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14. ABSTRACT Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death in the United States. The most common DNA alteration associated with prostate cancer is hypermethylation in the regulatory region of certain genes, particularly in the promoter of the pi-class glutathione-S-transferase (GSTP1) gene. Analysis of hypermethylation of other gene promoters in combination has demonstrated high sensitivity and specificity for prostate cancer diagnosis. In this project, we evaluate the feasibility of detection of prostate cancer by molecular urinalysis. Prostatic manipulation from sources such as a biopsy needle, transrectal ultrasound (TRUS) probe, or digital rectal exam (DRE), may cause prostatic DNA to appear in the urine by shedding of neoplastic cells or debris into the prostatic ducts and urethra. The specific impact of prostatic manipulation on the detection of DNA promoter hypermethylation in the urine is unclear, as there are no studies comparing urine obtained before and after prostatic manipulation in identical patients. We hypothesized that voided urine specimens from patients with prostate cancer would be more likely to have detectable DNA promoter hypermethylation immediately after prostate manipulation by TRUS-guided needle biopsy than after DRE. We have compared voided urine samples obtained after extended (15-second) DRE with voided urine samples obtained after TRUS-guided needle prostate biopsy from patients with suspected or confirmed prostate cancer using conventional methylation-specific PCR (MSP) analysis to examine the hypermethylation status of three different gene promoters: GSTP1, APC and EDNRB. These loci were chosen because of their high frequency of methylation in prostate cancer specimens. Methylation analysis at multiple genes has also been shown to have diagnostic and prognostic value in prostate cancer.					
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INTRODUCTION:

Serum prostate-specific antigen (PSA) and digital rectal examination (DRE) remain the standard of care for prostate cancer screening despite their limited ability to detect occult prostate cancer. It is estimated that 15% of men with a normal PSA and DRE harbor prostate cancer. The rate of false negative prostate biopsies is estimated to be between 20-35%. Clearly, more specific and sensitive tests are needed to spare unnecessary biopsies and better identify and prognosticate affected men with prostate cancer. The scope of this research is to study, develop, and optimize biomarkers for the detection and prognostication of prostate cancer by molecular urinalysis that may help discriminate benign from malignant conditions of the prostate.

BODY:

We have been collecting urine specimens for biomarker analysis since initial project funding. Optimized methods of urine collection and storage for prostate-specific biomarkers have been achieved. Specifically, conditions for the optimal collection of prostate cells shed after digital rectal exam (DRE) and voided into urine, as well as for the collection of shed DNA and protein, have been determined. Routine collection of initial urine post-DRE and post-prostate biopsy are processed to various fractions for cells, protein and DNA. The urine sediment is the most active fraction for our DNA, specific protein, and cellular analyses. Supernatants or whole urine are used for cytokine assays.

Urinary Protein Evaluation: We are continuing our work with alpha-Methylacyl-CoA racemase (AMACR) in order to determine if in a larger study this urinary protein will be a valuable addition to the current prostate cancer detection armamentarium (PSA and DRE) on Western blots. We have also begun to investigate the presence of other prostate-related proteins in urine as a marker for prostate disease by Western analyses: AGR-2 and MYO-6. Preliminary data demonstrate each of these to be detectable in urine. We are extending our protein work into cytokine analyses, and are currently analyzing data from a cytokine array done on expressed prostatic fluids post-prostatectomy in 42 patients. The most up- and down- regulated cytokines in cases with extensive cancers compared to cases with minimal cancer were catalogued, and ELISA assays performed to confirm these data quantitatively. HGF was the most promising marker of extensive cancer as detectable in fluid clearly of prostatic origin, and was found in vastly greater quantities than in serum. Work on HGF as a biomarker will continue as we pursue its detection in post-DRE and post-biopsy urine samples.

Urinary Cellular Evaluation: A new area of interest has been the search for prostate cells shed into urine post-DRE or biopsy. Cytospins of voided urine followed by FISH analyses for prostate-specific markers have been performed, and cells of apparent prostatic origin found in fluorescent analysis. The search for prostate cancer versus benign prostate cells in these urine samples continues using new markers and antibodies as we are able to obtain and optimize them.

Urinary DNA Evaluation: We are currently banking frozen urine sediments post-DRE and biopsy for subsequent hypermethylation analysis.

KEY RESEARCH ACCOMPLISHMENTS

- 1) Standardized and optimized methodology for the collection of clinical urine samples after prostate biopsies.
- 2) Creation of a urine bank from patients with prostate diseases.
- 3) Testing of novel biomarkers developed by colleagues using urine from this bank and from our clinic.

REPORTABLE OUTCOMES:

Our preliminary results demonstrated that there is a high concordance between the ability to collect epigenetically modified DNA from patients either post-biopsy or post-DRE, though certain cases had methylation detected only post-DRE or only post-biopsy. This result has been published as a manuscript.

Rogers CG, Gonzalgo ML, Yan G, Bastian PJ, Chan DY, Nelson WG, Pavlovich CP. High Concordance of Gene Methylation in Post-Digital Rectal Examination and Post-Biopsy Urine Samples for Prostate Cancer Detection. *Journal of Urology* 176 (5), pp 2280-2284

CONCLUSION:

Detection of prostate cancer by molecular urinalysis is feasible. We will continue to address our aims of collecting urine samples post-DRE and post-prostate biopsy, and will assess them for biomarker information. In addition, we are planning to continue analyzing the post-DRE and post-biopsy urine sediment (pellet) for intact prostate cells by cytoprep and immunohistochemistry now that prostate and prostate-cancer specific immunohistochemical markers are available. It appears that we are now able to detect prostate cells in the urine samples we collect, so the next step is to try and detect prostate cancer cells using relatively prostate cancer-specific immunohistochemical markers such as AMACR. The goal is to develop another modality of urine analysis into a specific diagnostic test for prostate cancer, and to compare this test with the others we are studying in terms of accuracy of diagnosis and prognostic relevance.

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APPENDICES:

Rogers CG, Gonzalgo ML, Yan G, Bastian PJ, Chan DY, Nelson WG, Pavlovich CP. High Concordance of Gene Methylation in Post-Digital Rectal Examination and Post-Biopsy Urine Samples for Prostate Cancer Detection. *Journal of Urology* 176 (5), pp 2280-2284

High Concordance of Gene Methylation in Post-Digital Rectal Examination and Post-Biopsy Urine Samples for Prostate Cancer Detection

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Purpose: We evaluated the concordance between post-digital rectal examination and post-prostate biopsy urine samples using conventional methylation specific polymerase chain reaction analysis of 3 gene promoters in patients with suspected or confirmed prostate cancer.

Materials and Methods: Voided urine specimens were collected from 17 men after 15-second digital rectal examination and again after transrectal ultrasound guided biopsy of the prostate for suspected malignancy or for followup biopsy as part of an expectant management protocol. Urine sediment DNA was isolated and subjected to bisulfite modification. Methylation of GSTP1, EDNRB and APC promoters was determined by conventional methylation specific polymerase chain reaction analysis in post-digital rectal examination and post-biopsy samples, and correlated with clinical information.

Results: Prostate cancer was detected on prostate biopsy in 12 of 17 patients (71%). Promoter methylation was detected in post-digital rectal examination urine specimens for GSTP1 (24%), APC (12%) and EDNRB (66%). Promoter methylation was detected in post-biopsy urine specimens for GSTP1 (18%), APC (18%) and EDNRB (77%). The concordance between post-digital rectal examination and post-biopsy urine samples was 94% for GSTP1 and APC, and 82% for EDNRB. Overall 100% of patients with biopsy proven prostate cancer had at least 1 gene methylated in urine vs 60% of those without evidence of prostate cancer on biopsy.

Conclusions: Gene analysis using conventional methylation specific polymerase chain reaction is a reliable method for detecting abnormal DNA methylation in voided urine samples obtained following digital rectal examination or prostate needle biopsy. The concordance between post-digital rectal examination and post-biopsy urinary samples for promoter methylation is high (82% to 94%), suggesting that urine collected after digital rectal examination may be used for genetic analysis with results similar to those in post-biopsy urine samples.

Key Words: prostate, prostatic neoplasms, biopsy, urine, methylation

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer related death in males older than 40 years in Western industrialized countries. The most common DNA alteration associated with prostate cancer is hypermethylation in the regulatory region of certain genes, particularly in the promoter of the π class GSTP1 gene.¹⁻⁴ Hypermethylation of gene promoter regions is associated with many human cancers.^{2,3} Aberrant GSTP1 methylation has been detected in the urine, ejaculate and prostatic secretions of men with prostate cancer.^{5,6} Analysis of hypermethylation of other gene promoters in combination has demonstrated high sensitivity and specificity for prostate cancer diagnosis.^{7,8}

Prostatic manipulation from sources such as a biopsy needle, TRUS probe or DRE may cause prostatic DNA to appear in urine by the shedding of neoplastic cells or debris into the prostatic ducts and urethra. The specific impact of prostatic

manipulation on the detection of DNA promoter hypermethylation in the urine is unclear because to our knowledge there are no studies comparing urine obtained before and after prostatic manipulation in identical patients. We hypothesized that voided urine specimens from patients with prostate cancer would be more likely to have detectable DNA promoter hypermethylation immediately after prostate manipulation by TRUS guided needle biopsy than after DRE.

We compared voided urine samples obtained after extended 15-second DRE with voided urine samples obtained after TRUS guided needle prostate biopsy from patients with suspected or confirmed prostate cancer. We used conventional MSP analysis to examine the hypermethylation status of the 3 gene promoters GSTP1, APC⁹ and EDNRB. These loci were chosen because of their high frequency of methylation in prostate cancer specimens.⁷ Methylation analysis at multiple genes has also been shown to have diagnostic and prognostic value for prostate cancer.^{10,11}

MATERIALS AND METHODS

Ten men undergoing prostate biopsy for suspected prostate cancer and 7 with previously diagnosed prostate cancer un-

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Study received Institutional Review Board approval.

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dergoing followup biopsy as part of an expectant management protocol were enrolled in the study. Approval was obtained from our Institutional Review Board before initiating the study and all patients provided written informed consent. Voided urine specimens (10 to 100 cc) were prospectively collected from 17 men with a mean age of 63.5 years immediately following 15-second DRE and again after transrectal ultrasound guided prostate biopsy during a single office visit. Molecular urinalysis of promoter methylation was performed by an investigator blinded to biopsy results.

Voided urine specimens were centrifuged for 10 minutes at $1,000 \times$ gravity to isolate cellular material and sediment. Total DNA was extracted from the urine pellet using a QIAamp® Viral RNA Mini Kit. The average DNA concentration yielded was approximately 100 ng/ μ l (range 68 to 150) with an average volume of approximately 80 μ l. DNA was then subjected to sodium bisulfite modification using a CpGenome™ Universal DNA Modification Kit. Concurrently modified were 1 μ g universally M DNA (Chemicon, Temecula, California) and 1 μ g genomic DNA from human male white blood cells (EMD Biosciences, San Diego, California). MSP was used to detect U and M alleles in each sample.¹² MSP was performed using 2 primer pairs, including 1 that detected U alleles and 1 that detected densely M alleles.^{7,13,14}

Table 1 lists the U and M specific primers used in PCR. PCR was performed with 3.0 μ l bisulfite modified DNA template in a 25 μ l reaction mixture containing 2.5 μ l $10 \times$ GeneAmp® reaction buffer II, 200 μ M of each deoxynucleoside triphosphate, $MgCl_2$ at the concentrations indicated, 0.25 μ M of each primer and 1.25 U AmpliTaq Gold® polymerase (table 1). PCR conditions were initial denaturation at 95°C for 10 minutes, followed by 40 to 50 cycles of denaturation at 95°C for 1 minute, annealing at the corresponding annealing temperature for 30 seconds, extension at 72°C for 1 minute and final extension at 72°C for 7 minutes (table 1). PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide staining under ultraviolet illumination. Each PCR reaction contained a water blank, a positive control (universally M DNA) and a negative control (white blood cell DNA). All urine samples were processed and analyzed in blinded fashion. Methylation status was then correlated with biopsy results and clinical information, and concordance was determined between post-DRE and post-biopsy urine samples.

Promoter methylation was analyzed in urine specimens after DRE and prostate biopsy to assess the concordance between matched pairs of post-DRE and post-biopsy urine specimens. Sensitivity was calculated to detect methylation

changes using primer sets separately and in combination. The concordance of hypermethylation results was determined for matched post-DRE and post-biopsy urine pairs, and an overall concordance rate was determined for each gene promoter assessed.

RESULTS

All urine samples yielded amplifiable DNA for most genes in our panel. The pattern of hypermethylation for our 3 gene panel was compared between post-DRE and corresponding post-biopsy urine samples. Table 2 shows clinicopathological and gene promoter methylation detection data on the 17 study patients. Prostate cancer was detected on prostate biopsy in 12 of 17 patients (71%). No cancer was identified on prostate biopsy in 5 men (29%). All cases of prostate cancer were clinical stage T1c with Gleason 6 disease on biopsy except in 1 of Gleason 7, clinical stage T2b disease. Four men with prostate cancer underwent RRP. RRP Gleason scores were identical to biopsy Gleason scores in all 4 patients.

We observed hypermethylation of the GSTP1 promoter in voided urine specimens of 4 of the 17 patients (24%) after DRE and in 3 (18%) after prostate biopsy. The frequency of promoter hypermethylation of APC was 2 of 17 patients (12%) for post-DRE urine samples compared to 3 of 17 (18%) for post-biopsy urine samples. The frequency of promoter hypermethylation of EDNRB was 8 of 12 informative post-DRE specimens (66%) compared to 10 of 13 informative post-biopsy urine samples (77%). The concordance between paired post-DRE and post-biopsy urine samples was 94% for GSTP1 and APC, and 82% for EDNRB.

The figure shows representative MSP analysis of paired post-DRE and post-biopsy urine specimens to evaluate promoter hypermethylation. Universally M DNA showed no band in the U lane and a strong band in the M lane, and vice versa for white blood cell DNA. Cases that demonstrated no appropriate PCR product in the U or M lane were classified as NI, possibly indicating an insufficient number of alleles amplifiable by the corresponding primers. Concordance for GSTP1 and APC promoter hypermethylation was seen in all paired post-DRE and post-biopsy samples (see figure). Concordance of hypermethylation at all 3 promoters was observed in certain post-DRE and corresponding post-biopsy urine specimens, including specimens 219, 227 and 230. Concordance between post-DRE and corresponding post-biopsy urine samples for EDNRB promoter hypermethylation was not present for specimen 213 (see figure). The post-DRE urine sample for patient 216 was NI for EDNRB.

TABLE 1. Primers used to amplify bisulfite converted DNA at GSTP1, APC and EDNRB promoter regions

Gene (specific primer)	5'-3' Primer		Amplicon Size (bp)	Annealing Temperature (°C)	$MgCl_2$ (mM)
	Forward	Reverse			
GSTP1:					
U	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCCAATACTAAATCACAACA	97	62	3.0
M	TTCGGGGTGTAGCGGTCGTC	GCCCCAATACTAAATCAGCAGC	91	62	3.0
APC:					
U	GTGTTTATTGTGGAGTGTGGGTT	CCAATCAACAACTCCCAACAA	108	60	3.5
M	TATTGCGGAGTGC GGTC	TCGACGAAC TCCGACGA	98	62	2.5
EDNRB:					
U	GGGTATTAGGAAGGAGTTTGTATTGTG	CACAACCTCTTACCAATATCCACA	111	62	3.5
M	GGTACGCGGGGAAGAAAAATAGTTG	ATACCGCCCGCAACCTCTTCG	149	65	2.0

TABLE 2. Clinicopathological characteristics and promoter hypermethylation detection status for *GSTP1*, *APC* and *EDNRB* in 17 study patients

Pt No.—Age	PSA (μg/ml)	DRE	Clinical Stage	Gleason	RRP Stage	Post-DRE/Post-Biopsy Urine		
						GSTP1	APC	EDNRB
Neg biopsy								
218—65	8.8	Neg	N/A	N/A	N/A	U/U	U/U	NI/U
219—60	11.3	Neg	N/A	N/A	N/A	M/M*	M/M*	M/M*
227—71	4.8	Neg	N/A	N/A	N/A	U/U	U/U	M/M
230—63	7.2	Pos	N/A	N/A	N/A	U/U	U/U	M/M
238—46	4.7	Pos	N/A	N/A	N/A	U/U	U/U	U/U
Pos biopsy								
228—64	6.1	Neg	T1c	6	N/A	U/U	U/U	M/M
226—58	1.2	Neg	T1c	6	N/A	U/U	U/U	M/M
60—72	0.4	Neg	T1c	6	N/A	U/U	U/U	M/M
105—52	12	Neg	T1c	6	T2NxMx	U/U	U/U	U/M*
176—59	4.4	Neg	T1c	6	T2cNxMx	U/U	U/U	NI/NI
213—59	5.9	Pos	T2b	4+3	T3aN0Mx	M/M	U/U	U/M*
214—67	3.5	Pos	T1c	6	N/A	M/U*	U/M*	U/U
215—62	5	Pos	T1c	6	N/A	U/U	U/U	M/NI
216—72	9.6	Pos	T1c	6	N/A	M/M*	M/M*	NI/M
217—72	5.4	Pos	T1c	6	Radiation	U/U	U/U	NI/NI
225—68	4.9	Pos	T1c	6	T2N0Mx	U/U	U/U	NI/NI
232—69	7.8	Pos	T1c	6	N/A	U/U	U/U	M/M

* Discordant urine pairs.

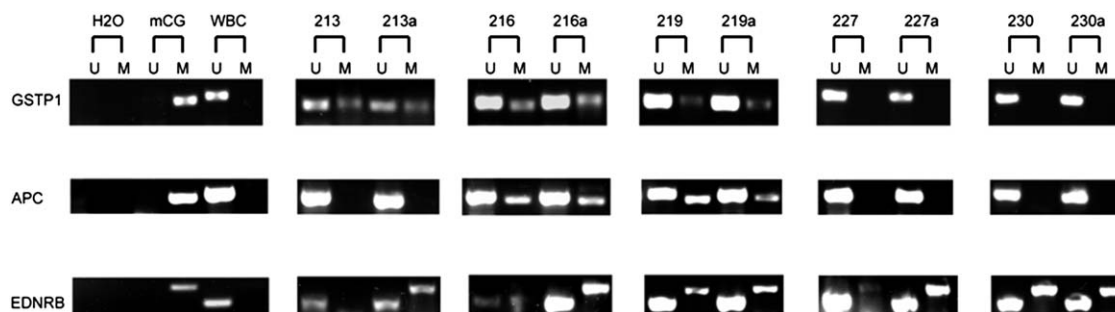
Hypermethylation of the *GSTP1* promoter was observed in 3 of 12 patients (25% sensitivity) with biopsy confirmed adenocarcinoma of the prostate and in 1 of 5 (20%) with no evidence of cancer on biopsy. Hypermethylation of the *APC* promoter was observed in 2 of 12 patients (17% sensitivity) with biopsy confirmed adenocarcinoma of the prostate and in 1 of 5 (20%) with no evidence of cancer on biopsy. Hypermethylation of the *EDNRB* promoter was observed in 6 of 7 informative cases (85% sensitivity) of biopsy confirmed adenocarcinoma of the prostate and in 3 of 5 (60%) with no evidence of cancer on biopsy. All 9 informative cases (100%) with prostate cancer showed at least 1 detectable hypermethylated gene promoter in the urine compared to 3 of 5 (60%) without evidence of cancer on biopsy.

DISCUSSION

Prostatic manipulation from sources such as a biopsy needle, transrectal ultrasound probe and/or DRE may cause DNA to appear in urinary tract fluids due to the shedding of neoplastic cells and debris into the prostatic ducts. The impact of prostatic manipulation on the detection of prostate cancer cells in urine has not previously been described in studies comparing detection rates in urine obtained before

and after prostatic manipulation in identical patients with cancer. To our knowledge our study is the first analysis of concordance between post-DRE and post-biopsy urine samples for promoter hypermethylation. We hypothesized that voided urine specimens from patients with prostate cancer would contain a higher amount of neoplastic cellular material for DNA analysis immediately after TRUS biopsy compared with a voided urine specimen after DRE and, hence, a higher rate of detection of promoter methylation. However, the concordance between post-DRE and post-biopsy urine samples for promoter hypermethylation in our study was high, suggesting that urine collected after DRE may be used for molecular urinalysis with results similar to those of urine samples obtained after more invasive techniques, such as prostate biopsy. Molecular analysis of urine obtained after prostate biopsy could potentially have a limited role for identifying patients with a false-negative biopsy who must undergo repeat biopsy. Post-DRE urine collection is more practical and less invasive than collection after prostate biopsy and it can be applied in a broader group of patients for prostate cancer detection, such as those undergoing routine prostate cancer screening.

Previous studies have shown the efficacy of *GSTP1* promoter hypermethylation for diagnosing prostate cancer in



Representative MSP analysis of paired post-DRE and post-biopsy (a) urine specimens for *GSTP1*, *APC* and *EDNRB* promoter hypermethylation. Prostate cancer was confirmed on prostate biopsy in patients 213 and 216. Prostate biopsy was negative in other patients. PCR product generation indicated U or M alleles. *H2O*, water blank PCR reaction contamination control. *mCG*, universally M DNA positive methylation control. *WBC*, white blood cell DNA negative methylation control.

various bodily fluids with sensitivity approaching 75%.^{6,13,15} We also included other gene promoters in our panel because the sensitivity and specificity of prostate cancer diagnosis in bodily fluids, such as urine, ejaculate and prostate secretions, and biopsy specimens has been shown to be as high as 92% to 100% when several markers are used in combination.^{7,11,15} The 3 genes chosen for our study panel (GSTP1, APC and EDNRB) have cancer and prostate cancer specific hypermethylation, and known biological significance. The diagnostic coverage of our 3 gene methylation panel for diagnosing prostate cancer in voided urine specimens was 100%.

Prostatic manipulation, ie massage, biopsy, etc, may increase the detection of prostate cancer DNA in voided urine from men harboring prostate cancer. Cairns et al noted GSTP1 hypermethylation in normally voided urine of 27% of men with early stage prostate cancer.¹⁶ After 1 minute of prostatic massage Goessl et al were able to detect GSTP1 hypermethylation in 68% of men with early stage prostate cancer.¹⁷ Gonzalgo et al used MSP to examine urine collected after prostate biopsy.¹³ They detected GSTP1 hypermethylation in 58% of patients with biopsy proven prostate cancer and in 33% of patients without prostate cancer or PIN. Gonzalgo et al also analyzed prostatic secretions from RRP specimens and detected GSTP1 hypermethylation in 86% of patients using a combinatorial MSP approach,¹³ which may represent close to the maximal sensitivity of this assay for detecting hypermethylation of this allele in patients with prostate cancer.¹⁶ In the current study DRE was performed for approximately 15 seconds per patient, which may be longer than typical diagnostic DRE. However, it is shorter than a typical prostatic massage of approximately 30 seconds to 1 minute. Our findings suggest that even with modest prostatic manipulation, such as may be performed by 15-second DRE, material of diagnostic value is shed into the prostatic urethra and into subsequently voided urine. Prostatic manipulation by DRE, biopsy, etc may cause spastic contractions of the smooth muscles of the prostate, which can cause nucleic acids and proteins to be trapped inside prostatic acini. Subsequent urination and smooth muscle relaxation may allow the evacuation of these compartments, thus, increasing prostate cancer DNA detection.

A limitation of our study is its small patient population. In addition, the prevalence of prostate cancer in our small cohort was high and it does not represent a typical screening population because we included patients with known prostate cancer who were on an expectant management program.¹⁸ We chose to include these patients because they routinely undergo prostate biopsy and already have a known cancer diagnosis, making them a reliable source for obtaining matched post-DRE and post-biopsy urine specimens for our proof of principle study. We emphasize that our current goal was to assess the concordance between matched post-DRE and post-biopsy urine samples, and so we did not design this study to optimize sensitivity and specificity since high sensitivity and specificity have already been demonstrated in other studies using MSP analysis. However, additional studies including patients with benign prostatic conditions and nonprostatic malignancies are warranted. The high proportion of patients on an expectant management protocol may actually have contributed to the low detection rate of prostate cancer in our study because these patients had low volume cancers on biopsy. Prostate cancer

detection could have potentially been improved in our study by increasing the number and volume of urine samples, and by performing more vigorous prostatic massage. It is unclear to what extent the TRUS probe influences prostatic manipulation during prostate biopsy and the subsequent shedding of cellular debris into urine. We did not analyze urine specimens after TRUS only without biopsy because this additional test would have limited the amount of urine available for analysis for each test and it is rarely clinically indicated. The detection of promoter hypermethylation in our study could theoretically have been biased in favor of post-biopsy urine samples since these patients underwent serial prostatic manipulation (DRE followed by biopsy). However, post-DRE urine samples still compared well to post-biopsy samples with a high concordance for promoter methylation between matched samples.

CONCLUSIONS

Our data suggest that voided urine samples obtained after DRE or after prostate biopsy contain similar epigenetic molecular information. The concordance between post-DRE and post-biopsy urine samples for promoter methylation is high (82% to 94%), suggesting that urine collected after DRE may be used to analyze genetic markers for prostate cancer with results similar to those of post-biopsy urine samples. Validation of this approach in larger, prospective trials and optimization of an appropriate panel of methylation markers may ultimately lead to widespread use of this technology for the early detection and prognostication of prostate cancer.¹⁹

Abbreviations and Acronyms

DRE	=	digital rectal examination
GSTP1	=	glutathione-S-transferase
M	=	methyated
MSP	=	methylation specific PCR
N/A	=	not applicable
NI	=	noninformative
PCR	=	polymerase chain reaction
RRP	=	radical retropubic prostatectomy
TRUS	=	transrectal ultrasound
U	=	unmethyated

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