lncRNA PDIA3P regulates cell proliferation and invasion in non-small cell lung cancer

XIAOGANG YANG and BAOJUN YANG

Department of Cardiac Surgery, Affiliated Hospital of Hebei University of Engineering, Handan, Hebei 056002, P.R. China

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Abstract. Increasing evidence has shown that long non-coding RNAs (lncRNAs) serve a critical role in tumor progression. The lncRNA protein disulfide isomerase family A member 3 pseudogene 1 (PDIA3P) is associated with the progression of oral squamous cell carcinoma and multiple myeloma. However, the roles of lncRNA PDIA3P in non-small cell lung cancer (NSCLC) remain unknown. In the present study, it was demonstrated that lncRNA PDIAP3 expression was significantly increased in patients with NSCLC, particularly in patients with an advanced Tumor-Node-Metastasis stages and lymph-node metastasis. The Gene Expression Profiling Interactive Analysis database revealed that patients with NSCLC with high lncRNA PDIA3P expression had a poorer disease-free survival rate. Furthermore, functional assays suggested that PDIA3P inhibition significantly suppressed the proliferation and invasion of NSCLC cells *in vitro* and reduced tumor growth *in vivo*. In addition, lncRNA PDIA3P enhanced the activity of the Wnt/β-catenin pathway in progression of NSCLC. The present study showed that lncRNA PDIA3P promoted NSCLC progression by regulating Wnt/β-catenin signaling, highlighting a potential therapeutic target for treating patients with NSCLC treatment.

Introduction

Lung cancer is the leading cause of cancer-associated death worldwide, of which non-small-cell lung cancer (NSCLC) accounts for >80% of lung cancer cases (1,2). Although there have been significant advances in treatment methods, including surgery combined with radiotherapy and/or chemotherapy, as a result of a high rate of local recurrence and metastasis, the 5-year survival rate remains poor (3,4). Therefore, a better understanding of the underlying molecular mechanisms involved in NSCLC progression is required.

Correspondence to: Dr Baojun Yang, Department of Cardiac Surgery, Affiliated Hospital of Hebei University of Engineering, 81 Congtai Road, Handan, Hebei 056002, P.R. China
E-mail: ybj_army@163.com

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Long non-coding RNAs (lncRNAs) are a type of non-coding RNA >200 nucleotides in length, with limited or no coding potential (5,6). There are an increasing number of studies which have demonstrated that lncRNAs are involved in multiple cellular processes, including in cancer (7,8). For example, Zhang *et al* (9) demonstrated that that upregulation of lncRNA metastasis associated lung adenocarcinoma transcript 1 was associated with tumor progression and poor prognosis in clear cell renal cell carcinoma. Gao *et al* (10) demonstrated that ZNFX1 antisense RNA 1 exhibited an oncogenic role in glioma progression by regulating epithelial-mesenchymal transition (EMT) and the Notch signaling pathway. Chen *et al* (11) showed that lncRNA colon cancer associated transcript 1 promotes the progression of multiple myeloma by acting as a molecular sponge of microRNA (miR)-181a-5p, thus modulating the expression of homeobox A1.

Protein disulfide isomerase family A member 3 pseudogene 1 (PDIA3P1) is a 2,099-nucleotide lncRNA that is mapped to human chromosome 1q21.1. Sun *et al* (12) reported that lncRNA PDIA3P was upregulated and interacted with miR-185-5p to promote the proliferation of oral squamous cell carcinoma cells by targeting cyclin D2. Yang *et al* (13) showed that lncRNA PDIA3P interacted with c-myc to regulate cell proliferation by activating the pentose phosphate pathway in multiple myeloma. The aim of the present study was to reveal the functions of lncRNA PDIA3P in the progression of NSCLC.

Materials and methods

Tumor specimens. A total of 73 pairs of NSCLC tissues and the adjacent normal tissues were obtained from patients who received surgery at The Affiliated Hospital of Hebei University of Engineering (Handan, China) between January 2013 and December 2016. All of the specimens were immediately frozen in liquid nitrogen and stored at -80˚C until they were used for RNA extraction. None of the patients received previous local or systemic treatment prior to the operation. Clinical information was obtained from the medical records of the patients. All the patients in the present study provided written informed consent. The present study was approved by The Ethics Committee of Hebei University of Engineering (approval no. HBU-2018-01127).

Cell culture and transfection. The human NSCLC cell lines A549, H1299, H1703, H520 and SK-MES-1, and the normal human bronchial epithelium cell line BEAS-2B were obtained

from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences) at 37˚C with 5% CO_2. The concentrations of siRNA and plasmids were 50 μM and 2 μg/ml, respectively. Small interfering (si)-PDIA3P and the negative control (NC) were purchased from Shanghai GenePharma Co., Ltd. The pcDNA3.1-PDIA3P plasmid was prepared in our previous study using RNA interference sequences (12) and an empty pcDNA3.1 vector (Shanghai GenePharma Co., Ltd.) was used as a negative control. The Wnt pathway inhibitor IWR-1-endo was obtained from Cayman Chemical Company. Aliquots of 2 mM in DMSO were stored at -20˚C and working concentrations (5 μM) were prepared prior to use. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection, according to the manufacturer's protocol (14). The sequences of si-PDIA3P were as follows: si-RNA-1, 5'-AACCACTGGGGAGGACTAGG-3'; si-RNA-2, 5'-TGG TAGCAGAGAATTTGAT-3'; and si-NC, 5'-AATTCTCCG AACGTGTCACGT-3'. Further experiments were completed 24 h following transfection.

Reverse transcription-quantitative (RT-q)PCR. Total RNAs were extracted from NSCLC tissues and cell lines using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR analysis was performed using a SYBR Green qPCR Master Mix kit (Promega Corporation), according to the manufacturer's instructions. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min followed by 40 cycles at 95˚C for 10 sec and 60˚C for 1 min. Relative expression was normalized to GAPDH and calculated using the $2^{-\Delta\Delta Cq}$ method (15). The sequences of the PCR primers were as follows: PDIA3P forward, 5'-AACCACTGG GGAGGACTAGG-3' and reverse, 5'-CAGTGCAGCTAA GAAATGGCT-3'; and GAPDH forward, 5'-ATGGGGAAG GTGAAGGTCG-3' and reverse, 5'-GGGTCATTGATGGCA ACAATATC-3'.

Cell proliferation assay. A Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) assay was performed to determine the proliferative rate of lung cancer cells. Briefly, cells were seeded into 96-well plates and cultured for the indicated times (24, 48 and 72 h). Subsequently, 10 μl CCK-8 solution was added and incubated for another 2 h at 37˚C. The absorbance at 450 nm was determined using a microplate reader (Bio-Rad Laboratories, Inc.).

Colony formation assay. A total of 2 ml complete medium containing $2x10^3$ transfected cells were added to 6-well plates and incubated for 2 weeks. Subsequently, the supernatant was discarded and the cells were washed with PBS, fixed using 500 μl methanol at room temperature for 20 min and stained with 0.1% crystal violet (Nanjing KeyGen Biotech Co., Ltd.) for 20 min at room temperature. The number of colonies >10 cells were counted using an optical light microscope at x50 magnification (Olympus Corporation).

Cell invasion assay. The invasive capability of the cells was examined using a Transwell chamber assay with an 8-μM pore (EMD Millipore). Briefly, $1x10^4$ cells were seeded in the upper chamber coated with Matrigel (Sigma-Aldrich; Merck KGaA) and incubated for 48 h. Cells which had not invaded were removed using a swab and the cells which had invaded through to the lower surface were fixed with methanol for 35 min at room temperature, stained with crystal violet for 50 min at room temperature, washed with PBS and counted under a light microscope (magnification, x50; Nikon Corporation).

Animal experiments. The transfected A549 cells were subcutaneously injected into 4 week-old female BALB/c nude mice (Beijing Experimental Animal Research Center, Beijing, China). Each group included 4 mice, and they were housed under specific pathogen free conditions at 20-26˚C, 40-70% humidity and a 12/12 h light/dark cycle. The mice had free access to food and water. At the end of the 7-week observation period, the mice were sacrificed by cervical dislocation, and the tumor tissues were removed for subsequent experiments (16). All experimental procedures were approved by the committee on animal experimentation of Hebei University of Engineering.

Western blotting. Proteins were lysed using RIPA buffer (Beyotime Institute of Biotechnology) and the concentrations were measured using a bicinchoninic acid protein assay (Beyotime Institute of Biotechnology). Proteins (20 μg/per lane) were separated using a 10% SDS-PAGE gel and transferred to a PVDF membrane (EMD Millipore). After incubation with antibodies against β-catenin (1:1,000; cat. no. ab32572), c-myc (1:1,000; cat. no. ab32072), glycogen synthase kinase (GSK)-3β (1:1,000; cat. no. ab32391) and GAPDH (1:5,000; cat. no. ab181602) (all from Abcam) overnight at 4˚C, the membranes were incubated with a goat anti-rabbit horse radish peroxidase-conjugated secondary antibody (1:5,000; cat. no. ab97051; Abcam) at room temperature for 1 h. The signals were visualized using an enhanced chemiluminescence reagent (EMD Millipore). Protein bands were visualized using ImageJ 1.48 software (National Institutes of Health).

Clinical databases. The online database Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancerpku.cn/index.html.) was used to analyze the RNA sequencing expression data relevant to the present study based on The Cancer Genome Atlas and the Genotype-Tissue Expression databases (17). GEPIA performs survival analyses based on gene expression levels and uses a log-rank test for hypothesis evaluation.

Immunohistochemistry. The expression of Ki-67 in nude mice injected with A549 cells was measured using immunohistochemistry. The immunohistochemistry assay was conducted according to previous report (18). The sections were treated with rabbit polyclonal anti-Ki-67 antibody (1:100; cat. no. ab833; Abcam) at 4˚C overnight. After successfully completing the previous steps, the sections were incubated with a secondary goat anti-rabbit antibody (1:1,000; cat. no. ab6721; Abcam) for 20 min at 37˚C. Image acquisition was performed by light microscope (magnification, x100; Nikon Corporation) and Image-Pro Plus version 6.0 (Media Cybernetics, Inc.) was used to analyze the integrated optical density values of the brown area.

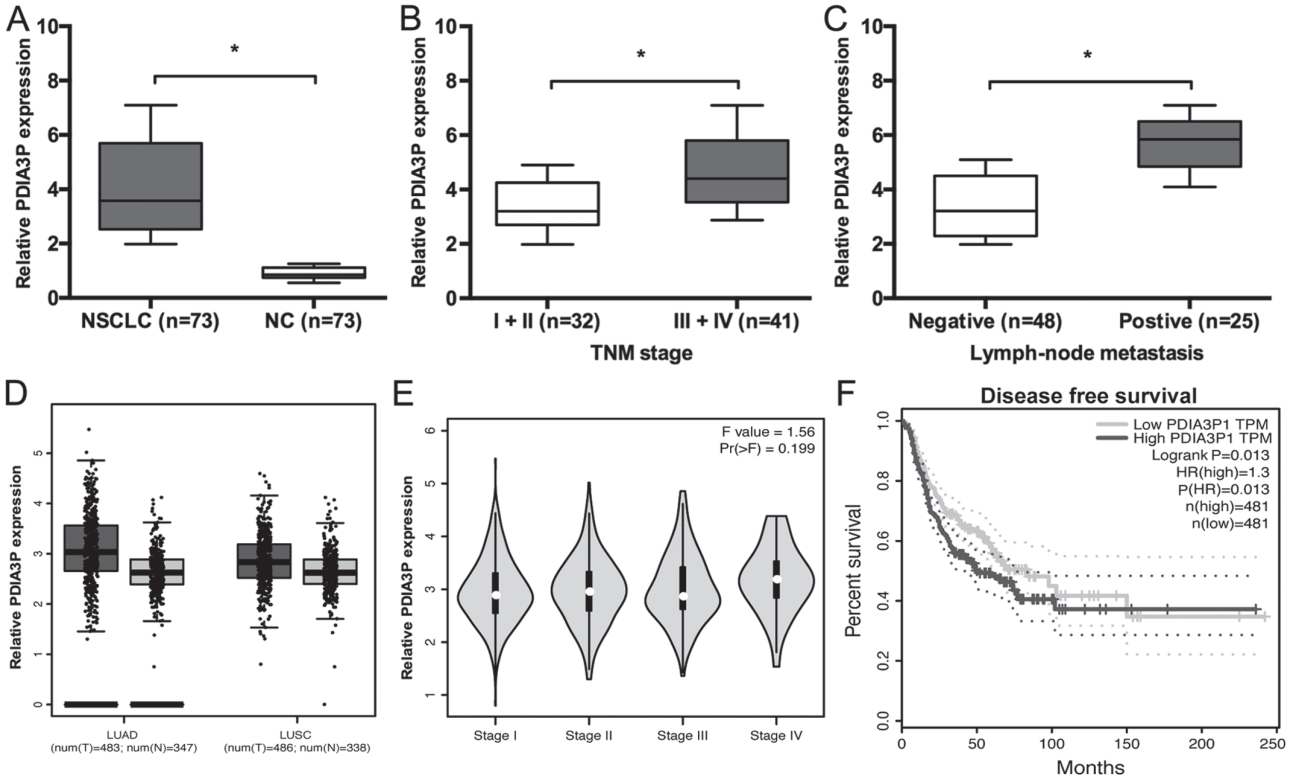

Figure 1. Expression of lncRNA PDIA3P is upregulated in NSCLC. (A) PDIA3P expression in 73 paired NSCLC tissues was analyzed by reverse transcription-quantitative PCR. *P<0.05. (B) PDIA3P expression was associated with advanced TNM stage in patients with NSCLC. *P<0.05. (C) PDIA3P expression was associated with advanced lymph node metastasis in patients with NSCLC. *P<0.05. (D) PDIA3P expression in NSCLC tissues and normal tissues was analyzed using GEPIA. (E) The GEPIA database indicated that high PDIA3P expression was associated with tumor stage. (F) The GEPIA database showed that high PDIA3P expression was associated with poor disease-free survival of patients with NSCLC. P=0.013. lncRNA, long noncoding RNA; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NSCLC, non-small cell lung cancer; NC, negative control; HR, hazard ratio; TNM, Tumor-Node-Metastasis; TPM, transcripts per million; GEPIA, Gene Expression Profiling Interactive Analysis; PDIA3P, protein disulfide isomerase family A member 3 pseudogene 1.

Statistical analysis. All data were analyzed using SPSS 18.0 software (SPSS, Inc.) and are expressed as the mean ± SD from at least three independent experiments. The differences between the two groups were evaluated using a Student's t-test or one-way ANOVA followed by a Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

lncRNA PDIA3P is upregulated in NSCLC. In the present study, PDIA3P expression in NSCLC tissues was examined. RT-qPCR analysis showed that PDIA3P expression was significantly higher in NSCLC tissues (Fig. 1A; P<0.05) compared with the control. High PDIA3P expression levels were inversely associated with TNM stages III and IV, and the presence of lymph node metastasis (Fig. 1B and C; Table I; P<0.05). Furthermore, data from the GEPIA database showed that PDIA3P expression was increased in NSCLC (lung adenocarcinoma and lung squamous cell carcinoma) tissues compared with normal tissues (Fig. 1D; P<0.05). In addition, high PDIA3P expression was associated with advanced tumor stage and poor disease-free survival of patients with NSCLC (Fig. 1E and F; P<0.05).

lncRNA PDIA3P-silencing suppresses the proliferation and invasion of NSCLC cells. To determine the biological functions of PDIA3P in NSCLC progression, the expression levels of PDIA3P were first determined in NSCLC cell lines (A549, H1299, H1703, H520 and SK-MES-1) and the normal human bronchial epithelium cell line BEAS-2B, and A549 and H520 cell lines were selected for further study as they exhibited high PDIA3P expression. RT-qPCR analysis showed that PDIA3P expression was significantly increased in all the NSCLC cell lines compared with BEAS-2B cells (Fig. 2A; P<0.05). Therefore, PDIA3P was silenced in both A549 and H520 cells by si-PDIA3P (Fig. 2B; P<0.05). A CCK-8 assay showed that PDIA3P knockdown decreased the proliferative rate of A549 and H520 cells compared with the negative control (Fig. 2C; P<0.05), and the cell colony formation was significantly decreased following PDIA3P silencing in A549 and H520 cells compared with the negative control (Fig. 2D; P<0.05). Furthermore, the migratory and invasive ability of A549 and H520 cells was reduced following silencing of PDIA3P compared with the control (Fig. 2E and F; P<0.05).

lncRNA PDIA3P silencing reduces tumor growth in vivo. The effects of PDIA3P on NSCLC growth *in vivo* were determined. The results showed that PDIA3P inhibition significantly reduced the tumor volume compared with the negative control (Fig. 3A; P<0.05). At 7 weeks after the injection, the mice were sacrificed, and tumor weight was determined. The results showed that PDIA3P silencing reduced the tumor

Table I. Associations between PDIA3P expression and clinical features of patients with NSCLC.

Characteristics	PDIA3P expression		P-value
	High, n=37	Low, n=36	
Age, years			0.415
>60	21	17	
≤60	16	19	
Sex			0.736
Male	22	20	
Female	15	16	
TNM stage			0.003[a]
I/II	10	22	
III/IV	27	14	
Tumor size, cm			0.295
>3	23	18	
≤3	14	18	
Lymph node metastasis			0.009[a]
Negative	19	29	
Positive	18	7	

[a]P<0.01. TNM, Tumor-Node-Metastasis.

weight (Fig. 3B; P<0.05). Furthermore, immunohistochemical staining showed that the Ki67 expression was decreased in PDIA3P-silenced xenograft tumor tissues (Fig. 3C; P<0.05).

lncRNA PDIA3P activates the Wnt/β-catenin signaling pathway. To determine the underlying mechanism by which PDIA3P affects NSCLC progression, RT-qPCR and western blotting were performed to examine the effects of PDIA3P on the Wnt/β-catenin pathway, which is frequently aberrantly activated in human cancer (19). The results showed that PDIA3P inhibition decreased both the mRNA and protein expression levels of β-catenin and c-myc, and increased the expression levels of GSK-3β in A549 cells (Fig. 4A and B; P<0.05). A549 cell line was used as it exhibited the highest expression. PDIA3P overexpression upregulated β-catenin and c-myc expression, and decreased GSK-3β expression in A549 cells, both at the mRNA and protein level (Fig. 4C-E; P<0.05). Furthermore, the Wnt pathway inhibitor IWR-1-endo partly reversed the effects of PDIA3P expression on proliferation (Fig. 4F; P<0.05) and invasion (Fig. 4G; P<0.05) in A549 cells. Therefore, PDIA3P may promote the progression of NSCLC, at least partly by regulating the Wnt/β-catenin pathway.

Discussion

There has been an increase in the number of studies demonstrating the potential roles of various lncRNAs as key regulators of cancer progression over the past decade (20-22), and genetic and epigenetic alterations of lncRNAs may serve key roles in tumorigenesis (23). Furthermore, abnormal expression of lncRNAs may serve important roles in cancer progression, including in NSCLC. For example, Xie *et al* (24) showed that the lncRNA gastric cancer associated transcript 2 is downregulated and associated with poor prognosis in patients with NSCLC. Cui *et al* (25) found that upregulation of the lncRNA small nucleolar RNA host gene 1 contributes to NSCLC progression via inhibition of miR-101-3p and activation of the Wnt/β-catenin signaling pathway. Gao *et al* (26) found that lncRNA FLVCR1 divergent transcript contributes to proliferation and invasion by sponging miR-573 to upregulate E2F transcription factor 3 expression in lung cancer.

In the present study, the function and underlying mechanisms of PDIA3P expression in NSCLC progression were determined. The results showed that PDIA3P expression was significantly increased in NSCLC tissues compared with adjacent non-tumor tissues. High PDIA3P expression was associated with advanced TNM stage cancer, lymph-node metastasis of cancer and poor disease-free survival of patients with NSCLC. PDIA3P functions in lung cancer were examined both *in vitro* and *in vivo*. The results showed that PDIA3P knockdown significantly inhibited the growth of NSCLC cells both *in vitro* and *in vivo*. Similarly, wound healing assays and Transwell assays showed that PDIA3P inhibition decreased the migration and invasion of lung cancer cells *in vitro*. In a further study the effects of PDIA3P on proliferation markers, such as proliferating cell nuclear antigen, and migration markers, such as matrix metalloproteinases, will be determined. Taken together, the results of the present study showed that PDIA3P may act as an oncogenic lncRNA in the progression of NSCLC.

The Wnt/β-catenin pathway is frequently activated in a wide range of different types of cancer, and is known to promote tumor invasion and metastasis through upregulation of factors regulating EMT (27,28). Recent studies demonstrated that certain lncRNAs affect the Wnt/β-catenin signaling pathway and thus regulate cancer progression. For example, Ma *et al* (29) showed that the lncRNA CCAL regulates the progression of colorectal cancer by activating the Wnt/β-catenin pathway through suppression of activator protein 2α. Zhao *et al* (30) demonstrated that upregulation of the lncRNA HNF1A antisense RNA 1 promotes cell proliferation and metastasis in osteosarcoma through activation of the Wnt/β-catenin pathway. However, the association between PDIA3P expression and the Wnt/β-catenin pathway in NSCLC remains unclear. In the present study, it was demonstrated that PDIA3P inhibition significantly reduced β-catenin and c-myc expression, and increased GSK-3β expression in NSCLC cells, while ectopic PDIA3P expression resulted in the opposite effects. In addition, *in vitro* functional assays showed that IWR-1 endo (Wnt pathway inhibitor) (31) attenuated the effects of PDIA3P on the proliferation and invasive ability of NSCLC cells, suggesting that PDIA3P promotes NSCLC progression at least partly through the Wnt/β-catenin pathway.

There are certain limitations to the present study. The effects of IWR-1 alone on b-catenin, or on cell viability and invasion ability, were not assessed.

In conclusion, PDIA3P is significantly increased in NSCLC, and promotes the proliferation and invasion of NSCLC by regulating the Wnt/β-catenin pathway. Future experiments should examine the effects of PDIA3P on EMT. The findings of the present study indicated that PDIA3P may serve as a potential therapeutic target for the treatment of patients with NSCLC.

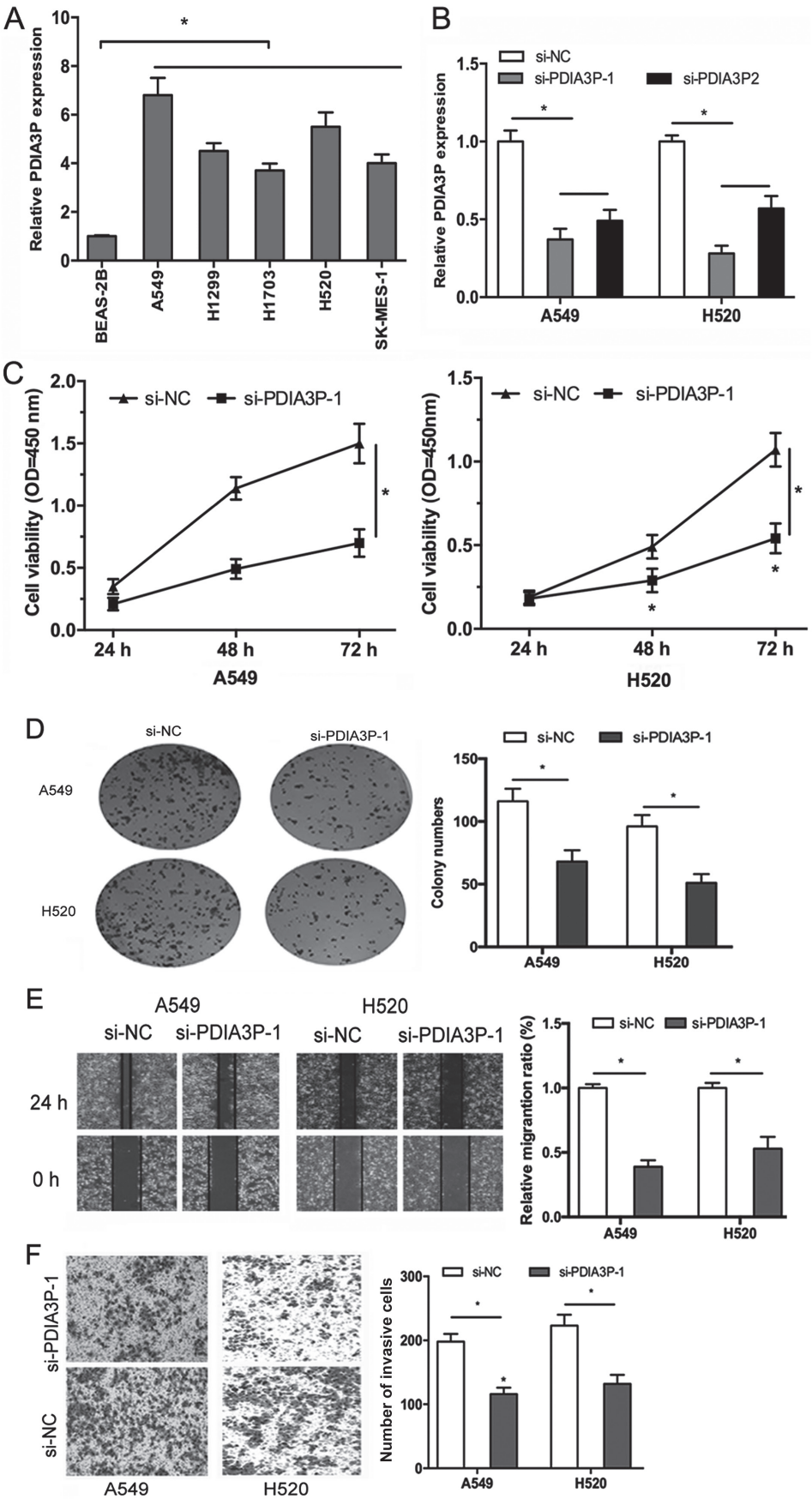

Figure 2. lncRNA PDIA3P inhibition suppresses the proliferation and invasion of NSCLC cells. (A) PDIA3P expression in NSCLC cells was detected by RT-qPCR. *P<0.05. (B) PDIA3P expression in NSCLC cells after treatment with si-PDIA3P using RT-qPCR. *P<0.05. (C) A Cell Counting Kit-8 and (D) colony formation assay were used to determine the viability of the NSCLC cells. *P<0.05. magnification, x50. (E) Wound healing and (F) Transwell invasion assays were performed to assess the migration and invasion of NSCLC cells. *P<0.05. magnification, x50. NSCLC, non-small cell lung cancer; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; OD, optical density; NC, negative control; PDIA3P, protein disulfide isomerase family A member 3 pseudogene 1.

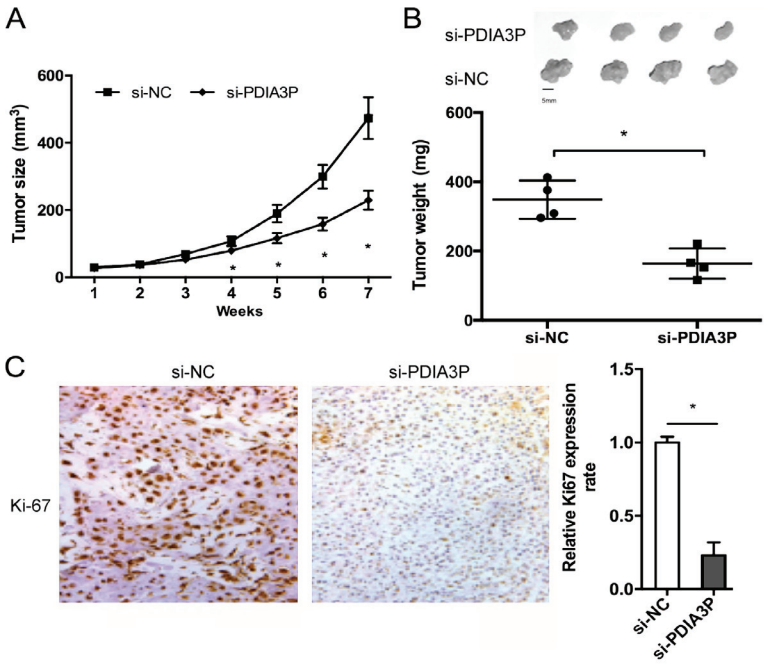

Figure 3. Long noncoding RNA PDIA3P downregulation reduces tumor growth *in vivo*. (A) Tumor volumes were analyzed every week for 7 weeks, and the tumor volume was significantly smaller in the si-PDIA3P group compared with the control from the 4th week onwards. *P<0.05 vs. respective si-NC group. (B) Tumor weight was measured 7 weeks after injection and was significantly lighter in the si-PDIA3P group compared with the control. *P<0.05. (C) Immunohistochemistry showed that Ki67 expression was reduced after PDIA3P inhibition. Magnification, x100. *P<0.05. si, small interfering; NC, negative control; PDIA3P, protein disulfide isomerase family A member 3 pseudogene 1.

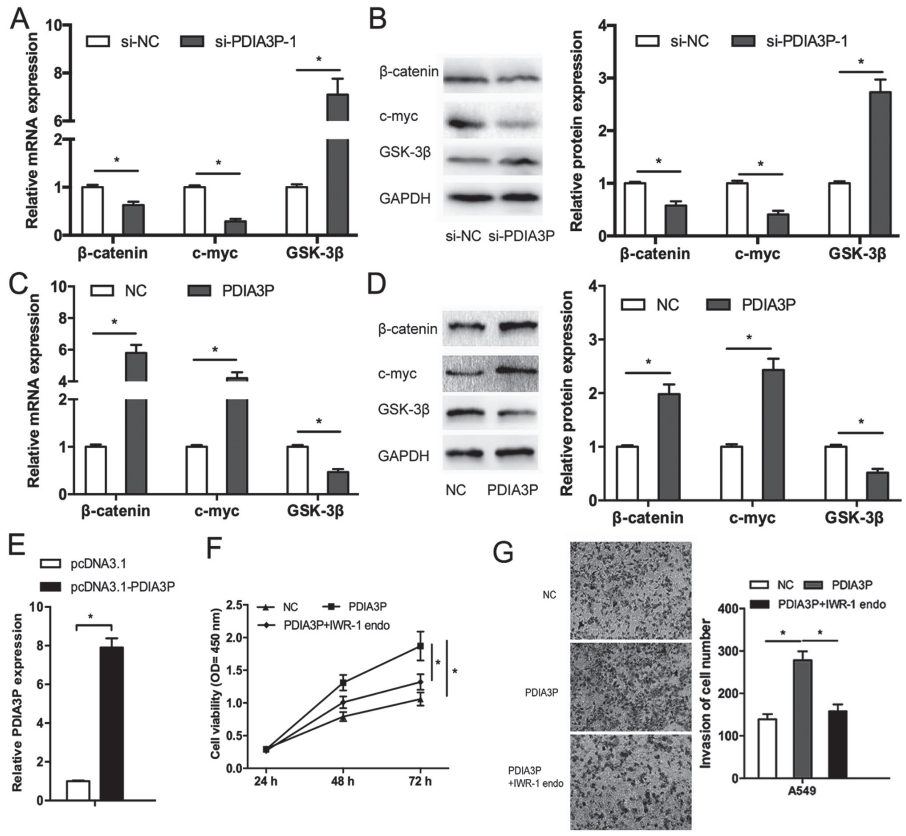

Figure 4. Long noncoding RNA PDIA3P promotes the Wnt/β-catenin pathway in NSCLC cells. (A) mRNA expression levels of β-catenin, c-myc and GSK-3β in A549 cells transfected with si-PDIA3P. *P<0.05. (B) Western blot analysis of the expression levels of β-catenin, c-myc and GSK-3β in NSCLC cells transfected with si-PDIA3P. *P<0.05. (C) mRNA expression levels of β-catenin, c-myc and GSK-3β in A549 cells transfected with PDIA3P. *P<0.05. (D) Western blot analysis of the expression levels of β-catenin, c-myc and GSK-3β in NSCLC cells transfected with PDIA3P. *P<0.05. (E) PDIA3P expression in NSCLC cells transfected with plasmid pcDNA3.1-PDIA3P. The Wnt pathway inhibitor IWR-1-endo partly rescued the effects of PDIA3P overexpression on the (F) proliferation and (G) invasion ability of NSCLC cells. Magnification, x50. *P<0.05. NSCLC, non-small cell lung cancer; si, small interfering; NC, negative control; OD, optical density; PDIA3P, protein disulfide isomerase family A member 3 pseudogene 1; GSK-3β, glycogen synthase kinase-3β.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

BY designed the current study. XY and BY performed the experiments and analyzed the data. BY drafted the manuscript. XY and BY wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All the patients in the present study provided written informed consent. The present study was approved by The Ethics Committee of Hebei University of Engineering (approval no. HBU-2018-01127). All experimental procedures involving animals were approved by the committee on animal experimentation of Hebei University of Engineering.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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