Original Article
Blocking platelet-derived growth factor-d inhibits human lung cancer progression

Feng Huang[1], Yongliang Yao[1], Jianhong Wu[1], Yanqing Zhang[1], Liya Yu[1], Xiongyong Pu[1], Longfei Xia[2]

[1]Department of Clinical Laboratory, The First People's Hospital of Kunshan Affiliated with Jiangsu University, Suzhou, Jiangsu Province, China; [2]School of Medicine, Jiangsu University, Zhenjiang, Jiangsu Province, China

Abstract: Platelet-derived growth factor-D (PDGF-D) is a kind of growth chemokine that regulates tumor growth and metastasis in various cancers. However, the function of this gene in lung cancer is little known. Here we found that PDGF-D is overexpressed in lung cancer tissue compared to adjacent tissue. Interestingly, the serum levels of PDGF-D in lung cancer patients were significantly decreased after operation. Knockdown of PDGF-D using siRNA in lung cancer cells inhibits cell proliferation *in vitro* and *in vivo* and decreased the activation of p-AKT signaling pathway. Moreover, silencing of PDGF-D in A549 cells remarkably reduced cell migration and invasion. Furthermore, PDGF-D knockdown significantly increased E-cadherin expression but decreased the expression of N-cadherin, MMP-2 and MMP-9. Taken together, our findings indicate that PDGF-D is involved in the progression of lung cancer growth, migration and invasion. Targeting PDGF-D with siRNA may provide a novel strategy for lung cancer therapy in patients with over-expressed PDGF-D.

Keywords: Lung cancer, PDGF-D, siRNA, cancer growth, migration, invasion

Introduction

Lung cancer, especially non-small-cell lung cancer (NSCLC), is one of the most malignant tumors with the highest mortality rate in the world. The prevalence of lung cancer is increasing rapidly, with more than 1.4 million deaths in the world every year [1, 2]. However, the prognosis of lung cancer is poor. It is estimated that the combined 5-year overall survival (OS) rate for all stages and subtypes of NSCLC remains as low as 15% [3]. Although various treatment regimens have been attempted in lung cancer patients, tumor metastasis is still the most difficult problem. Moreover, the molecular mechanisms of lung cancer metastasis are not well explored [4].

The platelet-derived growth factor (PDGF) family members are dimeric glycoproteins composed of two A, two B, or heterodimer AB. The known ligands include PDGF-A, PDGF-B, PDGF-C, PDGF-D, PDGF-AB, and their receptors PDGFR-α and PDGFR- [5] PDGF as an angiogenic pathway, can promote tumor cell growth, invasion and metastasis through various intra-cellular signaling pathways [6-9] PDGF-D, a newly identified isoform of PDGFs, specifically binds to and activates its cognate receptor PDGFR-β. Recent study found that PDGF-D was related to the enhancement of tumor heterogeneity, invasion and the curative effect of radiotherapy [10-12]. PDGF-D plays important roles in both autocrine stimulation of tumor cells and in paracrine signaling between tumor cells and surrounding stromal cells [13, 14]. Thus, targeting PDGF-D pathway may be of significance in the treatment of human lung cancer.

However, the expression and function of PDGF-D human lung cancer have been rarely studied. Therefore, this study aimed to investigate the functions of PDGF-D in lung cancer.

Materials and methods

Cell line culture

Lung cancer cell line A549 was obtained from Cell Bank of Shanghai Institute of Biological Sciences, and maintained in Dulbecco's modified Eagle's medium with low glucose (LG-DM-

EM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2. The cells were passaged every 2-3 days to maintain exponential growth.

siRNA transfection

Chemically synthesized PDGF-D siRNAs and scramble control siRNAs were purchased from Shanghai GenePharma (Shanghai, China). The siRNAs were transiently transfected into A549 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. A549 cells were plated in 6-well plates at a density of 1×10^5 cells/well.

RNA extraction and real-time RT-PCR

Total RNA was extracted from cells and tumor tissues using TRIZOL Reagent (Invitrogen). Equal amount of RNA was used for reverse transcription. The primers used in this study were synthesized by Invitrogen (Shanghai, China). Quantitative PCR analysis was performed to the expression of PDGF-D gene (Rotor-Gene 6000 Real-Time PCR Machine; Corbett Life Science, Sydney, Australia). An endogenous 'housekeeping' gene (β-actin) was used to normalize the results. The primers used in this study were as follows: PDGF-D forward, 5'-CTCAGGCGAGATGACTTGTA-3' and reverse 5'-CCACACCATCGTCCTCTAAT-3'.

Western blot analysis

The cells and tissues were collected and lysed in RIPA buffer on ice. Equal amount of protein was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto methanol pre-activated polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked by 5% w/v non-fat dry milk. Following sequential incubation with the primary and secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), the signal was visualized using HRP substrate (Millipore) and analyzed using MD ImageQuant Software. GAPDH (Kangcheng, China) was used as the loading control. Primary antibodies were as follows: anti-N-cadherin, anti-E-cadherin, anti-PDGF-D (Santa Cruz Biotechnology); AKT, p-AKT (phosphorylated AKT), anti-Bcl2, and anti-Bax (Bioworld Technology, Louis Park, MN,

USA); MMP-2, MMP-9 (Signalway Antibody Co., Ltd.).

Enzyme-linked immunosorbent assay

The serum level of PDGF-D in lung cancer patients before and after operation was measured using ELISA kit (R&D Systems, USA) following the manufacturer's instructions. Assays were performed in duplicate, and readings were compared with the standard curves from the standard protein provided with the kits.

Immunohistochemistry

Formalin-fixed paraffin-embedded lung cancer specimens containing adjacent tissues were deparaffinized in xylene, rehydrated through graded ethanol. The sections were boiled for 10 min in citrate buffer (pH 6.0, 10 mM) for antigen retrieval. The endogenous peroxidase activity was quenched with exposure to 3% hydrogen peroxide for 10 min. Then the sections were blocked with 5% BSA and incubated with appropriately diluted PDGF-D primary antibody (1:200) at 37°C for 1 h. After washes with PBS, the sections were then incubated with diluted secondary antibody for 20 min. Finally, sections were visualized with 3, 3'-diaminobenzidine (DAB) and then counterstained with hematoxylin for examination under a light microscope (Olympus, Tokyo, Japan).

Transwell migration assay

A549 cells treated with PDGF-D siRNA and scramble siRNA were seeded into the upper chamber (8 mm) (Corning, NY, USA) in serum-free medium. The complete medium was placed into the lower chamber. Following incubation at 37°C in 5% CO_2 for 12 h, the cells remaining on the top the polycarbonate membrane were wiped off with cotton swabs. The cells migrating to the low membrane were then fixed with methanol for 30 min. The migrated cells were stained with crystal violet and counted in six random fields under the microscope (Olympus) for each assay.

Cell colony formation assay

A549 cells treated with PDGF-D siRNA and negative scramble siRNA were trypsinized and resuspended to a concentration of 2,000 cells/3 ml HG-DMEM with 10% FBS and then
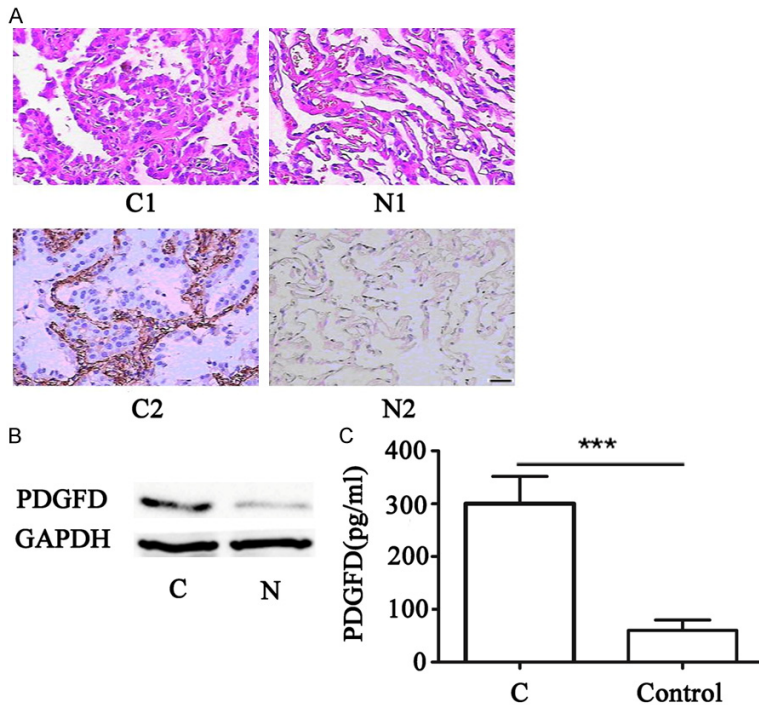
Figure 1. Overexpression of PDGF-D in human lung cancer patients. A. H&E staining of the lung cancer tissue and adjacent tissue, showing that cancer tissue was orderly and tightly organized (C1) whereas cancer adjacent tissue was obviously loose (N1) (Magnification, × 100; *scale bar* = 50 μm). PDGF-D is highly expressed human lung tissue samples (C2) compared to cancer adjacent tissue (N2) (Magnification, × 100; *scale bar* = 50 μm). B. Western blot analysis revealed that PDGF-D is overexpressed in cancer tissues (C) versus adjacent tissues (N). C. The level of PDGF-D in the serum of lung cancer patients before and after operation (***P < 0.001).

the modified ellipsoidal formula: (length × width^2)/2. Tumors were surgically removed, and photographed at 6 weeks after injection.

Statistical analyses

All experiments were conducted at least in triplicate. All data are expressed as the means ± SD. SPSS 11.0 software was used to analyze the data. The means in different treatment groups were compared by the Student's t-test. A *P*-value < 0.05 was considered statistically significant.

Results

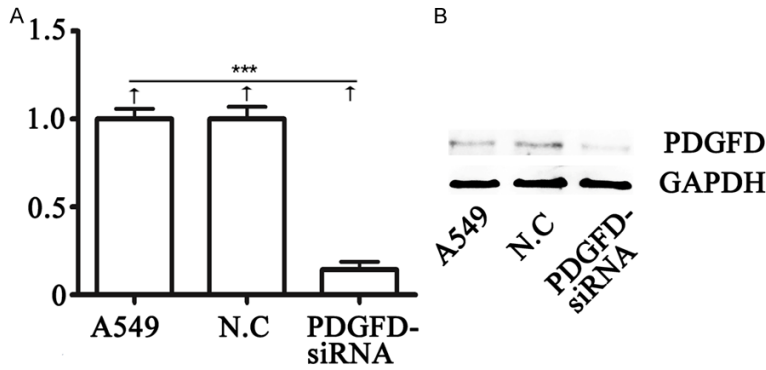*PDGF-D is highly expressed in tumor tissue and serum of lung cancer patients*

PDGF-D expression levels were examined in 86 primary lung cancer specimens and adjacent tissue. Immunohistochemical staining showed that PDGF-D was strongly expressed in the tumor tissues but weakly expressed in the tumor adjacent tissue (**Figure 1A**). Moreover, similar results were observed by Western blot analysis (**Figure 1B**). Furthermore, we detected the level of PDGF-D in the serum of 80 lung cancer patients before and after operation. Interestingly, our results showed that the serum levels of PDGF-D after operation were significantly lower than those before operation (**Figure 1C**).

PDGF-D siRNA inhibits the proliferation of lung cancer cells in vitro and in vivo

We further examined the gene and protein expression of PDGF-D in human lung cancer A549 cell line. QRT-PCR and Western blot analyses showed that PDGF-D was highly expressed in A549 cells at both mRNA and protein levels. (**Figure 2A** and **2B**) To further test the function of PDGF-D in A549 cells, PDGF-D expression in A549 cells was inhibited by PDGF-D siRNA. Compared with non-transfected group and scramble siRNA groups, the expression of

incubated in the plate for three weeks. At the end of the growth period, cells were fixed with methanol for 30 min and stained with crystal violet for 15 min. The cell colonies were photographed and the number of colonies was counted.

Animal model

Four-week-old BALB/c nude mice were purchased from Slac Laboratory Animal Center (Shanghai, China). Animals were maintained in accordance with institutional policies, and all studies were approved by the University Committee on Use and Care of Animals of Jiangsu University. Animals were randomly divided into 2 groups. The animals were injected subcutaneously with PDGF-D siRNA or scrambled siRNA-transfected A549 cells (2 × 10^6 in 200 μL PBS) into the backside of mice respectively. Tumor growth was evaluated by measuring the length and width of the tumor mass with caliper every 4 days. Tumor volume was calculated by

Figure 2. The efficiency of PDGF-D siRNA in A549 cells. A. Real-time PCR analysis of PDGF-D mRNA expression in A549 cells transfected with control and PDGF-D siRNAs. B. Western blot analysis of PDGF-D protein expression in A549 cells transfected PDGF-D siRNA and control siRNA (***$P < 0.001$).
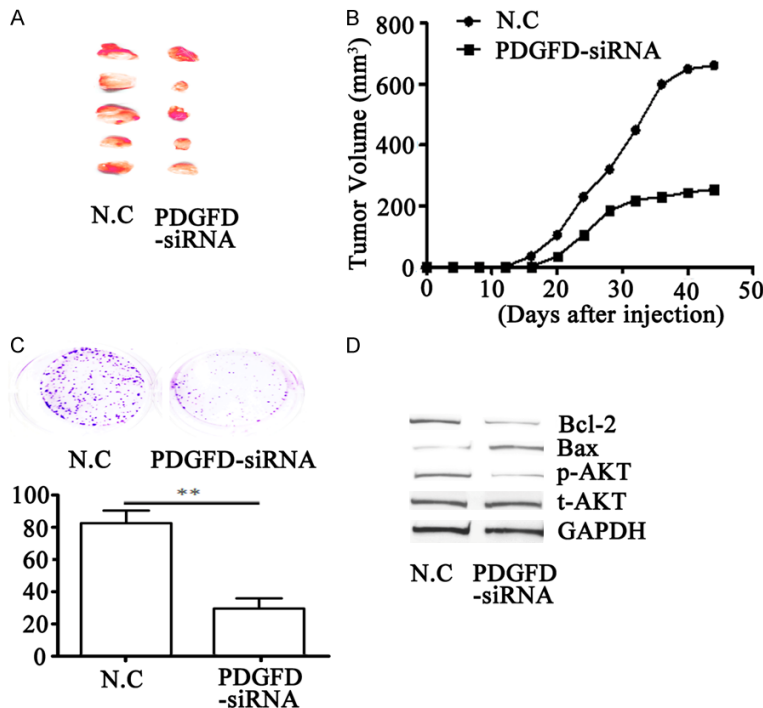
Figure 3. PDGF-D knockdown inhibits the growth of xenografted tumor. A. Representative photographs of tumor tissues in different groups. B. The tumor volume in different groups. C. Effect of PDGF-D siRNA on colony formation capacity of A549 cells. D. Effect of PDGF-D knockdown on the expression of p-AKT, AKT, Bcl-2 and BAX in A549 cells, GAPDH was used as an internal control (**$P < 0.01$).

PDGF-D mRNA and protein was significantly inhibited by PDGF-D siRNA (**Figure 2A** and **2B**). We injected PDGF-D siRNA- and scramble siRNA-transfected A549 cells into BALB/c nude mice. The xenograft tumors were removed, photographed and measured after six weeks. As shown in **Figure 3A** and **3B**, the size of tumors in the PDGF-D siRNA group was signifi-

cantly smaller compared to the control group. Moreover, PDGF-D siRNA reduced the capacity of cell colony formation in A549 cells *in vitro* (**Figure 3C**). We further examined the effect of PDGF-D siRNA on downstream signaling pathways relative to cell proliferation and apoptosis. Western blot showed that PDGF-D siRNA obviously inhibited the levels of p-AKT and Bcl-2 but increased Bax expression in A549 cells (**Figure 3D**).

*Inhibition of PDGF-D expression inhibits cell migration and invasion*

Tumor cell migration and invasion are important indicators for tumor malignancy. Here we observed that PDGF-D siRNA significantly decreased the migration capacity of A549 cells (**Figure 4A** and **4B**). Epithelial-messenchymal-transition (EMT) is closely related to tumor migration and invasion. We found that PDGF-D knockdown significantly decreased the expression of mesenchymal marker N-cadherin but increased the expression of E-cadherin, a marker of epithelial cells (**Figure 4C**). Matrix metalloproteases (MMP) are a class of proteolytic enzymes closely related to tumor invasion. Our results revealed that PDGF-D siRNA significantly decreased the protein expression of MMP-2 and MMP-9 compared to the control group (**Figure 4C**).

Discussion

With the increasing deterioration of air pollution and the environment in recent years, lung cancer is the most common cancer according to the global statistical data of cancer epidemiology worldwide [15]. PDGF-D has been found to be involved in cancer progression [16, 17].
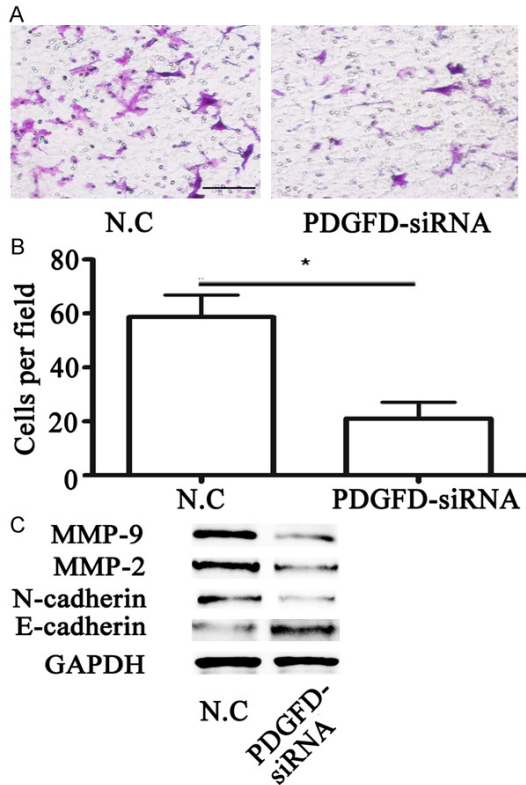
Figure 4. Effect of PDGF-D knockdown on cell migration and invasion. A, B. Transwell migration assay was used to examine cell migration capacity in A549 cells transfected with PDGF-D and negative scramble siRNAs. (magnification, × 100; scale bar = 50 μm). C. Effect of PDGF-D knockdown on the expression of E-cadherin, N-cadherin, MMP-2 and MMP-9 in A549 cells (*$P < 0.05$).

However, little is known about its function in lung cancer. The present study aimed to explore the expression and function of PDGF-D in the progression of human lung cancer, and demonstrated that inhibition of PDGF-D using siRNA can be used as a potential new therapy against lung cancer.

Recent studies showed that PDGF-D is overexpressed in mesangioproliferative glomerulonephritis and mediates mesangial cell proliferation [18, 19]. However, it is unclear for the expression of PDGF-D in lung carcinoma. Our study showed that PDGF-D was highly expressed in the tumor tissues compared to adjacent tissues. Moreover, we detected lower level of PDGF-D in the serum of lung cancer patients after operation compared to that before operation. These data indicate that PDGF-D may play important roles in lung cancer progression.

To explore the biological significance of PDGF-D overexpression in lung cancer, we silenced its expression in a PDGF-D-overexpressed lung cancer A549 cell lines. PDGF-D was reported to be involved in cancer cells proliferation and apoptosis [20]. Here we found that PDGF-D knockdown significantly the growth of xenografted tumor from A549 cells. AKT signaling pathway is one of the important pathways in the downstream of PDGF-D [14, 21]. The activation of AKT signaling pathway is closely related to cancer cells growth [22, 23]. Our results demonstrated that PDGF-D promotes cell proliferation but inhibits cell apoptosis, which is supported by the downregulation of p-AKT and Bcl-2 expression and upregulation of Bax expression in PDGF-D-ablated A549 cells.

It is known that EMT is essential for cancer progression. Recent studies demonstrated that PDGF-D plays an important role in governing EMT of cancer cells [24, 25]. Our results also found that PDGF-D knockdown significantly suppressed EMT change, supported by elevated E-cadherin expression but decreased N-cadherin expression. EMT is highly associated with the migration and invasion potentials of cancer cells. Emerging evidence reported that PDGF-D play a crucial role in promoting cancer cell invasion [20, 26]. In this study, we revealed PDGF-D is involved in the migration and invasion of lung cancer cells because PDGF-D siRNA significantly suppressed the migration of A549 cells and the protein expression of MMP-2 and MMP-9, two important factors for tumor cell invasion. The underlying mechanism of PDGF-D-mediated EMT, cell migration and invasion need further investigation.

In our study, we demonstrate that PDGF-D overexpression contribute to lung cancer progression (tumor cell proliferation, migration and invasion). Thus, targeting PDGF-D may represent a novel therapeutic strategy for lung cancer.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Longfei Xia, School of Medicine, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, Jiangsu Province, China. Tel: 86-15850327201; E-mail: xlfxiaolongfei@126.com

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