Identification of PMF1 Methylation in Association with Bladder Cancer Progression

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Abstract Purpose: Polyamines are important regulators of cell growth and death. The polyamine modulated factor-1 (PMF-1) is involved in polyamine homeostasis. After identifying an enriched CpG island encompassing the PMF1 promoter, we aimed at evaluating the clinical relevance of PMF1 methylation in bladder cancer.

Experimental Design: The epigenetic silencing of PMF1 by hypermethylation was tested in bladder cancer cells (n = 11) after azacytidine treatment. PMF1 methylation status was evaluated in 507 bladder tumors and 118 urinary specimens of bladder cancer patients and controls. PMF1 protein expression was analyzed by immunohistochemistry on tissue arrays containing bladder tumors for which PMF1 methylation was assessed (n = 218).

Results: PMF1 hypermethylation was associated with gene expression loss, being restored in vitro by a demethylating agent. An initial set of 101 primary frozen bladder tumors served to identify PMF1 hypermethylation in 88.1% of the cases. An independent set of 406 paraffin-embedded tumors also revealed a high PMF1 methylation rate (77.6%). PMF1 methylation was significantly associated with increasing stage (P = 0.025). Immunohistochemical analyses revealed that PMF1 methylation was associated with cytoplasmic PMF1 expression loss (P < 0.001), grade (P < 0.001), and poor overall survival using univariate (P < 0.001) and multivariate (P = 0.011) analyses. Moreover, PMF1 methylation in urinary specimens distinguished bladder cancer patients from controls (area under the curve = 0.800).

Conclusion: PMF1 was identified to be epigenetically modified in bladder cancer. The association of PMF1 methylation with tumor progression and its diagnostic ability using urinary specimens support including PMF1 assessment for the clinical management of bladder cancer patients.

Bladder cancer can be described as a genetic disease driven by the multistep accumulation of genetic and epigenetic factors (1, 2). The most common epigenetic event in the human genome is the addition of methyl groups to the carbon-5 position of cytosine nucleotides. CpG islands are present in one-half of human genes and typically overlap with promoters and first exons of genes (3–7). Transcriptional inactivation by CpG island promoter hypermethylation has been shown for critical bladder cancer-related genes (8–12).

The use of high-throughput profiling approaches accelerates the discovery of genetic and epigenetic events associated with tumorigenesis and tumor progression. CpG island arrays represent a high-throughput technology for the discovery of genes frequently hypermethylated during disease progression (13, 14). This technology has recently been applied to compare the methylation patterns of invasive bladder tumors and their respective normal urothelium counterparts (15). Using this epigenomic discovery approach, the polyamine modulated factor-1 (PMF-1) was identified as a potential hypermethylated candidate. An enriched 5’-CpG island was confirmed to be...
The novelty of our findings is high, because, to the best of clinical relevance of PMF1 along bladder cancer progression. Performed focused studies aiming to evaluate the potential but also potentially targeted therapeutically. In this report, we potentially useful as a tumor stratification biomarker and thus, PMF1 represents a methylated candidate not only has become a target for antineoplastic intervention (22–24). And the carcinogenic process (22, 23), polyamine metabolism expressed (20, 21). Because of its importance in cell growth a cell can directly or indirectly affect the spectrum of genes itssusceptibilitytobeepigeneticallymodified. Located around the transcription start site of PMF1, supporting its susceptibility to be epigenetically modified.

Polyamines are important regulators of cell growth and cell death. PMF1 is involved in the polyamine homeostasis (16–19). Variations in the intracellular polyamine content of a cell can directly or indirectly affect the spectrum of genes expressed (20, 21). Because of its importance in cell growth and the carcinogenic process (22, 23), polyamine metabolism has become a target for antineoplastic intervention (22–24). Thus, PMF1 represents a methylated candidate not only potentially useful as a tumor stratification biomarker and clinical outcome prognosticator of bladder cancer patients but also potentially targeted therapeutically. In this report, we performed focused studies aiming to evaluate the potential clinical relevance of PMF1 along bladder cancer progression. The novelty of our findings is high, because, to the best of our knowledge, PMF1 had not been reported to be epigenetically modified or differentially expressed in bladder malignancies.

Materials and Methods

Tumor samples. Primary bladder tumors were collected following the protection guidelines of human subjects at eight participating hospitals. Inclusion criteria of newly diagnosed bladder cancer patients were based on the histopathologic information, covering from early to advanced stages. It was also required to have tissue material available for obtaining both high-quality DNA for methylation analyses and high-quality matching paraffin blocks for microarray analyses using immunohistochemistry. An initial series of 101 frontline bladder tumors including a subset of paired normal urothelium served to screen PMF1 methylation rates among non-muscle-invasive tumors \( n = 56 \); \( pT1 \) \( ( n = 24 \) and \( pT2 \) \( ( n = 32 \) ) and muscle-invasive cases \( n = 45 \); \( pT2 \) \( ( n = 16 \) ), \( pT3 \) \( ( n = 25 \) ), and \( pT4 \) \( ( n = 4 \) ); ref. 25]. An independent set of 406 paraffin-embedded bladder tumors served to (a) validate PMF1 methylation rates; (b) assess the feasibility of PMF1 methylation analyses in paraffin-embedded material, and (c) evaluate the association of PMF1 methylation with clinicopathologic variables. This set comprised non-muscle-invasive tumors \( n = 250 \); \( pT1 \) \( ( n = 44 \) ), \( pT1 \) \( ( n = 197 \) ), and \( pT2 \) \( ( n = 9 \) ) and muscle-invasive cases \( n = 156 \); \( pT2 \) \( ( n = 88 \) ), \( pT3 \) \( ( n = 47 \) ), and \( pT4 \) \( ( n = 21 \) ) defined under standard criteria (26). OCT and paraffin-embedded bladder tumors were macro-dissected based on H&E evaluations to ensure a minimum of 75% of tumor cells (25, 27). Demographic information indicated the presence of 438 males and 69 females, with a median age of 66.0 years (range, 25–92 years).

Methylation analyses of the promoter of PMF1. The methylation status of PMF1 was analyzed by two PCR analysis strategies of bisulfite-modified genomic DNA, which induces chemical conversion of unmethylated, but not methylated, cytosine to uracil. First, genomic sequencing of both strands of PMF1 promoter was analyzed after bisulfite treatment of genomic DNA. A second strategy used PCR with primers specific for either the methylated or the modified unmethylated DNA [methylation-specific PCR (MSP, refs. 15, 28)]. Primer sequences for bisulfite sequencing and unmethylated and methylated reactions were designed encompassing its transcription start site distant at 50 bp after its ATG starting codon (Table 1). Genomic DNA was extracted using standard methods. DNA from normal lymphocytes treated in vitro with Ss1 methyltransferase was used as a positive control for methylated alleles. DNA from normal lymphocytes was used as a positive control for unmethylated alleles. PCR products were loaded onto nondenaturing 2% agarose gels, stained with ethidium bromide, and visualized under an UV transilluminator.

Mutational analyses of PMF1. The complete PMF1 coding sequence was screened for mutations in bladder cancer cell lines. The cDNA template (1 μL) of PMF1 was sequenced by PCR/denaturing high-performance liquid chromatography/sequencing. PCR amplification was done in a 30 μL reaction volume containing 0.2 μmol/L of each.

Table 1. Primer sequences and PCR conditions for bisulfite sequencing, methylated and unmethylated specific PCR, and reverse transcription-PCR for PMF1

<table>
<thead>
<tr>
<th>Sense primer (5′-3′)</th>
<th>Antisense primer (5′-3′)</th>
<th>Product size (bp)</th>
<th>Annealing temperature (PCR cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bisulfite sequencing</td>
<td>GGTATTATTTTTAGGAGTGAAAG</td>
<td>ACTAATCTCCCACTTCCCTAC</td>
<td>283</td>
</tr>
<tr>
<td>Methylated-specific PCR</td>
<td>CGTCGGTATTTAGTAGTGAG</td>
<td>TAAATACCCGTCCTCGAGACT</td>
<td>135</td>
</tr>
<tr>
<td>Unmethylated-specific PCR</td>
<td>GTGTTTAGGTAAGTAGGTGG</td>
<td>TAAATACCCGTCCTCGAGACT</td>
<td>135</td>
</tr>
<tr>
<td>Reverse transcription-PCR</td>
<td>AGGTGTTGTGAGCACT</td>
<td>GTTCCTGCCTTTTCTTCT</td>
<td>444</td>
</tr>
<tr>
<td>Sequencing</td>
<td>GGGAGGTTCAAGCTCAACATGCGCGC</td>
<td>GACTGGGCAATACCATTACCGCTTCCTGA</td>
<td>702</td>
</tr>
</tbody>
</table>
oligonucleotide (Table 1), 3.5 μmol/L MgCl₂, 200 μmol/L deoxynucleotide triphosphate, 1 unit EcoStart Taq polymerase (Ecogen), and 1 μL cDNA as template. PCR conditions were as follows: one cycle of hot-start at 95°C for 6 min followed by 35 amplification cycles (denaturation, 95°C for 30 s; annealing, 63°C for 30 s; extension, 72°C for 1 min) and a final elongation cycle at 72°C for 10 min. The PCR products of PMF1 were directly sequenced. PMF1 sequences were confirmed by analyzing the products of a second independent PCR and by sequencing both strands of PCR products.

**RNA and protein analysis of PMF1 in bladder cancer cell lines.** Human bladder cancer cell lines (n = 11) were treated with 1 and 5 μmol/L 5-aza-2’-deoxycytidine (AZA; Sigma) for 72 h to achieve demethylation (15, 28). RNA was isolated using standard methods. RNA (1 μg) was reverse transcribed using AMV reverse transcriptase (Promega) and amplified using specific primers and conditions for PMF1 (Table 1). PCR was done using a final volume of 15 μL containing 1× PCR Ecostart buffer (Ecogen), 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxynucleotide triphosphate, 0.25 μmol/L of each primer, 1.5 units Ecostart buffer (Ecogen), and 0.4 μg cDNA as template. Glyceraldehyde 3-phosphate dehydrogenase was used as internal control to ensure cDNA quality and loading accuracy. The amplification product was resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Cell lysates for protein analysis were analyzed by Western blotting using an anti-PMF-1 antibody (mouse monoclonal; BD Biosciences, 1:1,000 dilution). Equal loading was tested by reprobing with an α-tubulin antibody (mouse monoclonal; Sigma, 1:4,000 dilution).

**Immunofluorescence.** Cells were grown on coverslips in 6 dishes, fixed in 4% formaldehyde, and fluorescently stained (15, 28). To monitor AZA exposure, cells were stained for PMF1 at 1:500 dilution for 45 min using the antibody mentioned above. The secondary antibody was used at 1:250 dilution. Confocal optical sections were obtained using a Leica TCS SP microscope equipped with krypton and argon lasers. Images were acquired and processed using the Leica LCS Lite software.

**Tissue microarrays.** We constructed 6 different bladder cancer tissue microarrays including a total of 218 bladder tumors among the patients recruited for assessing PMF1 methylation mentioned above. Demographic information indicated the presence of 196 males and 22 females, with a median age of 65.0 years (range, 25-81 years). Tumor stage distribution was pT₁ (25), pT₂ (67), pT₃ (54), pT₄ (28), and pT₅ (18). Tumor grade distribution was grade 1 (19), grade 2 (10), and grade 3 (189). Clinicopathologic and annotated follow-up information allowed evaluation of associations of PMF1 methylation and protein expression patterns among them and with clinicopathologic variables.

**Immunohistochemistry.** Protein expression patterns of PMF1 were assessed at the microanatomic level by immunohistochemistry on these tissue microarrays using the antibody mentioned above at 1:800 dilution following standard avidin-biotin immunoperoxidase procedures (25, 27). p53 was also assessed using a mouse antibody at 1:50 dilution (clone D07; Novocastra). The biotinylated secondary antibody for PMF1 and p53 (Vector Laboratories) was used at 1:500 dilution. PMF1 staining was evaluated in the cytoplasm. p53 immunoreactivity
was evaluated in the nucleus (27). The individual scores were reviewed, and the agreement between two independent observers was calculated. Whenever a discrepancy was noted between the first and the second interpretations, the pathologist decided on the final scoring.

**Urinary samples.** Urine specimens of individuals presenting microscopic hematuria under first suspicion of bladder cancer were collected immediately before urinary cytology and/or cystoscopy. Samples were handled anonymously following ethical and legal guidelines at three participating hospitals. The presence of bladder cancer was confirmed by cystoscopy, the gold standard (26). Urinary DNA was extracted using standard methods. Genomic DNA (250 ng) was treated with bisulfite and analyzed by MSP (15, 28). Urinary specimens (n = 118) served to analyze the clinical utility of PMF1 methylation at discriminating bladder cancer patients (n = 34) from controls, including healthy individuals and patients with benign urologic diseases (n = 84). Demographic information of these individuals indicated the presence of 84 males and 34 females, with a median age of 62.0 years (range, 25.0-84.0 years). Histopathologic information after subsequent surgical interventions provided tumor stage distribution among cases: pTa (10), pT1 (11), and pT2 (10). Tumor grade distribution was grade 1 (10), grade 2 (5), and grade 3 (19).

**Statistical analysis.** Associations among PMF1 methylation and protein expression patterns of PMF1 and p53 with tumor stage and grade were evaluated using nonparametric Wilcoxon-Mann-Whitney and Kruskal-Wallis tests (29). Associations between PMF1 and p53 were analyzed using Kendall’s τ test (29). Associations of methylation and protein expression patterns with disease-specific overall survival were evaluated in those cases for which follow-up information were available using the log-rank test (29). Overall survival time was defined as the years elapsed between surgery and death from disease (or the last follow-up date). Patients who were alive at the last follow-up or lost were censored. To test whether PMF1 protein expression was an independent risk factor, a multivariate analysis was done using a Cox model including variables such as age, gender, stage, grade, and p53. Survival curves were plotted using Kaplan-Meier methodology. Receiving operating curve analyses defined the diagnostic performance of PMF1 methylation in urinary specimens, given by the area under the curve, estimating its 95% confidence interval (29).

**Results**

**PMF1 is epigenetically silenced in vitro.** PMF1 methylation was initially screened in bladder cancer cell lines (n = 11). PMF1 methylation patterns observed by bisulfite genomic sequencing highly confirmed the methylation of RT-112 found by MSP (Fig. 1A). Methylation analyses were then linked with PMF1 expression estimates. The hypermethylated RT-112 cells showed the lowest protein expression by Western blotting analysis. Bladder cancer cell lines were sequenced to evaluate the potential association of mutations with PMF1 silencing. The methylated cell line (RT-112) and those with low (UM-UC-3) and medium (TCCSUP, T24, and SW780) PMF1 expression by Western blotting were wild-type for PMF1 (Supplementary Table 1). A further link between hypermethylation and gene silencing was established by the treatment of methylated and unmethylated bladder cancer cells with a DNA-demethylating drug. Exposure of the methylated RT-112 cells to AZA restored transcript and protein expression of PMF1 (Fig. 1B). SW780 was used as a control cell line to assess the specificity of AZA exposure not to modify PMF1 expression in unmethylated cells. Overall, these results indicated a high correlation of PMF1 methylation with loss of protein expression in vitro, observations especially supported by AZA reactivation analyses.

**PMF1 is frequently hypermethylated in primary bladder tumors and associated with tumor progression.** Once the functional consequences of PMF1 hypermethylation were determined in vitro, we tested whether PMF1 hypermethylation was cancer specific. Comparison of methylation of 10 bladder tumors and their respective pairs of normal urothelium revealed that PMF1 methylation was found in 80% of the bladder tumors and in 10% of the normal urothelium analyzed (Fig. 2). Initial screening in frozen primary bladder tumors revealed that PMF1 hypermethylation was present in 88.1% of the cases (n = 101). A similar high methylation rate for PMF1 was observed (77.6%) in an independent larger set of paraffin-embedded bladder tumors (n = 406). The methylation status of PMF1 was then linked to clinicopathologic variables. Tumors displaying advanced T2+ disease were more frequently methylated than those with non-muscle-invasive lesions (Table 2). PMF1 methylation was significantly associated with tumor stage (P = 0.025, Kruskal-Wallis). Increased methylation rates

<table>
<thead>
<tr>
<th>Stage</th>
<th>n = 406, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT1a</td>
<td>Methylated: 6 (66.7)</td>
</tr>
<tr>
<td>pT1b</td>
<td>Methylated: 35 (79.5)</td>
</tr>
<tr>
<td>pT2</td>
<td>Methylated: 143 (72.6)</td>
</tr>
<tr>
<td>pT3</td>
<td>Methylated: 68 (77.3)</td>
</tr>
<tr>
<td></td>
<td>Methylated: 43 (91.5)</td>
</tr>
<tr>
<td></td>
<td>Methylated: 20 (95.2)</td>
</tr>
</tbody>
</table>
were found in high-grade bladder tumors (Table 3). However, the limited number of cases with low grade did not allow reaching statistically significant associations. Overall, these results indicated that PMF1 hypermethylation is a frequent event in bladder cancer in association with pathologic indicators of tumor progression.

**Loss of cytoplasmic PMF1 protein expression patterns correlated with tumor progression and clinical outcome of patients with bladder tumors.** The protein expression patterns of PMF1 by means of immunohistochemistry were evaluated in several tissue arrays containing bladder tumors for which PMF1 methylation had been assessed (n = 218). Tumors methylated for PMF1 had lower cytoplasmic protein expression than unmethylated cases (P = 0.032). High cytoplasmic PMF1 protein expression was found in low-grade and papillary lesions (Fig. 3A) compared with high-grade and invasive bladder tumors (Fig. 3B), respectively. The loss of cytoplasmic PMF1 protein expression was significantly associated with tumor stage (P < 0.001, Kruskal-Wallis) and tumor grade (P < 0.001, Kruskal-Wallis). PMF1 cytoplasmic expression inversely correlated with nuclear p53 protein expression (Kendall’s \( \beta = -0.132; P = 0.016 \)). p53 protein expression patterns were associated with tumor stage (P = 0.009). Lack of significant associations was found between p53 and tumor grade or disease-specific overall survival. In a subset of 171 bladder tumors with available follow-up, the presence of a cytoplasmic protein expression of PMF1 lower than 50% was significantly associated with shorter disease-specific overall survival (P < 0.001, log-rank; Fig. 3C). A multivariate Cox analysis was carried out to assess the prognostic value of PMF1 protein expression on patients’ survival. Among the potential prognostic factors adjusted for in this analysis, age, sex, p53, and tumor grade had no effect on patient survival time. Cox analyses indicated that tumor stage and PMF1 were the only independent

**Table 3.** Summary of the distribution of the promoter CpG island methylation status of PMF1 obtained by MSP regarding tumor grade

<table>
<thead>
<tr>
<th>Grade</th>
<th>( n = 406, n (%) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>46</td>
</tr>
<tr>
<td>Methylated</td>
<td>37 (80.4)</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>9 (19.6)</td>
</tr>
<tr>
<td>Grade 2</td>
<td>27</td>
</tr>
<tr>
<td>Methylated</td>
<td>23 (85.2)</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>4 (14.8)</td>
</tr>
<tr>
<td>Grade 3</td>
<td>333</td>
</tr>
<tr>
<td>Methylated</td>
<td>263 (79.0)</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>70 (21.0)</td>
</tr>
</tbody>
</table>

![Fig. 3. Protein expression patterns of PMF1 correlated with tumor progression and clinical outcome in bladder cancer. A and B, representative immunostainings of the differential protein expression patterns of PMF1 at the tissue level by immunohistochemistry on tissue arrays in non-muscle-invasive (A) and invasive (B) bladder tumors, respectively (P < 0.001). C, Kaplan-Meier curve disease-specific survival analysis indicating that a decreased protein expression of PMF1 measured by immunohistochemistry on tissue arrays was associated with poor disease-specific survival (P < 0.001, log-rank).](image)
prognostic factors ($P = 0.011$), having hazard ratios of death of 3.09 (95% confidence interval, 1.98-4.84) and 0.98 (95% confidence interval, 0.96-0.99), respectively. Overall, these analyses revealed that the loss of PMF1 cytoplasmic protein was associated with tumor progression and could be a likely predictor of poor outcome in bladder cancer patients.

**PMF1 hypermethylation in urinary specimens segregated bladder cancer patients from controls.** It was then tested whether PMF1 methylation could be detected in urinary specimens by means of MSP (Fig. 4A). PMF1 methylation provided a sensitivity and specificity of 64.7% and 95.2%, respectively. Receiving operating curve analyses gave an area under the curve of 0.800 (Fig. 4B). The majority of the positive cases unmethylated for PMF1 had pTa grade 1 tumors. Overall, these results indicated that the methylation of PMF1 was detectable in the urine and supported a diagnostic role for the methylation of PMF1 to discriminate between bladder cancer patients and controls with high sensitivity and specificity.

**Discussion**

Our study identified the epigenetic silencing of PMF1 and showed the clinical relevance of PMF1 methylation in uroepithelial tumors. The consequences of the novel hypermethylation of PMF1 along bladder cancer progression require to be assessed from the standpoint of mechanistic, biological, and translational implications. Mechanistically, it is important to evaluate the cellular consequences of PMF1 promoter methylation. Several techniques were used to link methylation analyses with expression estimates of PMF1. Methylation status and protein expression of PMF1 correlated to high extent among a variety of bladder cell lines representing the spectrum of bladder cancer progression in vitro. AZA exposure experiments confirmed the effect of methylation in PMF1 expression by specifically restoring PMF1 transcript and protein expression in methylated cells. Overall, in vitro analyses revealed that PMF1 was aberrantly silenced by CpG island promoter hypermethylation. In bladder tumors, the increased methylation rate together with the loss of cytoplasmic protein expression of PMF1 was associated with increasing tumor stage. Thus, increased methylation rates correlated with loss of PMF1 protein expression along bladder cancer progression also in human clinical material.

Biologically, PMF-1 was identified as one of the transacting factors involved in polyamine homeostasis (17–19, 30). Changes in the intracellular polyamine content of a cell can directly or indirectly affect the spectrum of genes expressed (16–19, 30). Polyamine depletions affect on the transcription rate and specific decrease in the expression of early growth-related genes including c-jun, c-myc, and c-fos (20–23). Thus, polyamines are considered important regulators of cell growth and cell death. Genes involved in cell cycle and cell growth (19–23) have been described to be transcriptionally regulated by PMF1. Although its role in cancer progression has not been fully elucidated, the epigenetic silencing of PMF1 might aid understanding as to how PMF1 may contribute to the regulation of polyamine metabolism and affect the transcriptional regulation of critical genes affecting tumorigenesis and tumor progression. Future studies are warranted to dissect such specific mechanisms in the context of bladder cancer and other neoplasias and the potential effect of risk factors such as tobacco, race, nutritional or environmental exposures, among others, into PMF1 methylation and expression using appropriate case-control experimental designs.

The translational implications of the discovery of PMF1 methylation have strongly been addressed in this work. The association of PMF1 with bladder cancer progression can be
References


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