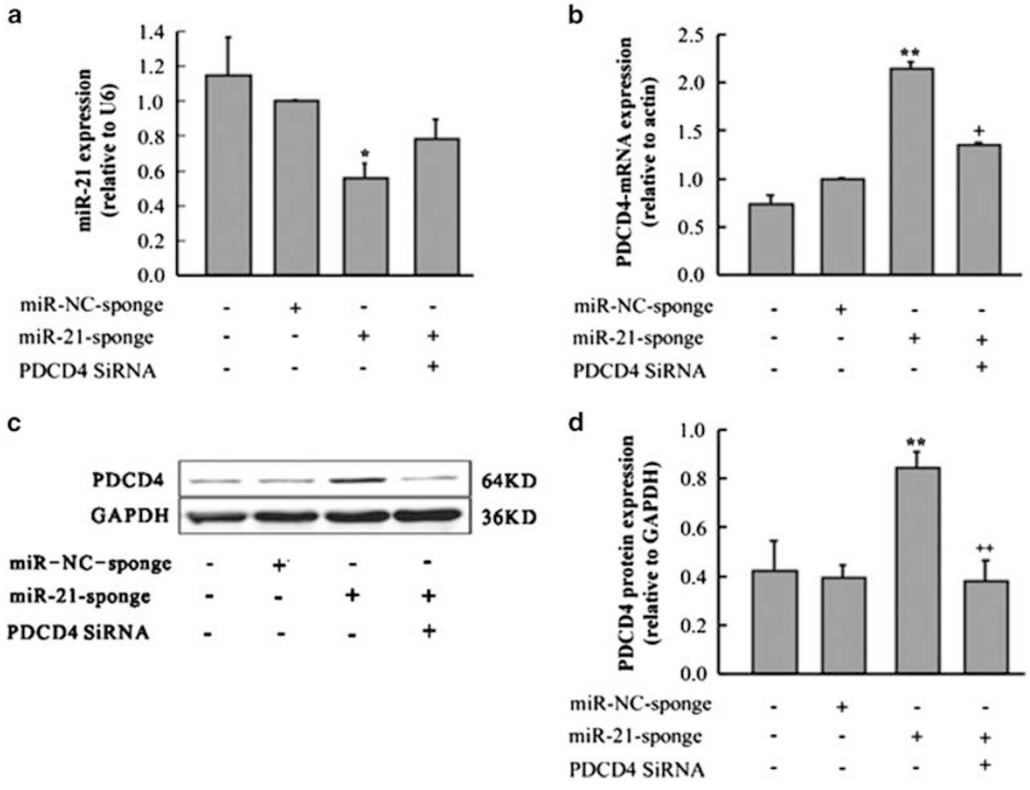

Figure 3. Expression and regulation of miR-21 and PDCD4 following transfection in A549 cells. Real-time PCR was performed to quantify miR-21 expression (**a**) and *PDCD4* mRNA expression (**b**) in A549 cells. (**c**) Western blot was performed to detect PDCD4 protein expression. (**d**) Densitometric western blot analysis. $^{*}P < 0.05$, $^{**}P < 0.01$ vs miR-NC-sponge; $^{+}P < 0.05$, $^{++}P < 0.01$ vs miR-21-sponge.

biomarkers that are associated with biological mechanisms contributing to malignancy may be important for understanding the pathologic attributes of NSCLC and for designing effective therapeutic strategies for this cancer.

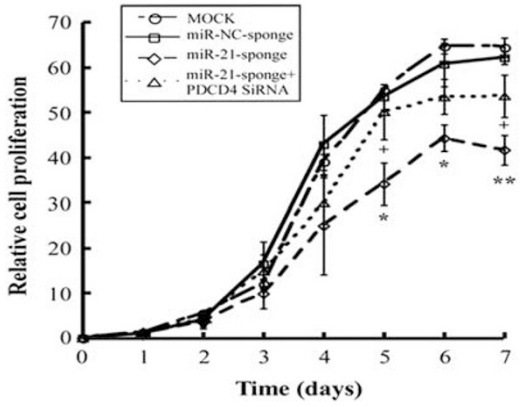

Figure 4. Effects of inhibition of miR-21 expression on A549 cell proliferation. Proliferation of cells transfected with miR-21-sponge was inhibited compared with cells transfected with miR-NC-sponge. Co-transfection of miR-21-sponge and PDCD4 siRNA reversed the inhibitory effect on cell proliferation $^{*}P < 0.05$, $^{**}P < 0.01$ vs miR-NC-sponge; $^{+}P < 0.05$ vs miR-21-sponge.

In NSCLC, it has been shown that miRNA expression profiles and specific miRNAs in lung tissue are correlated with tumor progression, disease prognosis, and clinical outcome.[21–23] In the present study, we observed overexpression of miR-21 in the serum of NSCLC patients. This finding suggests that serum miR-21 could be an independent molecular biomarker for NSCLC.

Previous work by others has demonstrated that miR-21 is a member of a class of miRNAs that are frequently overexpressed in solid tumors such as breast, prostate, pancreas, colon, kidney, and brain cancers.[24] However, little is known about the pathological role and the underlying mechanisms of miR-21 activity in NSCLC. We showed that transfection with miR-21-sponge, which can quench miR-21, reduced miR-21 expression in A549 NSCLC cells, while co-transfection with miR-21-sponge and PDCD4 siRNA upregulated miR-21 expression in these cells. Other reports reveal that PDCD4 inhibits Jun N-terminal kinase (JNK) activation by downregulating MAP kinase kinase kinase kinase 1, an upstream kinase of JNK.[25] Moreover, PDCD4 inhibits JNK1-dependent phosphorylation of c-Jun, which could regulate miR-21 expression. In other words, inhibition of PDCD4 by siRNA transfection results in activation of JNK and c-Jun,[26] leading to upregulation of miR-21.[27] Thus, there may exist a feedback-based regulatory mechanism for miR-21-PDCD4-JNK/c-Jun.

PDCD4 has been characterized as both a tumor suppressor and a pro-apoptotic gene that may function by binding eukaryotic initiation factor 4A and inhibiting translation initiation.[28] Such activity could facilitate the regulation of multiple proteins involved in tumor progression at the translational level. Studies have shown

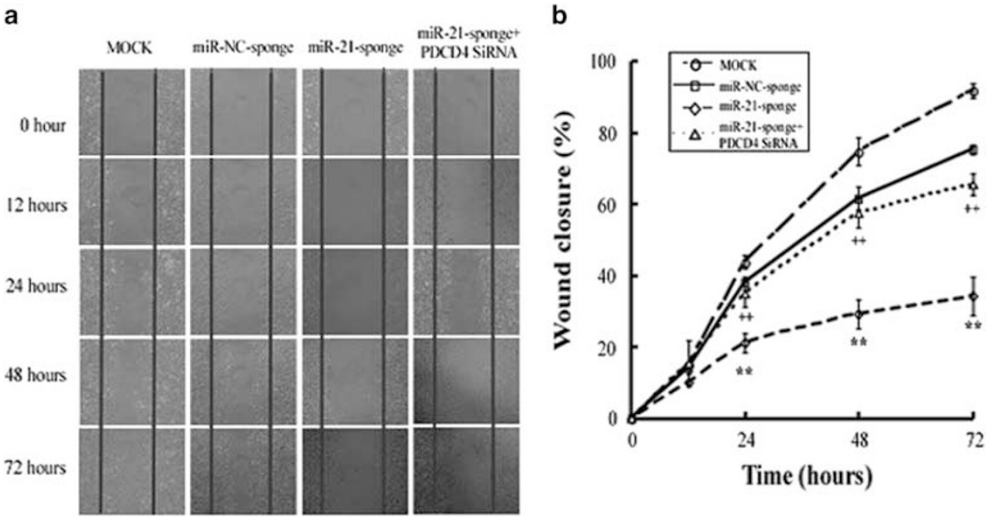

Figure 5. Effects of inhibition of miR-21 expression on A549 cell migration. Inhibition of miR-21 restrained the A549 cells migration while co-transfection of PDCD4 siRNA reversed the inhibitory effect on cell migration. (**a**) Migration of A549 cells transfected with miR-NC-sponge or miR-21-sponge without or with PDCD4 siRNA. (**b**) The migration was quantified as percentage of wound closure. $^{**}P < 0.01$ vs miR-NC-sponge; $^{++}P < 0.01$ vs miR-21-sponge.

Table 2. Percentages of wound closure in A549 cell monolayers

Compound	*Wound closure (%)*				
	0 h	*12 h*	*24 h*	*48 h*	*72 h*
MOCK	0	15.22 ± 0.68	43.78 ± 1.97	74.55 ± 3.88	91.58 ± 1.93
miR-NC-sponge	0	14.13 ± 1.59	37.80 ± 1.72	61.57 ± 3.13	75.17 ± 1.29
miR-21-sponge	0	9.98 ± 0.72	21.08 ± 2.69**	29.31 ± 4.08**	34.32 ± 5.32**
miR-21-sponge+PDCD4 siRNA	0	15.46 ± 6.45	34.83 ± 3.53^{++}	57.37 ± 4.07^{++}	65.37 ± 3.10^{++}

$^{**}P < 0.01$ vs miR-NC-sponge; $^{++}P < 0.01$ vs miR-21-sponge.

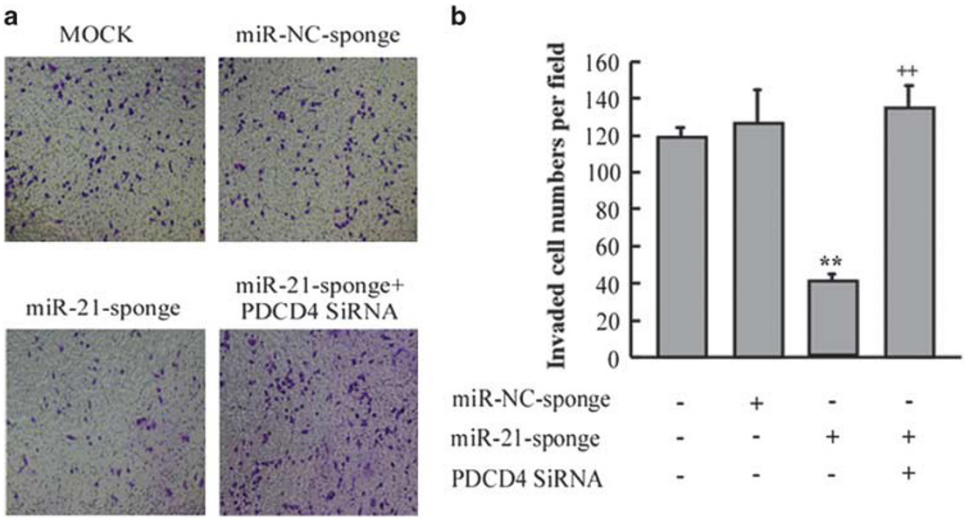

Figure 6. Effects of inhibition of miR-21 expression on A549 cell invasion. Inhibition of miR-21 restrained the A549 cells invasion, whereas co-transfection of PDCD4 siRNA reversed the inhibitory effect on cell invasion. (**a**) Cells transfected with miR-NC-sponge or niR-21-sponge with our without PDCD4 siRNA were subjected to Transwell assay. Cells that penetrated the membrane were stained with crystal violet on the lower chamber membrane. (**b**) Invaded cells were quantified under a microscope. $^{**}P < 0.01$ vs miR-NC-sponge; $^{++}P < 0.01$ vs miR-21-sponge.

that *PDCD4* is downregulated or lost in several tumor types[29,30] and that its ectopic expression reduces tumor progression.[30–32] In this study, we demonstrated that *PDCD4* was regulated by miR-21 in A549 cells. This is evidenced by the finding that *PDCD4* mRNA and protein levels were increased 2.14-fold and 2.16-fold, respectively, following inhibition of miR-21 expression in cells. Furthermore, we demonstrated that inhibition of miR-21 expression reduced cell proliferation, migration, and invasion of A549 NSCLC cells. Depletion of PDCD4 rescued these effects. These findings indicate that PDCD4 is an important tumor suppressor and a functional target of miR-21. Studies are needed to demonstrate the direct link between miR-21 and PDCD4 by overexpressing miR-21 or PDCD4 and to investigate the functional effects in other NSCLC cell lines in addition to A549 cell line. Luciferase activity assay may help to determine the direct interaction between miR-21 and PDCD4. More importantly, further investigation of *in vivo* effect of miR-21 using an animal model is warranted.

Certainly, a single miRNA can regulate hundreds of downstream genes with different biological functions and mechanisms. Targets of miR-21 other than PDCD4 are also likely to contribute to miR-21-induced biological effects in tumor cells, such as PTEN/PI3K/AKT and Ras/MEK/ERK.[13,33] On the other hand, one downstream molecule can be regulated by many upstream genes, including multiple miRNAs. Our present work does not exclude other mechanisms of PDCD4 regulation in tumors. Interestingly, inhibition of miR-21 had no effect on cell apoptosis (data not shown). A possible explanation for this finding is the specific characteristics of different cells. Further studies are needed to elucidate the molecular mechanism of such finding.

In conclusion, our results demonstrate for the first time that miR-21 is highly expressed in serum of patients with NSCLC and that inhibition of miR-21 expression can reduce tumor cell proliferation, migration, and invasion. These effects may be modulated through upregulation of PDCD4 expression. Our findings may provide a potential therapeutic approach for NSCLC through modulation of miR-21 and PDCD4 expression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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