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MicroRNA-421 promotes proliferation and invasion of non-small cell lung cancer cells through targeting PDCD4

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ABSTRACT

Recent evidence highlights that microRNAs serve as crucial regulators of tumorigenesis, including non-small cell lung cancer (NSCLC). The present study was designed to investigate the expression profile, clinical significance and biological role of miR-421 in NSCLC. The results showed that miR-421 expression was markedly increased in NSCLC tissues and cell lines. Further experimental data indicated that knockdown of miR-421 significantly inhibited NSCLC cell proliferation and induced cell cycle arrest in vitro. The migratory and invasive abilities of NSCLC cells were also attenuated following miR-421 knockdown. Furthermore, PDCD4 was identified as a direct target of miR-421, and its expression was inversely correlated with miR-421 expression in NSCLC tissues. PDCD4 also abrogated the oncogenic role of miR-421 in NSCLC cells. Collectively, our study revealed that miR-421 is significantly upregulated in NSCLC and might represent a potential therapeutic target for NSCLC patients.

1. Introduction

Currently, lung cancer remains the leading cause of cancer-associated mortality around the world [1], and non-small cell lung cancer (NSCLC), including adenocarcinoma and squamous cell carcinoma, accounts for approximately 80% of all lung cancer cases [2]. Despite great advances in the chemotherapy and molecular targeting therapy, the overall five-year survival rate of NSCLC patients remains poor [3]. Therefore, it is of great significance to understand the molecular mechanisms underlying NSCLC development and identify novel therapeutic targets for NSCLC patients.

MicroRNAs (miRNAs) are a class of endogenous small single-stranded, noncoding RNA molecules with approximately 22 nucleotides in length, which play important roles in regulation of target gene expression through binding to the 3′-untranslated region (3′-UTR), thus resulting in translational repression or mRNA degradation [4]. Numerous studies have demonstrated that aberrant expression or dysregulation of miRNAs may lead to many pathological processes, including tumorigenesis [5]. Among these miRNAs, miR-421 has been found to serve different roles in various human cancer types. miR-421 was significantly upregulated in gastric cancer and osteosarcoma tissues [6,7], but in breast cancer and glioma, miR-421 acts as a tumor suppressor [8,9]. However, up to now, little is known about the clinical and biological function of miR-421 in NSCLC.

In this study, we found that miR-421 was upregulated in NSCLC, and knockdown of miR-421 remarkably suppressed cell proliferation, migration and invasion of NSCLC cells. Moreover, PDCD4 was identified to be a direct functional downstream target of miR-421 in NSCLC.

2. Materials and methods

2.1. Clinical specimens

NSCLC tissues and the matched adjacent normal tissues were collected from 123 cases of patients who underwent surgical resection at Harbin Medical University Cancer Hospital (Harbin, China). The clinical data of these patients were listed in Table 1. No patients received chemotherapy or radiotherapy prior to surgery. All tissue samples were confirmed through histopathological evaluation, immediately frozen in liquid nitrogen, and stored at −80 °C until further use. The present study was approved by the Ethics Committee of Harbin Medical University Cancer Hospital, and written informed consent was obtained from all patients.

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Table 1
Correlation of miR-421 expression and clinicopathological characteristics in patients with NSCLC.

Characteristics	Total number (n = 123)	miR-421 expression		*P* value
		Low (n = 67)	High (n = 56)	
Age				0.346
< 65	54	32	22	
≥65	69	35	34	
Gender				0.872
Male	80	44	36	
Female	43	23	20	
Tumor location				0.855
Left lung	67	37	30	
Right lung	56	30	26	
Histology type				0.331
Adenocarcinoma	63	37	26	
Squamous	60	30	30	
Tumor size				0.025
< 5cm	81	50	31	
≥5cm	42	17	25	
TNM stage				0.009
I + II	87	54	33	
III + IV	36	13	23	
Lymph nodes metastasis				0.058
Yes	46	20	26	
No	77	47	30	

2.2. Cell culture and transfection

Four NSCLC cell lines (A549, H1299, H1975 and SPCA1) and a normal human bronchial epithelial cell line (16HBE), purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China), were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Loire, France) and 1% penicillin/streptomycin at 37 °C in an atmosphere containing 5% CO_2.

The miR-421 mimics, miR-421 inhibitor and their corresponding negative control were purchased from GenePharma (Shanghai, China). For PDCD4 overexpression, the full-length CDS sequence of PDCD4 was synthesized and inserted into the pcDNA3.1 (+) vector (Invitrogen). An empty pcDNA3.1 vector was used as a control. Cell transfection was performed using Lipofectamine 2000 reagent (Invitrogen). After transfection for 48 h, the following experiments were performed.

2.3. RNA extraction and RT-qPCR analysis

Total RNA was extracted from the tissues and cells using TRIzol reagent (Invitrogen). RNA (1 μg) was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara, Dalian, China). Then qPCR analysis was performed using SYBR® GreenMaster Mix (Takara) on an ABI PRISM 7300 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method [10], with U6 or GAPDH as an internal control. The sequences of the primers were showed in Table 2.

2.4. MTT assay

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, after transfection, 2000 cells were seeded in a 96-well culture plate and culture for 24, 48, 72, and 96 h, respectively. Then 20 μl MTT solution (5 mg/ml; Sigma, St. Louis, MO, USA) was added to each well, and the cells were incubated for another 4 h at 37 °C. Then the remaining formazan precipitates were dissolved in DMSO (Sigma) and the absorbance of each well at 570 nm was detected using a microplate reader (Bio-Tek Company, Winooski, VT, USA).

2.5. Cell cycle analysis

Cell cycle distribution was evaluated using the CycleTEST PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA, USA). Briefly, after transfection, cells were fixed in 70% ethanol at 4 °C for 48 h, incubated with 50 μg/ml RNase and stained with 50 μg/ml propidium iodide for 30 min at room temperature. Then, the proportion of cells in the G0/G1, S, and G2/M phases was determined by using a flow cytometer (BD Biosciences).

2.6. Transwell assay

Cell migration and invasion were evaluated by transwell assay using transwell chambers with 8-μm pores (Costar, Corning, NY, USA). In short, after transfection, cells were seeded with media containing 1% FBS into the uncoated (for migration analysis) or Matrigel-coated (for invasion analysis) upper chamber, and 600 μl of medium containing 10% FBS were added into the lower chamber as chemoattractant. After 48 h of incubation, the cells remaining on the upper surface of the chamber were carefully wiped out, and the cells that moved to the bottom surface were fixed with 95% ethanol and stained with 0.1% crystal violet for 20 min. Five random fields were counted per chamber under a microscope.

2.7. Western blot analysis

Tissue samples or cells were lysed with RIPA lysis buffer (Beyotime, Shanghai, China) containing protease inhibitor cocktail (Roche, Basel, Switzerland) and PMSF (Roche). Equal amounts of protein were fractionated by SDS-PAGE gel and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were then blocked with 5% non-fat milk and incubated overnight with primary antibodies against PDCD4 (1:500; Santa Cruz Biotechnology, Dallas, TX, USA) and GAPDH (1:1000; Cell Signaling Technology, Danvers, MA, USA), followed by incubation with the appropriate HRP-

Table 2
The sequences of the primers.

Gene name	Primer sequences
miR-421-RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCGCCC-3′
U6-RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATA-3′
miR-421 Forward primer	5′-ATCAACAGACATTAATT-3′
miR-421 Reverse primer	5′-GTGCAGGGTCCGAGGT-3′
U6 Forward primer	5′-CTCGCTTCGGCAGCACATATACT-3′
U6 Reverse primer	5′-ACGCTTCACGAATTTGCGTGTC-3′
PDCD4 Forward primer	5′-GGGAGGAGGAATCGGACAG-3′
PDCD4 Reverse primer	5′-TATGTTGGGAGGCGTGGC-3′
GAPDH Forward primer	5′-CGAGATCCCTCCAAAATCAA-3′
GAPDH Reverse primer	5′-TTCACACCCATGACGAACAT-3′

conjugated secondary antibodies (1:10000; Santa Cruz Biotechnology) at room temperature for 2 h. The signals were visualized using an enhanced chemiluminescent detection kit (Pierce, Rockford, IL, USA). GAPDH was considered as an inner loading control.

2.8. Dual-luciferase reporter assay

To construct a luciferase reporter vector, PDCD4 3′-UTR fragment containing putative binding sites for miR-421 was amplified by PCR, and the PCR product was subcloned in the pLUC Luciferase vector (Ruibo, Guangzhou, China). A mutant reporter construct was generated using the Strategene QuickChange Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Cells were cultured in 24-well plates and co-transfected with 50 ng of the recombinant plasmids together with 25 nM of miR-421 mimics or control using Lipofectamine 2000 reagent. Following 48 h of transfection, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

2.9. Xenografts in nude mice

Twelve BALB/c male nude mice of 5–6 weeks of age were obtained from Slac Laboratory Animal Co., Ltd (Shanghai, China) and housed under a natural day/night cycle with free access of foods and water. 2×10^6 A549 cells were implanted subcutaneously into the back of each nude mice. When the tumors reached approximately 100 mm^3, miR-421 antagomir or antagomir NC was directly injected into the tumors at a dose of 1 nmol (in 50 µl PBS) per mouse every 5 days. The tumor volume was measured every five days with the caliper and calculated as tumor volume = $0.5 \times$ length $\times$ width^2. The mice were killed on day 28 and the tumors were removed. The animal experiments were approved by the Ethics Committee of Harbin Medical University Cancer Hospital, and all efforts were made to minimize animal suffering.

2.10. Statistical analysis

Statistical analysis was performed using SPSS (version 18.0) software (SPSS Inc., Chicago, IL, USA) and Graphpad Prism (version 6.01) software (GraphPad Software Inc., La Jolla, CA, USA). Experimental data were expressed as the mean ± standard deviation (SD) from three independent experiments, in which the differences were analyzed by Student's *t* test (two-tailed) or one-way ANOVA. The Chi-Square test was used to determine the relationship between miR-421 expression and clinicopathological characteristics of NSCLC patients. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. miR-421 is upregulated in NSCLC

As demonstrated in Fig. 1A, miR-421 was significantly upregulated in NSCLC tissues compared with that in matched adjacent normal tissues. In addition, four NSCLC cell lines (A549, H1299, H1975 and SPCA1) showed significantly higher miR-421 expression levels than the normal human bronchial epithelial cell line (16HBE) (Fig. 1B). We then divided all NSCLC patients into two groups according to the cutoff value of miR-421 expression: the high expression group (n = 56) and the low expression group (n = 67), and we found that high miR-421 expression was closely associated with large tumor size ($P = 0.025$) and advanced TNM stage ($P = 0.009$) of NSCLC patients (Table 1).

3.2. miR-421 promotes NSCLC cell proliferation in vitro

To further investigate the biological role of miR-421 in NSCLC, miR-421 was then overexpressed in H1299 cells and knocked down in A549 cells (Fig. 2A). The results of MTT assay suggested that overexpression of miR-421 enhanced, whereas knockdown of miR-421 inhibited the proliferation of NSCLC cells (Fig. 2B). Besides, we analyzed the effects of miR-421 on the cell cycle distribution of NSCLC cells via flow cytometry. We observed a significant decrease in the percentage of cells in the G0/G1 phase and an increase in the percentage of cells in the S phase in miR-421-overexpressing H1299 cells, whereas knockdown of miR-421 induced cell cycle arrest in A549 cells (Fig. 2C).

3.3. miR-421 promotes NSCLC cell migration and invasion in vitro

We then evaluated the effects of miR-421 on the migratory and invasive capacities of NSCLC cells through transwell assay. As shown in Fig. 3, overexpression of miR-421 enhanced the migration and invasion of H1299 cells, whereas opposite results were observed in A549 cells with miR-421 knockdown.

3.4. PDCD4 is a direct target of miR-421 in NSCLC cells

Targetscan (http://www.targetscan.org) was then used to predict the target genes of miR-421 [11], and we found that PDCD4 contains a miR-421 binding site in its 3′-UTR (Fig. 4A). Then dual-luciferase reporter assay was performed to validate whether PDCD4 is a bona fide target of miR-421 in NSCLC cells. As demonstrated in Fig. 4B, co-transfection of both A549 and H1299 cells with pLUC-PDCD4-WT vector and miR-421 mimics remarkably reduced the luciferase reporter activity, and this effect was abrogated by binding site mutation. Moreover, we observed that inhibition of miR-421 expression led to a significant upregulation of PDCD4 in A549 cells, while miR-421 mimics significantly reduced PDCD4 expression in H1299 cells (Fig. 4C). Moreover, as expected, an obvious reduction of PDCD4 mRNA expression was also observed in NSCLC tissues (Fig. 4D), and the expression of PDCD4 mRNA was inversely correlated with miR-421 expression level in NSCLC tissues ($P = 0.037$; Fig. 4E).

3.5. Knockdown of miR-421 suppresses NSCLC tumor growth in vivo

To confirm the data obtained from the in vitro studies, the effect of

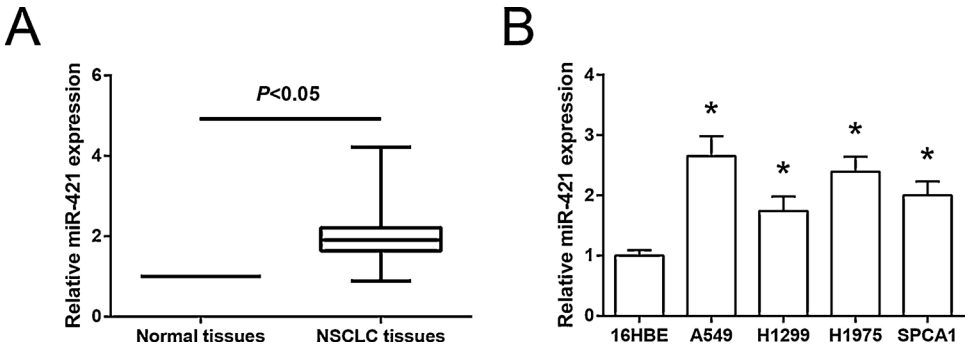

Fig. 1. miR-421 is upregulated in NSCLC. (A) RT-qPCR analysis of miR-421 expression levels in NSCLC tissues and matched adjacent normal tissues. (B) RT-qPCR analysis of miR-421 expression levels in four NSCLC cell lines (A549, H1299, H1975 and SPCA1) and a normal human bronchial epithelial cell line (16HBE). Data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ versus 16HBE cells.

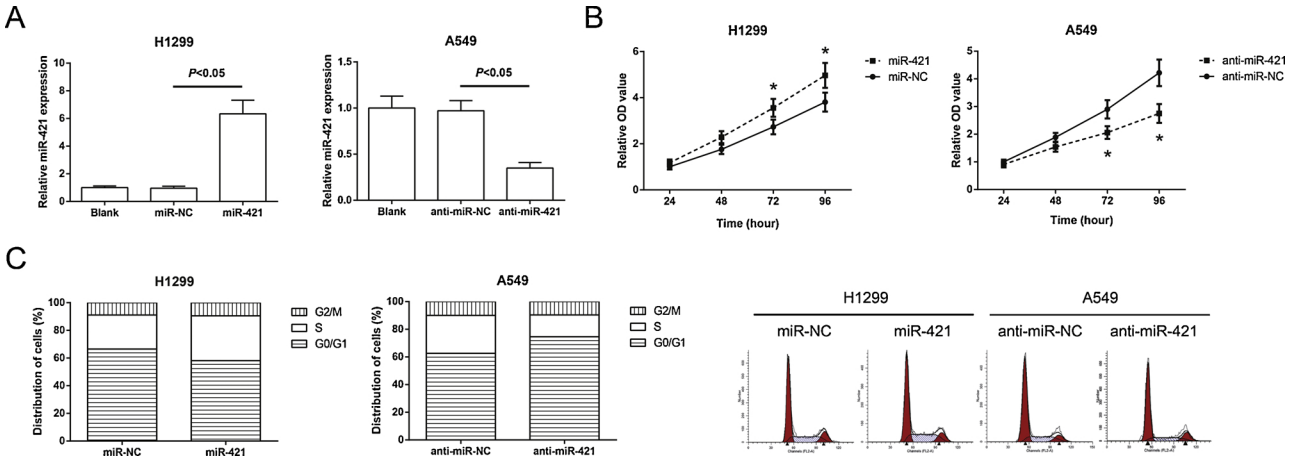

Fig. 2. miR-421 promotes NSCLC cell proliferation in vitro. (A) The expression levels of miR-421 in A549 and H1299 cells after transfection were detected by RT-qPCR analysis. (B) The proliferation of A549 and H1299 cells after transfection was detected by MTT assay. (C) The cell cycle distributions of A549 and H1299 cells after transfection were determined by flow cytometric analysis. Data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ versus miR-NC or anti-miR-NC-transfected cells.

miR-421 on NSCLC tumor growth was then assessed in vivo. We found that intratumoral injection of miR-421 antagomir significantly reduce the growth rate of A549 cells-derived tumor xenografts (Fig. 5A). On day 28 after cell inoculation, we found that the average weight of the tumors treated with miR-421 antagomir was significantly lower than that treated with antagomir NC (Fig. 5B). We further found that intratumoral injection of miR-421 antagomir indeed led to the down-regulation of miR-421 and upregulation of PDCD4 in the xenograft tissues (Fig. 5C–D).

3.6. PDCD4 abrogates the oncogenic role of miR-421 in NSCLC cells

We further investigated whether PDCD4 is involved in the oncogenic role of miR-421 in NSCLC. As indicated by MTT assay and transwell assay, co-transfection with pcDNA3.1-PDCD4 diminished the proliferation, migration and invasion of H1299 cells with miR-421 overexpression (Fig. 6A–B).

4. Discussion

Currently, the molecular mechanisms underlying the progression of NSCLC are still not fully understood. Over the past decades, more and more evidence has clearly depicted the important roles of miRNAs in NSCLC [12]. For example, miR-93-5p is upregulated in NSCLC and plays an oncogenic role [13], whereas exogenous overexpression of miR-615-3p inhibited the growth and metastasis of NSCLC cells [14]. Therefore, identification of NSCLC-related miRNAs and their direct target genes may provide new insights into the clinical treatment of this dangerous malignancy.

MiR-421 exerts regulatory functions in a number of human cancers.

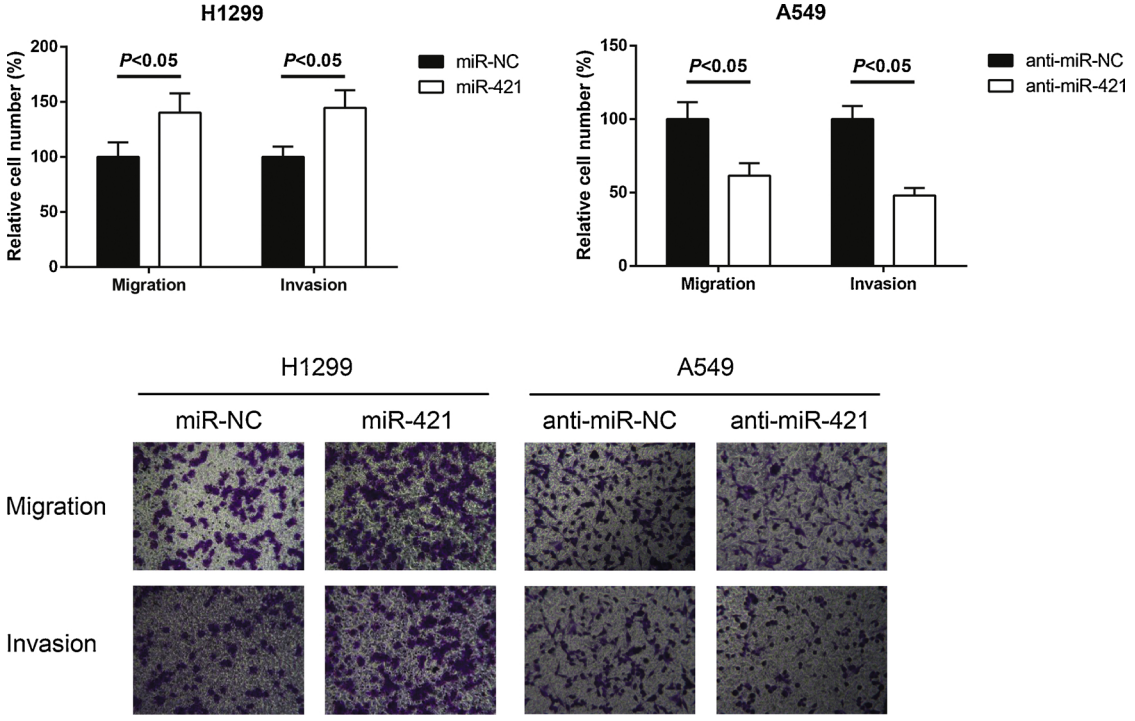

Fig. 3. miR-421 promotes NSCLC cell migration and invasion in vitro. The migration and invasion of A549 and H1299 cells after transfection were detected by transwell assay. Data are expressed as the mean ± SD from three independent experiments.

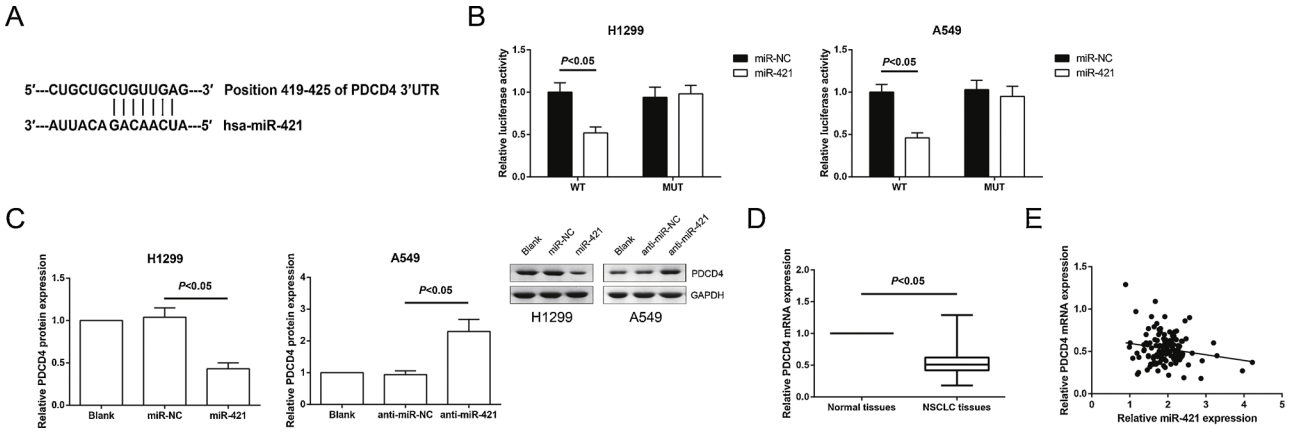

Fig. 4. PDCD4 is a direct target of miR-421 in NSCLC cells. (A) Predicted miR-421 target sequences in the 3′-UTR of PDCD4. (B) Dual-luciferase reporter assay was performed to confirm the direct binding efficiency of miR-421 and PDCD4 in A549 and H1299 cells. (C) The protein expression levels of PDCD4 in A549 and H1299 cells after transfection were detected by western blot analysis. Data are expressed as the mean ± SD from three independent experiments. (D) RT-qPCR analysis of PDCD4 mRNA expression levels in NSCLC tissues and matched adjacent normal tissues. (E) The correlation between miR-421 and PDCD4 mRNA expression in NSCLC tissues.

In the present study, the role of miR-421 in NSCLC progression was investigated. We first identified the high expression of miR-421 in both human NSCLC tissues and cell lines. We also found that high miR-421 expression was a valid factor associated with aggressive phenotypes of NSCLC patients. The gain-of-function and loss-of-function studies for miR-421 were then performed in two NSCLC cell lines, and the results demonstrated that overexpression of miR-421 promoted, whereas knockdown of miR-421 inhibited in vitro cell proliferation, migration and invasion. A reduction in cell proliferation is often induced by cell cycle arrest [15], and our results showed that miR-421 knockdown contributed to the cell cycle arrest of NSCLC cells in G0/G1 phase. Besides, inhibition of miR-421 also suppressed in vivo NSCLC tumor growth in a nude mice xenograft model. These data provided the potential involvement of miR-421 in NSCLC progression.

MiRNAs often regulate gene expression through binding to the 3'-UTRs of target mRNAs [16], and in this study, based on bioinformatics analysis, a complementary sequence for miR-421 was identified in the 3'-UTR of PDCD4. PDCD4, located on human chromosome 10q25.2, is a novel tumor suppressor involved in programmed cell death [17], and downregulated PDCD4 mRNA expression was found in NSCLC tissues [18]. Here, an inverse correlation between the expression of miR-421 and PDCD4 mRNA was also evidenced in our clinical NSCLC samples. More importantly, through rescue experiments, we further confirmed that the regulatory effects of miR-421 on NSCLC cell proliferation, migration and invasion may be partly due to its targeting of PDCD4.

In conclusion, this study showed that miR-421 is significantly

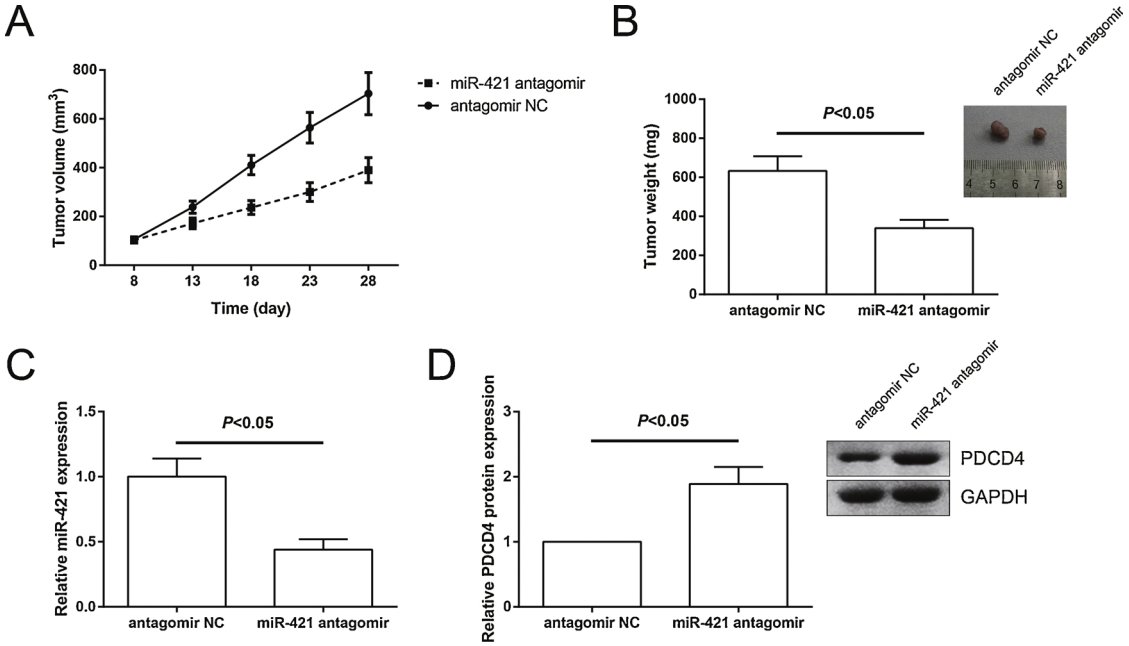

Fig. 5. Knockdown of miR-421 suppresses NSCLC tumor growth in vivo. (A) The volume of tumors was measured every five days to plot tumor growth curves. (B) The average weight of the tumors at the end of the experiment. (C) RT-qPCR analysis of miR-421 expression levels in the xenograft tissues. (D) Western blot analysis of PDCD4 protein expression levels in the xenograft tissues. Data are expressed as the mean ± SD (n = 6 per group).

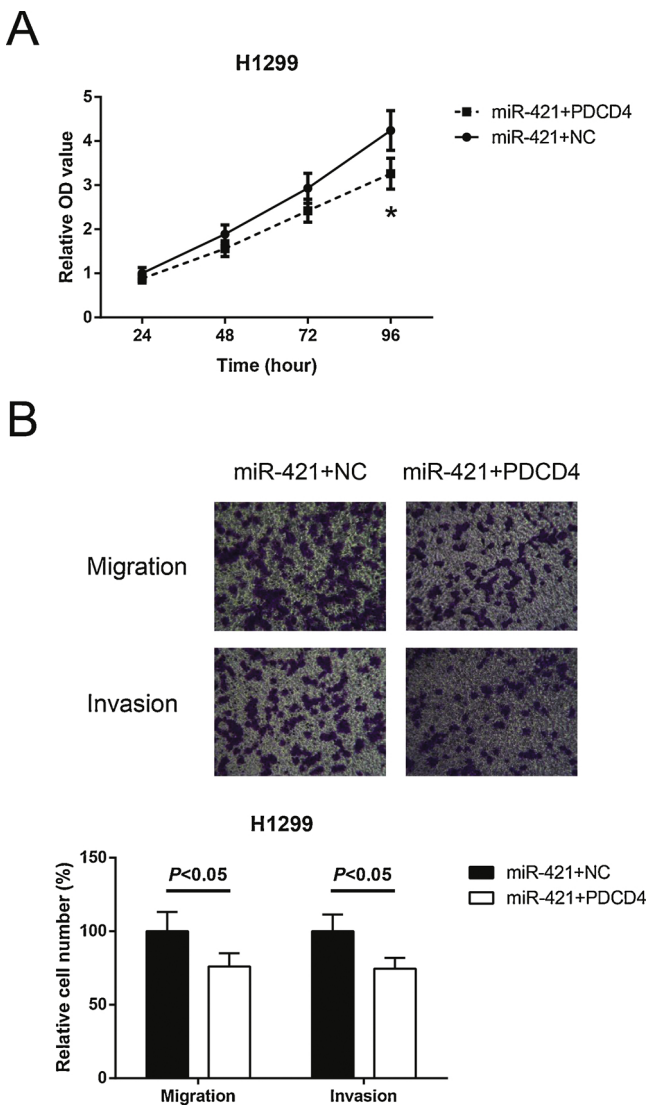

Fig. 6. PDCD4 abrogates the oncogenic role of miR-421 in NSCLC cells. (A) The proliferation of A549 and H1299 cells after transfection was detected by MTT assay. (B) The migration and invasion of A549 and H1299 cells after transfection were detected by transwell assay. Data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ versus empty vector-transfected cells.

upregulated in NSCLC, and its oncogenic role is largely achieved through one of its target, PDCD4. Although this study was limited by the number of NSCLC clinical samples and cell types, we still believed our findings will help to better understand the mechanisms of miR-421 in regulating NSCLC progression, and therefore provide a potential therapeutic target for the treatment of NSCLC.

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