Abstract. Protein phosphatase magnesium-dependent 1 delta (PPM1D) is involved in several types of cancer. The current study examined the role of PPM1D expression in prostate cancer (PCa) tissues and in PCa cell lines. Expression of PPM1D was evaluated using immunohistochemistry in 234 PCa tissues after radical prostatectomy and 80 benign prostatic hyperplasia (BPH) tissues. The associations of PPM1D expression with clinicopathological parameters and survival were analyzed. In vitro, tumor cells were transfected with small interfering RNA targeting PPM1D (siPPM1D) or si-Scramble, and the cell proliferation, migration and invasion were determined. We found that PPM1D expression was significantly higher in PCa tissues than that in BPH tissues. PPM1D expression was positively correlated with Gleason score (p=0.022), T stage (p=0.015) and lymph node status (p=0.016). Kaplan–Meier curve analysis showed that patients with positive PPM1D expression had shorter biochemical recurrence-free survival and overall survival. Furthermore, multivariate analyses showed that PPM1D expression was an independent predictor of both biochemical recurrence-free (hazard ratio=3.437, 95% confidence interval=1.154-6.209, p=0.016) and overall survival (hazard ratio=5.026, 95% confidence interval=2.545-8.109, p=0.007). Knockdown of PPM1D inhibited the proliferation, migration and invasion capabilities of PC-3 and LNCaP cells. PPM1D expression may predict for both overall and biochemical recurrence-free survival in patients after radical prostatectomy for PCa. Elevated PPM1D expression plays a key role in progression of PCa.

PPM1D as a Novel Biomarker for Prostate Cancer After Radical Prostatectomy

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Key Words: PPM1D, prostate cancer, biomarker, prognosis.

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Materials and Methods

Patients and prostate specimens. A total of 234 PCa samples were collected from patients with an average age of 64.5 years (50-86 years) at the Department of Urology, Shanghai Hospital, Second Military Medical University, China. All patients underwent radical prostatectomy from January 1997 to January 2011. The control group with BPH comprised of 80 age-matched patients, with an average age of 66.4 years (58-78 years), who were recruited during the same period. All slices from tumor samples were collected from areas of invasive adenocarcinoma and pathologically-identified according to hematoxylin and eosin staining. The tumor grade and clinical stage of these samples were assessed based on the 2002 TNM classification and Gleason system.

Follow-up. All patients were scheduled to have their serum prostate-specific antigen (PSA) level evaluated postoperatively every three months for the first year, every six months from the second to the fifth year and annually thereafter. Follow-up data were obtained by consulting the hospital medical records and the PCa database of our Department and by contacting the patients or their family members. Biochemical recurrence was defined as the sustained elevation of the serum total PSA level above 0.2 ng/ml on two or more occasions, and the date of biochemical recurrence was assigned as the date of the first value greater than 0.2 ng/ml. Follow-up time ranged from 3 to 176 months. This study was approved by the Second Military Medical University Ethical Committee (EC108-32).

Immunohistochemistry. Tissue slices (4 μm) were de-paraffinized and incubated in water with H₂O₂ for 30 min to remove endogenous peroxidase activity. The sections were then immersed in 10 mM citrate buffer (pH=6) and heated in a microwave oven for 30 min. Sections were blocked with 5% normal goat serum in phosphate-buffered saline for 1 h and then incubated overnight with antibody to PPM1D (Santa Cruz, CA, USA) at 4˚C. For the negative control, 5% normal goat serum was used to replace the primary antibody. Staining was detected using a standard avidin–biotin–peroxidase complex followed by hematoxylin counterstaining. Positive staining was defined as the presence of brown oxidized 3,3′-diaminobenzidine (DAB) in the given cellular compartments without background signal. The immunostaining scores of PPM1D were evaluated under a light microscope by two pathologists who were unaware of patients’ clinical information and determined using a combined scoring system based on the sum of the staining intensity and the percentage of positively stained cells. Scores from 0-3 were given in terms of the staining intensity and the percentage of positively stained cells as follows: score 0, no staining or fewer than 10% of the tumor cells; score 1+, weak staining in 10% or more of the tumor cells; score 2+, moderate staining in 10%- or more of the tumor cells; and score 3+, strong staining in 10% or more of the tumor cells. Scores 0 and 1+ were considered negative for PPM1D expression, while scores 2+ and 3+ were considered positive for PPM1D expression.

Cell culture and siRNA transfection. Human PCa cell lines PC-3 and LNCaP were obtained from the American Type Culture Collection (ATCC) repository (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone, USA) at 37°C in an incubator with 5% CO₂. PPM1D-specific siRNA and non-specific control siRNA (both from Invitrogen, USA) were transfected into PC-3 and LNCaP cells with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions.

Western blot. Total protein was isolated from PCa cells. Equal amounts of protein samples (60 μg) were subjected to a 10% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, USA). The membranes were blocked in 5% non-fat milk and then were incubated with primary antibody (anti-PPM1D, 1:1000; Abcam, CA, USA) or anti-GAPDH (1:2000; Santa Cruz, CA, USA) at 4°C overnight. The membranes were washed and incubated with specific peroxidase-conjugated secondary antibodies. Proteins were detected using the ECL system (Santa Cruz, CA, USA).

Cell vitality, invasion and migration assay. Cells were seeded into a 96-well plate at a density of 4000 cells per well and cultured for 5 days. Cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (Sigma-Aldrich, IL, USA) according to the manufacturer’s protocol. Cell migration and invasion ability were measured by transwell assay. Subsequently, 1×10⁴ transfected cells were plated in the upper chambers of the transwell (Santa Cruz, CA, USA) in fetal bovine serum-free medium and 10% fetal bovine serum-containing medium was deposited in the lower chambers. For cell invasion assays, transwell membranes were coated with matrigel (BD Biosciences, NJ, USA) before plating cells. After 24 h, cell were removed from the upper chambers, whereas cells on the lower chambers were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Five independent fields for each well were counted and photographed by microscope.

Statistical analysis. Pearson’s χ² test was used to analyze the association between PPM1D expression and clinicopathological parameters. Survival and PSA recurrence curves were evaluated using the Kaplan–Meier method, and the log-rank test. All clinical parameters were analyzed with univariate and multivariate Cox proportional hazards models. Data were analyzed with analysis of variance among three groups. All statistical analyses were performed using the SPSS16.0. A value of p<0.05 was considered significant.

Results

Correlations of PPM1D expression with clinical parameters in PCa. To investigate PPM1D protein expression levels in prostate tissues, 234 PCa patient samples and 80 age-matched control samples (BPH) were evaluated by PPM1D-specific immunohistochemical staining. As shown in Figure 1, results showed that 132 out of 234 PCa tissues (56.4%) were classified as PPM1D-positive. In contrast, 76 out of 80 (95%) BPH tissues were classified as PPM1D-negative. The PPM1D protein was observed predominately in the cytoplasm of PCa cells.

In addition, the associations between PPM1D expression and clinical and prognostic parameters were also analyzed. As shown in Table I, we revealed that PPM1D expression was positively correlated with Gleason score (p=0.022), T
Figure 1. Immunohistochemical staining for PPM1D in prostate cancer (PCa) and benign prostatic tissue (×200). A: Score 3+ PPM1D expression in PCa tissues. B: Score 2+ PPM1D expression in PCa tissues. C: Score 1+ PPM1D weak staining in benign prostatic tissue. D: Score 0 (negative) for staining of negative controls with the primary antibody omitted.

Figure 2. Prognostic value of PPM1D expression in prostate cancer. Kaplan–Meier curves showing the impact of PPM1D expression on biochemical recurrence-free (A) and overall (B) survival.
However, pretreatment PSA levels and positive surgical margin were not correlated with positive PPM1D expression.

### Correlation of PPM1D expression with patient survival

Using the Kaplan–Meier curve and log-rank test, we found that patients with positive PPM1D expression had shorter biochemical recurrence-free survival time compared to those with negative PPM1D expression ($p<0.001$, Figure 2A). In patients who had positive PPM1D expression, the overall survival was also significantly shortened in comparison with that of those with negative PPM1D expression ($p<0.001$, Figure 2B).

Cox proportional hazard model was used to further explore the relationship between PPM1D expression and survival of the 234 patients with PCa after radical prostatectomy. In both univariate and multivariate analyses, we found that positive PPM1D expression was significantly correlated with worse biochemical recurrence-free (hazard ratio=3.437, 95% confidence interval=1.154-6.209, $p=0.016$, Table I) and overall survival (hazard ratio=5.026, 95% confidence interval=2.545-8.109, $p=0.007$) (Table III). Our results suggest that PPM1D expression was an independent factor for predicting poor prognosis for patients with PCa in our study.

Table I. Clinicopathological features of 234 patients with prostate cancer.

<table>
<thead>
<tr>
<th>Feature</th>
<th>PPM1D-positive</th>
<th>PPM1D-negative</th>
<th>$p$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient (n)</td>
<td>108</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td>Serum PSA level (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>50</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>≥10</td>
<td>58</td>
<td>69</td>
<td>0.698</td>
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<tr>
<td>Gleason score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤6</td>
<td>37</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>≥7</td>
<td>71</td>
<td>50</td>
<td>0.022</td>
</tr>
<tr>
<td>T Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>70</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>T3-T4</td>
<td>38</td>
<td>18</td>
<td>0.015</td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>66</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>42</td>
<td>20</td>
<td>0.016</td>
</tr>
<tr>
<td>Surgical margin status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>70</td>
<td>86</td>
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</tr>
<tr>
<td>+</td>
<td>38</td>
<td>40</td>
<td>0.690</td>
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PSA: Prostate-specific antigen.

PPM1D silencing reduced cell proliferation, migration and invasion in PCa cells. Western blot analysis indicated that siPPM1D knocked-down PPM1D expression (Figure 3A). In both PC-3 and LNCaP cells, siPPM1D treatment significantly reduced cancer cell proliferation (Figure 3B and C). In cell migration assay (Figure 4A), siPPM1D treatment significantly reduced cell numbers of both PC-3 and LNCaP cells that migrated across the membranes when compared to si-Scramble treatment after 24 h incubation. Consistent results were observed in the cell invasion assay (Figure 4B). siPPM1D treatment significantly reduced invasion by PC-3 and LNCaP cells by more than a half. These results indicate that PPM1D expression is correlated with cell proliferation, migration and invasion of PCa cells.

### Discussion

Although PPM1D has been shown to have a pivotal role in the regulation of p53 signaling pathway in tumor biology, the prognostic role of PPM1D in PCa has not been fully-
explored. Here, we showed that positive PPM1D expression is present in 56.4% of PCa tissues in 234 cases in contrast to 5% of BPH tissues classified as PPM1D-negative. Importantly, we found that positive PPM1D expression was significantly correlated with worse biochemical recurrence-free and overall survival. Our results suggest that PPM1D expression was an independent factor for predicting poor prognosis for patients with PCa in our study.

Several lines of evidence have shown that PPM1D plays important roles in tumor biology. PPM1D is considered as an oncogene and overexpression of PPM1D contributes to development of human cancer by suppressing p53 activation (19). PPM1D is up-regulated in neuroblastoma, and pancreatic, lung, bladder, liver, ovarian and breast cancer (13, 14, 20, 21). PPM1D overexpression contributes to malignant progression by inactivating wild-type p53 and p38 MAPK as well as decreasing p16 protein levels in human breast tissues (16). PPM1D has been shown to be a prognostic marker for lung adenocarcinoma patients, pancreatic neuroendocrine tumors and medulloblastoma patients (12, 18, 22). In the present study, we demonstrated that PPM1D expression was positively correlated with Gleason score, T stage and lymph node status. Furthermore, we showed that patients with positive PPM1D expression had shorter biochemical recurrence-free survival time and overall survival in comparison with that in patients with negative PPM1D expression. We also performed in vitro experiments in PCa cells. The

Figure 4. Effect of siPPM1D-knockdown on prostate cancer cell migration and invasion. A: Cells transfected with scrambled siRNA (si-Scramble) or siRNA targeting PPM1D (siPPM1D) for 48 h and after another 48 h, migrated cells were stained and counted under a microscope (×10). Representative images are shown. B: The number of migrated cells is shown (×10). Data shown are the means±SD from five fields. *p<0.05 versus the si-Scramble group. 1: si-Scramble; 2: PPM1D.
Proliferation and invasion of PCa cell lines were significantly inhibited by PPM1D knockdown, indicating that PPM1D is not only a prognostic marker, but also a potential therapeutic target.

Taken together, the current study revealed that PPM1D was overexpressed in PCa tissues compared to BPH tissues and that high PPM1D expression was an indicator of poor prognosis for patients with PCa. Further studies are needed to investigate the underlying molecular mechanism of PPM1D in the progression of PCa.

Conflicts of Interest

The Authors have declared that no conflict of interest exists.

Acknowledgements

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References


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