PMEPA1, an Androgen-regulated NEDD4-binding Protein, Exhibits Cell Growth Inhibitory Function and Decreased Expression during Prostate Cancer Progression

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Abstract

PMEPA1 was originally identified as a highly androgen-induced gene by serial analysis of gene expression in androgen-treated LNCaP prostate cancer (CaP) cells. PMEPA1 expression is prostate abundant and restricted to prostatic epithelial cells. PMEPA1-encoded protein shows high sequence homology to a mouse N4wbp4-encoded protein that binds to Ned44 protein, an E3 ubiquitin–protein ligase involved in ubiquitin-dependent, proteasome-mediated protein degradation. Studies from our and other laboratories have suggested the role of PMEPA1 in cell growth regulation as noted by androgen induction of PMEPA1 expression, elevated PMEPA1 expression in nontumorigenic revertants of tumor cell lines after chromosome 8p transfer, and PMEPA1 expression alterations (up- or down-regulation) in human tumors. Here, we demonstrate that PMEPA1 protein through its PY motifs interacts with WW domains of the human NEDD4 protein. Exogenous expression of PMEPA1, in widely used CaP cell lines DU145, PC3, LNCaP, and LNCaP sublines (C4, C4-2, and C4-2B), conferred cell growth inhibition, and at least one of the PY motifs of PMEPA1 may be involved in its cell growth inhibitory functions. Quantitative expression analysis of PMEPA1 in paired normal and tumor cells of 62 patients with primary CaP revealed tumor cells associated decreased expression in 40 of 62 patients that were significantly associated with higher pathologic stage and serum prostate-specific antigen. Taken together, PMEPA1 negatively regulates growth of androgen responsive or refractory CaP cells, and these functions may be mediated through the interaction of PMEPA1 with the NEDD4 protein involved in the ubiquitin–proteasome pathway. Loss or reduced PMEPA1 expression in CaP further suggests its role in prostate tumorigenesis.

Introduction

Biological effects of androgens on target cells, e.g., prostatic epithelial cell proliferation and differentiation, as well as androgen ablation-mediated cell death, involve AR-mediated cell signaling (1). Systematic and comprehensive analysis of the ARGs should provide the biological reporters for androgen signaling in CaP. Our efforts to analyze ARGs by serial analysis of gene expression led to the discovery of PMEPA1 (2, 3). PMEPA1 expression was regulated by androgen in a dose- and time-dependent manner, and PMEPA1 was highly expressed in the prostate in comparison with other organs. PMEPA1 encodes a protein of 252 amino acids with a type Ib trans-membrane domain. PMEPA1 protein sequence homology search showed 83% identity to a recently reported mouse N4wbp4 protein, which was defined as one of the several proteins that bound to WW domain of the Ned44-encoded protein (4). Ned44, originally identified as a developmentally regulated gene in mice, now belongs to a family of ubiquitin–protein ligases characterized by two to four WW domains, a COOH-terminal homologous to E6-AP COOH terminus domain and an NH2-terminal C2 domain (5–7). Additional studies implicated the roles of Ned44 in diverse cellular functions through the ubiquitin-dependent, proteasome-mediated protein degradation (8–12). The WW domain of the Ned44 protein comprises of a module with two highly conserved tryptophan residues, which bind to target proteins that contain a PY motif, e.g., PPrxY (4, 13–17). The presence of two PY motifs in the predicted protein sequence of PMEPA1 and its similarity to the mouse Ned44-binding protein, N4wbp4, suggest that PMEPA1 is a potential binding partner of the Ned44, the human homologue of the Ned44.

Studies of PMEPA1 (2, 3, 18, 19) have suggested for its role in cell growth regulation as noted by the androgen induction of PMEPA1 expression, elevated PMEPA1 expression in nontumorigenic revertants of tumor cell lines after chromosome 8p transfer, and the PMEPA1 expression alterations (up- or down-regulation) in human tumors. Furthermore, the induction of PMEPA1 expression in nontumorigenic derivatives of multiple cancer cell lines, resulting from the introduction of chromosome 8p, suggested that PMEPA1 might be the downstream target of the critical cell growth regulatory genes on chromosome 8, the most frequently altered chromosomes in CaP.

The androgen-regulated nature of PMEPA1, the potential of PMEPA1 protein as a NEDD4-binding partner and suggested cell growth regulatory functions of PMEPA1, have now provided the impetus to study biochemical and cell biological functions of PMEPA1 and its CaP-associated alterations.

Materials and Methods

Plasmids. Mammalian expression vectors encoding PMEPA1-V5 and PMEPA1-GFP fusion proteins were generated by PCR amplification of the PMEPA1 open reading frame. For PMEPA1-V5-pcDNA3.1 vector, the primers 5’GCTGCTGGAGAACTGAAGGC3’ and 5’GTGTTCTTCTGTCTTTACCTC3’ were used. For PMEPA1-GFP-pEGFP vector, the primers used were 5’aagcttGCTGCTGGAGAACTGAAGGCG 3’ and 5’gaattcGTGTTCTTCTGTCTTTATC3’. The V5 tag or GFP protein was fused at the COOH terminus of the PMEPA1 protein. The PCR product for generating PMEPA1-V5 was inserted into pcDNA3.1-V5-His expression vector (Invitrogen, Carlsbad, CA). The PCR product for generating PMEPA1-GFP was
digested by HindIII and EcoRI and cloned into the same sites of pEGFP vector (Clontech, Palo Alto, CA). PMEPA1-PY motif mutants, in which the tyrosine residue (Y) was replaced with an alanine residue (A), were created by using QuickChange Site-directed Mutagenesis kit (Strategene, La Jolla, CA), using the PMEPA1-V5-pcDNA3.1 vector as a template. The plasmids of PMEPA1-PY motif mutants are as follows: (a) PMEPA1-PY1m-V5-pcDNA3.1, with the first PY motif mutation (Y126A); (b) PMEPA1-PY2m-V5-pcDNA3.1, with the second PY motif mutation (Y197A); and (c) PMEPA1-PY1mPY2m-V5-pcDNA3.1, with both the PY motif mutations (Y126A and Y197A). The sequences of all of the inserts in expression vectors were verified by DNA sequencing.

A bacterial expression plasmid of human NEDD4 gene (pNEDD4WW-GST-pGEX-2TK) encoding all four WW-domains (accession no. XM_046129) was fused to GST (GST-WW fusion protein) was generated by PCR amplification of the coding region of the four WW-domains with the primers 5’GCAG-GATCCCAACCGAGATGCCTGTCG-3’ and 5’GCAGAATTCTTTTTGAATCCCTGGAGTA-3’.

Normal prostate tissue-derived cDNA was used as a PCR template, and the amplified fragment was cloned into the BamHI/EcoRI sites of pGEX-2TK (Amersham Biotech, Piscataway, NJ). A mammalian expression vector (NEDD4-GFP-pEGFP) encoding NEDD4-GFP fusion protein was generated using the primers 5’GCACAGGTTCCCGGTTTGCCTGAAGC-3’ and 5’GCAGAATTCTTTTTTTTTTTTGTATTTGGTAGC-3’ to generate the NEDD4 gene by PCR.

PMEPA1 and NEDD4 Protein-binding Assays. In the *in vitro* binding of PMEPA1 and NEDD4 was assessed by GST pull-down assays. GST-WW fusion protein was purified with glutathione-Sepharose beads per the supplier. The LNCaP sublines C4, C4-2, and C4-2B (20–22) were transfected into the 50–70% confluent cells in triplicate in 60-mm Petri dishes with Lipofectamine (Invitrogen). TSG p53 (wt) and mt p53 (R175H and G245D) were also used in parallel as controls. Approximately 36 h later, with G418 at 800 μg/ml (DU145 and PC3) or 400 μg/ml (LNCaP and its sublines) was initiated. Cells were maintained with G418-containing medium that was changed every 3–4 days. After 2–4 weeks of selection, the cells were rinsed with 1× PBS, fixed with 2% formaldehyde in 1× PBS for 15 min, stained with 0.5% crystal violet in 1× PBS for 15 min,
PMEPA1 inhibits prostate cancer cell growth

and rinsed one to two times with distilled H2O. Colonies visible in each dish without magnification were counted by Open-Lab software. To assess the effects of PY motif mutations on the colony-forming ability of PMEPA1, LNCaP and PC3 cells were also transfected with PMEPA1 mutants: PMEPA1-PY1m-pcDNA3.1, PMEPA1-PY2m-pcDNA3.1, or PMEPA1-PY1m/PY2m-pcDNA3.1. PMEPA1-V5-pcDNA3.1 and expression vector without insert served as positive and negative controls, respectively, for the PMEPA1 mutants. Two independent colony-forming assays were performed as above.

To further evaluate the growth inhibitory effects of PMEPA1 on CaP cells, a stable PMEPA1-GFP-Tet LNCaP transfectant was generated. Expression of PMEPA1-GFP fusion protein in these cells was negatively regulated by tetracycline in the medium (Clontech). For cell proliferation assays, 3000 PMEPA1-GFP-Tet LNCaP cells were seeded in 96-well plates with or without 1 μg/ml tetracycline in the medium. CellTiter 96 Aqueous One Solution kit (Promega) was used to measure the cell proliferation according to the manufacturer’s instructions.

Prostate Tissue Samples, LCM, and QRT-PCR Assay. Matched CaP and normal tissues were derived from radical prostatectomy specimens from 62 CaP patients treated at Walter Reed Army Medical Center (under an Institutional Review Board-approved protocol). The procedures for collecting specimens were described previously (23). Ten-μm frozen sections were prepared and archived at −70°C. Histologically normal prostate epithelial and prostate tumor cells from each patient were harvested by a pathologist (W. Z.) using LCM equipment according to the protocol provided by the manufacturer (Arcturus Engineering, Mountain View, CA). Total RNA was prepared from the harvested normal and tumor prostate epithelial cells as described previously (23) and quantified with Fluorometer (Bio-Rad, Hercules, CA). QRT-PCR was conducted using 0.1 ng of total RNA from paired normal and tumor cells. PMEPA1 PCR primers were carefully designed that only amplify PMEPA1 but not STAG1, an alternatively spliced form of PMEPA1 (19). The PCR primers were 5’CATGATCCCGGAGCTCTG3’ and 5’TGATCGAAACAACTCAGCCTCC3’, and the FAM-labeled probe was 5’AGGCGGACAGTCTCTGCGAAAC3’.

GAPDH gene expression was detected as the internal control (PE Applied Biosystems, Foster, CA). Paired triplicate samples (one lacking reverse transcriptase and duplicate with reverse transcriptase) were amplified in 50-μl volumes using the manufacturer’s recommended universal reagent, proper primers, and probe of PMEPA1 or GAPDH using 7700 sequence detection system (PE Applied Biosystems). Results were plotted as average cT values for each duplicate sample minus the average duplicate cT values for GAPDH. Differences between matched tumor (T) and normal (N) samples were calculated using 2exp(cT tumor − cT normal) and expressed as fold changes in expression. The expression status of PMEPA1 was further categorized as overexpression in tumor tissue (T > N), defined as 1 + (1.5–3-fold), 2+ (3.1–10-fold), 3+ (10.1–20-fold), and 4+ (>20-fold) increased expression compared with matched normal tissue; reduced expression in tumor tissue (T < N), defined as 1− (1.5–3-fold), 2− (3.1–10-fold), 3− (10.1–20-fold), and 4− (>20-fold) decreased expression as compared with matched normal tissue; and no change (T = N) refers to the difference of PMEPA1 expression between T and N as <1.5 folds (0).

Statistical Analysis. Statistical analysis was performed with the SPSS software package. The association between PMEPA1 expression and clinicopathological features was analyzed using χ2 and t tests. P < 0.05 was considered as statistically significant.

Results and Discussions

PMEPA1-PY Motifs Interact with the WW Domains of NEDD4. The WW domains of NEDD4 protein facilitate its binding to the target proteins via interaction with the PY motifs of NEDD4-binding proteins (4, 13–17). Predicted PMEPA1 protein sequence comprises of two PY motifs, PY1 (PPPY) and PY2 (PPTY). PY1 is in the central region of the PMEPA1 protein, and PY2 is close to the COOH terminus of the PMEPA1 protein (Fig. 1A). PMEPA1 shares 83% sequence identity with the protein encoded by N4wbp4, a gene expressed in mouse embryo (Ref. 4; Fig. 1A). In vitro translated [35S] Methionine-labeled PMEPA1-V5 fusion protein, with the two intact PY motifs, showed binding to the GST-WW fusion protein (Fig. 1B, Lane 1). PMEPA1 with PY1 or PY2 mutations revealed significantly decreased binding to WW domains (Fig. 1B, Lanes 2 and 3). Furthermore, PMEPA1-V5 and NEDD4-GFP fusion proteins expressed in 293 cells showed strong association (Fig. 1C, Lane 1) and the mutant PMEPA1-V5 proteins having single mutation of PY1 or PY2 motif or double mutations of both PY1 and PY2 motifs exhibited significantly reduced binding to NEDD4 (Fig. 1C, Lanes 2–4). Thus, both in vitro and cell culture data support the prediction that PMEPA1 interacts with NEDD4, and these interactions are dependent on the binding of both PY motifs of PMEPA1 to WW domains. However, the PY2 motif mutation appeared to have a greater effect on binding of PMEPA1 to NEDD4 WW domain.

The high protein sequence identity of PMEPA1 with N4wbp4 suggests that PMEPA1 is the human homologue of N4wbp4. On the basis of the suggested role of Nedd4 in mouse development (4–12), the homology of PMEPA1 to a Nedd4-binding protein, experimental documentation of binding of PMEPA1 to NEDD4, and the androgen regulation and prostate abundance of PMEPA1 warrant evaluations of the roles of PMEPA1 in prostate development.

PMEPA1 is a Golgi-associated Protein. To gain additional insights into PMEPA1 cellular functions, its subcellular localization was determined. PMEPA1-GFP fusion protein showed perinuclear localization with a Golgi-like appearance (Fig. 2A). We performed the immunofluorescence assay to test the hypothesis that PMEPA1 local-

![Fig. 2. PMEPA1 localizes to cis-Golgi matrix. A, LNCaP transfectants expressing PMEPA1-GFP fusion protein. In B, LNCaP transfectants stained with anti-Golgi structure protein GM130. C, superimposed images of B and C. Arrows, the colocalization of PMEPA1-GFP fusion protein and GM130. Magnification: ×100.](image-url)
izes to the Golgi complex. The images of subcellular location of GM130, a cis-Golgi protein, showed a similar pattern as PMEPA1-GFP fusion protein (Fig. 2B). Superimposition of the images of PMEPA1-GFP fusion protein and GM130 in LNCaP-PMEPA1-GFP transfectants confirmed the localization of PMEPA1-GFP fusion protein on cis-Golgi structure (Fig. 2C). We did not see the colocalization of PMEPA1-GFP and TGN-38, which localizes to TGN (data not shown). In this regard, the subcellular localization of PMEPA1 is similar to other two newly identified Nedd4 WW domain-binding proteins, N4wbp5 and N4wbp5a, which were also localized to the Golgi complex (12, 17). As a highly ARG and NEDD4-binding protein, the localization of PMEPA1 on Golgi apparatus may suggest for the role of PMEPA1, an epithelial sodium channel, a known target down-regulated by NEDD4 (12).

Inhibits Growth of CaP Cells. To investigate the biological effects of PMEPA1 expression in the regulation of cell growth and contribution of PY motifs for such functions, we performed the colony formation assay by transfection expression vectors of the wt-PMEPA1 and PMEPA1-PY mutants. As shown in Fig. 3, A–F, colony-forming abilities of CaP cell lines DU145, PC3, LNCaP, and LNCaP sublines were significantly suppressed by transfection of the normal tissue; and T = N, similar expression between T and N, refers to the difference of PMEPA1 expression between T and N as <1.5-fold (0).

Table 1 PMEPA1 expression status in primary prostate cancers

<table>
<thead>
<tr>
<th>PMEPA1 expression</th>
<th>No. of patients/group (%)</th>
<th>Degree of PMEPA1 expression</th>
<th>Quantity</th>
<th>No. (%)</th>
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<tr>
<td>T &lt; N</td>
<td>40 (64.5)</td>
<td>1–</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2–</td>
<td>17 (42.5)</td>
<td></td>
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<td></td>
<td></td>
<td>3–</td>
<td>5 (12.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4+</td>
<td>7 (17.5)</td>
<td></td>
</tr>
<tr>
<td>T &gt; N</td>
<td>10 (16.1)</td>
<td>1+</td>
<td>6 (60.0)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>2+</td>
<td>4 (40.0)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>3+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T = N</td>
<td>12 (19.4)</td>
<td>4+</td>
<td>0</td>
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</table>

PMEPA1 gene expression in 62 matched LCM-derived prostatic cancer cells (T) and normal cells (N) was analyzed by QRT-PCR. The results were represented as differential expression in T and N as described in “Materials and Methods.” T < N, reduced expression in tumor tissue, defined as 1– (1.5–3-fold), 2– (3.1–10-fold), 3– (10.1–20-fold), and 4+ (>20-fold) decreased expression as compared with matched normal tissue; T > N, overexpression in tumor tissue, defined as 1+ (1.5–3-fold), 2+ (3.1–10-fold), 3+ (10.1–20-fold), and 4+ (>20-fold) increased expression as compared with matched normal tissue; and T = N, similar expression between T and N, refers to the difference of PMEPA1 expression between T and N as <1.5-fold (0).

Fig. 3. Effect of PMEPA1 on colony formation. Prostate tumor cell lines C4 (A), C4-2 (B), C4-2B (C), LNCaP (panel D), DU145 (E), and PC3 (F) were transfected with 3 μg each of PMEPA1-V5-pcDNA3.1 (PMEPA1) and pcDNA3.1 vector (Vector) in triplicate sets. In separate experiments, LNCaP (G) and PC3 (H) cells were transfected with control vector or expression vectors encoding wt-PMEPA1 or PMEPA1-PY mutants (1, PMEPA1-V5-pcDNA3.1; 2, PMEPA1-PY1m-pcDNA3.1; 3, PMEPA1-PY2m-pcDNA3.1; 4, PMEPA1-PY1mPY2m-pcDNA3.1; 5, pcDNA3.1). Transfected cells were selected for plasmid-containing cells with G418 for 3 weeks, and surviving cells were fixed and stained with crystal violet. Colonies were counted and displayed as histograms. For each cell line, a photograph of one dish of cells treated with 3 μg of each plasmid is also shown. In two independent experiments, mutations of PY1 motif appear to abolish the inhibition of colony formation by wt-PMEPA1, emphasizing the role of PY1 motif in PMEPA1 and NEDD4 interactions and the biological functions of PMEPA1 (Fig. 3, G and H). It is intriguing that both PY1 and PY2 mutations diminished the in vitro binding of PMEPA1 to NEDD4; however, in the context of biological function of PMEPA1 in the colony-forming assay, only mutation of PY1 motif has functional consequence. The growth inhibitory effect of PMEPA1 appears to be linked to the interactions of PY1 motif to NEDD4 WW...
Domain. This interpretation is based on the striking observations showing distinctively more colonies with PY1 motif mutant in comparison with wt-PMEPA1. Moreover, the growth inhibitory effect of PMEPA1 has been confirmed by the cell proliferation characteristics of stable PMEPA1-GFP-Tet-LNCaP cells, where exogenous PMEPA1 is up-regulated in the absence of tetracycline. The growth of the PMEPA1-GFP-Tet LNCaP cells in tetracycline-negative medium is significantly slower than that of PMEPA1-tet LNCaP transfectant in tetracycline-positive medium (Fig. 4). LNCaP cells with PMEPA1 overexpression also revealed increased retinoblastoma phosphorylation, further confirming cell growth inhibitory effect of PMEPA1 (data not shown).

PMEPA1 is expressed in AR-positive CaP cell lines: LNCaP and its sublines (C4, C4-2, and C4-2B). LNCaP cells are androgen dependent for growth. Although the growth of LNCaP sublines is androgen independent, AR is critical for their proliferation (24). We observed that overexpression of PMEPA1 by transfecting the PMEPA1 expression vector into LNCaP and its sublines significantly inhibited the cell proliferation. Because our preliminary observations showed that PMEPA1 overexpression in LNCaP cells resulted in altered expression of AR downstream genes, we hypothesized that the growth inhibitory effect of PMEPA1 on LNCaP and its sublines may be mediated through directly or indirectly affecting AR functions. Despite the growth inhibitory effect on AR-positive CaP cell lines, PMEPA1 was also found to inhibit the growth of AR-negative prostate tumor cells, DU145 and PC3, suggesting that the growth inhibitory effects of PMEPA1 on DU145 and PC3 could be mediated through alternative mechanisms, e.g., regulation of other nuclear steroid receptors by PMEPA1. Nonetheless, inhibition of CaP cell growth by PMEPA1 suggested that PMEPA1 might be involved in CaP development.

**Decreased PMEPA1 Expression in Prostate Tumor Tissues.**

CaP cell growth inhibitory functions of PMEPA1 led us to carefully evaluate the relationship of PMEPA1 expression alterations to the clinicopathologic features of CaP. The overall expression pattern of the PMEPA1 is shown in Table 1. Comparison of PMEPA1 expression between tumor and normal cells revealed tumor cell-associated decreased expression (T < N) in 64.5% tumor specimens (40 of 62), increased expression (T > N) in 16.1% specimens (10 of 62), and no change (T = N) in 19.4% specimens (12 of 62). When these nuclear alterations were stratified by organ-confined (pT2) and nonorgan-confined (pT3) disease, a higher percentage of PMEPA1 reduction was seen in pT3 (74%) versus pT2 (48%). Because the T > N group has a few cases, we combined the T > N and T = N groups (T ≥ N group). Comparison of the clinicopathologic parameters between the PMEPA1-T < N and PMEPA1-T ≥ N groups revealed that the PMEPA1-T < N group had a significantly higher percentage of patients with pT3 tumors (P = 0.035), and more patients in this group had a higher level of preoperative serum PSA (P = 0.023; Table 2). Of 62 patients whose tumors were analyzed for PMEPA1 expression, 14 patients showed CaP recurrence as defined by serum PSA level ≥ 0.2 ng/ml after prostatectomy. Of the 14 patients, 11 showed reduced tumor-associated PMEPA1 expression (78.5%). Reduced PMEPA1 expression seems to associate with a higher recurrence rate and shorter duration to recurrence after surgery, although the statistical analysis did not reveal a significant difference which might be attributable to the small number of patients (Table 2). It is worth noting these observations in the context of the report showing consistent high levels of induction of PMEPA1 in nontumorigenic revertants of tumor cell lines after the transfer of human chromosome 8p (18), which harbors putative TSGs (25, 26). Among chromosomal “hot spots” in CaP and other cancers, 8p loss is particularly frequent, occurring in ~80% of prostate tumors (27–29). Transfer of single human chromosome 8 has been shown to result in suppression of the malignant phenotype or the metastatic ability in a variety of cell lines (18, 30, 31). Therefore, it is possible that the reduced expression of PMEPA1 in prostate tumors might result from the loss of the putative TSGs or cell growth inhibitory genes on chromosome 8p in prostate tumor cells. Two very recent interesting reports have described PMEPA1 as a transforming growth factor-β-induced gene and marker of terminal colonocyte differentiation (32, 33).

In summary, we have demonstrated that PMEPA1 interacts with NEDD4 through its PY motifs. PMEPA1 has cell growth inhibitory effects when overexpressed in androgen-dependent or -independent CaP cells. PY1 motif of PMEPA1 appears to modulate its cell growth inhibitory function. PMEPA1 may function in the regulation of protein turnover via ubiquitination/proteasome pathways in cells. The biological effects of PMEPA1 in CaP cells and expression pattern of PMEPA1 in CaP indicate that PMEPA1 may function as the cell growth inhibitor regulated by androgen.

**Acknowledgments**

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**References**


**Table 2. PMEPA1 expression and clinicopathological features**

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<tr>
<th>PMEPA1 expression</th>
<th>Pathologic stage (%)</th>
<th>PSA range (%)</th>
<th>PSA recurrence (%)</th>
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<tr>
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<td>T3</td>
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<td>4.1–10 ng/ml</td>
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<td>11 (27.5)</td>
<td>29 (72.5)</td>
<td>1 (2.5)</td>
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<td>10 (45.5)</td>
<td>5 (22.7)</td>
<td>15 (68.2)</td>
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<tr>
<td>P</td>
<td>0.035</td>
<td></td>
<td>0.023</td>
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* L. L. Xu et al., unpublished data.
22. Wu, T. T., Sikes, R. A., Cui, Q., Thalmann, G. N., Kao, C, Murphy, C. F., Yang, H.,
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