Prostate Cancer Detection by *GSTP1* Methylation Analysis of Postbiopsy Urine Specimens[1]

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ABSTRACT

Purpose: **We assess the feasibility of a urinary test for prostate cancer detection in a high-risk patient cohort based on methylation-specific PCR analysis of the π class glutathione *S*-transferase (*GSTP1*) gene promoter.**

Experimental Design: **A total of 45 men underwent transrectal ultrasound-guided biopsy of the prostate for suspected malignancy. Clean-catch voided urine specimens were prospectively collected from each patient immediately after biopsy. Genomic DNA was isolated from urine specimens and subjected to sodium bisulfite modification. Methylation of the *GSTP1* promoter was examined in a blinded manner by methylation-specific PCR analysis and correlated with pathology results, and clinical information was obtained from the patient record.**

Results: **Methylation of *GSTP1* in the urine was detected in a total of 18 of 36 (50%) informative cases. A total of 7 of 18 (39%) patients with prostate adenocarcinoma identified on their initial biopsy had detectable urinary *GSTP1* methylation (58% sensitivity among informative cases). Abnormal urinary *GSTP1* methylation was also detected in 7 of 21 (33%) patients without evidence of cancer on biopsy and in 4 of 6 (67%) patients diagnosed with atypia or high-grade prostatic intraepithelial neoplasia.**

Conclusions: **We have demonstrated the feasibility of a novel, noninvasive molecular approach for the detection of epigenetic changes associated with prostate cancer. A screening test based on *GSTP1* methylation in the urine specimens of patients with suspected prostate malignancy may be a useful adjunct to serum screening tests and digital rectal examination findings for identification of men at increased risk of harboring cancer despite a negative biopsy. This molecular assay has potential application for stratification of patients into low- and high-risk groups for surveillance *versus* repeat biopsy.**

INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy and second leading cause of cancer death in the male population over the age of 40 in the United States. It is estimated that ~220,900 American men will be diagnosed with prostate cancer, and ~28,900 men will die from the disease in the year 2003 (1). Over the last 2 decades, there has been increased utilization of serum PSA[3] for disease diagnosis in addition to its use for monitoring recurrence or progression after definitive therapy. The utility of PSA and DRE for identification of patients at risk for harboring prostate cancer is limited by low specificity and results in a high frequency of unnecessary biopsies (2). TRUS-guided biopsy of the prostate is used to obtain a histological diagnosis of cancer in patients suspected of having disease. Approximately 20–36% of men who have a negative initial prostate biopsy are found to have prostate cancer on a subsequent biopsy (*i.e.,* a false negative result; Refs. 3 and 4). This observation is believed to be primarily the result of undersampling during the initial biopsy procedure. For this reason, traditional sextant biopsy has been replaced by 8–12 core biopsy regimens with greater attention to the posterolateral regions of the prostate (5–8). Such increased needle biopsy sampling may improve the sensitivity of TRUS-guided biopsy for early stage disease but has not increased the detection rate of clinically insignificant disease as assessed by tumor volume at radical prostatectomy (9). The detection of low volume prostate cancer remains a diagnostic challenge and has led some authors to advocate increasingly morbid saturation biopsy protocols (10, 11).

Indications for repeat biopsy and timing of the procedure are not well defined but may be prompted by identification of high-grade PIN on initial biopsy, a low percentage-free PSA, changes on DRE, an increase in PSA velocity, elevated PSA density, or development of urinary symptoms (12, 13). Many of these findings are not specific for the presence of cancer, and currently, no standard management algorithm exists for patients with suspected prostate malignancy and a negative initial biopsy. A conservative surveillance strategy for this group of men may include follow-up in 6 months to 1 year, or a more aggressive approach may be adopted in which a repeat biopsy is performed. Clinical management based on the biological properties of prostate cancer and molecular alterations associated with disease progression may provide a rational approach for risk stratification.

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[3] The abbreviations used are: PSA, prostate-specific antigen; DRE, digital rectal examination; BPH, benign prostatic hyperplasia; PIN, prostatic intraepithelial neoplasia; MSP, methylation-specific PCR; CI, confidence interval; GST, glutathione *S*-transferase; TRUS, transrectal ultrasound; NI, noninformative.

Alterations in the patterns of DNA methylation are among the earliest and most common events in tumorigenesis. Many gene promoters contain GC-rich regions of DNA known as CpG islands. Methylation of CpG islands located within the promoter region of genes may result in down-regulation of transcriptional activity via local effects on DNA-binding proteins and alteration in chromatin structure. Abnormal methylation of genes, including those involved with control of cellular growth (*i.e.,* tumor suppressor genes), has been reported in a wide spectrum of human cancers (14). DNA methylation therefore serves as a useful molecular marker for cancer detection.

GSTs constitute a superfamily of enzymes responsible for detoxification of a wide range of xenobiotics. These enzymes catalyze the nucleophilic attack of reduced glutathione on electrophilic compounds and have evolved as a cellular protection system against their toxic effects. Methylation of regulatory sequences at the GSTP1 gene locus is found in the vast majority (>90%) of prostate carcinomas and is associated with transcriptional down-regulation (15–17). Methylation of *GSTP1* is the most common molecular alteration described in prostate cancer, and it has also been demonstrated in high-grade PIN but not in normal prostate tissue (15, 18). Methylation of *GSTP1* has also been reported in the voided urine of patients with prostate cancer but not in patients with BPH (19, 20). We prospectively evaluated the methylation status of *GSTP1* in the urine of men undergoing prostate biopsy to assess the feasibility of this molecular test for prostate cancer detection in patients at elevated risk for prostate cancer.

MATERIALS AND METHODS

Patient Enrollment and Specimen Collection. After written informed consent was obtained, 45 men (mean age, 64 years) undergoing TRUS-guided biopsy of the prostate for suspected malignancy provided urine samples for molecular analysis. This protocol was approved by the Johns Hopkins University Institutional Review Board. Clean catch-voided urine specimens (~50 ml) were collected from each patient immediately after completion of the biopsy procedure. Urine specimens were then stored at 4°C.

DNA Isolation, Bisulfite Modification, and MSP. Urine specimens were centrifuged for 10 min at 2000 × *g* to isolate cellular material and sediment. Total DNA was extracted from the urine pellet (QIAamp Viral RNA Mini Kit; Qiagen, Valencia, CA) and then subjected to sodium bisulfite modification (CpGenome DNA Modification Kit; Intergen, Purchase, NY). MSP was used for the detection of unmethylated and methylated *GSTP1* alleles in each sample (21). Primers used for amplification of unmethylated alleles were: 5′-GAT GTT TGG GGT GTA GTG GTT GTT-3′ and 5′-CCA CCC CAA TAC TAA ATC ACA ACA-3′. Primers used for amplification of methylated alleles were: 5′-TTC GGG GTG TAG CGG TCG T-3′ and 5′-GCC CCA ATA CTA AAT CAC GAC G-3′. The PCR conditions were: initial denaturation of 95°C for 10 min followed by 94°C for 15 s, 62°C for 30 s, and 72°C for 30 s, for a total of 45 cycles. PCRs were performed in 25-μl volumes under the following conditions using GeneAmp reaction buffer II (Applied Biosystems, Foster City, CA): (*a*) 2.5 mM $MgCl_2$; (*b*) 250 μM each deoxynucleotide triphosphate; (*c*) 1 μM final concentration of each primer; and (*d*) 1 unit of AmpliTaq Gold polymerase (Applied Biosystems). PCR products were electrophoresed on 4% agarose gels and visualized with ethidium bromide staining. All urine samples were collected and analyzed without knowledge of biopsy results until completion of experimental procedures and assignment of *GSTP1* methylation status.

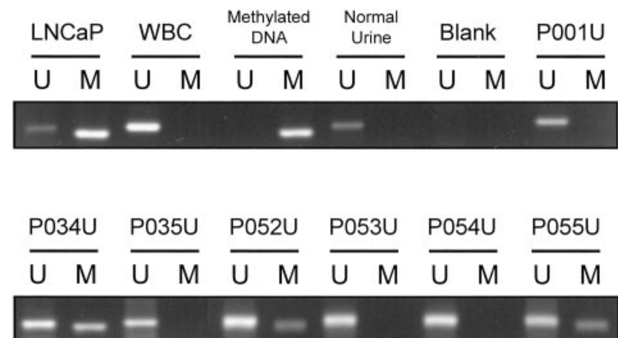

Fig. 1 Molecular urinalysis of *GSTP1* by MSP in postbiopsy urine specimens of patients with suspected prostate malignancy. Generation of a PCR product indicates the presence of unmethylated (*U*) or methylated (*M*) *GSTP1* alleles. *LNCaP,* cell line DNA (positive control for methylation); *WBC,* WBC DNA (negative control for methylation); *Methylated DNA,* positive control for methylation; *Normal Urine,* negative control for methylation; *Blank,* water contamination control; *P001U* to *P055U,* urine specimen identification numbers.

Statistical Analysis. Sensitivity values and their 95% CIs were calculated to assess the feasibility of using *GSTP1* methylation as a diagnostic tool for prostate cancer detection in postbiopsy urine specimens. We examined the overall performance of urinary *GSTP1* methylation for detection of prostate cancer among all patients enrolled in our study and included NI cases. Analyses were also performed excluding patients with NI *GSTP1* methylation results (cases that did not have a sufficient amount of DNA suitable for PCR amplification). Specificity values were not calculated in this study because of the 20–36% false negative rate that has been reported among men with a negative initial prostate biopsy. All analyses were performed using STATA 7.0.

RESULTS

MSP analysis of postbiopsy urine specimens is shown in Fig. 1. LNCaP prostate cancer cell line DNA was used as a positive control for methylation and is almost exclusively methylated at the sites examined by MSP as demonstrated by a faint band in the "U" (unmethylated) lane and a strong intensity band in the "M" (methylated) lane. Genomic DNA isolated from human leukocytes was used as a negative control for methylation at the *GSTP1* locus. Universally methylated DNA (Intergen) was used as another positive control for methylation. A routine-voided urine specimen from a patient at low risk for harboring prostate cancer and without evidence of disease demonstrated the presence of only unmethylated alleles. Cases that demonstrated no PCR product in either the "U" or "M" lanes were NI, indicating that an insufficient amount of DNA was present in the urine specimen after processing and sodium bisulfite modification (data not shown). Abnormal *GSTP1* promoter methylation was observed in the following postbiopsy urine specimens shown in Fig. 1: (*a*) P034U; (*b*) P052U; and (*c*) P055U. The following specimens shown in Fig. 1 did not show

Table 1 Clinical characteristics, biopsy results, and *GSTP1* methylation status in postbiopsy urine specimens

Patient no.	Age	DRE	PSA	Biopsy result	*GSTP1* methylation
P007U	69	+[a]	6.0	Cancer	Yes
P008U	80	+	8.9	Cancer	Yes
P009U	53	−	10.1	Cancer	Yes
P014U	68	−	3.0	Cancer	Yes
P019U	73	−	9.5	Cancer	Yes
P050U	46	+	0.5	Cancer	Yes
P055U	80	−	3.4	Cancer	Yes
P001U	66	+	2.9	Cancer	No
P004U	62	+	6.4	Cancer	No
P030U	68	−	7.5	Cancer	No
P035U	79	+	5.6	Cancer	No
P051U	69	−	6.2	Cancer	No
P010U	65	+	12.1	Cancer	NI
P015U	52	−	4.5	Cancer	NI
P032U	65	+	7.4	Cancer	NI
P037U	75	−	10.3	Cancer	NI
P047U	77	+	86.6	Cancer	NI
P049U	54	−	5.4	Cancer	NI
P013U	53	−	3.7	Atypia	Yes
P034U	59	−	7.7	Atypia	Yes
P038U	68	−	2.1	Atypia	Yes
P039U	54	−	9.0	Atypia	Yes
P028U	55	−	17.8	Atypia	No
P054U	48	−	5.5	PIN	No
P002U	66	+	1.7	No Cancer	Yes
P006U	59	−	5.2	No Cancer	Yes
P033U	72	−	11.5	No Cancer	Yes
P036U	65	+	0.7	No Cancer	Yes
P042U	71	−	8.3	No Cancer	Yes
P048U	65	+	8.0	No Cancer	Yes
P052U	66	−	4.5	No Cancer	Yes
P003U	59	+	2.0	No Cancer	No
P005U	60	−	5.0	No Cancer	No
P011U	63	−	7.3	No Cancer	No
P012U	53	−	8.3	No Cancer	No
P016U	57	−	1.5	No Cancer	No
P020U	70	−	0.6	No Cancer	No
P021U	64	+	6.5	No Cancer	No
P043U	54	−	7.6	No Cancer	No
P044U	55	−	18.5	No Cancer	No
P045U	62	−	6.1	No Cancer	No
P053U	65	−	17.0	No Cancer	No
P022U	65	−	8.2	No Cancer	NI
P029U	72	+	1.4	No Cancer	NI
P040U	77	−	11.3	No Cancer	NI

[a] +, induration or palpable nodule, −, normal.

evidence of *GSTP1* methylation: (*a*) P001U; (*b*) P035U; (*c*) P053U; and (*d*) P054U.

Clinical characteristics (age, DRE, and PSA), biopsy result, and urinary *GSTP1* methylation status of the 45 men who were prospectively enrolled in our study are shown in Table 1. Patients were stratified into three groups according to the results of their prostate biopsy: (*a*) cancer; (*b*) no cancer; or (*c*) atypia/PIN. A total of 18 of 45 (40%) patients in our cohort had a diagnosis of prostate adenocarcinoma confirmed by biopsy. No cancer was identified by histological analysis in 21 of 45 (47%) men, and atypia/PIN was identified in 6 of 45 (13%) patients. There was no significant association between detection of *GSTP1* methylation and Gleason score or percentage of cancer identified on biopsy (data not shown).

A total of 18 of 45 (40%) patients with suspected prostate malignancy was found to have detectable *GSTP1* methylation in their postbiopsy-voided urine specimens. Methylation of the *GSTP1* promoter was observed in 7 of 18 (39%; 95% CI: 17–64%) patients with biopsy-confirmed adenocarcinoma of the prostate. Abnormal urinary *GSTP1* methylation was detected in a total of 7 of 21 (33%; 95% CI: 15–57%) patients with no evidence of cancer on biopsy and in 4 of 6 (67%; 95% CI: 22–96%) patients with a diagnosis of atypia or PIN. NI methylation test results were obtained in 9 of 45 (20%) patients because of an insufficient amount of PCR-amplifiable DNA being present in these urine samples. There were 6 of 18 (33%) NI *GSTP1* methylation tests among patients with a diagnosis of cancer, 3 of 21 (14%) in men with a negative biopsy, and 0 of 6 (0%) among patients with a diagnosis of atypia or PIN.

Patient characteristics and frequency of abnormal urinary *GSTP1* stratified according to prostate biopsy, excluding NI cases, are summarized in Table 2. A total of 18 of 36 (50%) informative cases had detectable *GSTP1* methylation in the urine. Among informative cases with evidence of prostate adenocarcinoma on initial biopsy, 7 of 12 (58%; 95% CI: 28–85%) patients had detectable urinary *GSTP1* methylation. There were 7 of 18 (39%; 95% CI: 17–64%) informative cases with no evidence of cancer on biopsy that had abnormal *GSTP1* methylation present in the urine. A total of 4 of 6 (67%; 95% CI: 22–96%) patients with atypia or PIN identified on biopsy had abnormal urinary *GSTP1* methylation. In a previous study, Goessl *et al.* (22) observed only 1 case of *GSTP1* methylation in voided urine collected after prostate massage among 45 patients with a diagnosis of BPH. Results from this study are shown in Table 2 for comparison to the frequency of *GSTP1* methylation observed in the postbiopsy urine specimens of our cohort.

Table 3 shows follow-up results for a limited number of patients who have undergone radical prostatectomy for cancer or repeat biopsy for an initial diagnosis of atypia. At the present time, a total of 4 of 18 (22%) patients with a cancer diagnosis has undergone radical prostatectomy. No significant association between postbiopsy urinary *GSTP1* methylation and tumor volume was observed. A total of 2 of 6 (33%) patients with a diagnosis of atypia or PIN has undergone a second biopsy. Both of these patients (100%) were diagnosed with cancer on repeat biopsy.

DISCUSSION

We describe a novel approach using postbiopsy-voided specimens to assess the feasibility of prostate cancer detection based on the presence of abnormal urinary *GSTP1* methylation. We hypothesized that a higher amount of cellular material, including prostatic epithelium, would be shed in the urine immediately after a TRUS-guided needle biopsy procedure, compared with a routine voided urine specimen. The sensitivity of *GSTP1* methylation in postbiopsy-voided urine specimens among informative cases was 58% (95% CI: 28–85%) for the diagnosis of cancer. This percentage was significantly higher than reported in previous studies that used preoperative voided or catheterized urine specimens from patients with known prostate cancer (58 *versus* 27–36%; Refs. 19 and 20). Consistent with our present data, detection of *GSTP1* methylation in voided urine specimens after prostatic massage in patients with a prostate cancer diagnosis had a sensitivity as high as 76% (23).

Table 2 Patient characteristics and frequency of abnormal urinary *GSTP1* methylation stratified according to prostate biopsy results
The number of patients (%) in each category is shown.

	Age mean (SD)	Abnormal DRE *n* (%)	PSA > 4 *n* (%)	*GSTP1* methylation *n* (%)
Postbiopsy urine[a]				
All patients (n = 36)	63.2 (8.5)	11 (31)	25 (69)	18 (50)
Adenocarcinoma (n = 12)	67.8 (10.4)	6 (50)	8 (67)	7 (58)
Atypia or PIN (n = 6)	56.2 (6.8)	0 (0)	4 (67)	4 (67)
No cancer (n = 18)	62.6 (5.7)	5 (28)	13 (72)	7 (39)
Postprostate massage urine[b]				
BPH controls (n = 45)	NA[c]	NA	NA	1 (2)

[a] Excludes NI *GSTP1* methylation test results.
[b] Postprostate massage urine from patients with a diagnosis of BPH is shown for comparison (data from Ref. 22).
[c] NA, data not available.

Table 3 Follow-up results of patients who have undergone radical prostatectomy or repeat biopsy

A. Radical prostatectomy	Initial biopsy result	*GSTP1* methylation[a]	Final pathological stage[b]
P050U	Cancer	Yes	pT2A
P010U	Cancer	NI	pT2C
P015U	Cancer	NI	pT2B
P032U	Cancer	NI	pT3A
B. Repeat biopsy			**Repeat biopsy result**
P013U	Atypia	Yes	Cancer
P039U	Atypia	Yes	Cancer

[a] From postbiopsy-voided urine.
[b] American Joint Committee on Cancer staging.

Another recent study using prostate biopsy washings demonstrated *GSTP1* methylation in 10 of 10 (100%) patients diagnosed with adenocarcinoma, 4 of 6 (67%) patients with PIN, and 0 of 10 (0%) patients with BPH (24).

The combination of serum PSA and DRE is the most widely used clinical tool for identification of patients at risk for harboring prostate cancer. Pathologic confirmation of malignancy via prostate biopsy is required for establishing a definitive diagnosis and is an essential component of the clinical decision-making process. However, TRUS-guided biopsy is inherently limited by sampling error that can be problematic particularly in patients with larger prostates or low-volume disease (25, 26). Previous evidence suggests that ~20–36% of these patients will actually have prostate cancer that was missed on initial biopsy (3, 4). Because there is currently no standard of care for management of patients with a negative initial biopsy and persistently elevated PSA or abnormal DRE, surveillance options for this high-risk patient population include assaying for percentage-free PSA or monitoring for changes in either PSA velocity or density (12, 13). The presence of atypia or high-grade PIN on initial biopsy warrants close follow-up, and at our institution, we recommend repeat biopsy for patients with these findings.

A total of 11 of 24 (46%) informative cases with a diagnosis of morphologically normal tissue, atypia, or PIN was found to have *GSTP1* methylation in the urine. Aberrant methylation of the *GSTP1* promoter is found almost exclusively in prostate cancer or PIN and is not typically present in normal cells or BPH (15, 19). Hence, abnormal *GSTP1* methylation serves as a robust molecular marker for prostate cancer detection because of its extremely high specificity (18). Methylation of urinary *GSTP1* has been reported previously in 1 of 45 (2%) patients with BPH, yet it is possible that even this patient may have had occult prostate cancer that was not diagnosed (22). Other studies have reported a higher frequency of *GSTP1* promoter methylation in morphologically normal tissue and BPH that could be discriminated using a quantitative real-time assay but not MSP (17, 27). The group of men in our series with a negative biopsy and urinary *GSTP1* methylation thus represents a population that may benefit from repeat biopsy rather than surveillance as part of a clinical trial for early prostate cancer detection.

Limitations of our study include the small patient population and the lack of long-term follow-up. Repeat biopsy results of the 27 patients in our cohort with a diagnosis of no cancer, atypia, or PIN are needed to validate the utility of *GSTP1* methylation for detection of early prostate cancer. Repeat biopsies to date have only been performed on 2 of 6 (33%) patients with a diagnosis of atypia or PIN. Both of these patients (100%) were found to have cancer on repeat biopsy. These findings provide evidence for the potential utility of this test for clinical decision making. Additional follow-up will be useful to determine whether the remaining 9 patients with positive urinary *GSTP1* methylation and no evidence of cancer on initial biopsy actually have disease. NI methylation test results were obtained in 9 of 45 (20%) patients in our study and were likely attributable to an insufficient amount of cells contained in some small volume urine specimens, as well as to loss of material during subsequent DNA isolation and sodium bisulfite treatment. The collection of larger urine volumes and improvements in laboratory technique may help reduce the frequency of future NI test results. We found no significant association between postbiopsy urinary *GSTP1* methylation and tumor volume in the four radical prostatectomy specimens that were available for histological analysis. A single MSP assay was used in our study and may not provide sufficient discrimination for this purpose. Analysis of multiple sites with MSP (*i.e.,* more than one primer set) or utilization of quantitative methods for detection of urinary methylation may be more effective in yielding this information.

We could not determine the specificity of urinary *GSTP1* methylation for prostate cancer detection in this study, because men in our cohort without evidence of cancer on initial biopsy

cannot be considered to be truly disease negative at this point in time. We also cannot completely exclude the possibility of false-positive results obtained from urine specimens because methylation analysis of actual biopsy tissue was not performed. However, the high specificity of *GSTP1* methylation for prostate cancer has been confirmed by a number of previous studies (15, 19). Detection of urinary *GSTP1* methylation in patients with suspected prostate malignancy and a negative biopsy should therefore be sufficient evidence to warrant concern for the presence of occult disease. A diagnostic test for *GSTP1* methylation in postbiopsy urine specimens could be used to determine whether or not a repeat biopsy is indicated, whereas other strategies (*i.e.,* analysis of voided urine after prostate massage) could be used to prompt an initial biopsy as well as a repeat procedure in the event of a negative initial result.

We have demonstrated the feasibility of a new clinical strategy based on a noninvasive molecular test that can be used as an adjunct to current methods for prostate cancer detection. Abnormal *GSTP1* methylation found in postbiopsy urine specimens may be helpful for identification of patients at risk for harboring malignancy despite a negative biopsy. This test may also improve detection of cancer in patients with low-volume disease. Urinary *GSTP1* methylation may therefore be used to stratify patients with negative initial biopsy results into low- and high-risk groups. The efficacy of this noninvasive molecular test for early prostate cancer detection may be important in developing future clinical management algorithms and for establishing indications for surveillance or repeat biopsy. Larger prospective trials and longitudinal follow-up are needed to validate the clinical utility of our observations.

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