Diagnostic and Prognostic Information in Prostate Cancer with the Help of a Small Set of Hypermethylated Gene Loci

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Abstract

Purpose: Our study was designed to evaluate promoter CpG island hypermethylation in the diagnosis and prognosis of prostate cancer.

Experimental Design: Primary prostate cancers from 53 patients, pelvic lymph nodes, noncancerous prostate tissues, and prostate cell lines were analyzed. Real-time methylation-specific PCR was used to identify CpG island hypermethylation at five promising gene loci (i.e., *GSTP1*, *APC*, *PTGS2*, *MDR1*, and *RASSF1a*).

Results: At three gene loci (*GSTP1*, *APC*, and *PTGS1*) and CpG island, hypermethylation was highly prevalent in prostate cancers (71-91%), and analysis of receiver operator curves showed that hypermethylation at these three gene loci can distinguish between prostate cancer and noncancerous prostatic tissue (i.e., benign hyperplasia) with a sensitivity of 71.1% to 96.2% and a specificity of 92.9% to 100%. Using sensitive SYBR green methylation-specific PCR technology, we observed a respective 28% and 71% hypermethylation rate at the *RASSF1a* and *MDR1* loci in benign prostate hyperplasia, which may represent early nonaggressive carcinogenesis. Methylation characteristics in prostate cancer metastases (i.e., pelvic lymph nodes) were comparable to the respective primary cancer. Statistical analysis showed no correlation between the methylation status of a single gene locus and clinicopathologic variables (e.g., preoperative prostate specific antigen levels, Gleason score, capsular penetration, involvement of seminal vesicle, and age). In contrast, the methylation of two (*GSTP1*/*APC*; *GSTP1*/*PTGS2*) or three (*GSTP1*/*APC*/*PTGS2*) gene loci correlated with prognostic indicators (i.e., pathologic stage, extraprostatic extension, and Gleason score, but not with prostate specific antigen levels).

Conclusions: Our data suggest that the evaluation of DNA hypermethylation at three gene loci (i.e., *GSTP1*, *APC*, and *PTGS2*) is of diagnostic and prognostic value in prostate cancer.

Prostate cancer is the most common cancer in men and the second leading cause of cancer-related deaths in the United States and Western Europe. Since the beginning of the prostate specific antigen (PSA) testing era in the late 1980s, there has been a significant increase in the diagnosis of men with nonpalpable prostate cancer (1, 2). Autopsy studies and the recent Prostate Cancer Prevention Trial have revealed a higher prevalence of prostatic cancer than anticipated by PSA screening alone (3–5). The lifetime risk of developing prostate cancer in the United States is 1 in 6, whereas the lifetime risk of death due to metastatic prostate cancer is 1 in 30 (6). It is estimated that 230,000 to 240,000 new cases will be diagnosed in 2004/2005 and about 30,000 men will die of the disease in the United States at the same time (6). In Western European countries without prostate cancer screening, a somewhat lower prevalence is found, with age-standardized incidence rates of 54.92 per 100,000 (United States: 104.33 per 100,000; ref. 7).

PSA screening, regardless of the threshold value, has certain well-documented limitations with regards to sensitivity and specificity for the detection of prostate cancer (8). Therefore ultrasound-guided prostate biopsy is still the gold standard method for diagnosing prostate cancer. The precise biopsy strategy in terms of the number of samples and the sampled area, however, remains controversial (9, 10). Moreover, nondiagnostic but clinically suspicious lesions, such as a small focus of atypical glands or high-grade prostatic intraepithelial neoplasia, necessitate further evaluation in the absence of obvious cancer in the specimen (11).

Molecular studies have revealed important information about prostate cancer development and progression (8). Furthermore, multiple immunohistochemistry tools to aid the diagnosis of prostate cancer have been developed. As of yet, none of these procedures, alone or in conjunction, have

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been able to definitively diagnose prostate cancer (12–16). Presently, the emergence of new molecular tests may improve identification of early neoplastic alterations in prostatic cells, but only a few have been adequately validated for clinical use (8, 17, 18).

Epigenetic alterations (i.e., aberrant DNA methylation patterns, generalized hypomethylation, and regional CpG island hypermethylation) are characteristics of tumor cells (19). The earliest and most common somatic genome alteration during prostate cancer development seems to be CpG island hypermethylation in the regulatory region of the π-class glutathione *S*-transferase (*GSTP1*) gene (>90%; refs. 8, 20). The detection of *GSTP1* CpG island hypermethylation in bodily fluids like urine or serum even reaches sensitivities up to 76% (21, 22). However, the use of a single gene locus has several drawbacks. First, the maximum sensitivity can only be as high as the frequency of hypermethylation at a specific CpG locus. Second, noncancerous tissues can in some cases harbor CpG island hypermethylation at the same gene locus. Third, methylation of a single gene locus may occur in other cancers and thus be misleading in prostate cancer (23). Furthermore, it has been shown that the number of hypermethylated genes increases as prostate cancer progresses; thus, the investigation of several genes may provide additional diagnostic information (24, 25).

To establish a small set of reliable diagnostic investigations in prostate cancer, we quantitatively assessed the methylation status of CpG islands in the regulatory regions of *GSTP1* and four other genes we thought promising. Reports in the literature have shown that these genes are either silenced or activated by hypermethylation/hypomethylation mechanisms and/or play important roles in many human neoplasms:

1. APC (adenomatosis polyposis coli): a well-characterized tumor suppressor involved in down-regulating WNT/β-catenin signaling and central to development and the mature organism. Initially identified in colorectal cancer, APC is inactivated in various malignancies including prostate cancer by genetic and epigenetic mechanisms (23, 26–29).
2. *PTGS2* [prostaglandin-endoperoxide synthase 2 or cyclooxygenase 2 (COX-2)]: involved in the regulation of inflammation (e.g., T-helper-2 response) and mitogenesis/carcinogenesis, presumably inactivated in apoptosis. It is presumably up-regulated in proliferative inflammatory atrophy of the prostate, but not in prostate carcinoma, by genetic/epigenetic alterations (30).
3. *MDR1* (multidrug resistance 1): encodes the P-glycoprotein and is a member of the ATP-binding cassette transporter gene superfamily that regulates the trafficking of drugs, peptides, xenobiotics, and ions across cell membranes. Its expression correlates with resistance to hormone therapy and is thought to be important in the progression of primarily hormone-sensitive malignancies like prostate cancer. Activation of MDR1 occurs rapidly in response to varied cellular stresses, and epigenetic hypomethylation may hereby play a role (28, 31–35).
4. *RASSF1a* (Ras association domain family 1 isoform A): a tumor suppressor that interacts with Cdc20, an activator of the anaphase-promoting complex, resulting in the inhibition of activity of complexes and the prevention of mitotic progression. It conserves cyclins A and B, and its expression correlates with proapoptotic activity. Epigenetic inactivation of RASSF1a is frequently observed in a number of solid tumors and epithelial cancers including prostate cancer (24, 28, 29, 36–43).

Our data show that combining the CpG island methylation status of three of these genes (i.e., *GSTP1*, *APC*, and *PTGS2*) provides the clinician with valuable diagnostic and prognostic information. The significance of the hypermethylation of *RASSF1a* and *MDR1* that was observed in benign prostate hyperplasia (BPH) as well as prostate cancer is discussed.

Materials and Methods

Patients and sample collection. A total of 53 consecutive patients with prostate cancer and 14 consecutive patients with BPH were recruited into this study. All tissue samples were obtained during urological standard procedures (radical or open prostatectomy, radical cysto-prostatectomy) done at the Department of Urology, University of Bonn, Germany, between 2000 and 2003. All patients had given written informed consent for the collection of tissue and further analysis according to the institutional ethical guidelines before surgery. Pelvic lymph nodes were obtained during radical prostatectomy for clinically localized prostate cancer in every patient. All cancerous and noncancerous samples were meticulously surveyed by pathologists and microdissected to ensure a high grade of prostatic epithelia purity (>70% cancer cells by microscopic examination). Clinical information is listed in Table 1. Follow-up information was available for 23 of the 53 patients (3-38 months; median, 21 months). Biochemical recurrence (i.e., PSA > 0.2 ng/mL) was observed in two patients (mean onset, 24 months).

Three prostate cancer cell lines (LNCaP, DU-145, and PC-3) and one BPH cell line (BPH-1) were studied. The cell lines were obtained from the "Deutsche Sammlung für Mikroorganismen und Zellkultur," Braunschweig, Germany, and cultured according to the protocol of the vendor (http://www.dsmz.de). Briefly, cell lines were grown in RPMI 1640 containing 10% fetal bovine serum and 35 units/mL Pen/Strep (all ingredients from PAA Laboratories, Coelbe, Germany). Additionally, BPH-1 medium contained 1% insulin, transferrin, and selenium supplement and 0.1% testosterone (Sigma, Seelze, Germany). Cell lines were grown in 5% CO_2/95% air at 37°C.

DNA isolation. The archival, formalin-fixed, paraffin-embedded tissues were cut in 20 μm serial sections. At the beginning and end of each section, a 7.5-μm-thick cut was taken for H&E staining. The area of prostate cancer or BPH was marked by an experienced pathologist and subsequently microdissected. After deparaffinization using xylol and ethanol, we isolated genomic DNA using the Qiagen DNA Mini Kit (Qiagen GmbH, Hilden, Germany). DNA isolation from pelvic lymph nodes revealed sufficient yield for further analysis in only 15 of 53 patients. Only 3 of these 15 lymph nodes had metastatic involvement. Additionally, cell lines and healthy volunteers' WBC DNA were isolated using the Qiagen DNA Mini kit. Universal methylated DNA was prepared by treatment of WBC-DNA with *Sss*I CpG methylase (New England Biolabs, Frankfurt, Germany).

Sodium bisulfite treatment. Sodium-bisulfite modification was done according to Grunau et al. (44). In short, four aliquots of 2 μg DNA and 2 μg tRNA carrier were dissolved in 100 μL distilled water and denatured in a final concentration of 0.3 mol/L NaOH for 20 minutes at 37°C. Afterwards, 520 μL of a 3.87 mol/L sodium bisulfite solution (pH 5) containing hydroquinone at a final concentration of 10 mmol/L were added and incubated at 55°C for 18 hours in the dark. The modified DNA was desalted with QIAEx II (Qiagen) and the four aliquots were dissolved in 100 μL of 1 mmol/L Tris-HCl (pH 8). Final desulfonation was done (0.3 mol/L NaOH for 20 minutes at 37°C), followed by neutralization with 47 μL of 10 mol/L ammonium acetate

Table 1. Clinical information of patients

Clinical variable	Prostate cancer patients (%)	BPH patients (%)
Age (y)		
Mean	63.26	64.87
Median	62	63
Range	50-75	50-81
Preoperative PSA (ng/mL)		
Mean	13.8	13.68
Median	9.805	13.25
Range	2-58.4	3.65-21.62
Pathologic stage		
Organ confined	30 (56.6)	
Extraprostatic extension	20 (37.3)	
Seminal vesicle involvement	6 (11.3)	
Lymph node/distant metastases	5 (9.4)	
pT_2	31 (58.5)	
pT_3	20 (37.7)	
pT_4	2 (3.8)	
Cumulative Gleason score		
3	3 (5.7)	
4	2 (3.8)	
5	11 (20.8)	
6	13 (24.5)	
7	19 (35.8)	
8	2 (3.8)	
9	1 (1.9)	
10	2 (3.8)	
Total	53 (100)	15 (100)

and addition of 1 μg tRNA. Finally, after overnight ethanol precipitation, these were washed in 70% ethanol and the DNA was resuspended in 140 μL of 1 mmol/L Tris-HCl (pH 8). Modified DNA was stored for less than 6 weeks. All reagents not otherwise specified were from Sigma-Aldrich (Seelze, Germany).

Real-time methylation-specific PCR. We did real-time methylation-specific PCR amplification of the promoter/regulatory regions of *GSTP1*, *APC*, *PTGS2*, *MDR1*, and *RASSF1a*. *MYOD1* served as internal reference. The primer sequences to amplify bisulfite-converted CpG islands are listed in Table 2. Primers sequences were obtained from previously published data [*MYOD1* (45), *RASSF1a* (46), or self-designed (*GSTP1*, *APC*, *PTGS2*, and *MDR1*) with MethPrimer (http://www.ucsf.edu/urogene/methprimer; ref. 47)]. All PCR experiments were carried out in a volume of 10 μL with 384-well plates and an Applied Biosystems 7900HT Sequence Detector (Perkin-Elmer, Foster City, CA). The fluorescence signal of the quantitative methylation-specific PCR was generated by SYBR Green I, a dye included in any double-stranded DNA during PCR amplification (SYBR Green PCR Master Mix, Applied Biosystems, Foster City, CA).

Samples (2.7 μL bisulfite-treated DNA) were run in triplicate containing 5 μL SYBR Green Master Mix (Applied Biosystems) and 5.5 pmol of each forward and reverse primer. Every PCR experiment included serial dilutions of a positive control for construction of the calibration curve, a positive and a negative DNA sample, and water blanks. PCR amplification was done by means of the following procedure: 95°C for 15 minutes, followed by 40 cycles at 95°C for 30 seconds, 56°C for 20 seconds, and 72°C for 30 seconds, followed by a final 10-minute extension step at 72°C. A subsequent dissociation curve analysis checked the specificity of products. The same reaction conditions were applied to all six genes.

Relative levels of methylated DNA in each sample were calculated and described as the normalized index of methylation (NIM), as suggested by Yegnasubramanian et al. (28). The NIM defines the ratio of the normalized amount of methylated templates at the promoter to the amount of converted *MYOD1* templates in any given sample and serves as an index of the percentage of bisulfite-converted input copies of DNA that are fully methylated at the primer site (28). The NIM scale from white (no methylation detected) to black (>99% of input DNA methylated) was designed with Microsoft Visual Basic (Microsoft Corporation, Seattle, WA).

Statistical analysis. Differences in the NIM between benign and cancerous tissue were tested by means of the Mann-Whitney test. Correlations between the NIM, on one hand, and between age, preoperative PSA, and Gleason score, on the other hand, were determined by Spearman's correlation coefficient. The relationship between various gene combinations and clinicopathologic variables was examined by χ^2 or Fisher's exact test, as indicated. All tests were two sided. Statistical analysis was done with SPSS software (SPSS, Inc., Chicago, IL). The optimal threshold value that distinguishes cancer and noncancerous tissue was calculated via receiver operator curve analysis. Using the threshold value, we determined the specificity, sensitivity, positive predictive value, and negative predictive value for each gene and for multiple combinations.

Table 2. Primers of gene regulatory regions used in hypermethylation studies

Gene	Forward primer	Reverse primer	Gene bank
MYOD1	5′ CCA ACT CCA AAT CCC CTC TCT AT 3′	5′ TGA TTA ATT TAG ATT GGG TTT AGA GAA GGA 3′	AF027148
GSTP1	M: 5′ GTC GTG ATT TAG TAT TGG GGC 3′	M: 5′ CTA ATA ACG AAA ACT ACG ACG ACG 3′	X08058
	U: 5′ TGT GAT TTA GTA TTG GGG TGG 3′	U: 5′ TCA CTA ATA ACA AAA ACT ACA ACA ACA AA 3′	
APC	M: 5′ GAG GGT ATA TTT TCG AGG GGT AC 3′	M: 5′ AAT AAA AAA CGC CCT AAT CCG 3′	U02509
	U: 5′ GAG GGT ATA TTT TTG AGG GGT ATG 3′	U: 5′ AAT AAA AAA CAC CCT AAT CCA CA 3′	
MDR1	M: 5′ CGT TGT TAG ATT TTT AAT TTT GTT TTC 3′	M: 5′ CCA ACT ACT CTA ACC GCG AT 3′	X58723
	U: 5′ TGT TGT TAG ATT TTT AAT TTT GTT TTT GT 3′	U: 5′ TAC CCC AAC TAC TCT AAC CAC AAT 3′	
RASSF1a	M: 5′ GCG TTG AAG TCG GGG TTC 3′	M: 5′ CCC GTA CTT CGC TAA CTT TAA ACG 3′	AF132675
	U: 5′ GTG TTG AAG TTG GGG TTT 3′	U: 5′ CCC ATA CTT CAC TAA CTT TAA ACA 3′	
PTGS2	M: 5′ TTT TTT TCG GTA TTT TAT TTA AGG C 3′	M: 5′ AAA CGC ACA AAT TTC CGC 3′	AF044206
	U: 5′ TTT TTT TTG GTA TTT TAT TTA AGG TGA 3′	U: 5′ CCC AAA CAC ACA AAT TTC CA 3′	

NOTE: M, methylated sequence; U, unmethylated sequence.

Results

Gene hypermethylation in prostate cell lines and noncancerous prostatic tissue. Figure 1A shows that the five gene loci (CpG islands) we studied are regularly hypermethylated in prostate cancer cell lines (i.e., DU-145, LNCaP, and PC-3). In the prostate hyperplasia cell line, BPH-1, CpG island hypermethylation was only observed at the *RASSF1a*. Figure 1B and Table 3 show that CpG island hypermethylation in BPH also occurs rather frequently at the *MDR1* and *RASSF1a* gene loci, whereas *GSTP1*, *APC*, and *PTGS2* are rarely hypermethylated.

Gene hypermethylation in clinically localized prostate cancers. Figure 2 and Table 3 show the CpG island hypermethylation status in 53 different prostate cancer tissue specimens. In all cases, sufficient amounts of DNA were extracted and thus no uninformative cases had to be taken out of the analysis. A large degree of CpG island hypermethylation was detected in all five gene loci and all tissue samples. CpG island hypermethylation was significantly different in prostate cancer and BPH, except in the case of the *MDR1* locus where no distinct difference in methylation frequency was observed (Table 3).

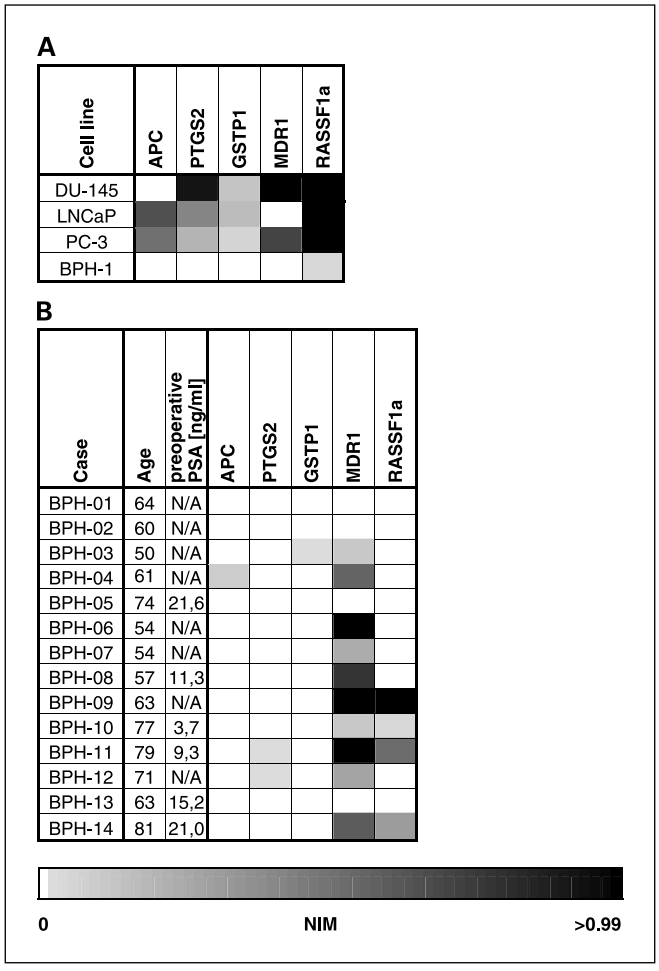


Fig. 1. Gene hypermethylation (expressed as NIM) at five gene loci (CpG-islands) in prostate cell lines and tissues. Methylation studies were done using real-time methylation-specific PCR as described in Materials and Methods. *A,* prostate cancer cell lines (LNCaP, DU-145, and PC-3) and benign prostatic hyperplasia cell line (BPH-1). *B,* tissue samples from various benign prostatic hyperplasias as specified in the graph. The NIM was scaled between white (no methylation) and black (>99% of input DNA was methylated).

Table 3. Frequencies of methylation at various gene loci (%)

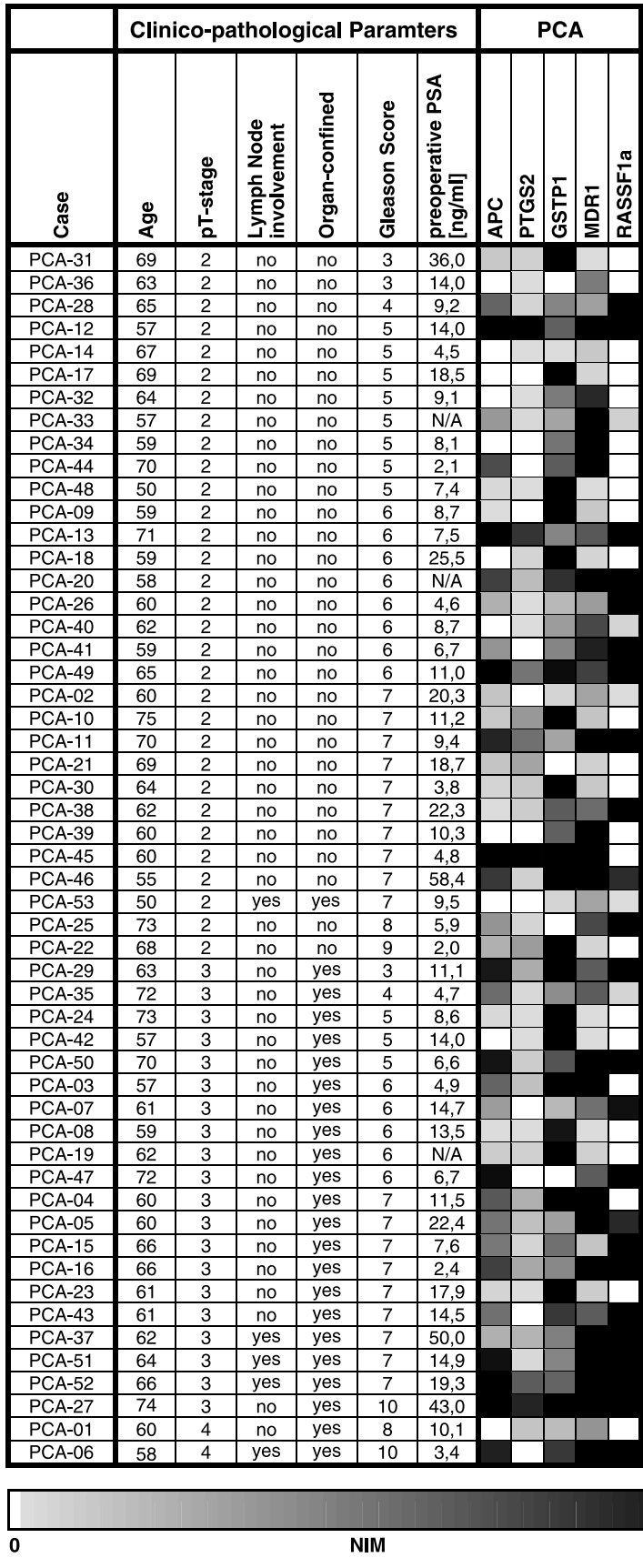
Group	*GSTP1*	*PTGS2*	*APC*	*MDR1*	*RASSF1a*
BPH	0 (0)	0 (0)	1 (7.1)	10 (71.4)	4 (28.6)
Prostate cancer	48 (90.6)	38 (71.1)	44 (83)	53 (100)	36 (67.9)
*P**	<0.001	<0.001	<0.001	0.112	0.009

*Mann-Whitney *U* test.

Gene hypermethylation in lymph nodes of clinically localized prostate cancer. Lymph nodes with extractable/intact DNA were available from only 15 patients with prostate cancer (see Fig. 3). Histopathologic examinations revealed metastatic disease in the lymph nodes of only three of these patients. Of the five gene loci we studied, distinct CpG island hypermethylation was observed in the lymph nodes of only two of these patients. In these cases, the methylated CpG islands were similar, but the methylation index (NIM) of these loci was only vaguely related to the findings in the primary tumor (Fig. 3). On the other hand, no noteworthy hypermethylation of gene loci was observed in lymph nodes that showed no histopathologic involvement. A recent report shows that the frequency and quality (fingerprint) of CpG island hypermethylation in the metastases of prostate cancer may be similar to that in the primary tumor (28). Our small number of lymph node samples with metastasis prohibits any weighty statistical conclusions.

Sensitivity and specificity of gene hypermethylation in the diagnosis of prostate cancer. To assess the usefulness of gene hypermethylation so as to discriminate between noncancerous and cancerous tissues, the sensitivity, specificity, as well as the positive and negative predictive values of hypermethylation were determined via receiver operator curve analysis (Fig. 4; Table 4). This procedure revealed that hypermethylation at *GSTP1*, *APC*, and *PTGS2* gene loci was the most powerful means to distinguish between cancerous and noncancerous prostatic tissue. Each gene locus had a sensitivity of >71%, a specificity >92%, and an area under the curve of >0.89. Furthermore, combining the last mentioned genes resulted in even more diagnostic power. For example, hypermethylation at *GSTP1* in combination with hypermethylation at *PTGS2* yields a diagnostic sensitivity of 96.2%, maintaining a specificity of 100%.

Correlation of hypermethylation and clinicopathologic variables. With the help of the χ^2 and Fisher's exact test, correlation between the hypermethylation of gene loci and various previously established, clinicopathologic variables was evaluated (i.e., preoperative serum PSA level, pathologic stage of surgical specimen, organ-confined tumor, seminal vesicle involvement, pelvic lymph node involvement, pathologic Gleason score, surgical margin status, and patient age; cf. Table 5; refs. 48, 49). Hypermethylation of a single gene did not correlate with any of the examined clinicopathologic variables to a statistically significant extent. On the other hand, the simultaneous or alternative hypermethylation of two and three genes (see Table 5) significantly correlated with the pathologic stage, extraprostatic extension of the tumor, and the Gleason score. For instance, the simultaneous hypermethylation at *GSTP1* and *APC* correlated with the



Case	Age	pT-stage	Lymph Node involvement	Organ-confined	Gleason Score	preoperative PSA [ng/ml]
PCA-31	69	2	no	no	3	36,0
PCA-36	63	2	no	no	3	14,0
PCA-28	65	2	no	no	4	9,2
PCA-12	57	2	no	no	5	14,0
PCA-14	67	2	no	no	5	4,5
PCA-17	69	2	no	no	5	18,5
PCA-32	64	2	no	no	5	9,1
PCA-33	57	2	no	no	5	N/A
PCA-34	59	2	no	no	5	8,1
PCA-44	70	2	no	no	5	2,1
PCA-48	50	2	no	no	5	7,4
PCA-09	59	2	no	no	6	8,7
PCA-13	71	2	no	no	6	7,5
PCA-18	59	2	no	no	6	25,5
PCA-20	58	2	no	no	6	N/A
PCA-26	60	2	no	no	6	4,6
PCA-40	62	2	no	no	6	8,7
PCA-41	59	2	no	no	6	6,7
PCA-49	65	2	no	no	6	11,0
PCA-02	60	2	no	no	7	20,3
PCA-10	75	2	no	no	7	11,2
PCA-11	70	2	no	no	7	9,4
PCA-21	69	2	no	no	7	18,7
PCA-30	64	2	no	no	7	3,8
PCA-38	62	2	no	no	7	22,3
PCA-39	60	2	no	no	7	10,3
PCA-45	60	2	no	no	7	4,8
PCA-46	55	2	no	no	7	58,4
PCA-53	50	2	yes	yes	7	9,5
PCA-25	73	2	no	no	8	5,9
PCA-22	68	2	no	no	9	2,0
PCA-29	63	3	no	yes	3	11,1
PCA-35	72	3	no	yes	4	4,7
PCA-24	73	3	no	yes	5	8,6
PCA-42	57	3	no	yes	5	14,0
PCA-50	70	3	no	yes	5	6,6
PCA-03	57	3	no	yes	6	4,9
PCA-07	61	3	no	yes	6	14,7
PCA-08	59	3	no	yes	6	13,5
PCA-19	62	3	no	yes	6	N/A
PCA-47	72	3	no	yes	6	6,7
PCA-04	60	3	no	yes	7	11,5
PCA-05	60	3	no	yes	7	22,4
PCA-15	66	3	no	yes	7	7,6
PCA-16	66	3	no	yes	7	2,4
PCA-23	61	3	no	yes	7	17,9
PCA-43	61	3	no	yes	7	14,5
PCA-37	62	3	yes	yes	7	50,0
PCA-51	64	3	yes	yes	7	14,9
PCA-52	66	3	yes	yes	7	19,3
PCA-27	74	3	no	yes	10	43,0
PCA-01	60	4	no	yes	8	10,1
PCA-06	58	4	yes	yes	10	3,4

Fig. 2. Gene hypermethylation (expressed as NIM) at five gene loci (CpG islands) in prostate cancer tissues specified in the graph and arranged according to pT stage and Gleason score. See Fig. 1 for further details.

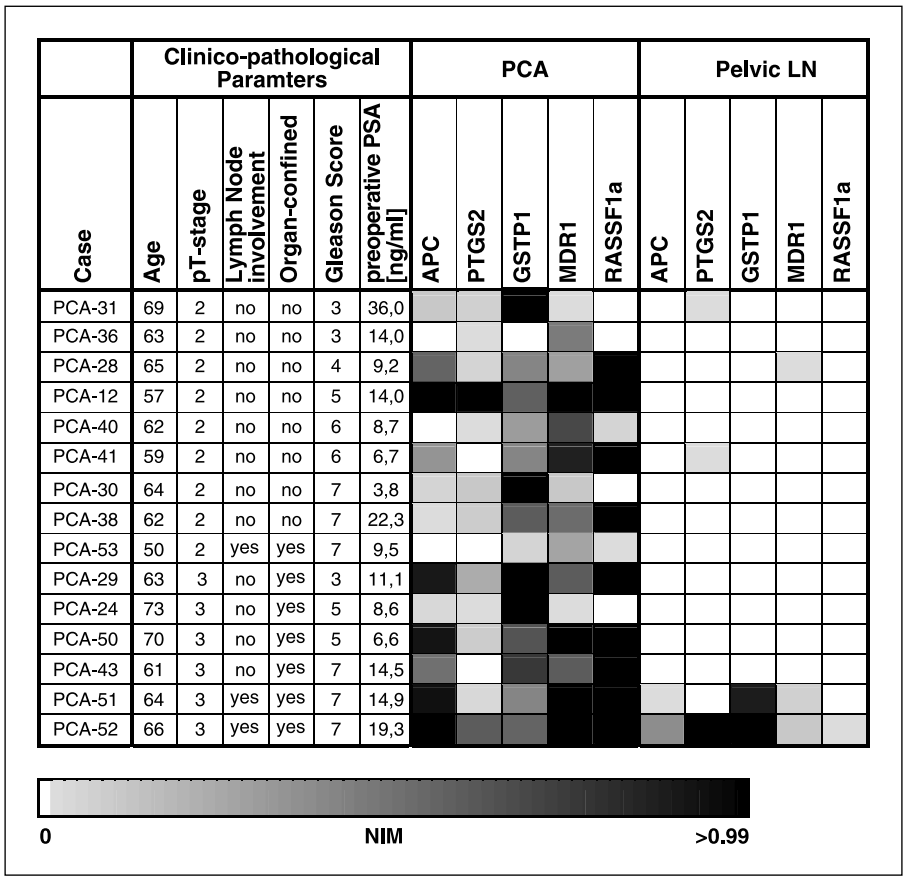


Fig. 3. Gene hypermethylation (expressed as NIM) at five gene loci (CpG islands) in tissues from various pelvic lymph nodes (*LN*) and the corresponding primary prostate cancer as specified in the graph. See Fig. 1 for further details.

pathologic stage (P = 0.049), whereas the alternative hypermethylation at *GSTP1* or *APC* correlated with the Gleason score (P = 0.017). Furthermore, the simultaneous hypermethylation of *GSTP1* and *PTGS2* correlated with extraprostatic extension (P = 0.036), and the simultaneous hypermethylation of *GSTP1*, *PTGS2*, and *APC* correlated with the pathologic stage (P = 0.35) and extraprostatic extension (P = 0.046). Interesting but not significant was our finding that higher pathologic staging was usually accompanied by a higher gene methylation index (i.e., NIM; cf. Figs. 2 and 3).

Biochemical recurrence after radical prostatectomy (PSA > 0.2 ng/mL) was seen in 2 of 23 patients with follow-up examinations (PCA-01 and PCA-20; see Fig. 2). The Gleason score was 6 and 8, respectively. Neither of these two patients had histologic infiltration of the pelvic lymph nodes. Unfortunately, no lymph node material was available to reassess DNA hypermethylation. Others have suggested (28) that a higher than median/mean methylation index (NIM) at the *PTGS2* gene locus predicts recurrence. Our small data set does not underline this finding.

Discussion

We quantitatively assessed CpG island methylation in the regulatory regions of five genes (i.e., *GSTP1*, *APC*, *PTGS2*, *MDR1*, and *RASSF1a*) that were likely to be silenced or activated by epigenetic alterations in prostate cancer. During the late course of our study, Yegnasubramanian et al. (28) published an article showing that our choice of genes was indeed correct and coincided with their findings that these genes are all frequently hypermethylated in prostate cancer. Technically, the main goal of our study was to show that a small set of gene loci is sufficient for a reliable diagnosis of prostate cancer.

Earlier studies with archival, formalin-fixed, paraffin-embedded tissues that were used exclusively in our project showed that the average DNA template length present in these tissues is on the order of 300 to 400 bp. A PCR product length less than 160 bp is adequate for applying methylation-specific PCR technology.[3] Microdissection methods ensured a high grade of prostatic epithelia purity. Therefore, our results should not be subject to large amounts of disturbing (i.e., stromal) cells. Additionally, by using the NIM as reported by Yegnasubramanian et al. (28), we were able to approximate the fraction of DNA methylation at a given gene locus for each and every tissue sample. To our knowledge, we are the first group to use the fluorescent DNA-binding dye SYBR green in real-time methylation-specific PCR hypermethylation studies. For our purposes, SYBR green proved more sensitive in detecting hypermethylation than other techniques (i.e., quantitative real-time methylation-specific PCR using a Taqman probe; data not shown). The group of Jeronimo et al. (50) showed that conventional methylation-specific PCR is also more sensitive compared with a Taqman based real-time methylation-specific PCR assay.

[3] Unpublished data.

Our results show and confirm that hypermethylation occurs at the chosen *GSTP1*, *APC*, *PTGS2*, *MDR1*, and *RASSF1a* gene loci. In the case of *GSTP1* and *APC*, we expected and found a high rate of gene silencing by epigenetic hypermethylation in prostate cancer and practically no methylation in BPH or control tissue. In contrast, we initially expected the *MDR1* gene loci to be activated and therefore hypomethylated in prostate cancer because its expression correlates with resistance to hormone sensitivity and is thought to be important in the progression of primarily hormone/androgen-sensitive malignancies like prostate cancer (31, 34, 35). Surprisingly, we found that the *MDR1* locus is hypermethylated in prostate cancer and BPH. These findings confirm the results of Yegnasubramanian et al. (28) who also observed hypermethylation at the *MDR1* gene loci in prostate cancer and the report of Kawai et al. (51) who showed by immunohistochemical staining that the expression of a P-glycoprotein isoform is lower in prostate cancer than in normal prostate tissues. Our finding that *MDR1* is highly methylated in BPH has not been reported before and may represent early carcinogenesis or clinically relevant benign hyperplasia. The rationale behind the hypermethylation/silencing of the *MDR1* gene in BPH as well as in prostate cancer awaits clarification. Possibly, a yet unidentified member of the ATP-binding cassette transporter gene superfamily is stress induced in BPH and prostate cancer, thus shutting down the expression of P-glycoprotein (32).

PTGS2 encodes COX-2 and its overexpression is associated with proinflammatory actions and the progression of various malignancies, including prostate cancer. Reports also show that COX-2 inhibitors exert antiproliferative and proapoptotic actions in prostate cancer cell lines (30). Seemingly contrary to these findings, other reports show that COX-2 may not be expressed or is down-regulated in prostate carcinoma (28, 32). Our findings support the latter reports (i.e., that the *PTGS2*

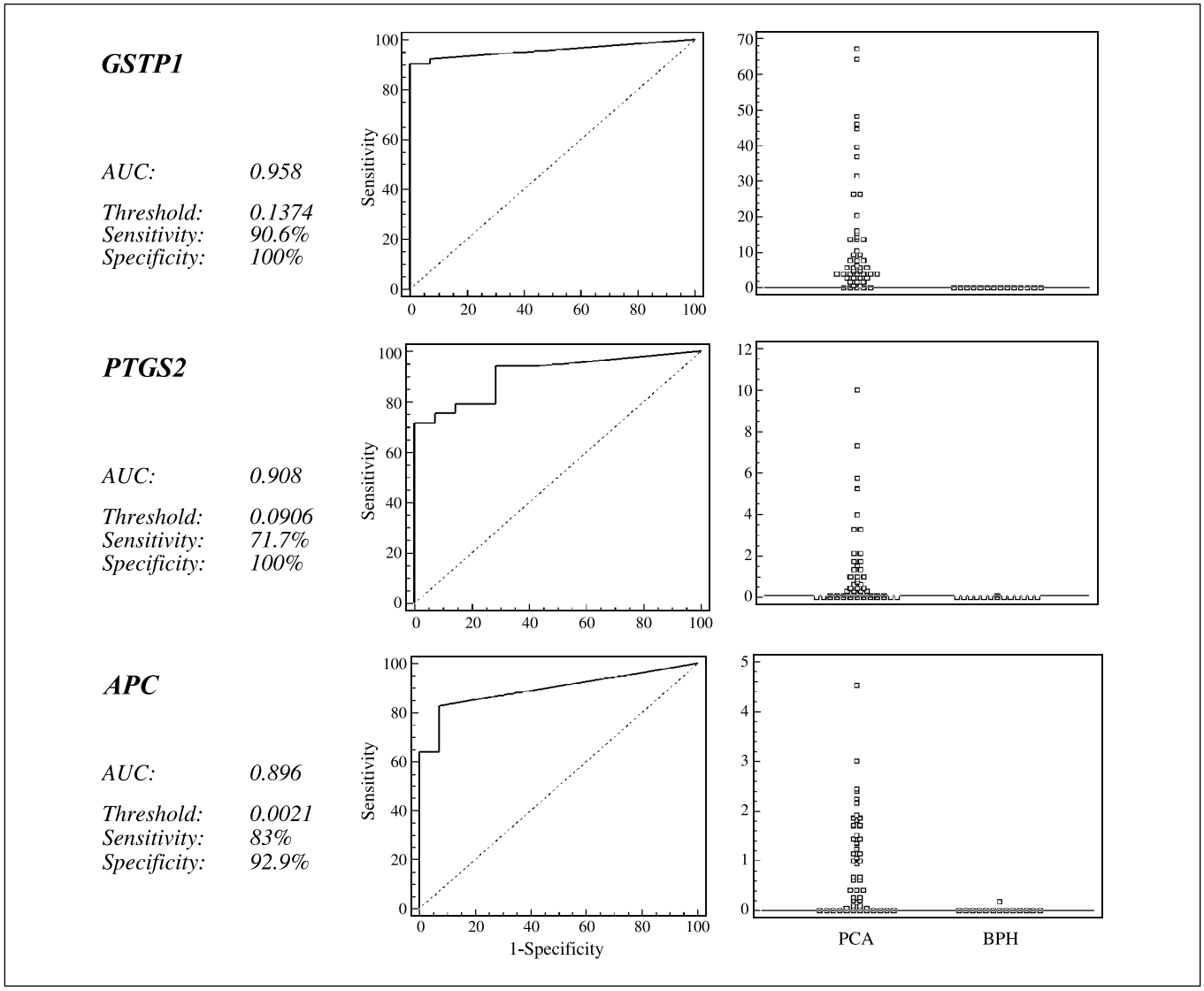


Fig. 4. Optimal sensitivity and specificity determination for the three hypermethylated gene loci (CpG islands) in primary prostate cancer that were most promising in diagnosis. Left, receiver operator curve analysis was used to determine the methylation (NIM) threshold that yields the optimal sensitivity and specificity for each of the three hypermethylated gene loci. Right, histograms of the NIM of all prostate cancers and benign specimens are shown for each gene locus. The threshold NIM, the optimal sensitivity and specificity, and the area under the receiver operator curve (*AUC*) are shown for each gene locus.

Table 4. Diagnostic information on various hypermethylated gene loci, single and in combination

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
GSTP1	90.6 (82.7-98.4)	100	100	73.7 (61.9-85.6)
PTGS2	71.7 (59.7-83.9)	100	100	48.3 (35-61.9)
APC	83.0 (72.9-93.1)	92.9 (85.8-99.7)	97.8 (93.8-100)	59.1 (46-72.5)
MDR1	100	28.6 (16.6-40.9)	84.1 (74.1-93.8)	100 (24.-50.1)
RASSF1a	67.9 (55.4-80.5)	71.4 (59.1-83.4)	90 (81.9-98)	37 (24.1-50.1)
GSTP1 or *PTGS2*	96.2 (90.9-100)	100	100	87.5 (78.7-96.6)
GSTP1 or *APC*	96.2 (90.9-100)	92.9 (86.1-99.9)	98.1 (84.4-100)	86.7 (77.7-96)
GSTP1 or *RASSF1a*	98.1 (94.3-100)	71.4 (59.3-83.6)	92.9 (85.9-99.7)	90.9 (83.3-98.8)
APC or *PTGS2*	88.7 (80.3-97.4)	92.9 (85.9-99.7)	97.9 (93.9-100)	68.4 (55.9-81)
GSTP or *APC* or *PTGS2*	98.1 (94.4-100)	92.9 (86.1-100)	98.1 (94.3-100)	92.9 (85.6-99.7)
GSTP1 and *PTGS2*	66 (53.4-78.9)	100	100	43.8 (30.5-57.2)
GSTP1 and *APC*	77.4 (66.2-88.7)	100	100	53.8 (40.4-67.3)
GSTP1 and *RASSF1a*	60.4 (47.3-73.6)	100	100	40 (26.9-53.2)
APC and *PTGS2*	66 (53.1-78.6)	100	100	34.8 (22-47.7)
GSTP1 and *APC* and *PTGS2*	62.3 (49.4-75.5)	100	100	41.2 (27.9-54.4)

NOTE: See text for further details.
Abbreviations: PPV, positive predictive value; NPV, negative predictive value; 95% CI, confidence interval.

gene is silenced in prostate cancer by epigenetic hypermethylation). The pathophysiologic/biochemical significance of the hypermethylation of *PTGS2* in prostate cancer remains as yet unclear. However, these findings do suggest that the beneficial action of COX-2 inhibitors observed in prostate cancer is not due to COX-2 enzyme inhibition.

As mentioned before, the epigenetic silencing of the tumor suppressor *RASSF1a* in prostate cancer has been observed by others (24, 28, 29, 36–43). Song et al. recently showed that *RASSF1a* contributes to the spatiotemporal regulation of mitosis through a new mechanism. By interacting with Cdc20, the *RASSF1a* protein inhibits the anaphase-promoting complex and prevents the degradation of cyclins A and B until the spindle checkpoint becomes fully operational (42). Exactly how *RASSF1a* affects mitotic progression is not known. Our results show that *RASSF1a* is not only silenced in prostate cancer but

Table 5. Correlation of clinicopathologic variables and the hypermethylation of gene loci

	Total	*GSTP1*	*PTGS2*	*APC*	*MDR1*	*RASSF1a*	*GSTP1* or *APC*	*GSTP1* or *PTGS2*	*GSTP1* and *PTGS2*	*GSTP1* and *APC*	*GSTP1* and *PTGS2* and *APC*
Pathologic stage											
pT_2	31	27	21	23	31	20	29	30	18	21	17
pT_3	20	19	16	20	20	14	20	19	16	19	16
pT_4	2	2	1	1	2	2	2	2	1	1	0
*P**		0.691	0.826	0.129	†	0.433	0.478	0.911	0.241	0.049	0.035
No extraprostatic extension	33	29	21	25	33	22	31	32	18	23	17
Extraprostatic extension	20	19	17	19	20	14	20	19	17	18	16
P‡		0.639	0.123	0.129	†	1.000	0.521	1.000	0.036	0.105	0.046
Gleason score											
2-3	3	2	3	2	3	2	2	3	2	2	2
4-6	26	24	17	21	26	15	25	24	17	16	16
7-10	24	22	18	21	24	19	24	24	16	17	15
*P**		0.344	0.402	0.605	†	0.267	0.017	0.340	0.995	0.786	0.985

NOTE: Apart from those listed in the table, the following clinicopathologic variables were tested without showing significant correlations to hypermethylation ($P < 0.05$): the patient's age at the time of surgery; the preoperative PSA levels (i.e., three patient groups with PSA <4 ng/mL, from 4 to 10 ng/mL, and >10 ng/mL); organ-confined versus non-organ-confined PCA; involvement of seminal vesicles or pelvic lymph nodes.
* χ^2 test was applied.
† No *P* value available.
‡ Fisher's exact test was applied.

also partially in BPH, as well as in the respective cell lines. It has been suggested by others that the detection of epigenetic silencing of *RASSF1a* in BPH represents early carcinogenesis (37). However, in view of the mentioned reports, *RASSF1a* can act as a guardian of mitosis (42); therefore, the appearance of hypermethylation at the *RASSF1a* gene locus may also be a sign of clinically relevant but still benign hyperplasia.

Briefly, hypermethylation at the *GSTP1*, *APC*, and *PTGS2* gene loci was significantly different in prostate cancer than in BPH and could reliably distinguish between cancerous and noncancerous tissue, whereas hypermethylation at *MDR1* and *RASSF1a* could not. On the other hand, hypermethylation at the latter two gene loci may turn out to be useful as an early sign of carcinogenesis. In a work that was published after we had finished our investigations, the group of Tokumaru et al. (52) showed that another combination of four hypermethylated gene loci (i.e., *TIG1*, *APC*, *RARβ2*, and *GSTP1*) can also detect prostate cancer with a very high sensitivity and specificity in fresh-frozen sextant biopsies from excised prostates. Two other workgroups also using real-time methylation-specific PCR reported that hypermethylation at the *EDNRB* gene locus encoding the endothelin receptor type B correlates with prostate cancer disease severity (Gleason score and pathologic stage; refs. 28, 43). This implies that *EDNRB* is a candidate for consideration in a small diagnostic hypermethylation test panel in prostate cancer.

In spite of the fact that we used a unique set of primers for *GSTP1*, *APC*, and *PTGS2*, our hypermethylation results are similar to recent data reported by others who used comparable real-time methylation-specific PCR methods to evaluate hypermethylation in BPH and prostate cancer (26, 28, 43, 52, 53). This suggests that hypermethylation at these genes is widespread and present at different CpG islands in the gene promoter region. In contrast, hypermethylation at *RASSF1a* and *MDR1* gene loci seems more fickle, and recent reports by others (26, 40, 43, 53, 54) who used a similar methylation-specific PCR technology but different primer sets show varying CpG island hypermethylation in prostate cancer and BPH, which differs from our results. This underlines the fact that *RASSF1a* and *MDR1* are not reliable in identifying cancerous prostatic tissue.

CpG island hypermethylation at a single gene locus revealed no correlation with established clinicopathologic variables (i.e., preoperative PSA level, Gleason score, pathologic stage, seminal vesicle or lymph node involvement, surgical margin status, age, etc.; refs. 48, 49). On the other hand, by using the χ^2 and Fisher's exact test, we were able to show that important prognostic indicators, such as the pathologic stage, extraprostatic extension, and the Gleason score, correlated with the combined hypermethylation results at *GSTP1*/*APC*, *GSTP1*/*PTGS2*, and *GSTP1*/*APC*/*PTGS2* (see Table 5). To our knowledge, no such gene combination studies with similar results have been reported. We conclude that a small test panel that quantitatively evaluates hypermethylation at *GSTP1*, *APC*, and *PTGS2* (possibly including *MDR1*, *RASSF1a*, and *EDNRB*) may suffice to diagnose and prognosticate prostate cancer. However, this hypothesis must be further tested in clinical specimens from a much larger cohort of consecutive patients.

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