Prostate Cancer Detected by Methylated Gene Markers in Histopathologically Cancer-Negative Tissues from Men with Subsequent Positive Biopsies

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Abstract

The goal of this retrospective, multicenter study was to evaluate the ability of a newly developed refinement of a quantitative methylation-specific PCR assay to detect prostate cancer in histopathologically negative biopsy samples collected from men who were later positively diagnosed during a follow-up biopsy procedure. Biomarkers tested in the assay included the much-studied glutathione-S-transferase P1 gene and others reported to be frequently methylated in prostate cancer. Core biopsy tissue from subjects with serial negative biopsies served as a negative control to assess assay specificity. As a positive control, biopsy core tissue from patients histopathologically diagnosed with prostate cancer was used to gauge true marker sensitivity in known cancer-containing specimens. Testing was completed in 971 archived paraffin-embedded tissue blocks from 264 men screened for prostate cancer. More samples were initially tested, but due to the advanced age of the paraffinized tissue, DNA quality for quantitative methylation-specific PCR analysis was insufficient in 34% of the available blocks. The glutathione-S-transferase P1 gene has been confirmed as a powerful indicator of the presence of prostate cancer cells. A sensitivity of 52% was observed in the "potentially false-negative first biopsies," with a corresponding specificity of 85% and the sensitivity in biopsy tissue cores containing histopathologically confirmed prostate cancer was 95%. An even higher sensitivity can be reached with RAR-2β (84%) and APC (72%), with respective specificities of 48% and 50%. Gene methylation was detected in initial, negative biopsy tissue in men who were later diagnosed with prostate cancer. Testing for methylation in histopathologically negative biopsies could improve the early detection of prostate cancer. (Cancer Epidemiol Biomarkers Prev 2009;18(10):2717–22)

Introduction

Prostate cancer remains a major medical health issue representing the most frequent cancer among men. The discovery and widespread utilization of serum prostate-specific antigen (PSA) monitoring for early detection has greatly changed the way prostate cancer is diagnosed, and has resulted in the downstaging of the disease. However, the development of new tumor markers with better sensitivity and specificity to detect early cancer remains vitally important. Although we recognize that a distinction has been made between clinically significant and histologic prostate cancer, our objective was to determine if DNA methylation status would increase the accuracy of the histopathologic diagnosis of prostate cancer. It is estimated that up to 1,000,000 men are biopsied in the United States each year with ~200,000 incident cases of prostate cancer (1). The biopsy process itself, although the gold standard for diagnosis, has a false-negative rate as high as 30% and is affected by the number of cores obtained (2, 3). Therefore, a negative set of core biopsies does not exclude the presence of cancer, and a high index of suspicion for prostate cancer might exist in the face of an initially negative set of prostate biopsies (4). Repeat biopsies of the same patient would be expected to decrease the number of false negatives and increase the number of cancers. A prospective single-institution study recently reported on 1,051 subjects who were biopsied on up to four separate occasions over time (5). Cancer rates detected on the first, second, third, and fourth biopsies were 22% (231/1051), 10% (83/820), 5% (36/737), and 4% (4/94), respectively. In this study therefore, ~15% of men with a negative diagnosis after a first set of core biopsies were false negatives. In the population-based, multi-site Prostate Cancer Prevention Trial, repeat biopsy of men enrolled in the placebo arm showed that 22% (1,074/4,921) were positive on first biopsy and 16% (112/687) of men who were negative on a first biopsy were positive on the second (6).

Due to the substantial false-negative rate, needle biopsy of the prostate does not perform well at excluding a cancer diagnosis. The need for repeat biopsies in this group can affect patient care in two ways: it can delay cancer detection in patients with disease (and delay effective therapy) or subject cancer-free men to additional

Table 1. Origin and classification of the entire sample collection

Center	No. of tissue blocks	No. of subjects per diagnostic class			
		Neg/neg (1)	Neg/pos (2)	Pos (3)	Total
UTHSC*	463	64	91		155
UCHSC[†]	415	45	23		68
UMaas[‡]	365	55	26	116	197
Comm[§]	219	—	30		30
Total	1,462	164	170	116	450

NOTE: The samples belonged to three different histopathologic classes: (1) neg/neg, two negative biopsies at least 24 mo apart (if three consecutive negative biopsies were recorded, no time limit was required); (2) neg/pos, samples with a first negative biopsy followed by a positive biopsy <24 mo later; (3) pos, samples from patients diagnosed with prostate cancer in the tested biopsy series.
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invasive biopsy procedures. A molecular-based test that complements pathologic review whereas maintaining high specificity would be a great benefit. The study reported here tested tissue from men with serial prostatic biopsies to determine the utility of quantitative methylation marker testing to meet this objective.

Methylation of the promoter CpG gene regulatory sequences of several genes has been shown to control gene expression associated with the development of prostate cancer (7). Methylation of the glutathione-*S*-transferase P1 gene (*GST-Pi*) is associated with loss of expression of this critical DNA-detoxifying protein and has been extensively studied as a marker for prostate cancer (8, 9). A high prevalence of promoter methylation in *GST-Pi* and other pertinent epigenetically silenced genes is observed in prostate cancer cells as opposed to that seen in normal and benign prostatic hyperplasia cells within the prostate (10, 11). A new refinement of a PCR-based assay has been developed for DNA methylation based on real-time measurement of the fluorescent signal created by the products of the PCR reaction in which the degree of methylation is proportional to the amount of fluorescent product (12, 13).

The study reported here evaluated archived biopsy samples from subjects ultimately diagnosed with prostate adenocarcinoma, as well as controls who have remained cancer-free upon re-biopsy. Biopsy core tissues determined to be negative by histopathology were tested to determine the ability of methylated gene markers to specifically detect cancer in subjects later confirmed to have a prostate cancer diagnosis. The demonstration of increased sensitivity of molecular testing, whereas maintaining high specificity, indicates that methylated gene testing could assist clinical decision-making in men with negative prostate biopsies but a high index of suspicion.

Materials and Methods

Biopsy Core Material. This retrospective study was approved by the institutional review board at each participating site and strict subject confidentiality was maintained. Archived tissues were used from biopsies collected from men considered to be at risk for prostate cancer who underwent needle core biopsy tissue extraction for histopathologic evaluation. Biopsy cores were formalin-fixed and embedded in paraffin blocks as part of standard hospital procedures. Following the sectioning and pathologic review, tissue blocks were stored in the hospitals' pathology laboratories. Study sites reviewed prostate screening records to identify tissue samples that met the protocol entry criteria. Tissue block samples were categorized into one of three different classes. Two classes of clinical outcome (cases and controls) from subjects with serial biopsies were identified. "Neg/pos" refers to samples from men with an initial negative biopsy series followed by a positive biopsy outcome within 24 mo of the initial negative findings. "Neg/neg" refers to samples from men with two histopathologically negative biopsies separated by at least 24 mo or at least three negative biopsy findings regardless of time. Because previous publications (7, 10-13) reported low to moderate levels of hypermethylation in biopsy tissues containing suspicious cells [high-grade prostatic interstitial neoplasm (HGPIN) and atypical small acinar proliferation (ASAP)], only biopsy specimens reported to have no evidence of cellular or glandular abnormalities were used in analyses of marker specificity (neg/neg samples). In both outcome categories, only the initial biopsy tissue was tested. Blocks obtained from the University of Texas San Antonio and the University of Colorado Health Science Centers were cut and sent for blinded histopathologic review by a centralized pathologist (D. Troyer). The second biopsy samples were also blindly re-read. Assessment of the cancer status of these samples from the neg/pos cohort provided an index of how superficial or deep the cancer cells permeated through the biopsy cores. The third class of samples was comprised of positive cases, and these samples were obtained from the University of Maastricht. "Pos" refers to histologically positive samples in the initial tested biopsy series. Biopsy tissues from the University of Maastricht or a commercial supplier of tissues were not re-read.

Depending on the site contributing the samples, different numbers of tissue blocks were available per patient. In most cases, multiple cores were a typical tissue block containing three cores.

Quantitative Methylation-Specific PCR Analysis. Fifty microns of all the available tissue blocks were cut and sent to the OncoMethylome Sciences Laboratory in Liege, Belgium for testing. After deparaffinization, DNA was extracted using the classic phenol/chloroform extraction method, and resuspended in 45 μL of LoTE (3 mmol/L Tris, 0.2 mmol/L EDTA; pH 8.0). DNA was quantified using the Picogreen dsDNA quantitation kit (Molecular

Probes, Invitrogen) following the directions of the manufacturer, and up to 1.5 μg of DNA was input to the modification reaction.

The bisulfite reaction was done using the EZ DNA Modification Kit (Zymo). This reaction selectively deaminates unmethylated cytosine residues resulting in a conversion to uracil, whereas 5-methyl cytosine residues were not modified. The modified DNA was eluted into 40 μL of Tris-HCl (1 mmol/L; pH 8.0) and then stored at −80°C. These modified DNA samples were used for direct, real-time methylation-specific PCR.

Analyte (m_*GST-Pi* and *ACTB*) quantification was done by real-time methylation-specific PCR assays. These consisted of parallel amplification/quantification processes using specific primer and primer/detector pairs for each analyte using a fluorescent label assay format on an ABI Prism 7900HT instrument (Applied Biosystems).

Five microliters of the modified DNA was added to a PCR mix (total volume, 25 μL) containing buffer [16.6 mmol/L $(NH_4)_2SO_4$, 67 mmol/L Tris (pH 8.8), 6.7 mmol/L $MgCl_2$, and 10 mmol/L β-mercaptoethanol], deoxynucleotide triphosphates (5 mmol/L), forward primer (6 ng), reverse primer (18 ng), fluorescent label (0.16 μmol/L), and Jumpstart DNA Taq polymerase (0.4 units; Sigma Aldrich). Specific primers for the bisulfite-modified version of the methylated gene were used which bind adjacent to the probe-binding region. The primer sequences and fluorescent label sequences used for each of the genes are summarized in Supplementary Table S1. The cycle program used was as follows: 5 min at 95°C, followed by 45 cycles of 30 s at 95°C, 30 s at 57°C (51°C for APC; plateau data collection), and 30 s at 72°C followed by a final stage of 5 min at 72°C. A standard curve of 2×10^6 to 20 copies of plasmid material were included to determine copy numbers of unknown samples by interpolation of their Ct values to the standard curve. The reference gene β-actin (*ACTB*) was used as a reference gene in the assay, using primers which are outside any CpG islands.

The results were generated using the SDS 2.2 software (Applied Biosystems), exported as Ct values (cycle number at which the amplification curves cross the threshold value, set automatically by the software), and then used to calculate copy numbers based on a linear regression of the values plotted on a standard curve of 20 to 2×10^6 gene copy equivalents, using plasmid DNA containing the bisulfite-modified sequence of interest. Cell lines MCF7 and HCT116 were included in each experiment as positive and negative controls, respectively, and entered the procedure at the DNA extraction step.

To compensate for variations in copy number due to differences in sample volume, sample handling, DNA isolation, and tumor heterogeneity, the copy numbers derived were divided by the ACTB copy numbers for

Table 2. PSA values in nonevaluable vs. evaluable subjects

	No. of subjects	Mean ± SD	Max. PSA	Min. PSA
Not evaluable	100	7.8 ± 6.5	58.2	0.2
Evaluable	121	8.1 ± 7.2*	63.3	0.3
Total	221	8.0 ± 6.9	63.3	0.3

*$P = 0.71$ nonevaluable vs. evaluable.

Table 3. Cancer detection by re-read pathology review of newly cut paraffin tissue sections

Sites providing tissue	Class	Prostate cancer confirmed in re-cut of second (positive) biopsy		Total
		Yes	No	
UTHSC and UCHSC	Neg/pos	64 (57%)	50 (43%)	114
	Neg/neg	0	109 (100%)	109

Abbreviations: UTHSC, University of Texas Health Science Center at San Antonio, TX; UCHSC, University of Colorado Health Science Center, Denver, CO.

that sample. This figure was multiplied by 1,000 for convenient handling, and the result referred to as the ratio-value. Cutoffs could then be applied to this ratio-value to determine if a sample was methylated or not. Scatter plots and receiver operator curves were done with MedCalc V9.6.

Results

Sample Histopathology Classifications. The samples belonged to three different histopathology classes shown in Table 1: (1) negative histology followed by a positive biopsy less than 24 months later, (2) negative biopsy followed by a negative biopsy more than 24 months later or a third negative biopsy at any time point, and (3) histologically positive samples in the tested biopsy series. Except for class 3, only tissues that were originally classified to be histopathologically cancer-free (initial biopsy) were tested.

In order to meet the time requirements for neg/neg and neg/pos criteria, only patients with multiple biopsies could be considered for testing. This led to the acquisition of samples that were relatively old. It is a known phenomenon that tissue embedded in paraffin loses DNA integrity over time. The biopsy samples were collected, formalin-fixed, paraffin-embedded, and archived between January 1997 and July 2005. DNA recovery yields are reduced in older tissues. Digital rectal exam results were available from 111 subjects, 52 cases (pos or neg/pos), and 59 controls (neg/neg). For cases, 26 (50%) had normal digital rectal exam results, 17 (33%) had benign prostatic hyperplasia, and 9 (17%) were suspicious. For controls, 28 (47%) patients were normal, 20 (34%) showed benign prostatic hyperplasia, and 11 (19%) were suspicious.

The number of evaluable tissue blocks, determined by a threshold of β-actin copy number, was 971 from a total of 264 subjects. Of these 264, we retrieved the PSA values of 221 subjects from the clinical records closest to but before the original tested biopsy (Table 2).

Due to this DNA degradation effect, not all subjects had all their biopsy core tissue tested for methylation abnormalities. We defined a minimum amount of two-thirds of the available tissue blocks per subject to have at least 200 copies of *ACTB* to yield an evaluable result for that subject. The number of collected biopsy cores, as well as the number of cores stored per paraffin block, varied among patients and across institutions. The final methylated state of each gene was based on
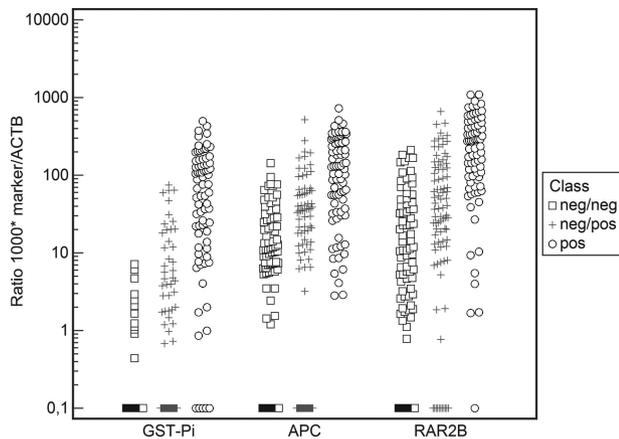
Figure 1. Methylation results for each subject. Each point is expressed as 1,000 times the methylated marker value/ACTB (the highest marker copy number from any tissue block from each subject was used for the calculation).

the aggregated results of all cores per subject. A positive gene methylation result in any tissue sample resulted in that subject being designated as positive for that gene.

From testing done in biopsy cores in other trials (14), fresher FFPE tissue yields higher levels of viable DNA. If all tissue blocks per subject had been tested for methylation, the detection of occult cancer cells would likely be improved. Additionally, recent improvements of DNA extraction methods and the use of a multiplexed assay (several genes within a single PCR run) would improve the evaluability rate by requiring less input DNA. These new techniques are presently being used or explored by OncoMethylome Sciences.

Histopathologic Re-Reads of Paraffin Sections. The subset of neg/neg and neg/pos samples contributed by the University of Texas and University of Colorado were histopathologically re-read blindly by a centralized pathologist (D. Troyer). Cancer was found in none of the originally classified negative biopsies, confirming their negative status. However, 43% of the re-read series of sections prepared from the second, untested positive diagnostic biopsy did not detect the presence of prostate cancer (Table 3). This likely reflects the small foci of prostate cancer cells in these cores. We maintained the original neg/pos classification and did not reclassify these as neg/neg. The corresponding information for the other sites is not available as the re-reading was done on the original slides at these sites.

The initial biopsy tissue from the neg/pos group contained either no evidence of abnormal cells or suspicious cells (HGPIN or ASAP). Of the 170 neg/pos samples from the four contributing sites listed in Table 1, a subset of 75 (44%) had both a suspicious first biopsy (HGPIN or ASAP) and an evaluable methylation result. Thus, 44% of the neg/pos class had evidence of atypia in the first biopsy.

Results of Methylation Markers. DNA from 971 tissue blocks from 264 subjects was blindly tested for methylated GST-Pi, APC, and RAR-2β gene promoter levels. The results are shown in Fig. 1. Receiver operator curves for

the three methylation markers are shown in Fig. 2, areas under the curve were 0.624 for APC, 0.683 for GST-Pi, and 0.712 for RAR-2β.

Using a ratio cutoff of 0 for GST-Pi yielded a sensitivity of 52% for the prediction of the presence of prostate cancer in the second biopsy when testing the histologically negative first biopsy (neg/pos group). The corresponding specificity was 85% (neg/neg group), whereas 95% sensitivity was obtained for the cases (pos group). The markers RAR-2β and APC required a higher cutoff to decrease the number of false positives in the neg/neg group (Table 4). However, a cutoff of 10 yielded a high sensitivity (84% for RAR-2β and 72% for APC) for the prediction of the presence of cancer in the follow-up biopsy in first biopsy tissue which was histopathologically negative (neg/pos group). Combining the three markers did not improve accuracy (data not shown).

Discussion

Early detection of cancer and subsequent treatment is usually associated with improved patient outcomes compared with late stage diagnosis. Active prostate cancer screening using serum PSA level monitoring and digital rectal examination is now standard practice in several countries. The gold standard for prostate cancer diagnosis is histopathologically reviewed prostatic biopsy cores. This technique, although generally effective, is subject to sampling errors and a significant false-negative rate (15). A molecular-based assay may improve the precision to detect cancer compared with microscopic examination alone.

Gene promoter methylation profiling has repeatedly shown its value as a sensitive and specific diagnostic tool for many human cancers. In particular, methylation of the DNA detoxifying gene, GST-Pi, is associated with specific prostate cancer detection in a number of clinical
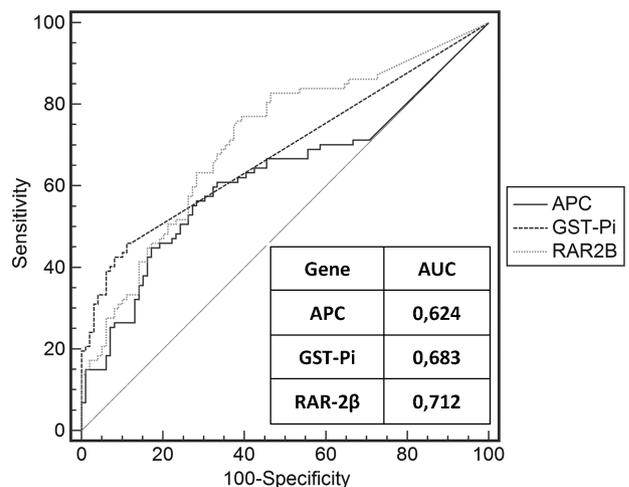

Figure 2. Methylation ratios. Receiver operator curves for APC, GST-Pi, and RAR-2β methylation ratios. For receiver operator curve analyses, cases were defined as subjects with a first histologically negative biopsy but with a positive follow-up biopsy less than 24 mo later (neg/pos class). Neg/neg controls were as defined in Materials and Methods.

Table 4. Sensitivity and specificity values obtained for GST-Pi, RAR-2β, and APC

Marker	Cutoff (for ratio)	Sensitivity cases, pos class (%)	Specificity, neg/neg class (%)	Sensitivity to predict cancer in subsequent biopsy, neg/pos class (%)
GST-Pi	0	95	85	52
RAR-2β	10	95	48	84
APC	10	93	50	72

sample-blinded testing series (7-13). Because of these initial reports, *GST-Pi* has become the most widely reported hypermethylated gene in prostate cancer (16). The ideal set of genes for analysis might be those which are robust in both predicting a histologic diagnosis of cancer and prognosis. *APC* has promise as both a diagnostic and prognostic marker (17). Finally, *GSTP1, APC,* and *RAR-2β* have all been detected in the urine following digital rectal examination, and may therefore offer the possibility of confirmatory testing of urine when biopsies are positive (18).

The present study took advantage of this DNA-based technology to test archived, routinely collected clinical specimens. Such retrospective testing (more problematic for protein or RNA-based assays) provides important, time-saving insights into the clinical utility of an assay. By knowing the cancer/no cancer status of follow-up biopsies, we were able to examine initial, cancer-negative biopsy tissues for the markers' ability to detect cancer perhaps missed by histopathology. This enhanced precision could improve the earlier detection of prostate cancers.

Testing of more recently collected and stored biopsy samples in another clinical study resulted in a much higher DNA recovery rate than observed here (19). In this retrospective study, we tested only the initial, older sample, and DNA degradation had occurred in the archived formalin-fixed, paraffin-embedded tissue blocks. This degradation in older tissue was more pronounced in these very small (1 mm diameter) tissue specimens than what has been observed for tumor blocks or fresher biopsies. Up to one-third of the originally histopathologically reviewed biopsy cores remained untested. This very likely lessened the sensitivity of the assay to find occult cancer cells.

The majority of initial, negative biopsy tissues probably did not contain cancer, even if cancer was diagnosed in a subsequent biopsy. In order to determine the "true" sensitivity of the methylation markers to detect disease, we included biopsy specimens that contained histopathologically confirmed cancer. Such testing was done blindly in sample batches including cancer-free specimens. GST-Pi and the other methylation markers showed impressive sensitivity and specificity in the three types of biopsy tissues tested.

Assessing any improvements in diagnostic performance of an ancillary test such as DNA methylation requires knowledge of the true prevalence of prostate cancer. If we were to eliminate ascertainment biases, sampling, and processing variables, what would be the true prevalence of prostate cancer? Because most clinical studies involve "for cause" biopsies as the means for disease ascertainment, reliable data on the true incidence of prostate cancer are surprisingly scarce, especially for men 30 to 50 years of age. An exception to this pattern was the recently completed Prostate Cancer Prevention Trial which required an end of study biopsy in which the incidence was 24.4% in the control group (20). It is well

known that the incidence of prostate cancer varies according to serum PSA levels, and early and influential studies of PSA showed an incidence of 22% to 26% in the PSA range of 4.0 to 10.0 ng/mL and >50% when PSA levels were >10.0 ng/mL (21, 22). Further analysis of the placebo group in the Prostate Cancer Prevention Trial study indicated that there is no PSA value below which a man is free of risk of prostate cancer. The prevalence of prostate cancer was 6.6%/PSA 0.0 to 0.5 ng/mL; 10.1%/PSA 0.6 to 1.0 ng/mL; 17.0%/PSA 1.1 to 2.0 ng/mL; 23.9%/PSA 2.1 to 3.0 ng/mL; and 26.9%/PSA 3.1 to 4.0 ng/mL (23).

A recent study by Sakr approaches this problem with a robust sample size combined with near-complete sampling of the prostate (24). For the third to eighth decades of life, the incidence of histologic prostate cancer in this cohort of 1,051 subjects was 7%, 23%, 39%, 44%, 65%, and 72%. All prostates were examined in detail using whole-mount processing. Although these results cannot be indiscriminately applied to data from core needle biopsies, they do suggest that the true prevalence of histologic cancer in men 50 to 60 years of age is 40% to 45%. What is the incidence of cancer in first biopsies as compared with this? Two retrospective studies which analyzed large numbers of biopsies from multiple sites and practices indicate that 30% to 35% of initial biopsies are positive for cancer (25, 26). This suggests that histologic cancer is underdetected by biopsy.

Methylation marker testing of negative prostate biopsy core tissues may be particularly useful for advising men who are clinically at risk for prostate cancer but who have a negative biopsy. Whether and when to re-biopsy is a difficult decision. Histologic features can be of some help in determining the risk of cancer in a repeat biopsy. When HGPIN is present in the first biopsy, 23% to 24% of the second biopsies show cancer (27, 28). When the first biopsy shows histologic atypia (e.g., ASAP), the repeat biopsy is positive in ~40% of cases. If both PIN and atypia are present on the initial biopsies, the incidence of cancer in repeat biopsies can exceed 50% ranging up to as high as 60% (29). Thus, the risk of prostate cancer upon repeat biopsy is ~20% in the absence of PIN or atypia, and as high as 60% in the presence of atypia. An algorithmic approach that includes methylation status may allow the stratification of these men into high-risk and low-risk categories. Several nomograms have been published that are used to determine both risk (30) of disease and the stage of disease.[5] Incorporation of methylation status into the decision-making algorithms may aid clinical decision-making.

In summary, ~1 million men are biopsied annually in the United States. If 30% of these men are diagnosed with cancer on the first biopsy, this leaves 700,000 men with a negative biopsy at risk. Counseling these men may be

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[5] http://urology.jhu.edu/prostate/partintables.php

aided by DNA methylation status. These numbers are large enough to potentially justify an ancillary test such as DNA methylation that would minimize anxiety and additional biopsies if the information were sufficiently specific and accurate. The test would be done on the biopsies themselves, and would therefore be correlated with the histologic data. The choice of which blocks to assay and what would trigger the decision to perform the assay remains a subject for future studies.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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