Tumor Suppressor Function of PGP9.5 Is Associated with Epigenetic Regulation in Prostate Cancer—Novel Predictor of Biochemical Recurrence after Radical Surgery

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Abstract

Background: The expression level of protein G product 9.5 (PGP9.5) is downregulated because of promoter CpG hypermethylation in several tumors. We speculated that impaired regulation of PGP9.5 through epigenetic pathways is associated with the pathogenesis of prostate cancer.

Methods: CpG methylation of the PGP9.5 gene was analyzed in cultured prostate cancer cell lines, 226 localized prostate cancer samples from radical prostatectomy cases, and 80 benign prostate hyperplasia (BPH) tissues.

Results: Following 5-aza-2'-deoxycytidine treatment, increased PGP9.5 mRNA transcript expression was found in the LNCaP and PC3 cell lines. With bisulfite DNA sequencing, partial methylation of the PGP9.5 promoter was shown in LNCaP whereas complete methylation was found in PC3 cells. After transfection of PGP9.5 siRNA, cell viability was significantly accelerated in LNCaP but not in PC3 cells as compared with control siRNA transfection. Promoter methylation of PGP9.5 was extremely low in only one of 80 BPH tissues, whereas it was found in 37 of 226 prostate cancer tissues. Expression of the mRNA transcript of PGP9.5 was significantly lower in methylation (+) than methylation (−) prostate cancer tissues. Multivariate analysis of biochemical recurrence (BCR) after radical prostatectomy revealed pT category and PGP9.5 methylation as prognostically relevant. Further stratification with the pT category in addition to methylation status identified a stepwise reduction of BCR-free probability.

Conclusion: This is the first clinical and comprehensive study of inactivation of the PGP9.5 gene via epigenetic pathways in primary prostate cancer.

Impact: CpG methylation of PGP9.5 in primary prostate cancer might become useful as a molecular marker for early clinical prediction of BCR after radical prostatectomy. Cancer Epidemiol Biomarkers Prev; 21(3); 487–96. ©2012 AACR.

Introduction

Prostate cancer is one of the most common malignant disorders found in men throughout the world. Recent advances in prostate-specific antigen (PSA) testing as a screening strategy coupled with increased public awareness of prostate cancer as a slow-growing tumor more likely to be cured after appropriate treatment strategy has led to increases in the numbers of cases detected in the early stage such as organ confined disease (i.e., under stage T2; ref. 1). Despite the recent drastic stage migration of prostate cancer cases, locally advanced prostate cancer (i.e., stage T3 or T4) continues to occupy nearly 10% of all newly diagnosed cases (2). For organ confined prostate cancer, a radical prostatectomy or brachytherapy are primary therapeutic options. However, up to one quarter of those patients may experience biochemical recurrence (BCR) after radical surgery (3).

To improve the prognosis of affected patients, especially those with clinically localized prostate cancer, an adjuvant setting of radiotherapy and/or hormonal therapy seems to be promising to prevent BCR and the following metastatic progression (4–6). Thus, the capability to precisely predict BCR after such radical treatments is critical for determining whether additional treatment options will be mandatory. The most common parameters used to predict BCR after radical prostatectomy are Gleason score, pathologic T stage, tumor volume, and positive surgical margin (7). However, those are rarely effective, even when combined, probably due to the biologically heterogeneous nature of the disease. Therefore, a novel approach to predict BCR more precisely after a radical prostatectomy than the present pathology-based conventional strategy is
Translational Relevance

Previous studies have shown that protein G product 9.5 (PGP9.5) is a putative tumor-suppressor gene and the association of promoter CpG hypermethylation of PGP9.5 with an increase in the malignant potential of several cancers. In this study, we found that impaired regulation of PGP9.5 through epigenetic pathways is associated with the pathogenesis of localized prostate cancer. This is the first clinical and comprehensive investigation of inactivation of the PGP9.5 gene through epigenetic pathways in primary prostate cancer. Our results indicate that the epigenetics of promoter CpG hypermethylation of PGP9.5 might be one of the mechanisms associated with downregulation and functional loss of PGP9.5 in human prostate cancer. We concluded that CpG methylation of PGP9.5 in primary prostate cancer may be an attractive molecular marker for early clinical prediction of biochemical recurrence after a radical prostatectomy.

desirable and would be applicable to determining the necessity of additional treatments.

Protein gene product 9.5 (PGP9.5) is a neuron-specific protein with opposing functions of both ubiquitin carboxyl-terminal hydrolase and ligase (8, 9) and thought to be a common antigen in both neuroendocrine and secretory cells in the prostate (10–12). Recent progress in molecular biology techniques has shown that promoter hypermethylation of CpG islands is significantly related to downregulation or silencing of gene expression (13–15). CpG hypermethylation involving the promoter of the tumor suppressor gene seems to be a common mechanism underlying gene silencing, which leads to functional loss as a tumor suppressor. In human solid tumors, the expression level of PGP9.5 is frequently downregulated because of promoter CpG hypermethylation, which is considered to have a tumor suppressor function (16–19). Unfortunately, only limited information is available for PGP9.5 in human prostate glands, making it difficult to evaluate its functional role as a tumor suppressor. In human solid tumors, the expression level of PGP9.5 is frequently downregulated because of promoter CpG hypermethylation, which is considered to have a tumor suppressor function (16–19). Unfortunately, only limited information is available for PGP9.5 in human prostate glands, making it difficult to evaluate its functional role as a tumor suppressor and its active involvement in neuroendocrine differentiation.

The aim of the present study was to assess the correlation between expression of the PGP9.5 gene and epigenetic mechanisms (CpG hypermethylation). In addition, we evaluated the associations between the expression of PGP9.5 or CpG methylation of PGP9.5 status and clinical parameters or BCR in cases of localized prostate cancer following a radical prostatectomy.

Materials and Methods

Tissue samples and preparation

Two hundred and twenty-six primary localized prostate cancer (from a radical prostatectomy) and 80 pathologically proven benign prostate hyperplasia (BPH; from transurethral resection) specimens were obtained from the stocks of Shimane University Hospital ( Izumo, Shimane, Japan). Pathologic findings for the localized prostate cancer samples were determined using the General Rules for Clinical and Pathological Studies on Prostate Cancer provided by the Japanese Urological Association and Japanese Society of Pathology (20), which are essentially based on World Health Organization criteria and Gleason pattern (21). The 226 localized prostate cancer specimens comprised 166 cases of pT2, 56 cases of pT3, and 4 cases of pT4 disease. Our routine diagnostic strategy for prostate cancer includes serum PSA level, transrectal ultrasonography, color Doppler ultrasonography, and MRI findings, which enable us to detect the localization of prostate cancer before carrying out a radical prostatectomy (22). Control prostate specimens were taken from radical prostatectomy samples according to the following criteria: (i) prostate cancer presented as a single cancer focus, (ii) preoperative prostate cancer localization identical to postoperative pathologic findings, and (iii) prostate cancer localized on one side of the prostate. Finally, we selected and used 60 samples as controls, none of which contained any cancerous areas. Written informed consent was obtained from all patients. All of the BPH samples and half of each prostate cancer tissue specimen were fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin wax. Five-micrometer thick sections were subjected to hematoxylin and eosin staining for histologic evaluation and microdissection. The remaining half of each prostate cancer sample was immediately frozen and stored at −80 °C for RNA extraction. Microscopically dissected samples were analyzed for methylation. In the BPH samples, high-grade prostate intraepithelial neoplasia and cancer were ruled out by microscopic analysis.

Cell cultures and nucleic acid extraction

The human prostate cancer cell lines LNCaP and PC3 were obtained from American Type Culture Collection, but no authentication was done by us. The medium used in this study was RPMI-1640 supplemented with 10% fetal calf serum. Genomic DNA from all prostate samples was extracted by a QIAGEN Tissue kit (Qiagen) after microdissection (23) and precipitated with ethanol. Genomic DNA from cell line samples was extracted with DNAzol reagent (Invitrogen Life Technologies), and total RNA was extracted with TRI reagent (Molecular Research Center). The RNA pellet obtained after isopropanol and ethanol precipitation was dried, resuspended in 25 μL of RNase-free water, and stored in aliquots at −80 °C until reverse transcribed. The concentrations of DNA and RNA were determined with a spectrophotometer, and their integrity was checked by gel electrophoresis.

MTT assay

LNCaP and PC3 cells (5 × 10^3) were plated in 96-well plates in RPMI-1640 containing 10% FBS at a final volume of 0.1 mL. The next day, the cells were treated with PGP9.5 siRNA, then MTT was added (20 μL/well of 5 g/L solution in PBS) after culturing for 24, 48, or 72 hours. After incubation at 37 °C for 4 hours, the reaction was...
stopped by addition of 100 mL of dimethyl sulfoxide. The reaction product was quantified by measuring absorbance at 490 nm with an ELISA reader (Wallac 1420 Victor2, Victor Co.) and HT-Soft software (Perkin-Elmer). All samples were assayed in 6 wells and repeated.

**Treatment with 5-aza-2’-deoxycytidine**

To screen for epigenetic alterations in the PGP9.5 gene, LNCaP and PC3 cells were treated with 5-aza-2’-deoxycytidine (5-aza-dC, 5 µmol/L) in duplicate. Cultured cells were harvested after 4 days of treatment. Using cDNA, the difference in expression level of the PGP9.5 mRNA transcripts before and after 5-aza-dC treatment was analyzed with a StepOne quantitative RT-PCR system (Applied Biosystems).

**Treatment of LNCaP and PC3 cells with siRNA of PGP9.5**

Oligonucleotides representing siRNA against the expression of human PGP9.5 and mismatch control oligonucleotides were purchased from Ambion. The sense and antisense strands for siRNA-PGP9.5 were 5’-AAC-UAGUCCUAAGUGUATT-3’ and 5’-UACACUUUGGACUAACUUCT-3’, respectively. Cells were seeded at 5 × 10^5 cells in each well of 6-well plates with 2 mL of growth medium 18 hours before transfection and were 70% confluent at the time of transfection. For inhibition of PGP9.5, 5 µL of siRNA oligonucleotides (siRNA-PGP9.5 or siRNA-control) and 5 µL of Lipofectamine RNAiMAX reagent (Invitrogen Life Technologies Inc.) were separately diluted with 250 µL of Opti-MEM (Gibco). Cells were then transfected with Lipofectamine + siRNA-PGP9.5, Lipofectamine + siRNA-control, or left untransfected. Transfection was terminated after 5 hours by aspirating the transfection medium and adding fresh RPMI-1640 containing 10% FBS. Nonadherent cells were washed off and remaining cells were incubated at 37°C. Cell viability was measured with an MTT assay at 24, 48, and 72 hours after treatment.

**cDNA preparation and quantitative PCR of PGP9.5**

Using 1 µg of RNA, 0.5 µg of oligo(dT) primer, and 0.5 units of RNase inhibitor, cDNA was converted by reverse transcriptase (Promega). The expression level of the mRNA transcript of PGP9.5 was measured with a StepOne quantitative RT-PCR system (Applied Biosystems), with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) used as the reference gene. The reaction protocol recommended by the manufacturer (Applied Biosystems) was strictly followed. For each run, a standard curve was generated using a serial dilution of the external standard. The level of expression was calculated as the ratio of the target gene to that of the reference G3PDH.

**Methylation analysis and bisulfite DNA sequencing**

Genomic DNA (100 ng) was modified with sodium bisulfite by a commercial kit (Invitrogen Life Technologies). On the basis of the functional promoter sequence (24), methylation- and nonmethylation-specific primers were designed using MethPrimer. The regions amplified by these primers have 16 CpG sites and their relationship to the CpG sites are shown in Fig. 1A. For methylation-specific PCR (MSP), a second round of nested PCR [MSP and unmethylation-specific PCR (USP)] was done using the universal PCR product amplified by Uni-S and Uni-AS primers as a template. The first universal primer set had no CpG sites in either the forward or reverse primer. In each assay, the absence of a DNA template served as a negative control. The primer sequences for universal, MSP, and USP are shown in Fig. 1B. The MSP and USP products were analyzed by 2% agarose gel electrophoresis. For bisulfite DNA sequencing, 1 µL of bisulfite-modified DNA was amplified using a pair of universal primers (Uni-S and Uni-AS) in a total volume of 20 µL. Sequencing of the PCR products using either a forward or reverse universal primer was done according to the manufacturer’s instructions (Applied Biosystems).

**Apoptosis assay**

PC3 and LNCaP cells transfected with PGP9.5 siRNA were seeded in 96-well plates (3,000 cells per well), whereas those transfected with control siRNA were used as controls. After 48 hours, the culture medium was replaced with fresh RITC medium containing APOPercentage Dye Label (Biocolor Ltd.) and incubated for 1 hour at room temperature. Purple red–stained cells were identified as apoptotic cells. The number of purple red–stained cells per 100 cells was determined.

**Immunohistochemistry**

Two radical prostatectomy samples were evaluated for immunoreactivity of PGP9.5 protein, focusing on its location in the specimen, and the pathologic difference in the immunoreactivity between prostate cancer and BPH. Immunostaining of PGP9.5 was done using 5-µm thick consecutive sections obtained from paraffin-embedded materials using the mouse monoclonal antibody for PGP9.5 (1:250; Dako). Slides were prepared with antigen retrieval with citrate buffer (10 mmol/L, pH 6.0) before incubation with the primary antibody. For negative controls, the primary antibody was replaced with nonimmune serum. 3,3’-Diaminobenzidine (Sigma-Aldrich) was used as the chromogen, and counterstaining was done using methyl green.

**Statistical analysis**

Parametric data were statistically analyzed using an ANOVA test followed by a posthoc test. Nonparametric data were analyzed by a χ² test. BCR-free probability was estimated using the Kaplan–Meier method, and survival curves for the groups were compared statistically using a log-rank test. An age-adjusted logistic regression model was applied to determine independent factors contributing to BCR. A P value of less than 0.05 was considered to be statistically significant.
Results

Effects of PGP9.5 siRNA transfection on cell growth of LNCaP and PC3 cells

As shown in Fig. 2A, after LNCaP cells were transfected with PGP9.5 siRNA, the cellular growth of those cells was significantly accelerated, as compared with control siRNA–transfected LNCaP cells. On the other hand, there was no significant difference in cellular growth between PGP9.5 siRNA transfection and control siRNA transfection in PC3 cells (Fig. 2B). The difference in the effect of PGP9.5 siRNA

Figure 1. Schema of PGP9.5 promoter and locations of the primers. A, the putative region of the PGP9.5 promoter is depicted, with special reference to the primer location used. Universal primers (F, forward and R, reverse) do not contain any CpG sites within the primer sequence. MSP primers of F and R do contain 4 and 2 CpG sites, respectively, within their sequences, and USP primers are designed in the same manner. Regions A, B, and C are putative regions without special significance. The dashed arrows indicate CpG sites that have a different methylation status between LNCaP and PC3 cells. B, oligonucleotide sequences of universal (F and R), MSP (F and R), and USP (F and R) primers.

Results

Effects of PGP9.5 siRNA transfection on cell growth of LNCaP and PC3 cells

As shown in Fig. 2A, after LNCaP cells were transfected with PGP9.5 siRNA, the cellular growth of those cells was significantly accelerated, as compared with control siRNA–transfected LNCaP cells. On the other hand, there was no significant difference in cellular growth between PGP9.5 siRNA transfection and control siRNA transfection in PC3 cells (Fig. 2B). The difference in the effect of PGP9.5 siRNA

Figure 2. Effects of PGP9.5 siRNA transfection on LNCaP and PC3 cells. A, in LNCaP cells, cell viability was significantly increased after transfection of PGP9.5 siRNA as compared with control siRNA transfection. A detectable level of the mRNA transcript of PGP9.5 was found in LNCaP cells even before 5-aza-dC treatment (see Fig. 3A). B, in PC3 cells, there was no significant difference in cell viability between those transfected with PGP9.5 siRNA and control siRNA. No expression of the mRNA transcript of PGP9.5 was found in PC3 cells before 5-aza-dC treatment (see Fig. 3A). C, the number of LNCaP cells undergoing apoptosis was significantly decreased after transfection with PGP9.5 siRNA as compared with control siRNA. Pink-colored cells were considered to have undergone apoptosis.
transfection on cellular viability between LNCaP and PC3 cells appeared to be dependent on the presence of the PGP9.5 mRNA transcript. Figure 2C shows differences in apoptosis between PGP9.5 siRNA transfection and control siRNA transfection in LNCaP cells, which suggests suppression of apoptosis by PGP9.5 siRNA transfection.

**Promoter CpG methylation of PGP9.5 and mRNA transcript level in LNCaP and PC3 cells**

In LNCaP cells, the expression level of the PGP9.5 mRNA transcript was significantly increased after 5-aza-dC treatment, though PGP9.5 mRNA expression was also detected before treatment (Fig. 3A). On the other hand, the PGP9.5 mRNA transcript was significantly restored after 5-aza-dC treatment in PC3 cells, whereas that transcript was not clearly detectable before 5-aza-dC treatment. These findings suggest that promoter CpG methylation is associated with PGP9.5 expression in LNCaP and PC3 cells.

To confirm the relationship between CpG methylation and expression of the mRNA transcript of PGP9.5, we designed a primer sequence within the putative promoter CpG methylation region.
PGP9.5 promoter region to analyze the methylation status of the PGP9.5 promoter. As shown in Fig. 3B, partial methylation of CpG, in which the C peak was likely covered with the T peak, was detectable in LNCaP cells and this result seems to be compatible with that showing the effect of 5-aza-dC treatment. Likewise, the CpG promoter of PGP9.5 was completely methylated in PC3 cells (Fig. 3C), which in turn was in line with our finding of a loss of the PGP9.5 mRNA transcript in PC3 cells. In clinical samples, PGP9.5 protein expression was detectable in the stroma adjacent to prostate cancer tissue or nerve fibers, as shown in Fig. 3D and E.

Association of promoter CpG methylation of PGP9.5 with clinicopathologic findings of prostate cancer

We designed primers to discriminate between methylated and nonmethylated alleles following bisulfite treatment (Fig. 1B). Representative MSP and USP bands are shown in Fig. 4A. Of the 226 patients with prostate cancer who underwent a radical prostatectomy, 34 were found to be positive for PGP9.5 methylation. On the other hand, only 1 of the present cases with BPH was found to have a positive PGP9.5 methylation status (Fig. 4B). As shown in Table 1, a significant association of PGP9.5 methylation was found with pathologic findings showing pT category, perineural invasion, and venous and lymphatic vessel involvement. No significant association of PGP9.5 methylation was found with Gleason score or preoperative PSA value. Twenty-four patients with positive PGP9.5 methylation among 178 patients with prostate cancer who underwent no additional treatment before and after a radical prostatectomy showed a significantly worse BCR-free probability than those with nonmethylation (Fig. 4C). Interestingly, as shown in Fig. 4D, further stratification with the pT category (organ confined vs. locally advanced) in addition to methylation status identified a stepwise reduction of BCR-free probability.
As shown in Fig. 5A, the expression level of the PGP9.5 mRNA transcript was significantly lower in prostate cancer tissues than in noncancerous tissues and lower in methylated than nonmethylated prostate cancer tissues (Fig. 5B). When the expression level of the PGP9.5 mRNA transcript was divided into 2 groups based on the median value, a lower expression was significantly correlated with locally advanced disease or venous involvement (Fig. 5C), whereas no significant association with perineural invasion was found (Table 1). Similar to the findings of CpG methylation of the promoter, a lower expression of the PGP9.5 mRNA transcript was significantly associated with BCR after a radical prostatectomy (Fig. 5D).

Although both promoter CpG methylation and PGP9.5 mRNA transcript level were significantly associated with BCR after a radical prostatectomy in univariate analysis, PGP9.5 methylation was shown to be a more significantly independent predictor for BCR than mRNA expression in multivariate analysis. Because inclusion of the pT category as a variable in multivariate analysis resulted in loss of statistical power of PGP9.5 methylation as a predictor of BCR (Table 2), the essential role of PGP9.5 as a BCR predictor was in addition to the pT category. Thus, the combination of PGP9.5 methylation and pT category established a more attractive stratification for risk of BCR in a stepwise manner (Fig. 4D).

### Discussion

In the prostate, PGP9.5 protein expression is mostly detected in neuroendocrine cells and likely regulates both prostatic growth and differentiation (10–12, 25). Neuroendocrine differentiation in prostate cancer is closely associated with disease progression and unfavorable clinical outcome, with chromogranin A (CHGA) considered to be the best candidate marker (26–28). A previous investigation found that CHGA-positive neuroendocrine cells that also showed PGP9.5 immunoreactivity were significantly decreased in BPH (11). In addition, another showed that PGP9.5 expression in prostate cancer was restricted to neuroendocrine cells and likely lost in malignant epithelium (12), whereas downregulation of the PGP9.5 mRNA transcript caused by promoter CpG hypermethylation was frequently found in cancer tissues (16–19). However, the functional role of PGP9.5 in prostate cancer remains unclear, due to the lack of comprehensive analyses of human prostate specimens.

### Table 1. PGP 9.5 methylation and expression in relation to clinicopathologic findings

<table>
<thead>
<tr>
<th>Variables</th>
<th>PGP 9.5 methylation</th>
<th></th>
<th></th>
<th></th>
<th>PGP 9.5 expression</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Positive (N = 34)</td>
<td>Negative (N = 192)</td>
<td>P</td>
<td>Higher (N = 70)</td>
<td>Lower (N = 78)</td>
<td>P</td>
<td></td>
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<tr>
<td>Age, y</td>
<td>71</td>
<td>71</td>
<td>NS</td>
<td>71</td>
<td>68</td>
<td>NS</td>
<td></td>
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<tr>
<td>PSA, ng/mL</td>
<td>9.3</td>
<td>8.5</td>
<td>NS</td>
<td>9.3</td>
<td>8.0</td>
<td>NS</td>
<td></td>
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<tr>
<td>pT category, n (%)</td>
<td>&lt;pT2</td>
<td>19 (56)</td>
<td>147 (77)</td>
<td>&lt;0.005</td>
<td>57 (81)</td>
<td>50 (64)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>&gt;pT3</td>
<td>15 (44)</td>
<td>45 (23)</td>
<td></td>
<td>13 (19)</td>
<td>28 (36)</td>
<td></td>
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<tr>
<td>Gleason score, n (%)</td>
<td>&lt;7</td>
<td>24 (71)</td>
<td>158 (82)</td>
<td>NS</td>
<td>59 (84)</td>
<td>61 (78)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>&gt;8</td>
<td>10 (29)</td>
<td>34 (18)</td>
<td></td>
<td>11 (16)</td>
<td>17 (22)</td>
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<td>Perineural invasion, n (%)</td>
<td>Positive</td>
<td>5 (15)</td>
<td>63 (33)</td>
<td>&lt;0.05</td>
<td>19 (27)</td>
<td>23 (29)</td>
<td>NS</td>
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<tr>
<td></td>
<td>Negative</td>
<td>20 (65)</td>
<td>129 (67)</td>
<td></td>
<td>51 (73)</td>
<td>55 (71)</td>
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<td>Vascular invasion, n (%)</td>
<td>Positive</td>
<td>18 (53)</td>
<td>135 (70)</td>
<td>&lt;0.05</td>
<td>55 (79)</td>
<td>49 (63)</td>
<td>&lt;0.05</td>
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<td></td>
<td>Negative</td>
<td>16 (47)</td>
<td>54 (29)</td>
<td></td>
<td>15 (21)</td>
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<td></td>
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<td>3 (1)</td>
<td></td>
<td>0</td>
<td>1 (1)</td>
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<td>Lymphatic invasion, n (%)</td>
<td>Positive</td>
<td>11 (32)</td>
<td>98 (51)</td>
<td>&lt;0.05</td>
<td>35 (50)</td>
<td>30 (38)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>8 (24)</td>
<td>27 (14)</td>
<td></td>
<td>11 (16)</td>
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<td>15 (44)</td>
<td>67 (35)</td>
<td></td>
<td>20 (34)</td>
<td>39 (50)</td>
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androgen production (29, 30). It is considered that the function of PGP9.5 in the prostate is as a regulator of neuroendocrine differentiation. Early identification of prostate cancer cells with neuroendocrine differentiation or confirmation of the high probability of developing treatment-resistant neuroendocrine differentiation following radical surgery can surely reveal the best treatment strategy to improve the outcome of patients with prostate cancer. In the present study, we speculated that the expression of PGP9.5 in prostate cancer tissues is regulated by epigenetic alteration of CpG methylation and the functional role of PGP9.5 in prostate cancer is that of a tumor suppressor. Irrespective of the androgen-dependent potential of the prostate cancer cell lines used in our study, 5-aza-dC treatment significantly increased PGP9.5 mRNA transcript expression as compared with

![Figure 5](image-url)

**Figure 5.** Effects of PGP9.5 methylation status on expression level of PGP9.5 mRNA transcript in association with clinicopathologic findings. A, the expression level of the PGP9.5 mRNA transcript was significantly lower in prostate cancer tissues than that in noncancerous prostate cancer tissues \(P < 0.005\). B, in prostate cancer tissues, PGP9.5 methylation (+) resulted in a significantly lower level of expression of the PGP9.5 mRNA transcript than PGP9.5 methylation (−; \(P < 0.01\)). C, a higher level of expression of the PGP9.5 mRNA transcript was significantly associated with a lower prevalence of cases with advanced disease (greater than pT3; \(P < 0.05\)) and venous involvement \(P < 0.05\). D, a higher level of expression of the PGP9.5 mRNA transcript was significantly associated with higher BCR-free probability after a radical prostatectomy \(P < 0.05\).

### Table 2. Age-adjusted multivariate analysis for predicting BCR using logistic regression model

<table>
<thead>
<tr>
<th>Variables</th>
<th>Coefficient</th>
<th>(\chi^2)</th>
<th>(P)</th>
<th>Relative risk (95% CI)</th>
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<td>Gleason score</td>
<td>0.444</td>
<td>0.865</td>
<td>NS</td>
<td>1.559 (0.612–3.971)</td>
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<tr>
<td>PGP9.5 methylation</td>
<td>1.260</td>
<td>6.280</td>
<td>&lt;0.02</td>
<td>3.526 (1.316–9.446)</td>
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<td>Perineural invasion</td>
<td>−2.508</td>
<td>5.841</td>
<td>&lt;0.02</td>
<td>0.081 (0.011–0.623)</td>
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<tr>
<td>pT category</td>
<td>1.677</td>
<td>14.67</td>
<td>&lt;0.005</td>
<td>5.352 (2.268–12.629)</td>
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</table>

Abbreviation: CI, confidence interval.
nontreatment, suggesting the probability of CpG hyper-methylation as a regulator of PGP9.5 gene expression in human prostate cancer cells. As shown in Fig. 3, bisulfite DNA sequencing of the PGP9.5 promoter resulted in complete methylation in PC3 cells whereas only partial methylation was dominant in LNCaP cells. This may explain why a detectable level of PGP9.5 mRNA transcript was found even before 5-aza-dC treatment in LNCaP cells.

We also found that the prevalence of CpG methylation of PGP9.5 was significantly higher in prostate cancer than BPH tissues. As for the latter, only one of the present cases with BPH was found to have a positive PGP9.5 methylation status. Inversely, the expression level of the PGP9.5 mRNA transcript was significantly lower in prostate cancer than noncancerous prostate tissues, suggesting epigenetic regulation of PGP9.5 in the clinical samples as well. Our results showed that neither CpG methylation of the PGP9.5 promoter nor the expression level of the mRNA transcript of the PGP9.5 gene had a correlation with Gleason score, which likely indicate that PGP9.5 alteration is an early event in the pathogenesis of prostate cancer. Our findings also showed that siRNA transfection of PGP9.5 in LNCaP cells, in which partial methylation was found in the CpG promoter with a weak level of PGP9.5 mRNA transcript detected, significantly enhanced cell viability through acceleration of the inhibitory effect on cultured cells, despite of a significantly lower amount of PGP9.5 mRNA transcript in LNCaP. In contrast, in PC3 cells, in which the PGP9.5 mRNA transcript was not absolutely detectable because of complete methylation of the CpG promoter, no inhibitory effect of siRNA transfection of PGP9.5 on the viability of PC3 cells was found. From these findings, we thought that PGP9.5 has a tumor suppressive potential in human prostate cancer tissues, though the mechanism underlying that tumor suppressive function remains unknown. Using an in vitro model, PGP9.5 was shown to be a critical downstream effector of p53 involving inactivation of the Akt/Pi3 pathway (19). Considering that the Akt/Pi3 pathway is frequently activated in prostate cancer tissues, and p21 expression at the mRNA transcript level was detectable in both LNCaP and PC3 cells (data not shown), coupled with the PGP9.5 siRNA transfection findings mentioned earlier, it is quite reasonable that PGP9.5 functions as a tumor suppressor in prostate cancer.

Next, we investigated the prognostic relevance of PGP9.5 methylation and expression by focusing on the probability of early detection of BCR after radical surgery. Univariate analysis showed that higher BCR-free probability was significantly associated with higher PGP9.5 expression or negative PGP9.5 methylation (Figs. 4D, 5D). Furthermore, age-adjusted multivariate analysis clearly showed the prognostic relevance of PGP9.5 methylation to predict early BCR after surgery, following pT category, and also identified PGP9.5 methylation as an independent factor superior to Gleason score. Some cancer tissues have shown increased expression of PGP9.5 (31–33), leading to confusion about whether PGP9.5 serves as a tumor suppressor or oncogene. Provided that one allele might be affected by methylation and the remaining allele with the shared characteristics of the oncogenic property, the expression level of the PGP9.5 mRNA transcript might become quite ambiguous and result in far from what can be speculated by methylation analysis. We concluded that this explains why the statistical power of mRNA expression of PGP9.5 to predict BCR after a radical prostatectomy was lost when compared with the methylation status of the PGP9.5 promoter itself.

In prostate cancer tissues, perineural invasion is thought to be a substantial gateway for prostate cancer cells to spread out from the prostate. Interestingly, as shown in Table 2, perineural invasion of prostate cancer cells was found to be related to BCR after surgery and showed a significant correlation with PGP9.5 methylation. Management of high-risk, localized prostate cancer remains an extraordinary challenge, because of the high recurrence rate exceeding 50% after primary therapy (34). Gleason score, pathologic T stage, tumor volume, and positive surgical margins are all major concerns for BCR and may constitute independent factors for predicting BCR following radical prostatectomy (7). However, these are not able to always predict BCR due to the biologic variability of prostate cancer. In this study, we found that further stratification with pT category in addition to methylation status identified a stepwise reduction of BCR-free probability, in which the highest BCR-free probability was found in prostate cancer tissues both methylation-negative and with a pT category of less than 2, whereas the lowest was in prostate cancer tissues shown to be methylation-positive and with a pT category greater than 3a. On the basis of our findings, we concluded that CpG methylation of PGP9.5 status in combination with pathologic T stage can more accurately predict BCR and may help to identify and select patients for additional treatments following radical prostatectomy. In future study, the potential difference in the PGP9.5 expression found between prostate cancer tissue and adjacent stromal tissue affecting BCR after radical prostatectomy should be also analyzed to make clear the essential role of PGP9.5 as tumor suppressor in prostate cancer.

This is the first clinical and comprehensive study of inactivation of the PGP9.5 gene via epigenetic pathways in primary prostate cancer. The epigenetics of promoter CpG hypermethylation of PGP9.5 may be one of the mechanisms associated with downregulation and functional loss of PGP9.5 in human prostate cancer. We consider that CpG methylation of PGP9.5 in primary prostate cancer might become useful as a molecular marker for early clinical prediction of BCR after radical prostatectomy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Received October 13, 2011; revised January 6, 2012; accepted January 9, 2012; published OnlineFirst January 13, 2012.

Received October 13, 2011; revised January 6, 2012; accepted January 9, 2012; published OnlineFirst January 13, 2012.
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