

RETRACTED ARTICLE: PP4R1 accelerates cell growth and proliferation in HepG2 hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC), as the fifth most common cancer worldwide, has become the third leading cause of cancer-related deaths. It is reported that protein phosphatase 4 (PP4) is an essential protein for nucleation, growth, and stabilization of microtubules in centrosomes/spindle bodies during cell division. Besides, previous studies have identified protein phosphatase 4 regulatory subunit 1 (PP4R1) as a constitutive interaction partner of PP4 catalytic subunit PP4C. The PP4C-PP4R1/PP4 complex plays a role in dephosphorylation, regulation of histone acetylation, and NF- κ B activation. However, little is known about the pathological functions of PP4R1 in human cancers. Thus, in order to investigate how PP4R1 functions in human HCC, two common hepatocarcinogenesis Hep cell lines HepG2 and SMMC-7721 were employed, transduced with recombinant lentivirus expressing PP4R1 short hairpin RNA. Compared with the controls, the cells treated with Lv-shPP4R1 showed a significant decrease in cell proliferation and colony formation. The results of flow cytometry showed that the knockdown of PP4R1 caused HepG2 cells arrest at G₂/M phase in the cell cycle. Furthermore, the transduction of Lv-shPP4R1 into HepG2 cells led to the inactivation of two major mitogen-activated protein kinase signaling cascades: p38 and c-Jun N-terminal kinase (JNK), indicating that PP4R1 could promote cell proliferation, which might be regulated by p38 and c-Jun N-terminal kinase pathways. In a word, this study highlights the crucial role of PP4R1 in promoting HCC cell growth, which might elucidate the pathological mechanism of HCC.

Keywords: hepatocellular carcinoma, PP4R1, shRNA, proliferation, apoptosis

Introduction

Hepatocellular carcinoma (HCC), a common primary liver cancer, which is the fifth most common cancer worldwide, has become the third leading cause of cancer-related deaths.^{1,2} HCC is a complex and heterogeneous malignancy that arises in the context of progressive underlying liver dysfunction. Therefore, no single dominant or pathognomonic molecular mechanism exists in HCC. Given the asymptomatic nature of early disease and the limited use of surveillance, the majority of HCC cases are present at advanced or incurable stages. Even among patients with liver cancer detected earlier, there are still very few candidates for surgery because of the coexisting disease. In addition, the prognosis of advanced-stage HCC is poor, with an overall survival rate of <5%.³ Besides, the 5-year recurrence rates of over 70% have been reported despite surgical or locoregional therapies in earlier stages.⁴ Thus, new treatment modalities must be pursued.

It has been suggested that many physiopathologic processes are controlled through the balance between protein phosphorylation and dephosphorylation. Protein phosphatases (PPs) are the primary effectors of dephosphorylation, and can be grouped into three main classes based on sequence, structure, and catalytic function. The largest class of PPs is the phosphoprotein phosphatase family comprising PP1, PP2A, PP2B,

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PP4, PP5, PP6, and PP7, and the PP Mg²⁺- or Mn²⁺-dependent family, composed primarily of PP2C.

PP4, which belongs to the PP2A family, is a protein complex comprised of a catalytic subunit PP4C plus regulatory subunits.⁵ PP4 has been reported to be involved in many processes such as microtubule organization at centrosomes, resistance to apoptosis induced by ultraviolet irradiation and cisplatin, and recovery from the DNA damage checkpoint,⁵⁻⁸ maturation of spliceosomal small nuclear ribonucleic proteins (snRNPs),⁹ DNA repair,¹⁰ tumor necrosis factor- α signaling, activation of c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) 8,¹¹ regulation of histone acetylation,¹² NF- κ B activation,¹³ and division. A major form of PP4 in organisms from yeast to humans comprises PP4C in complex with a core regulatory subunit R2, and a variable regulatory subunit R3.¹⁴ Moreover, serine/threonine-protein phosphatase 4 regulatory subunit 1 (PP4R1), a unique non-catalytic regulatory phosphatase subunit, was first identified as a constitutive interaction partner of PP4C.¹⁵ The PP4C-PP4R1 PP4 complex plays a role in the dephosphorylation and regulation of HDAC3.¹² Furthermore, PP4R1 was also identified as a negative regulator of NF- κ B activity in T lymphocytes.¹⁶ It was demonstrated that PP4R1 formed part of a distinct PP4 holoenzyme and directed PP4C activity to dephosphorylate and inactivate NF- κ B signaling. At last, a recent study identified a novel mechanism that PP4R1 targeted TRAF2 and TRAF6 to mediate the inhibition of the NF- κ B pathway.¹⁷ However, the functional role of PP4R1 in human cancers remains unclear yet.

In this study, a lentivirus vector was successfully constructed to introduce PP4R1 short hairpin RNA (shRNA) into two human HCC cell lines HepG2 and SMMC-7721. Lentivirus-mediated silencing of PP4R1 inhibited the proliferation and colony-forming ability of HepG2 cells, and induced cell cycle arrest in the G₂/M phase, which might elucidate the pathological mechanism of HCC.

Materials and methods

Cell lines and cell culture

Human HCC cells, HepG2, SMMC-7721 (Catalogue number TCHu 72), and human embryonic kidney 293T cells (Catalogue number GNHu17) were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, People's Republic of China). HepG2, SMMC-7721, and 293T cells were maintained in Dulbeccos' Modified Eagle's Medium (DMEM; Hyclone [GE Healthcare Life Sciences, Piscataway, NJ, USA], Catalogue number SH30243.01B+) supplemented with 10% fetal bovine serum (Biowest [Kansas City, MO, USA], Catalogue number S1810). All cell lines

were cultured at 37°C in a 5% CO₂ humidified incubator. No ethics statement was required from the institutional review board for the use of this cell line.

Lentivirus-mediated shRNA knockdown of PP4R1 expression

The shRNA sequence 5'-GCTTGAATCTCGGTGTC TTCTCGAGGAAAGACACCGAGATTCAAGCT TTTT-3' was designed targeting human PP4R1 gene (NM_001042388.2). The negative control shRNA was 5'-GCGGAGGGTTTGAAAGAATTTCTCGAGAT ATTCTTTCAAACCCCTCCGCTTTT-3'. The double-stranded DNA fragments were formed in the annealing reaction system. The pFH⁺ vector (Shanghai Hollybio, Shanghai, People's Republic of China) was linearized by *NheI* and *PacI* restriction enzyme digestion. Pure linearized vector fragments and double-stranded DNA fragments were collected and combined together during a 16-hour reaction. Each DNA was used to transform the *Escherichia coli* strain DH5 α and was purified with a plasmid purification kit (Qiagen, Valencia, CA, USA). The ligation product was confirmed by polymerase chain reaction (PCR) and sequencing. The generated plasmids were named pFH-Lv-shPP4R1 or Lv-shCon. Recombinant lentiviral vectors and packaging plasmids (pVSVG-I and pCMV Δ R8.92) (Shanghai Hollybio) were cotransfected into 293T cells. Supernatants containing lentivirus expressing PP4R1 shRNA (Lv-shPP4R1) or control shRNA (Lv-shCon) were harvested 48 hours after transfection. The lentiviruses were then purified via ultracentrifugation, and the viral titer was determined by counting green fluorescent protein (GFP)-positive cells. The viral titer was determined by the method of end point dilution through counting the numbers of infected GFP-positive cells at 100 \times magnification under a fluorescence microscope (Olympus, Tokyo, Japan). Titer in IU/mL = (the numbers of green fluorescent cells) \times (dilution factor) / (volume of virus solution). For lentivirus infection, HepG2 cells (50,000 cells/well) were seeded in six-well plates and transduced with Lv-shPP4R1 or Lv-shCon at a multiplicity of infection of 10. Infection efficiency was determined through counting GFP-positive cells under a fluorescence microscope 96 hours after infection, and the knockdown efficiency of PP4R1 was evaluated by real-time quantitative PCR (qPCR) and Western blot analysis.

RT-PCR and qPCR

Total cellular RNA was extracted using TRIzol[®] reagent (Invitrogen [Thermo Fisher Scientific, Waltham, MA, USA], Catalogue number 15596-018). RNA quantity and quality

were determined by spectrophotometry and agarose gel electrophoresis, respectively. Approximately 1 µg of extracted total RNA samples was reverse transcribed into cDNA with oligodT primers according to the manufacturer's protocol for RT-PCR (Promega [Fitchburg, WI, USA], Catalogue number M1705). RT-qPCR was carried out by the GXD kit iqSYBR Green (BioRad [Hercules, CA, USA], Catalogue number 1708882AP) according to the manufacturer's instructions with regard to the CFX96 Touch™ Real-Time PCR Detection System (BioRad). Primer sets used were as follows: for β -actin, 5'-GTGGACATCCGCAAAGAC-3' (forward), 5'-AAAGGGTGTAACGCAACTA-3' (reverse) and for PP4R1, 5'-ACGTCCCATTGCTCTGAATC-3' (forward), 5'-CTTGGGACATCTGCCAAAGT-3' (reverse). The cycling conditions were: initial denaturation 95°C for 60 seconds, 95°C for 5 seconds, 60°C for 20 seconds, 40 cycles. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method.

Western blot

After 8 days of lentivirus infection, cells were harvested and lysed in ice-cold lysis buffer (50 mM Tris, 2% sodium dodecyl sulfate [SDS], 5% glycerinum, 100 mM NaCl, 1 mM EDTA, pH=6.8). Total protein concentrations of the lysate were determined using the BCA Protein Assay Kit (Pierce Biotechnology [Life Technologies = ThermoFisher], Catalogue number 23235). Approximately 30 µg of protein in each lane was electrophoresed on 4–20% SDS-polyacrylamide gel electrophoresis and transferred to a poly(vinylidene fluoride) (PVDF) membrane (BioRad, Catalogue number 162-0134), and incubated with rabbit anti-PP4R1 (Sigma-Aldrich [St Louis, MO, USA], Catalogue number HPA041089; 1:1,000 dilution) and rabbit anti-GAPDH (Proteintech [Chicago, IL, USA], Catalogue number 10494-1-AP; 1:10,000 dilution) overnight at 4°C. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat antirabbit IgG antibody (Santa Cruz Biotechnology Inc [Dallas, TX, USA], Catalogue number SC-2054; 1:5,000 dilution). Signals were detected using the ECL-PLUS/Kit (Amersham [GE Healthcare Life Sciences], Catalogue number RPN2132) according to the manufacturer's protocol. GAPDH served as the internal standard.

MTT assay

The effect of PP4R1 on cell viability was analyzed using the MTT assay based on growth curves of HepG2 cells in vitro. Briefly, cells were reseeded in 96-well plates at a concentration of 2.25×10^4 /mL in 200 µL/well after 4 days

of lentivirus infection. Cells were then further cultured in this manner for 1–5 days. Four hours before the termination of culture, MTT (5 mg/mL; Sigma) was added at a volume of 20 µL/well. Later, the entire supernatant was discarded, and solubilization solution (0.01 M HCl, 10% SDS, 5% isopropanol) was added at a volume of 100 µL/well and incubated in an air bath shaker at 37°C for 10 minutes. The absorbance at 595 nm of each well was determined using the Epoch Microplate Spectrophotometer (BioTek, CA, USA).

Colony formation assay

After 4 days of lentivirus infection, HepG2 cells (1,500 cells/well) were reseeded in 96-well plates and incubated for 10 days to form normal colonies. The media were replaced every 3 days. Then the cells were fixed with paraformaldehyde for 30 minutes at room temperature. The fixed cells were washed twice with phosphate-buffered saline (PBS), stained by 1% crystal violet (Beyotime [Jiangsu, People's Republic of China], Catalogue number C0121) for 10 minutes, washed with ddH₂O and air dried. The total number of colonies that contain more than 50 cells was counted under light microscopy. Image analysis was conducted using Metamorph version 7.6.0 software (Molecular Devices, Sunnyvale, CA, USA).

Cell cycle analysis

After 4 days of lentivirus infection, HepG2 cells were seeded in 6 cm dishes at a density of 200,000 cells/dish and cultured for 40 hours. Cells were then released by digestion with trypsin and harvested. After centrifugation, the cell pellet was washed twice with precooled PBS and fixed with precooled 70% ethanol overnight at 4°C. Then the cells were resuspended in PBS containing 10% fetal bovine serum, filtered through a 400-mesh sieve, and stained with propidium iodide solution (PI, 50 µg/mL, 100 µg/mL RNase in PBS) at 37°C in the dark for 30 minutes. Finally, the cells were analyzed for the cell cycle phase by FACScan (Beckman Coulter, Pasadena, CA, USA). The percentages of cells at various phases of the cell cycle were analyzed using the ModFit software (Verity Software House, Topsham, ME, USA).

Intracellular signaling array

Cell lysate was prepared as mentioned earlier. The phosphorylated or cleaved signaling molecules were detected using a PathScan® Intracellular Signaling Array Kit (Chemiluminescent Readout) (Cell Signaling Technology [Beverly, MA, USA], Catalogue number 7323) according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using Prism 5 for Windows software (GraphPad Software, San Diego, CA, USA). Statistically significant differences between groups were evaluated using the Student's *t*-test. The results were determined to be significantly significant when $P < 0.05$ was obtained.

Results

Suppression of PP4R1 by shRNA in HepG2 cells and SMMC-7721 cells

To explore the role of PP4R1 in human HCC, lentivirus-mediated shRNA targeting PP4R1 was used to silence its endogenous expression in HepG2 cells and SMMC-7721 cells. In parallel, a negative control (Lv-shCon) and wild-type (Con) cells were run. The infection efficiency of recombinant

lentivirus was above 80% as revealed by fluorescence microscopy in both HepG2 cells and SMMC-7721 cells (Figure 1A). RT-qPCR assay showed that Lv-shPP4R1 could significantly downregulate PP4R1 gene expression in HepG2 cells, while no knockdown effect was observed following Lv-shCon infection (Figure 1B). Western blot analysis also confirmed the silencing of PP4R1 protein expression by Lv-shPP4R1 (Figure 1C). These results revealed that recombinant lentivirus could efficiently be transduced into HepG2 cells and SMMC-7721 cells, and silenced PP4R1 expression.

PP4R1 silencing inhibited the proliferation of HepG2 cells and SMMC-7721 cells

The effects of PP4R1 knockdown on HepG2 cell viability and proliferation were further investigated. MTT assay showed

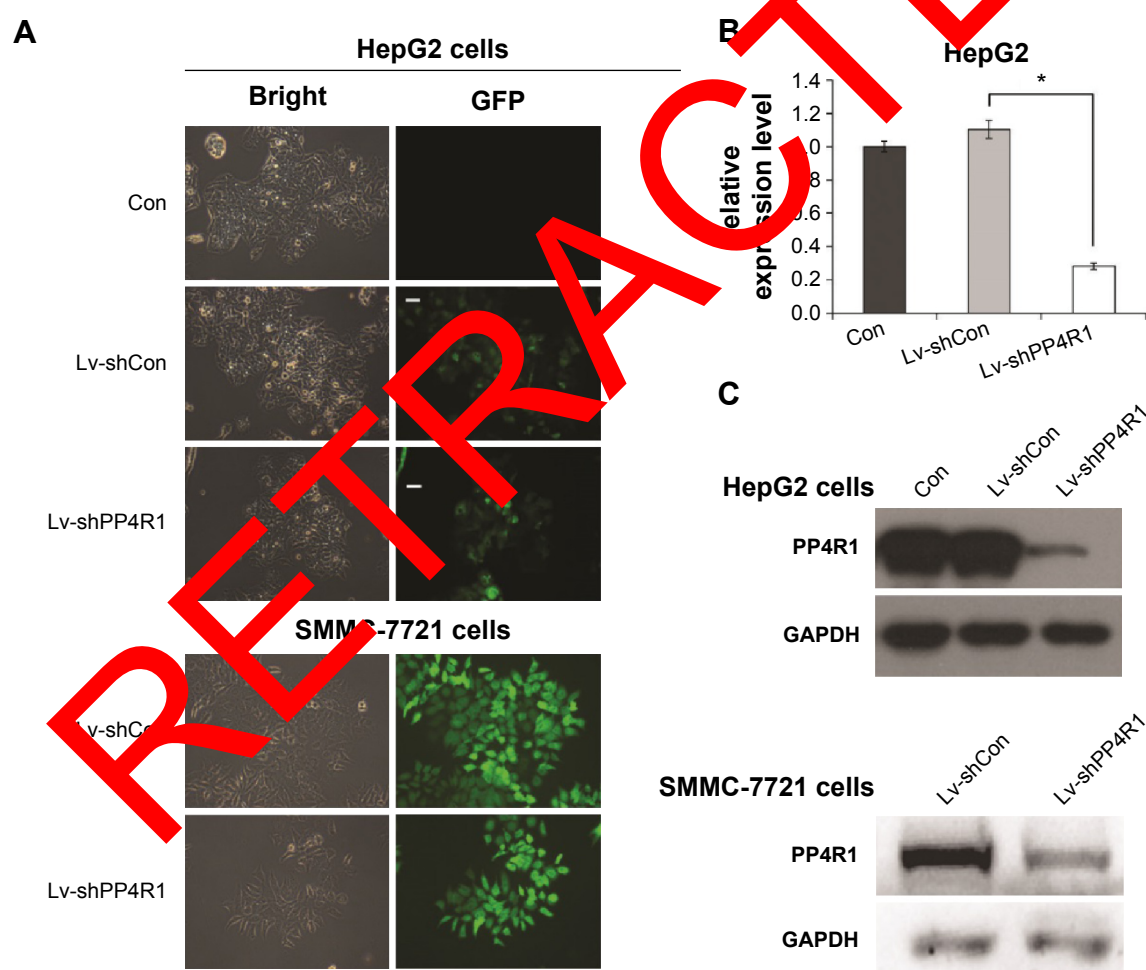


Figure 1 Silencing of PP4R1 expression in HepG2 cells and SMMC-7721 cells by Lv-shPP4R1.

Notes: (A) Fluorescence microscopy examination of lentivirus infection efficiency in HepG2 cells and SMMC-7721 cells (scale bar: 100 μ m). (B) RT-qPCR analysis of the PP4R1 mRNA level in HepG2 cells after lentivirus infection. (C) Western blot analysis of the PP4R1 protein level in HepG2 cells and SMMC-7721 cells. After lentivirus infection, data represent as means \pm SD from three independent experiments. $*P < 0.001$.

Abbreviations: Con, control; Lv-shCon, control shRNA; Lv-shPP4R1, lentivirus expressing PP4R1 shRNA; PP4R1, protein phosphatase 4 regulatory subunit 1; RT-qPCR, real-time quantitative polymerase chain reaction; shRNA, short hairpin ribonucleic acid; SD, standard deviation.

that PP4R1 silencing markedly inhibited the viability of HepG2 cells and SMMC-7721 cells (Figure 2A). The proliferative rate of the Lv-shPP4R1 group started to drop on day 3 as compared with Lv-shCon and control groups, and the gap reached the maximum on day 5 ($P<0.001$).

Colony formation assay was conducted to gain an insight into the long-term effect of PP4R1 on cell proliferation. The colony-forming efficiency of the Lv-shPP4R1 group was much less than that of the Lv-shCon and control groups (Figure 2B). We can see from Figure 2C that the size of a single colony was shrunk and the number of colonies was diminished in HepG2 cells following Lv-shPP4R1 infection.

PP4R1 silencing blocked the cell cycle progression of HepG2 cells

Next, we performed flow cytometry assay to determine whether the pro-proliferative effect of PP4R1 in HepG2 cell

line is mediated via cell cycle control. We could see from Figure 3A that the cell cycle distribution of HepG2 cells was visibly changed after PP4R1 knockdown. Compared with the Lv-shCon and control groups, the cell percentage of the G_0/G_1 phase was declined, while the cell population in the G_2/M phase was elevated in the Lv-shPP4R1 group (Figure 3B). Thus, PP4R1 might regulate cell cycle progression to control cell growth.

PP4R1 knockdown inhibited the activity of SAPK/JNK and p38 pathways

To gain insight into how PP4R1 knockdown alters the phenotype of HCC cells, we examined the expression of intracellular signaling molecules using a PathScan® Intracellular Signaling Array Kit (Cell Signaling Technology), as shown in Figure 4A, compared with the Lv-shCon group, PP4R1 knockdown obviously downregulated the phosphorylated levels of p38 (Thr180/Tyr182) and SAPK/JNK (Thr183/Tyr185). To verify

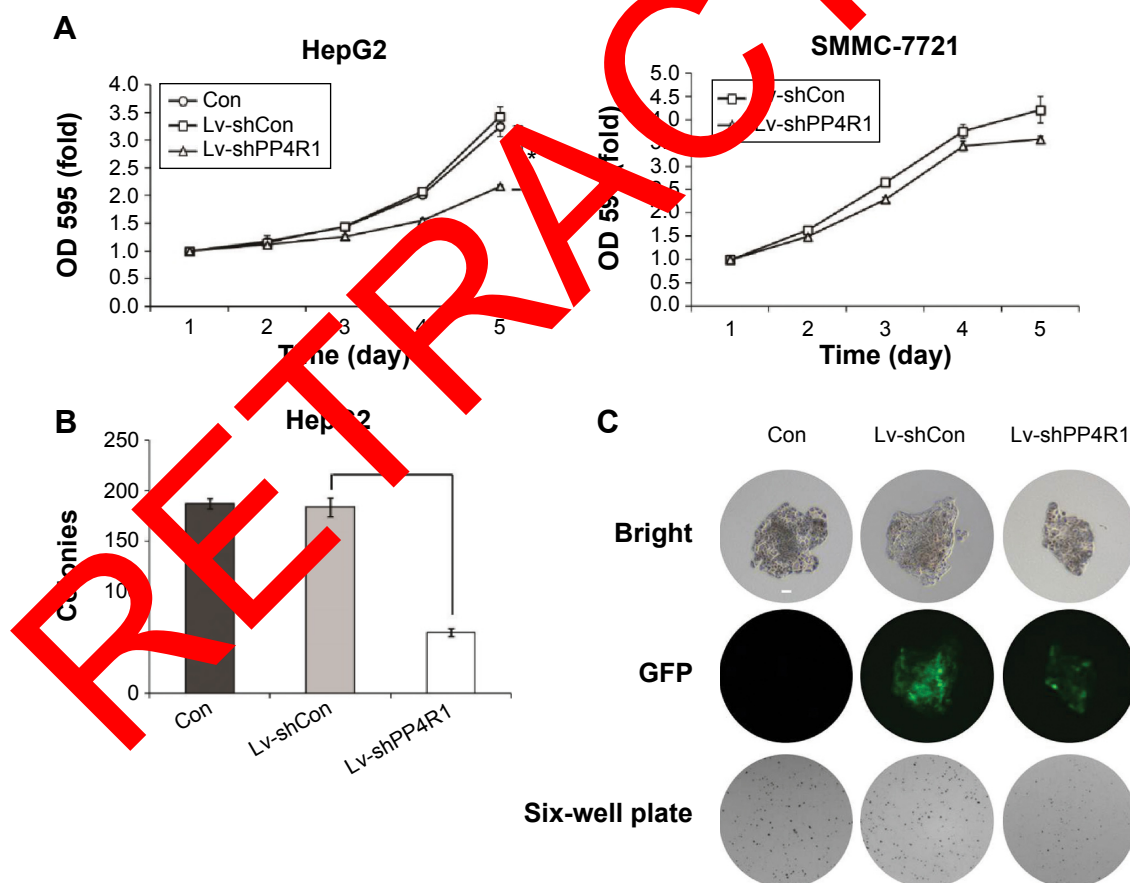


Figure 2 Lv-shPP4R1 inhibited the viability and proliferation of HepG2 cells.

Notes: (A) Growth curves of HepG2 cells and SMMC-7721 cells in three groups (Con, Lv-shCon, and Lv-shPP4R1) measured by MTT assay. (B) Statistical analysis revealed that the number of colonies in the Lv-shPP4R1 group was significantly smaller than that in the control groups. (C) Images recorded under micro and macro views, representing the size and the number of colonies in each group (scale bar: 250 μ m). Data represented as means \pm SD from three independent experiments. $*P<0.001$.

Abbreviations: Con, control; GFP, green fluorescent protein; Lv-shCon, control shRNA; Lv-shPP4R1, lentivirus expressing PP4R1 shRNA; OD, optical density; PP4R1, protein phosphatase 4 regulatory subunit 1; SD, standard deviation; shRNA, short hairpin ribonucleic acid.

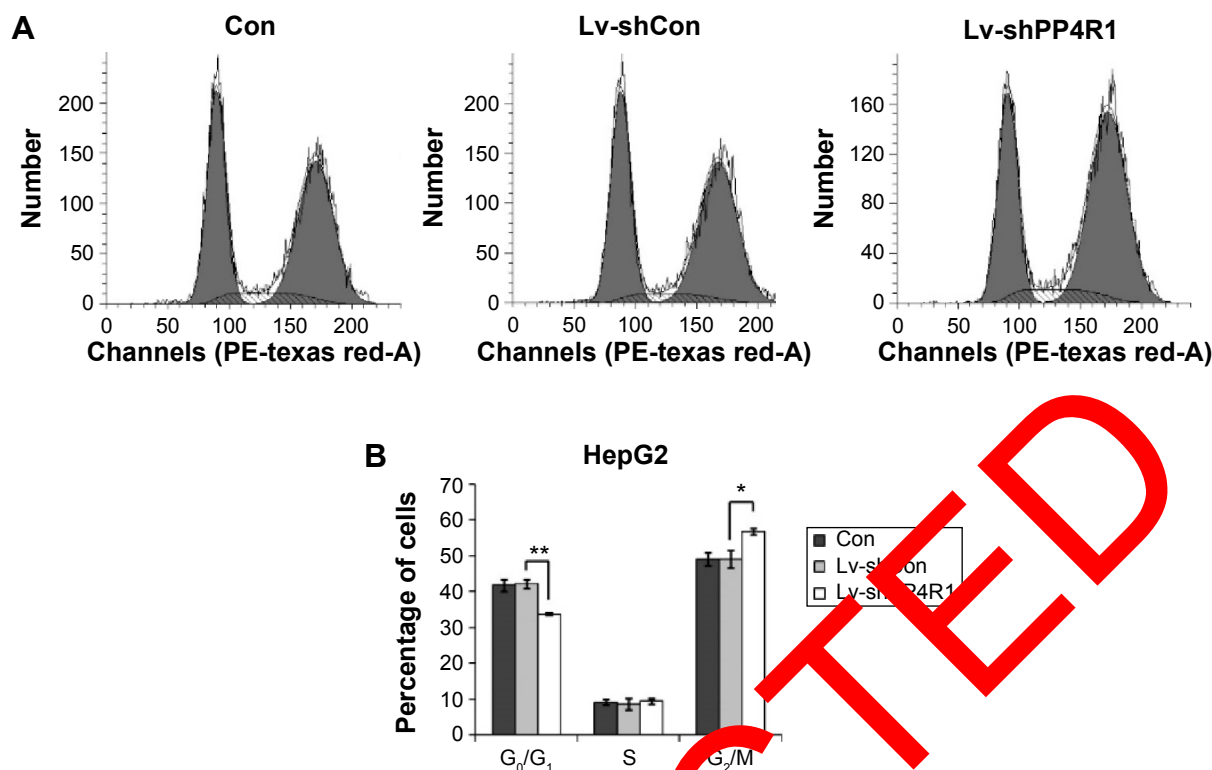


Figure 3 Lv-shPP4R1 blocked the cell cycle progression of HepG2 cells.

Notes: (A) FACS analysis of cell cycle distribution of HepG2 cells in three groups (Con, Lv-shCon, Lv-shPP4R1). (B) Knockdown of PP4R1 in HepG2 cells led to an increase of cells in the G₂/M phase and, concomitantly, a decrease of cells in the G₀/G₁ phase. Data represent means \pm SD from three independent experiments. * $P < 0.05$, ** $P < 0.01$. **Abbreviations:** Con, control; FACS, fluorescence activated cell sorting; Lv-shCon, control shRNA; Lv-shPP4R1, lentivirus expressing PP4R1 shRNA; PP4R1, protein phosphatase 4 regulatory subunit 1; SD, standard deviation; shRNA, short hairpin ribonucleic acid.

the results further, we found that compared with the Lv-shCon group, PP4R1 knockdown obviously downregulated the phosphorylated levels of p38 through Western blot analysis (Figure 4B). All these results revealed that PP4R1 silencing could suppress p38 and SAPK/JNK pathways.

PP4R1 knockdown had no effect on the activity of NF- κ B pathway

To investigate whether PP4R1 knockdown affects the activity of NF- κ B in HCC cells, we separated the nucleus from the cytoplasm and detected the expression of p65 in them

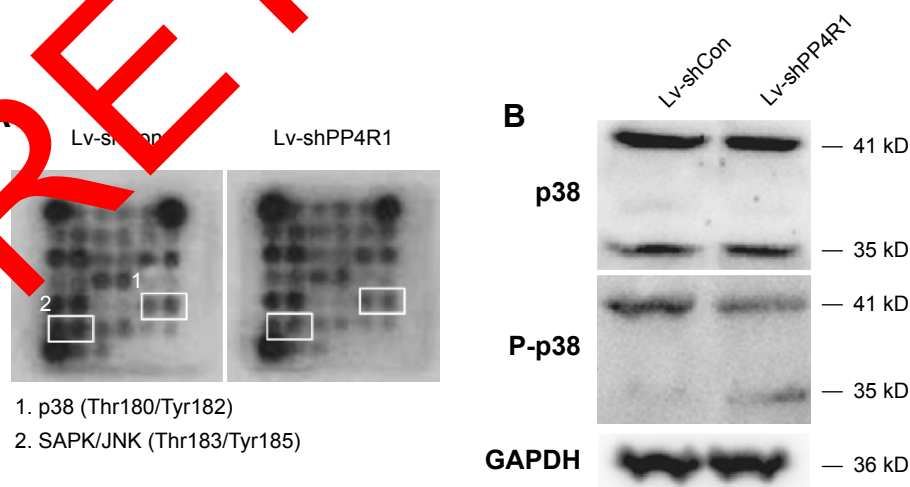


Figure 4 Lv-shPP4R1 caused inactivation of p38 and JNK.

Notes: (A) Cell extracts were prepared and analyzed using the PathScan® Intracellular Signaling Array Kit (Chemiluminescent Readout). Images were acquired by briefly exposing the slide to standard chemiluminescent film. (B) Western blot analysis of p38 and JNK protein level in HepG2 cells and SMMC-7721 cells after lentivirus infection. **Abbreviations:** JNK, c-Jun N-terminal kinase; Lv-shCon, control shRNA; Lv-shPP4R1, lentivirus expressing PP4R1 shRNA; PP4R1, protein phosphatase 4 regulatory subunit 1; shRNA, short hairpin ribonucleic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

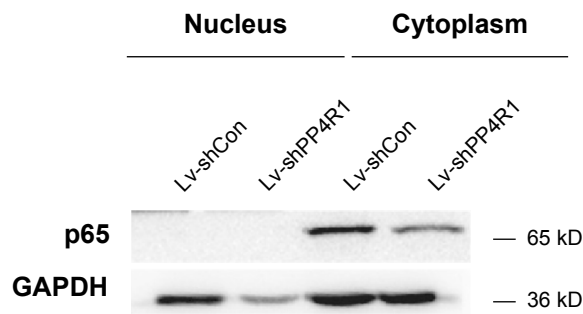


Figure 5 Lv-shPP4R1 had no effect on the activity of NF- κ B.

Notes: Western blot analysis of p65 protein level in HepG2 cells and SMMC-7721 cells after lentivirus infection.

Abbreviations: Lv-shCon, control shRNA; Lv-shPP4R1, lentivirus expressing PP4R1 shRNA; PP4R1, protein phosphatase 4 regulatory subunit 1; shRNA, short hairpin ribonucleic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

separately through Western blot. The results show that p65 was not expressed in the nucleus, and PP4R1 knockdown downregulated the expression of p65 (Figure 5).

Discussion

Hepatocarcinogenesis is considered to be a process originating from hepatic stem cells (however, the role of liver stem cells as HCC cells of origin is debated)¹⁸ or mature hepatocytes, and evolving from chronic liver disease driven by oxidative stress, chronic inflammation, and cell death followed by unrestricted proliferation/restricted regeneration, and permanent liver remodeling. In recent years, improved knowledge of the oncogenic processes and signaling pathways that regulate tumor cell proliferation, differentiation, angiogenesis, invasion, and metastasis has contributed to the identification of several potential therapeutic targets, which has driven the development of molecularly targeted therapies. Herein, we identified PP4R1 as an essential player in HCC cell growth *in vitro*, which might serve as a potential therapeutic target in HCC.

PP4 is an essential protein for nucleation, growth, and stabilization of microtubules, centrosomes/spindle bodies during cell division.¹⁹ In this study, we found that knockdown of PP4R1 by lentivirus-mediated stable gene silencing in HepG2 cells and SMMC-7721 cells caused a significant reduction in cell viability and proliferation.

A previous study showed that silencing of PP4C in HEK293 cells led to cell cycle arrest at the M phase, with some cells displaying aberrant chromosomal organization and loss of microtubules near the centrosomes.²⁰ Thus, to examine whether PP4R1 knockdown affects cell cycle progression, we conducted flow cytometry assay in HepG2 cells and SMMC-7721 cells, and found PP4R1 silencing caused a block at G₂/M phase of the cell cycle, which was similar to the outcome of PP4C deficiency. Furthermore, transduction

of Lv-shPP4R1 into HepG2 cells led to inactivation of two major MAPK signaling cascades: p38 and JNK, indicating that the p38 and JNK pathways might be involved in the inhibition of cell growth by PP4R1. Our ongoing study should further validate the antiapoptosis role of PP4R1 in HCC cells. Besides, it has been suggested that PP4R1 targeted TRAF2 and TRAF6 to mediate the inhibition of the NF- κ B pathway.¹⁷ In our study, we found p65 was not expressed in the nucleus and PP4R1 knockdown downregulated the expression of p65, which suggested that the NF- κ B pathway might have no effect on the cell growth inhibition by PP4R1 in HCC cells.

In conclusion, this is the first report on the involvement of PP4R1 in HCC. Moreover, the inhibition of HCC cell growth by PP4R1 silencing could be linked with the induction of cell cycle arrest as well as apoptosis. Our findings have led us to believe that further research on PP4R1 will increase the understanding of the pathological mechanism of HCC.

Disclosure

The authors report no conflicts of interest in this work.

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