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Original Article

Knockdown of protein phosphatase 4 regulatory subunit 1 inhibits growth of lung cancer cells in vitro

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Abstract: Lung cancer is the leading cause of death from cancer around the world. Cancer patients undertaking chemotherapy or radiotherapy would experience a wide range of distressing side effects. Hence, exploring a promising new and safe therapeutic strategy to fight against cancer was urgent. Protein phosphatase 4 catalytic subunit (PP4c) is a member of the Protein phosphatase 2 (PP2A) family and play an essential role in biological processes of microtubule (MT) growth/organization, apoptosis, and tumor necrosis factor signaling. Protein phosphatase 4 regulatory subunit 1 (PPP4R1), a protein that co-purifying with PP4c from bovine testis extracts, has been reported to be involved in tumor progression. However, its functional role in lung cancer remains unclear. In this study, we firstly evaluated the expression levels of PPP4R1 in three lung cancer cell lines by quantitative real-time PCR (qRT-PCR) analysis and found that PPP4R1 was expressed highest in 95D cells. Then PPP4R1 was knocked down by lentivirus-based system in 95D cells. Proliferation and colony numbers were evaluated in lung cancer 95D cells by tetrazolium (MTT) and colony formation assay, respectively. As a result, stable knockdown of PPP4R1 led to suppression of proliferation and colony-forming ability in 95D cells. Subsequently, the cell cycle analysis showed knockdown of PPP4R1 arrested cell cycle at G0/G1 phase in 95D cells. Further investigation indicated that knockdown of PPP4R1 down-regulated cell-cycle activators, including CDK2, CDK4 and CDK6, as determined by western blot. Our results suggest that PPP4R1 knockdown inhibited lung cancer cell proliferation via down regulating cell-cycle activators. The identification of PPP4R1 may provide a potential therapeutic strategy for lung cancer treatment.

Keywords: Cell cycle, cell proliferation, lung cancer, protein phosphatase 4 regulatory subunit 1, cyclin-dependent kinase

Introduction

Lung cancer is the leading cause of death from cancer around the world [1-3]. As with other epithelial malignancies, the occurrence of lung cancer is associated with a series of pathological changes in the bronchial epithelium [1]. Lung cancers are defined into two major histological groups, small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) [2]. Chemotherapy, radiotherapy, and surgical resection offer symptomatic relief and modest improvement for survival in lung cancer [3]. However, cancer patients undertaking chemotherapy or radiotherapy would experience a wide range of distressing side effects including nausea, vomiting, dysphoria, decline in immunity, easy metastasis, etc [4, 5]. Hence, exploring a promising new and safe therapeutic strategy to fight against cancer was urgent.

Protein phosphatase 4 catalytic subunit (PP4c) is a member of the Protein phosphatase 2 (PP2A) family and play an essential role in biological processes of microtubule (MT) growth/organization, apoptosis, and tumor necrosis factor signaling [6, 7]. Protein phosphatase 4 regulatory subunit 1 (PPP4R1), a ~125 kDa protein that co-purifying with PP4c from bovine testis extracts [8]. It encodes a regulatory subunit of the protein phosphatase PP4 which belongs to the highly conserved protein phosphatases family of serine/threonine phosphatases and is most closely related to PP2A and PP6 phospha-

tases [9]. At the same time, PP2A has revealed roles in cell growth and the inhibition of nuclear telomerase activity in human breast cancer cells [10]. However, a few studies indicated that the abundance of PP2A components provides a large target for mutations that might derail proper enzyme function and could contribute to the process of tumorigenesis [11]. For PPP4R1, it is also a noncatalytic molecule that acts as a novel interaction partner of PP4c, which is overexpressed in human breast and lung cancers contrast with benign breast and lung tissue lesions [12]. Additionally, PPP4R1 was proposed to be associated with the occurrence of gastric cancer [13]. It was also found that PPP4R1 accelerate growth and proliferation in HepG2 hepatocellular carcinoma and breast cancer cells [14, 15], but was unrevealing in lung cancer.

The development of gene therapy provides a number of innovative treatments that depends on delivery of foreign materials into target malignant [16, 17]. RNA interference (RNAi), mediated by short interfering RNAs (siRNAs) or short hairpin RNAs (shRNA), is a powerful approach to eliminate gene products to facilitate understanding the gene biological function [18, 19].

To investigate the implication between PPP4R1 and lung cancer, PPP4R1 was knocked down by lentivirus-based shRNA system in lung cancer cells. Then, proliferation and colony numbers were evaluated in PPP4R1-knockdown lung cancer cell lines. Subsequently, the cell cycle progression was analyzed by flow-cytometric, as well as the implicit mechanism. The identification of potential markers involved in the development and growth of lung cancer will benefit for gene therapeutic against this disease.

Materials and methods

Cell culture

Human lung cancer cells 95D, H1299 and A549 and human embryonic kidney cells 293T were purchased from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China) for this study. H1299 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). Both A549 and 95D cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) (SH30243.01B, Hyclone) supplemented with 10% FBS (S1810, Biowest). All these cells were maintained in a in a humidified atmosphere of 5% CO_2.

Construction of recombinant lentivirus and infection

The corresponding short hairpin RNA (shRNA) sequence of PPP4R1 (Genbank accession number NM_001042388.2) and negative control shRNA sequence were 5'-GCTTGAATCTCGGTGTCTTTCCTCGAGGAAAGACACCGAGATTCAAGCTTTTTT-3' and 5'-GCGGAGGGTTTGAAAGAATATCTCGAGATATTCTTTCAAACCCTCCGCTTTTTT-3'. The nucleotide sequences were inserted into the pFH-L plasmid (Shanghai Hollybio, China) between NheI and PacI sites and named as pFH-L-shPPP4R1 or pFH-L-shCon. For the packaging of lentivirus, pFH-L-shPPP4R1 or pFH-L-shCon were transfected into HEK293T cells along with the packing auxiliary helper pVSVG-I and PCMVΔR8.92 (Shanghai Hollybio, China) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. For lentivirus infection, human lung cancer 95D cells were plated in 6-well plates at a density of 5×10^4 cells, and then transduced with PPP4R1 shRNA (Lv-shPPP4R1) or control shRNA (Lv-shCon) expressing lentivirus at a MOI of 10. The infection efficiency was observed after 96 h under a fluorescence microscope for the green fluorescence protein (GFP) expression.

Quantitative real-time PCR (qRT-PCR) analysis

Total cellular RNA was extracted from 95D, A549 and H1299 lung cancer cells using the AB Gene Total RNA Isolation Reagent (Advanced Biotechnologies Ltd., Epsom, Surrey, UK) after 5 days of infection. The RNA was converted to cDNA by using the Superscript cDNA synthesis kit according to the manufacturer's protocol. Forward and reverse primer sequences for PPP4R1 are 5'-ACGTCCCATTGCTCTGAATC-3' and 5'-CTTGGGACATCTGCCAAAGT-3'. Actin was used as an internal control and its primers were forward: 5'-GTGGACATCCGCAAAGAC-3' and reverse: 5'-AAAGGGTGTAACGCAACTA-3'. QRT-PCR was carried out in a 25 μl reaction volume: 10 μl of 2 × SYBR® Premix EX Taq™, 0.8 μl of forward and reverse primers, 5 μl of cDNA, and 4.2 μl of ddH_2O. Amplifications were performed out on the BioRad Connet Real-time PCR platform using the following procedure: initial denaturation at 95°C for 1 min, denaturation 95°C for 5 s, and then annealing extension of 60°C

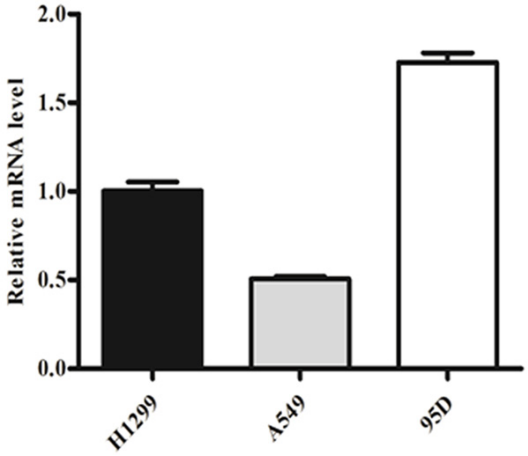

Figure 1. The mRNA expression level of PPP4R1 in three lung cancer cell lines using qRT-PCR analysis. Actin was used as an internal control.

for 20 s for 40 cycles. The relative expression of PPP4R1 levels compared with actin was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot assay

After infection for 7 days, 95D cells were collected after lentivirus infection and lysed in 2X SDS Sample Buffer (10 mM EDTA, 100 mM Tris-Hcl (pH 6.8), 4% SDS and 10% Glycine). The protein concentration was determined by bicinchoninic acid (BCA) protein assay. Subsequently, the proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked in using 5% nonfat milk powder in TBS and incubated with the following primary antibodies: rabbit anti-PPP4R1 (1:1,000, Sigma, HPA041089), rabbit anti-CDK2 (1:1,000, Cell signaling, #2546), rabbit anti-CDK4 (1:1,000, Proteintech, 11026-2-AP), rabbit anti-CDK6 (1:1,000, Proteintech, 19117-1-AP), and rabbit anti-GAPDH (1:500,000, Proteintech Group, Inc, 10494-1-AP) overnight at 4°C followed by incubation with secondary antibody horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:5,000, Santa Cruz, SC-2054) for 1 h at room temperature. The western blot bands were detected using the Luminescent Image Analyzer LAS-3000 (Fujifilm).

Tetrazolium (MTT) assay

Cells were plated in 96-well tissue culture plates at a density of 2,000 cells/well after lentivirus infection. Total 20 μl of MTT solution (5 mg/ml) was added to each well and the incubation was continued for 1, 2, 3, 4 and 5 days at 37°C for 4 h. Medium and MTT were then removed from the wells and replaced with 100 μl acidic isopropanol (10% SDS, 5% isopropanol and 0.01 mol/L HCl). Optical densities (OD) at 595 nm were measured using an automated microplate reader (EL 312e Biokinetics reader, Biotek Instruments).

Colony formation assay

A vitro colony-forming assay was performed on 95D cells after lentivirus infection. Briefly, 95D cells (500 cells/well) were seeded in 6 well plates. The medium was changed two times per week for 8 days and the cells were washed twice with PBS and fixed with 4% paraformaldehyde. The cells were then stained with freshly prepared diluted Crystal for 20 min. The cells were rinsed with distilled water and colonies (more than 50 cells) were counted under a fluorescence microscope.

Cell cycle analysis

Cell cycle distributions were carried out by staining with propidium iodide, followed by flow cytometric analysis. At four days after transduction, cells were seeded into 6-cm dishes at a density of 200,000 cells per dish and synchronized by serum starvation for 72 h. After being washed with ice-cold PBS, cells were then suspended in 1 ml ice-cold 70% ethanol and incubated at 4°C for 30 min. The supernatant solution was removed by centrifugation and the remaining cell pellets were suspended in 10 μg/ml of DNase-free RNase for 30 min. Finally, PI solution (25 μg/ml, 180 U/ml RNase, 0.1% Triton X-100, and 30 mg/ml polyethylene glycol in 4 mM citrate buffer, pH 7.8; Sigma Chemical) was added to the suspension. Then the suspension was subjected FACSCalibur flow cytometer analysis.

Statistical analysis

The data were presented as means ± SD from 3 or more experiments. Student's t test was used to examine whether there was significant differences between PPP4R1 silenced cells and control cells in SPSS 13.0 software. A p value of < 0.05 was considered statistically significant

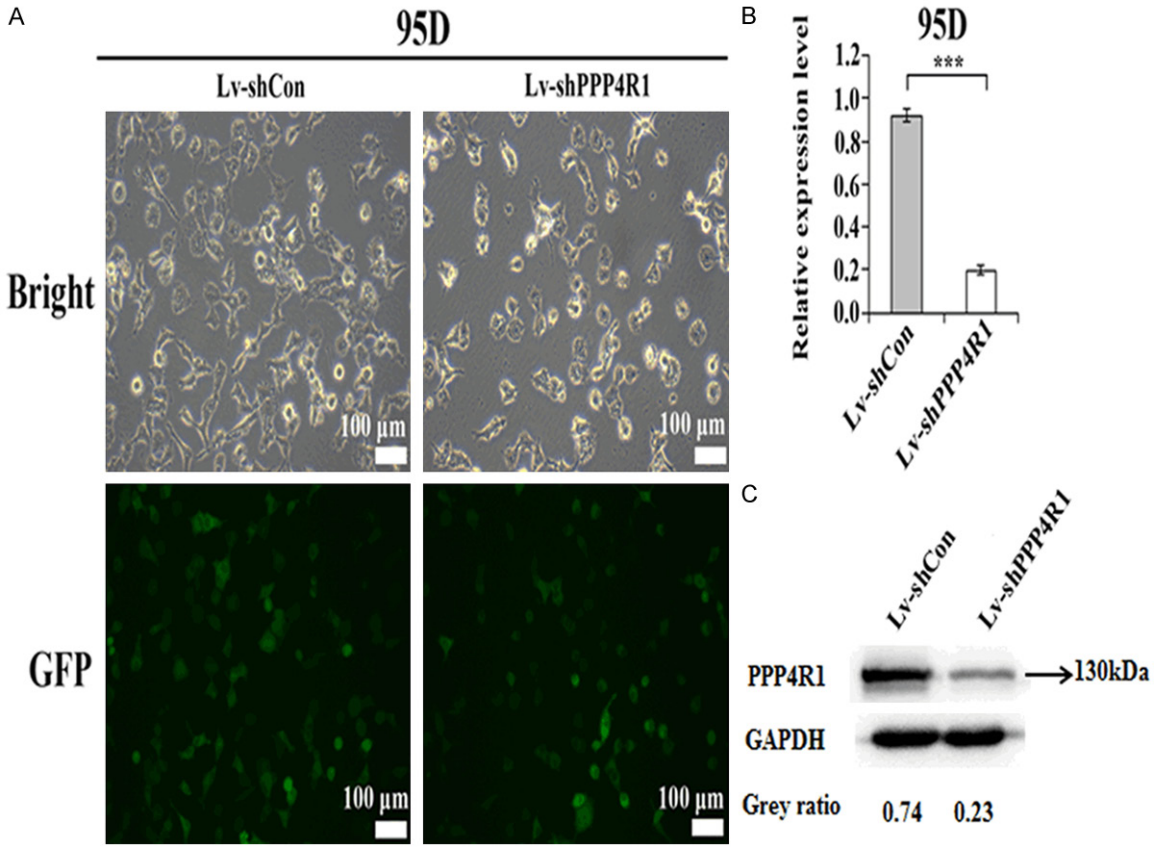

Figure 2. Knockdown of PPP4R1 suppressed PPP4R1 mRNA and protein expression in 95D cells. A. The efficiency of infection was determined by fluorescence microscopic. Scale bar = 100 μm. B. Relative mRNA expression of PPP4R1 in 95D cells transduced with lentiviral particles (Lv-shPPP4R1 or Lv-shCon) by qRT-PCR. All data represent the mean ± SD of three independent experiments. ***$P < 0.001$. Actin was used as a control. C. PPP4R1 protein expression level in Lv-shPPP4R1 or Lv-shCon infected 95D cells by western blot immunoassay. GAPDH was used as internal control.

Results

Expression of PPP4R1 in different lung cancer cell lines

The 95D, A549 and H1299 lung cancer cell lines were cultured to measure the expression levels of *PPP4R1* using qRT-PCR assay. *PPP4R1* mRNA was detected in all the three lung cancer cell lines (**Figure 1**) and the result showed that the mRNA expression of *PPP4R1* in 95D cells was the highest in the three cell lines.

Targeted disruption of PPP4R1 in 95D cells mediated by lentivirus vectors

To investigate the biological effects of PPP4R1, lung cancer 95D cells with higher levels of PPP4R1 were chosen to be infected with Lv-shPPP4R1 and Lv-shCon vectors, respectively. As shown in **Figure 2A**, more than 80% of the total cells were observed GFP positive, suggesting a satisfactory efficient infection. Furthermore, the relative mRNA expression level of *PPP4R1* (**Figure 2B**) was confirmed to be downregulated by 78.4% in 95D cells after infection with lentivirus vectors, as determined by qRT-PCR. Additionally, western blot analysis showed that the PPP4R1 protein level in cells with Lv-shPPP4R1 was obviously lower than that of Lv-shCon (**Figure 2C**). These results demonstrated that successful infection of *PPP4R1* targeted shRNA could significantly reduce the *PPP4R1* expression in 95D cells at both transcriptional and translational levels.

Knockdown of PPP4R1 suppressed 95D cell proliferation and colony-forming ability

To clarify the effects of PPP4R1 on proliferation of lung cancer 95D cells, the MTT cell viability assay was used to determine cell survival and

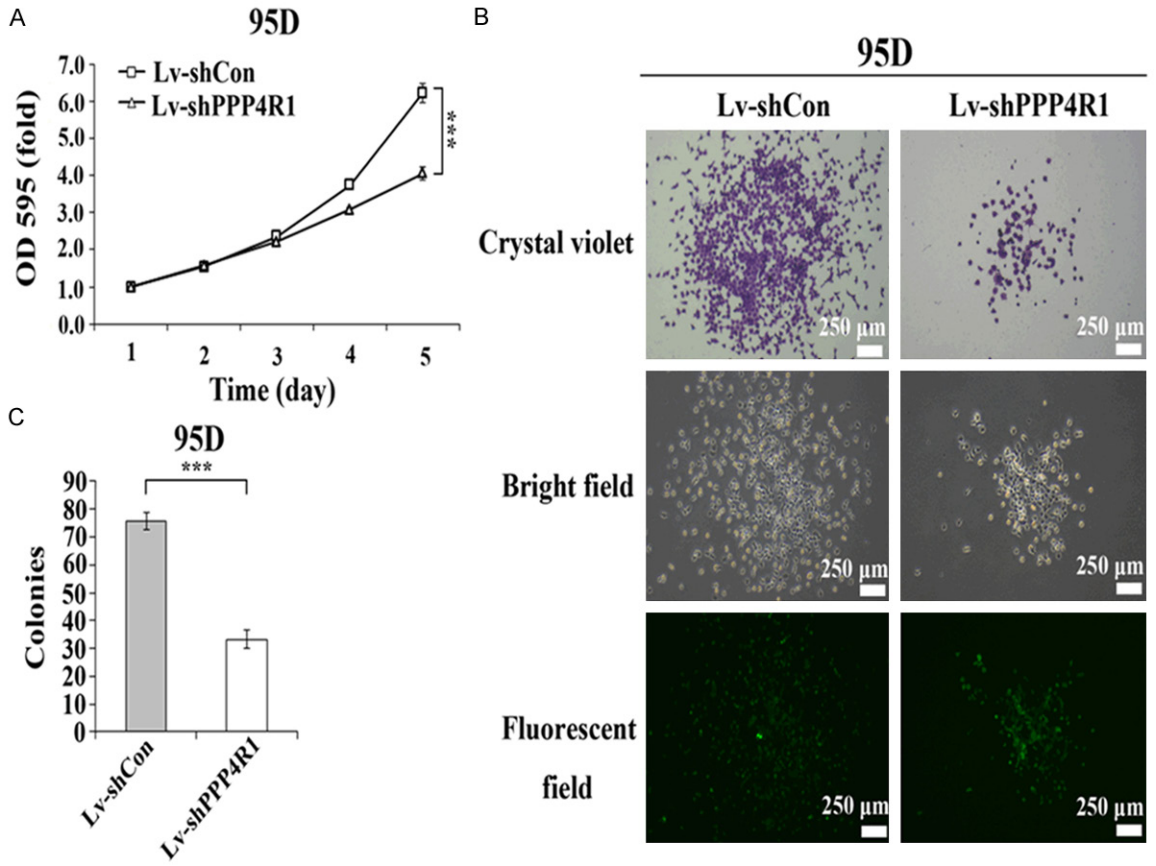

Figure 3. PPP4R1 knockdown affects cell proliferation and colony formation in 95D cells. A. The cell proliferation levels in 95D cells with PPP4R1 knockdown and control. B. Representative images of colonies after crystal staining under bright field and fluorescent microscopy. Scale bar = 250 μm. C. Numerical representation in colonial in PPP4R1 knockdown group and control group. All data represent the mean ± SD of three independent experiments. ***indicates $P < 0.001$.

proliferation at each time point. Indeed, the transfection of PPP4R1 shRNA into 95D cells was shown to affect the number of viable cells after incubation for 3 days. The cell numbers are decreased by 17.8% ($P < 0.001$) and 34.7% ($P < 0.001$) at the 4th and 5th day in Lv-shPPP4R1 group compared with the Lv-shCon (**Figure 3A**). These results showed that sequence-specific knockdown of PPP4R1 inhibited proliferation of 95D cells.

Next, we investigated the effect of PPP4R1 on colony forming ability by using colony formation assay following crystal violet staining. As shown in **Figure 3B**, the number of the colonies was decreased markedly in 95D cells after PPP4R1 knockdown. Further analysis showed there was a decrease of about 56.6% in total colonies formed in Lv-shPPP4R1 group compared with control suggesting that the colony forming ability of lung cancer 95D cells was suppressed by knockdown of PPP4R1.

PPP4R1 knockdown induced disturbance of cell cycle of 95D cells

Flow cytometry was used to detect the mechanism of inhibition of cell proliferation and colony formation by knockdown of PPP4R1. As shown in **Figure 4A**, the cell cycle distribution of PPP4R1 shRNA infected cells was different from that of control shRNA infected cells. The percentage of 95D cells of the G0/G1 phase increased by 22% in the Lv-shPPP4R1 groups compared with Lv-shCon groups (**Figure 4B**). During S and G2/M phases, the percentage of cells decreased about 7.1% and 34.4% in Lv-shPPP4R1 groups in comparison with control. These results showed that PPP4R1 knockdown in human lung cancer 95D cells caused cell cycle arrest at the G0/G1 phase.

To further explore the molecular mechanism of PPP4R1 on the lung cancer cell proliferation, the expression alterations of some cell cycle

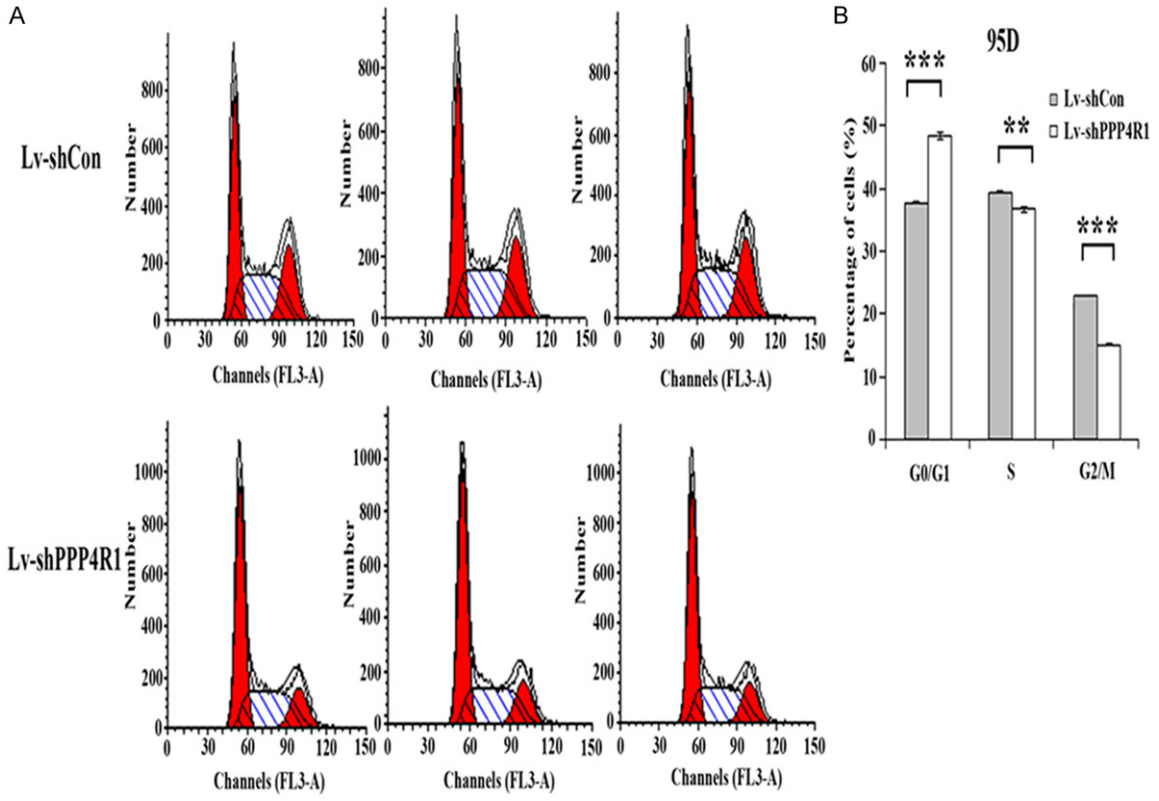

Figure 4. Knockdown of PPP4R1 disturbed cell cycle of 95D cells. A. Cell cycle distribution patterns of 95D cells were determined by flow cytometry. B. Percentage of 95D cells at G0/G1, S, and G2/M phases following Lv-shPPP4R1 or Lv-shCon infection. All data represent the mean ± SD of three independent experiments. $**P < 0.005$, $***P < 0.001$.

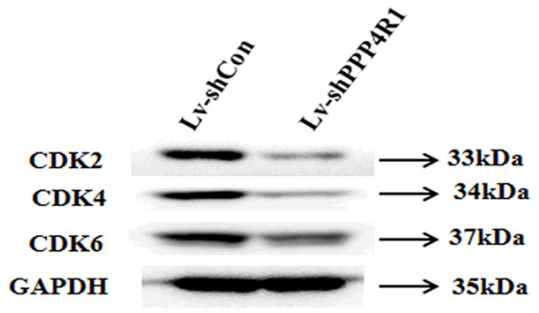

Figure 5. The effect of PPP4R1 silencing on cell cycle regulators and apoptotic markers in 95D cells. Western blot was used to evaluate the expression of CDK2, CDK4 and CDK6 in 95D cells after Lv-shPPP4R1 infection. GAPDH was used as the internal control.

regulators were detected using western blot assay. As shown in **Figure 5**, the expression of cell cycle regulators, including CDK2, CDK4 and CDK6, were significantly down regulated in Lv-shPPP4R1 group compared to those in shCon group.

Discussion

Lung cancer is one of the most common cancer related deaths around the world, with an average 15 percent five-year survival rate [20]. Despite treatment advances in recent years, a better understanding of the potential biomarkers will hopefully lead to improve in survival and quality of life for this challenging disease. This study identified and characterized a potential lung cancer-associated gene PPP4R1, which has been shown as a regulatory subunit of the PP2A family serine and threonine phosphatase PP4C in T cells and T-cell lymphoma [21]. Lentivirus-mediated RNA interference was successfully performed to delete PPP4R1 expression in human lung cancer 95D cells (**Figure 2**). MTT and crystal violet staining assays showed that knockdown of PPP4R1 caused a significant reduction in the proliferation and colony formation (**Figure 3**). Flow cytometry analysis has demonstrated that the PPP4R1 depletion arrested cell cycle at G0/G1 phase (**Figure 4**).

Furthermore, to illuminate the molecular mechanisms by which PPP4R1 affects 95D cells growth, we detected some molecules associated with cell cycle control after PPP4R1 knockdown. Western blot analysis showed knockdown of PPP4R1 suppressed the expression levels of CDK2, CDK4 and CDK6 in 95D cells (**Figure 5**). It is widely accepted that uncontrolled proliferation is the hallmark of cancer, which promotes tumor metastatic growth [22, 23]. Cell cycle progression plays an essential role in regulating cell proliferation. Cyclin dependent kinases (CDK), including CDK 2, CDK 4 and CDK 6, have been demonstrated to be closely associated with the cell cycle progression [24]. CDK 2 was considered to be a unique kinase bound to cyclin E, essential for the G1-to-S transition and progression and its expression can be controlled by CDK 4 [25]. Down-regulation of CDK 2 expression could significantly inhibit the proliferation of 95D cells [26]. Zhu et al. [27] demonstrated that Cdc42 and CDK 6 deficiency suppresses the proliferation of NSCLC A549 cells and promotes G1 arrest. Recent studies show PPP4R1 was found to accelerate growth and proliferation in HepG2 hepatocellular carcinoma and breast cancer cells [14, 15]. Consistent with previous studies, our data demonstrated knockdown of PPP4R1 in 95D cells significantly downregulated CDK 2, CDK 4, and CDK6 proteins expression. Therefore, we speculate that the PPP4R1-knockdown depresses the proliferation and colony formation in lung cancer cells, mainly though arresting the cells at the G0/G1 phase. Based on the above evidences, we can conclude PPP4R1 plays a vital role in the process of lung cancer progression. PPP4R1 may be directly or indirectly modulate cell cycle division through regulating CDKs expression in lung cancer. It is worth pointing out that this study for the first time has identified PPP4R1 as a novel potential therapeutic target for human lung cancer.

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Disclosure of conflict of interest

None.

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