Targeting PPM1D by lentivirus-mediated RNA interference inhibits the tumorigenicity of bladder cancer cells

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Abstract

Protein phosphatase magnesium/manganese-dependent 1D (PPM1D) is a p53-induced phosphatase that functions as a negative regulator of stress response pathways and has oncogenic properties. However, the functional role of PPM1D in bladder cancer (BC) remains largely unknown. In the present study, lentivirus vectors carrying small hairpin RNA (shRNA) targeting PPM1D were used to explore the effects of PPM1D knockdown on BC cell proliferation and tumorigenesis. shRNA-mediated knockdown of PPM1D significantly inhibited cell growth and colony forming ability in the BC cell lines 5637 and T24. Flow cytometric analysis showed that PPM1D silencing increased the proportion of cells in the G0/G1 phase. Downregulation of PPM1D also inhibited 5637 cell tumorigenicity in nude mice. The results of the present study suggest that PPM1D plays a potentially important role in BC tumorigenicity, and lentivirus-mediated delivery of shRNA against PPM1D might be a promising therapeutic strategy for the treatment of BC.

Key words: Protein phosphatase magnesium/manganese-dependent 1D; Bladder cancer; Gene silencing; RNA interference; Proliferation

Introduction

In 2012, bladder cancer (BC) was the fourth most common cancer in males and the eighth most common in females in the United States (1). Approximately 20% of tumor node metastasis (TNM) stage T1 primary tumors that undergo re-resection progress to invasive BC (2,3). In addition, overexpression of p53, p21, and p16 is associated with increased risk of recurrence and poor long-term survival in BC, suggesting that targeted treatment in the early stages of the disease could be a useful strategy (4,5).

Protein phosphatase magnesium/manganese-dependent 1D (PPM1D), also called wild-type p53-induced phosphatase (Wip1), is a member of the magnesium-dependent serine/threonine protein phosphatase (PPM) family (6,7). It was first identified as a phosphatase induced by p53 in response to ultraviolet and ionizing radiation (8). The PPM1D gene is located on chromosome 17q23.2 and is a negative regulator of stress response pathways. PPM1D plays a variety of roles in cellular processes, including abrogation of cell cycle checkpoints and inhibition of senescence, apoptosis, and DNA repair (9). Studies have shown that PPM1D possesses oncogenic properties (10,11). Amplified levels of the PPM1D gene have been found in several cancer cell lines including neuroblastoma and lung, breast, pancreatic, bladder, and liver cancers (11,12). Moreover, PPM1D is overexpressed in a number of human primary tumors, such as breast cancer (13), ovarian cancer (14,15), neuroblastoma (16), hepatocellular cancer (17) and lung cancer (18), and is associated with poor prognosis.

RNA interference (RNAi) is an endogenous protein suppression mechanism by which short double-stranded RNA (dsRNA) mediates sequence-specific degradation of mRNA, thereby preventing translation of the protein encoded by the target mRNA (19,20). RNAi can be used to specifically target mutant genes, cancer-associated genes or receptors involved in oncogenic pathways, thereby opening new avenues in anticancer therapy (21,22). RNAi has been successfully used to control cell proliferation and the invasive ability of BC cells (23).

To elucidate the role of PPM1D in BC, we used lentivirus-delivered shRNA to knock down PPM1D expression. This
model system was used to examine the effect of PPM1D silencing on BC cell proliferation and growth and the antitumor potential of PPM1D shRNA in vivo and in vitro.

Material and Methods

Cells lines and cell culture

The human urinary BC cell lines 5637 and T24 and the human renal epithelial cell line HEK293T were purchased from the American Type Culture Collection (USA) and maintained at 37°C and 5% CO2. The HEK293T and T24 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), and the 5637 cell line was cultured in RPMI-1640 (Invitrogen) supplemented with 10% FBS.

Lentiviral plasmid construction, lentivirus production, and cell infection

The human PPM1D (Gen-Bank accession no. NM_003620.3) specific small interfering RNA (siRNA) sequence, which was designed with online software from Invitrogen, was 5’-CCCTTCTCGTGTGCTTTAAA-3’. The nonsilencing (NS) sequence (5’-TTCTCCGAACGTGTACGC-3’) was used as a scrambled control (24). Pairs of complementary oligonucleotides with these sequences were synthesized, annealed, and ligated into a linearized pGCSIL-GFP plasmid vector. These plasmids were amplified in E. coli DH5 and purified using a QIAGEN Plasmid Maxi Kit (Qiagen, The Netherlands). Lentivirus was generated in 293T cells by cotransfection of the recombinant pGCSIL-GFP vector, together with pHelper 1.0 and pH helper 2.0 plasmids using Lipofectamine 2000 (Invitrogen). The lentiviral particles were harvested 48 h after transfection and purified by ultracentrifugation (2 h at 50,000 g) (25), and are hereafter referred to as Lv-si-PPM1D (a specific interference construct for PPM1D) or Lv-si-CTRL (negative control). For cell infection, 30% confluent 5637 and T24 cells were incubated with lentiviruses for 48 h, and the medium, which contained puromycin (10 μg/mL; Sigma-Aldrich, USA), was replaced to select stable clones. Each cell line was divided into two experimental groups. A total of 5

Quantitative real-time polymerase chain reaction (PCR) and Western blotting

Total RNA was extracted and reverse-transcribed as described previously (26). Quantitative real-time PCR reactions were carried out with an ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, USA) using 25 μL of a reaction mixture that consisted of 0.1 μM primers, 10 μL 2 x SYBR Premix Ex Taq (Takara, Japan), and 20-100 ng cDNA sample. The following primers were used: PPM1D, 5’-AGAGAATGTCCAAGGCTAGTC

Flow cytometric assay

Cells were harvested and fixed with cold 70% ethanol for 1 h. The cells were sequentially centrifuged (5 min at 100 g) and resuspended with phosphate-buffered saline (PBS). Cells were stained with propidium iodide (Sigma-Aldrich) at 4°C for 30 min in the dark and analyzed using flow cytometry. Each experiment was performed in triplicate.

Animal experiments

Five-week-old male BALB/c mice were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (China) and received humane care in compliance with the Guidelines for the Care and Use of Experimental Animals in Research. Mice were divided into 2 groups of 10 mice each, referred to as the si-CTRL and si-PPM1D groups. A total of 5 x 10^6 Lv-si-CTRL or Lv-si-PPM1D infected cells were suspended in Eagle’s minimal essential medium (EMEM) and injected subcutaneously into the right flank of each mouse in the
corresponding group. The tumor diameter was measured, and the volume was calculated using the formula $V = 0.4 \times a \times b^2$ (V = volume, $a$ = largest diameter, $b$ = smallest diameter) on days 10, 14, 18, and 24. Mice were photographed and humanely killed on day 24, and the tumors were dissected and weighed.

**Statistical analysis**

All data are reported as means ± SE. Statistical analysis was performed using the Student two-tailed unpaired $t$-test for comparisons between two groups. In all cases, $P < 0.05$ was considered to be statistically significant.

**Results**

**Lentivirus-mediated shRNA inhibited the expression of PPM1D in BC cells**

The 5637 and T24 cell lines were infected with Lv-si-PPM1D; the highest infection efficiency was ≈90%, as determined by detecting the expression of green fluorescent protein (GFP) 96 h after infection (Figure 1A). Quantitative real-time PCR analysis showed that the PPM1D mRNA level was significantly lower in the si-PPM1D group compared to the si-CTRL group (Figure 1B). The protein level of PPM1D in the si-PPM1D group was also strongly decreased compared with the si-CTRL group (Figure 1C).

**Knockdown of PPM1D inhibited BC cell growth**

To examine the effect of PPM1D knockdown on BC cell growth, Lv-si-PPM1D- or Lv-si-CTRL-infected 5637 and T24 cells were subjected to MTT and colony formation assays. As shown in Figure 2, cell proliferation in the si-PPM1D group was significantly inhibited compared with that in the si-CTRL group. Colony formation ability was significantly lower in the si-PPM1D group compared to the si-CTRL group (Figure 3). Similar results were obtained in 5637 and T24 cells.

Flow cytometric analysis showed that the proportion of cells in the G1 phase was markedly increased in the si-PPM1D group compared with the si-CTRL group (Figure 4), partly explaining the growth suppression mediated by Lv-si-PPM1D.

**Knockdown of PPM1D inhibited BC tumorigenicity in vivo**

The 5637 cells infected with Lv-si-CTRL or Lv-si-PPM1D were subcutaneously implanted into nude mice to examine
the effect of PPM1D knockdown on BC tumorigenicity in vivo. All of the mice in the si-CTRL group displayed steadily and progressively growing tumors, whereas the BC cells in the si-PPM1D group showed weaker tumorigenicity, and the mice developed smaller tumors (Figure 5).

**Discussion**

Gene dysregulation is frequently observed in cancer, and gene expression profiles vary among different cancers. PPM1D or Wip1 is a serine/threonine phosphatase that is overexpressed and shows oncogenic activity in multiple human cancers (10). However, the role of the PPM1D gene in BC has not been investigated to date. To elucidate the function of PPM1D in BC, we used lentivirus-mediated RNAi to inhibit PPM1D expression in T24 and 5637 cells and investigated the effects of PPM1D knockdown in these BC cell lines. A lentiviral vector carrying PPM1D shRNA and a GFP reporter gene was constructed, which showed high infection efficiency in 5637 and T24 cells and effectively silenced PPM1D expression. These results indicated the successful construction of an effective shRNA vector targeting the PPM1D gene.

Lentivirus-mediated PPM1D silencing strongly inhibited the growth and proliferation of T24 and 5637 BC cells in vitro, as demonstrated by MTT and colony formation assays. The data showed that the role of PPM1D in BC was consistent with that in other cancers (16,27-29). Flow cytometric cell cycle analysis showed that PPM1D knockdown increased the proportion of T24 and 5637 BC cells in the G0/G1 phase, indicating that PPM1D downregulation blocked cell cycle progression. This could be a mechanism by which PPM1D silencing suppresses proliferation.

To determine the therapeutic value of lentivirus-mediated RNAi of PPM1D for BC treatment, we analyzed its effect in a xenograft model. The results showed that sh-PPM1D lentivirus inhibited 5637 BC cell proliferation and suppressed their tumorigenic potential, indicating that targeting PPM1D may be a potential therapeutic strategy for the treatment of BC.

Previous studies have shown that PPM1D promotes tumorigenesis in a p53-dependent manner. PPM1D is induced by p53 in response to various environmental stresses and facilitates the return of cells to the pre-stress state (6). In addition, PPM1D inhibits p53 activity by directly dephosphorylating p53 or its regulators such as ATM, Chk1, and Chk2, which indirectly inhibit p53 activity (10,30). However, T24 and 5637 BC cells have p53 mutations, which implies that PPM1D may function in a p53-independent manner in BC cells. PPM1D is a target of p53 and other transcription factors, including the estrogen receptor-α and nuclear factor-κB (NF-κB) (9). The p38

![Figure 4](image-url) - Knockdown of PPM1D increases the proportion of 5637 cells in G1 phase. Cell cycle distribution was analyzed by flow cytometry. A, Representative images of three independent FACS analyses are shown. B, Proportion of cells in the different cell cycle phases. Data are reported as means±SD of three independent experiments. **P<0.01, compared to si-CTRL (t-test).

![Figure 5](image-url) - Knockdown of PPM1D inhibited the tumorigenicity of bladder cancer cells in vivo. Representative photographs of nude mice (A) and tumors (B) dissected from nude mice 24 days after injection of lentivirus-infected 5637 cells. C, Tumor volumes were recorded on days 10, 14, 18, and 24. D, Mice were humanely killed and tumors were weighed on day 24. **P<0.01, compared to si-CTRL (t-test).
mitogen-activated protein kinase (MAPK) (31) and Akt (28) signaling pathways may be downstream mediators of PPM1D activity in BC. Further studies are required to distinguish these mechanisms. In conclusion, the results of the present study provided evidence that PPM1D plays a potentially important role in BC tumorigenicity and could be a promising target for therapeutic intervention.

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