Disruption of serine/threonine protein phosphatase 5 inhibits tumorigenesis of urinary bladder cancer cells

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Abstract. Serine/threonine protein phosphatase 5 (PPP5C) is a member of the protein serine/threonine phosphatase family and has been shown to participate in multiple signaling cascades and tumor progression. We found that PPP5C was highly expressed in bladder cancer tissues compared to normal urothelial tissues, and positively correlated to tumor stages through ONCOMINE microarray data mining. Knockdown of PPP5C via a lentivirus-mediated short hairpin RNA (shRNA) markedly inhibited cell proliferation and colony formation. Flow cytometric analysis showed that PPP5C-deficient T24 and BT5637 bladder cancer cells were arrested in G0/G1 phase and induced apoptosis. In addition, tumor growth was inhibited in vivo in a xenograft nude mouse model. Further studies indicated that knockdown of PPP5C downregulated c-myc and CDK4, whereas upregulated p27, BAD and Beclin1. These results suggest that PPP5C is associated with bladder cancer (BCa) and plays an oncogenic role in the development and progression of bladder cancer.

Introduction
Bladder cancer (BCa) is a common malignant tumor in the urinary tract, ranking the first in urologic tumors and twelfth in all cancers among the Chinese populations (1). BCa is diagnosed yearly in estimated 429,800 patients, of whom about 160,000 succumb to death (2). Other than age, a series of environmental factors contributes to the onset of BCa, which emphasizes the importance of prevention of the disease. Besides, because of the recurrent feature of bladder cancer needing repeated treatment and lifetime surveillance, thus imposing tremendous economic burdens on both society and families. It is therefore urgent to identify molecular candidates involved in the disease and targets for gene therapy.

Protein phosphorylation and dephosphorylation not only affect cell life in multiple ways but play an important part during cancer progression (3). Serine/threonine protein phosphatase 5 (PPP5C) as a member of the protein serine/threonine phosphatase family is ubiquitously expressed in mammalian cells (4). Several studies have demonstrated that PPP5C is associated with human cancers, among which liver and breast cancers are the most studied (5,6). Moreover, constitutive expression of PPP5C also accelerates tumor growth in mice (7). Despite the discovery of the role of PPP5C in the development and progression of other cancers, whether PPP5C participates in BCa remains unknown.

As a powerful tool in identifying the function of candidate molecules, RNA interference (RNAi) system has been utilized in cancer gene therapy (8,9). Thus, the present study explored the biological function of PPP5C in bladder cancer using ONCOMINE microarray datasets analysis and lentivirus-mediated shRNA system.

Materials and methods
Cell culture. Human bladder cancer cell lines T24 and BT5637, and human embryonic kidney cell line 293T were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). BT5637 cells were cultured in RPMI-1640 medium (HyClone Laboratories, Inc., Logan, Utah, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The T24 cell line was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.
UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). T-24 cells were cultured in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Gibco). 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories) supplemented with 10% FBS. Cells were maintained at 37°C in a humidified incubator with a constant air flow of 5% CO₂.

Construction of lentivirus. Small interfering RNA (siRNA) targeting PPP5C (NC_000019.10) (5'-GAGACAGAGAGAT TACAGTACTCGGATCAGTACTTCTCTGTCTCTTT TT-3') and the non-silencing control sequence (5'-CTAG CCCCCTTTCTCCGAACGGTGTCACGTATCTCGGAGATAC GTGACACGTCCGGAGATTTTTTAT3') was transfected into short hairpin RNA (shRNA) and cloned into pFH-L green fluorescent protein (GFP) vector (Shanghai Hollybio, Shanghai, China) by double digestion with NheI and PacI. The generated plasmid shPPP5C-pFH-L or shCon-pFH-L, together with two pHelper plasmids pSVVG-I and pCMV.R8.92 (Shanghai Hollybio) was transfected into 293T cells. After 72-h transfection, the lentiviruses (Lv-shPPP5C or Lv-shCon) in the culture medium were collected and purified by ultracentrifugation and the titer of each lentivirus was determined.

Cell infection of lentivirus. T24 and BT5637 cells were seeded in 6-well plates with a concentration of 5x10⁴ and 4x10⁴ respectively, and then infected with lentivirus Lv-shPPP5C or Lv-shCon at a multiplicity of infection (MOI) of 25 and 35, respectively. The percentage of GFP-positive cells represented the infection efficiency.

Quantitative real-time PCR. After 120-h infection with lentivirus, T24 and BT5637 cells were collected and total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, 2 µg total RNA was reverse transcribed with Oligo (dT) primer and the M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. The complementary DNA was used for real-time PCR and a final volume of 20 µl was adopted as follows: 10 µl 2X SYBR Premix Ex Taq (Takara, Dalian, China), 0.8 µl forward and reverse primers, 2.5 µM, 5 µl cDNA, 4.2 µl ddH₂O. PCR and data collection were performed on CFX Connect™ Real-Time PCR detection system (Takara) and data analysis was operated with the 2ΔΔCT method normalized to the endogenous control β-actin. Primers used in real-time PCR are as follows:

β-actin forward, GTGGACATCCGCAAAGAC and β-actin reverse, AAAGGGTGTAACGCAACTA; PPP5C forward, GGTGAGGTGAAGGCCAAGTA and PPP5C reverse, TGT GGATCTGAGCAGCGAG.

Western blot assay. One hundred and twenty hours after infection, T24 and BT5637 cells were collected and lysed in 2X SDS lysis buffer (100 mM Tris-HCl, pH 6.8, 10 mM EDTA, 4% SDS, 10% glycerine). Cell lysates were separated on SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in TBST buffer with 5% milk. Then, the membranes were incubated with the following primary antibodies: PPP5C (#ab105752; Abcam, Cambridge, UK), β-actin (A3853; Sigma-Aldrich) and GAPDH (2118; Cell Signaling Technology, Danvers, MA, USA) and detected at 595 nm using a microplate reader. Each group had five duplicate and the experiment was performed in triplicate.

Colony formation assay. Five hundred T24 cells and BT5637 cells were seeded in 6-well plates and infected with the indicated lentivirus for 72 h. The supernatant was then discarded and culture medium was replaced every 3 days. The cells were cultured for another 8 days, fixed with 4% paraformaldehyde and stained with purple crystals. The number of colonies was analyzed statistically. The experiment was performed three times and each group had three repetitions.

Cell cycle analysis. T24 (1x10⁵) and BT5637 cells were seeded in 6-cm dishes and infected with Lv-shCon and Lv-shPPP5C lentivirus for 3 days, followed by 40-h culture without the virus. The cells were then collected and fixed with 75% ethanol for 24 h at 4°C. Propidium iodide (20 mg/ml) (PI; Sigma-Aldrich) was used to treat the cells and DNA content was recorded with a flow cytometer (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The experiment was performed in triplicate.

Cell apoptosis analysis. T24 (1x10⁵) and BT5637 cells were cultured in 6-cm dishes and infected with Lv-shCon and Lv-shPPP5C lentiviruses for 3 days. Then, the cells were reseeded on 6-cm dishes. After 3 days, Annexin V/PI (BD Biosciences) double staining was performed to analyze cell apoptosis by flow cytometry. Each experiment was repeated at least three times.

Animal experiments. Twelve six-week-old nude mice were randomly divided into two groups and then injected subcutaneously in the right flank with 5x10⁴ T24 cells pre-infected with Lv-shCon and Lv-shPPP5C lentiviruses. Twenty days later, the tumor size was measured every three days and calculated using the formula: (length x width²)/2. At the end of the experiment, the mice were euthanized and the tumors were removed from each nude mouse, imaged and weighed.
Bioinformatic analysis of the ONCOMINE microarray datasets. The expression level of PPP5C in BCa was analyzed using the ONCOMINE microarray datasets (www.oncomine.org), which contain a great deal of public and published microarray datasets with tens of thousands of samples. By using ‘PPP5C’, ‘BCa’ and ‘mRNA’ as the search terms, 17 datasets were acquired, of which 3 datasets with 7 subunits included PPP5C expression in BCa and normal urothelial tissues. Then, a meta-analysis was carried out to compare the level of the PPP5C between the BCa and the normal tissue. Besides, we compared the expression of the PPP5C from T stage and grade malignancy of the BCa in the Blaveri Bladder 2 and Dyrskjot Bladder datasets. Information on survival time in ALS Bladder was obtained for survival analysis. All data are reported log2 Median-Centered intensity in the ONCOMINE microarray datasets.

Statistical analysis. Data are presented as mean ± SD from at least three experiments. Statistical comparison between the Lv-shCon and Lv-shPPP5C was performed using Student’s t-test. The PPP5C expression and patient characteristics of the BCa were analyzed using the Chi-square test in the BCa tissue microarray. In addition, the Kaplan-Meier analysis was used to evaluate the relation between the prognosis and the PPP5C expression in ALS microarray datasets. The SPSS version 19 (IBM Corp., Armonk, NY, USA) was used to calculate the statistics and P<0.05 was considered statistically significant.

Results

PPP5C expression of the bladder cancer on the ONCOMINE microarray datasets. To evaluate the expression level of the PPP5C in BCa, the public online ONCOMINE microarray database was used to perform meta-analysis for evaluation of PPP5C expression in BCa. A total of 3 datasets including 7 groups comparing PPP5C expression between the BCa and normal urothelial tissue were included in this study, which comprised 343 BCa and 130 normal urothelial tissues. As shown in Fig. 1B, the expression level of PPP5C in BCa was higher than that in the normal tissue in all three datasets. Besides, the results of the meta-analysis of the 7 groups in the 3 datasets also support that the PPP5C was overexpressed in BCa (Fig. 1A), and the difference was statistically significant between BCa and normal urothelial tissues. Furthermore, the correlation between PPP5C expression and T stage and malignant grade of BCa was analyzed. It was found that PPP5C was highly expressed in T stages ≥T1 compared to that in Ta of BCa (Fig. 1D and E), and in high-grade BCa than that in low-grade BCa (Fig. 1F). Patients with low-level PPP5C expression indicated a better prognosis in ALS Bladder microarray dataset (Table I). Similarly, Kaplan-Meier survival analysis of ALS dataset showed that overall survival (OS) was poor in patients with upgraded PPP5C (Fig. 1C).

Table I. PPP5C expression and patient characteristics of ALS Bladder dataset.

|                | No. of patients | PPP5C |          |          | P-value |
|----------------|-----------------|-------|----------|----------|---------|
| Overall, n (%) | 30 (100.0)      | 15    | 15       |          | 0.277<sup>a</sup> |
| Mean patient age, years (range) | 60.4±7.3 | 58.9±6.8 | 61.9±7.7 |          | 1.000<sup>b</sup> |
| Bergkvist grade, n (%) |       |       |          |          |         |
| Grade 1 and 2 | 3               | 1     | 2        |          | 1.000<sup>b</sup> |
| Grade 3 and 4 | 27              | 14    | 13       |          |         |
| T stage, n (%) | | | |          |         |
| ≤T2b           | 3               | 2     | 1        |          | 0.466<sup>b</sup> |
| ≥T3a           | 27              | 13    | 14       |          |         |
| M stage, n (%) | | | |          |         |
| M0             | 15              | 9     | 6        |          | 0.042<sup>b</sup> |
| M1             | 15              | 6     | 9        |          |         |
| Overall survival status, n (%) |      |       |          |          |         |
| Alive          | 5               | 5     | 0        |          |         |
| Dead           | 25              | 10    | 15       |          |         |

<sup>a</sup>Kruskal-Wallis test; <sup>b</sup>Chi-square test.

Lentivirus-mediated interference downregulates PPP5C expression in bladder cancer cells. To determine the role of PPP5C in BCa, a lentivirus-mediated shRNA system was performed using Student’s t-test. The PPP5C expression and patient characteristics of the BCa were analyzed using the Chi-square test in the BCa tissue microarray. In addition, the Kaplan-Meier analysis was used to evaluate the relation between the prognosis and the PPP5C expression in ALS microarray datasets. The SPSS version 19 (IBM Corp., Armonk, NY, USA) was used to calculate the statistics and P<0.05 was considered statistically significant.
downregulated the endogenous PPP5C expression in urinary BCa cell lines.

**Lentivirus-mediated knockdown of PPP5C suppresses the viability and proliferation of bladder cancer cells in vitro.** To identify whether PPP5C played a role in tumorigenesis, MTT and colony formation assay were adopted. Compared with Lv-shCon-infected T24 cells, the viability of cells infected with Lv-PPP5C and Lv-shPPP5C was significantly reduced (Fig. 3A and B).

To further elucidate the role of PPP5C in cancer cell proliferation, colonies formed in T24 cells after PPP5C knockdown were analyzed. As shown in Fig. 3C, colony formation in Lv-shPPP5C-infected cells was obviously impaired with respect to morphology and quantity, as compared with Lv-shCon-infected cells. In addition, the number of colonies...
in Lv-shPPP5C-infected cells was reduced by nearly 50% (Fig. 3E). Similar result was also observed in BT5637 cells (Fig. 3D and F). In summary, our data demonstrate that PPP5C expression was of importance for the viability and proliferation of BCa cells.

Knockdown of PPP5C inhibits bladder cancer cell growth in vivo. To further validate the oncogenic function of PPP5C in vivo, Lv-shPPP5C-infected and Lv-shCon-infected T24 cells were injected subcutaneously to the six-week-old nude mice, and the tumor volume and weight were measured after tumor formation. As shown in Fig. 4, tumor size and weight in Lv-shPPP5C-infected mice were significantly reduced as compared with those in mice treated with Lv-shCon-infection. These results further support that PPP5C functions as an oncogene in vivo.

Disruption of PPP5C expression leads to cell cycle arrest of the bladder cancer cells. Tumorigenesis largely depends on accelerated or at least normal cell cycle of cancer cells. Knowing that PPP5C played an important role in BCa cell proliferation, flow cytometric analysis of cell cycle distribution through PI staining to see whether PPP5C participated in the regulation of cancer cell cycle showed that after infection with Lv-shPPP5C, T24 cells were arrested in G0/G1 phase compared with Lv-shCon cells (65.61±0.86 vs. 56.49±0.57%), and the number of cells in G2/M phase was decreased (11.54±0.24 vs. 19.37±0.49%) (Fig. 5A and B). Similarly, the proportion of BT5637 cells in G1/G0 phase in Lv-shPPP5C group was increased (74.6±0.88 vs. 67.66±1.74%) and the number of cells in G2/M phase was reduced significantly (4.55±2.28 vs. 10.08±2.03%). These findings suggest that knockdown of PPP5C could cause T24 and BT5637 cell cycle arrest in G0/G1 phase.

Downregulation of PPP5C expression induces apoptosis of the BCa cells. To further confirm the effect of PPP5C on BCa cell apoptosis, flow cytometric analysis through Annexin V/PI
double staining was performed in T24 and BT5637 cells after lentivirus infection. The four domains divided by Annexin V and PI plots represent the different states of cells. Viable cells were located in Annexin V-/PI-, necrotic cells in Annexin V-/PI+, early apoptotic cells in Annexin V+/PI-, and late apoptotic cells in Annexin V+/PI+ (Fig. 6A). As shown in Fig. 6B, early and late apoptosis were increased significantly in lv-shPPP5C as compared with lv-shCon both in T24 and BT5637 cells. Above all, PPP5C silencing promoted apoptosis of BCa cells.

shRNA-mediated depression of PPP5C regulates the protein level of CDK4, c-Myc, p27, BAD and Beclin1. To further clarify the mechanism of the PPP5C in regulating the biological function of the bladder cancer, the expression of downstream signaling molecule were analyzed by western blot analysis. The level of CDK4 and c-Myc was decreased whereas the level of p27, BAD and Beclin1 was increased (Fig. 7). These results provide evidence that interfering with the expression of PPP5C could regulate apoptosis, cell cycle and other biological functions.

Discussion

Compared with other solid cancers, much less is known about the molecular mechanism of BCa. Despite a variety of recent genome-wide profiling studies, there is still no valid strategy for early diagnosis or therapeutic intervention of BCa (10-12). Therefore, the confirmation of critical molecular signatures is badly needed for the effective treatment of the malignancy.
Figure 4. Knock-down of the PPP5C level inhibits the growth of BCa in vivo. (A and C) The subcutaneous xenograft nude mice and the tumor sample were collected and imaged. (B and D) The tumor size and weight were significantly decreased in nude mice infected with lv-shPPP5C-T24 cell (n=6) as compared with those in nude mice infected with lv-shCon-T24 (n=6) (*P<0.05, **P<0.01, ***P<0.001).

Figure 5. Disruption of PPP5C expression causes cell cycle arrest in bladder cancer cells. (A) Flow cytometric analysis showing cell cycle distribution of T24 and BT5637 cells infected by Lv-shCon or Lv-shPPP5C. (B) Cell percentages in G0/G1, S and G2/M of T24 and BT5637 cells treated as in (A) were analyzed statistically (*P<0.05, **P<0.01, ***P<0.001).
PPP5C is a ubiquitously-expressed protein with only one form rather than two or more isoforms of other phosphoprotein phosphatase members (13,14). The biological function of PPP5C remains largely unknown. Some studies have reported its role in cancer development. Elevation of PPP5C has been found both in human cancer tissues and human breast cancer cell line MCF-7 (5). PPP5C is also associated with other cancers such as liver cancer and several other cellular signaling cascades (6,14,15). However, few studies have focused on the role of PPP5C in BCa progression. Given the severe consequences and economic burden brought by BCa, we aimed to find out whether PPP5C was its candidate biomarker and provide a novel therapeutic target for BCa.

The ONCOMINE microarray datasets is a cancer microarray database and integrated data-mining platform (16) that can be used to analyze differential expressions between cancer and paired normal tissues. Besides, the relation between gene expression and various clinical data with respect to pathology, prognosis, metastasis and drug resistance can also be analyzed using the ONCOMINE microarray dataset. Increased numbers of studies have begun utilizing the datasets to validate and explore their research results (17-19). Using the ONCOMINE microarray dataset, the present study found that PPP5C was upregulated in BCa, which was correlated with T stage, malignant grade and survival of BCa patients (Fig. 1).

Figure 6. Apoptotic cells are increased after PPP5C silencing. (A) Apoptosis of T24 and BT5637 was analyzed in lv-shCon and lv-shPPP5C groups using flow cytometry and Annexin V/PI staining. (B) Early apoptosis (V−/PI−) and late apoptosis (V+/PI+) of the T24 and BT5637 cells were statistically analyzed (*P<0.05, **P<0.01, ***P<0.001).

Figure 7. The protein level transformation by western blot analysis. As shown CDK4 and c-Myc were decreased, and BAD, Beclin1 and § were increased after infection with lv-shPPP5C vs. lv-shCon. GAPDH was used as loading control.
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In conclusion, we demonstrated that lentivirus-mediated shRNA targeting PP5C played a crucial role in viability, proliferation, apoptosis and migration of BCa cells, suggesting that PPP5C may prove to be a potential therapeutic target for the treatment of urinary BCa.

Acknowledgements

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