Molecular Detection of Localized Prostate Cancer Using Quantitative Methylation-Specific PCR on Urinary Cells Obtained Following Prostate Massage

Morgan Rouprêt,1 Vincent Hupertan,2 David R. Yates,1 James W.F. Catto,1 Ishtiaq Rehman,1 Mark Meuth,1 Sylvie Ricci,3 Roger Lacave,3 Géraldine Cancel-Tassin,2 Alexandre de la Taille,5 François Rozet,6 Xavier Cathelineau,6 Guy Vallancien,6 Freddie C. Hamdy,1 and Olivier Cussenot2,4

Abstract

Purpose: The diagnosis of localized prostate cancer is difficult due to a lack of cancer-specific biomarkers. Many patients require repeat prostate biopsies to diagnose the disease. We investigated whether aberrant promoter hypermethylation in prostatic fluid could reliably detect prostate cancer.

Experimental Design: Urine samples were collected after prostate massage from 95 patients with localized prostate cancer undergoing radical prostatectomy (63 pT1, 31 pT2, and 1 pT3) and from 38 control patients. Ten genes (GSTP1, RASSF1a, ECDH1, APC, DAPK, MGMT, p14, p16, RARβ2, and TIMP3) were investigated using quantitative real-time methylation-specific PCR. Receiver operator curves were generated.

Results: The frequency of gene methylation ranged from 6.3% (p14) to 83.2% (GSTP1) in prostate cancer patients. At least one gene was hypermethylated in 93% of cancer patients. The specificity of methylation was 0.74. Methylation was significantly more frequent (P < 0.05) in cancer than control patients for all genes except p14 and p16. According to receiver operator curve analysis, the four-gene combination of GSTP1 (0.86), RASSF1a (0.85), RARβ2 (0.80), and APC (0.74) best discriminated malignant from nonmalignant cases. The sensitivity and accuracy of this four-gene set were 86% and 89%, respectively.

Conclusions: The presence of aberrant methylation in urinary cells obtained after prostate massage is significantly associated with prostate cancer. A panel of four genes could stratify patients into low and high risk of having prostate cancer and optimize the need for repeat prostatic biopsies.

Prostate cancer is the most commonly detected male cancer and the second leading cause of male cancer deaths in the United States and in Europe (1). Its prognosis is directly related to stage at diagnosis and treatment (2). However, the early and specific diagnosis of prostate cancer is difficult due to lack of cancer-specific biomarkers. The most common marker used is prostate-specific antigen (PSA), whose specificity and sensitivity

for the disease are low, regardless of the threshold value used (either 2.5 or 4.0 ng/mL; refs. 3–5). PSA may in fact be a better marker of benign prostate hypertrophy than of prostate cancer. To confirm the diagnosis of cancer, the gold standard method is ultrasound-guided prostate biopsy (6). This is an uncomfortable procedure with frequent morbidity. Markers that distinguish benign from clinically silent malignant disease are urgently needed to improve the care of men with prostate cancer and reduce the number of unnecessary biopsies.

Cancer arises through the accumulation of multiple molecular genetic events (7). These include changes in DNA sequence or content (inherited and acquired) and changes in gene expression through epigenetic mechanisms (8–10). The development of diagnostic and screening tools that detect these alterations (and the corresponding neoplastic cells) with a high specificity and sensitivity would constitute a major step forward in oncological care (7, 8). One mechanism of tumor suppressor gene inactivation seems to be methylation of the cytosine-guanine dinucleotides (CpG islands) within gene promoter regions. Hypermethylation at various gene loci has been detected in several urologic malignancies, including prostate cancer. Most studies have been done on prostate tissue (11–14) but some have used urine (15, 16), sometimes obtained after prostate massage (17, 18). To our knowledge, there are, however, few studies that have investigated an

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Received 10/11/06; revised 12/1/06; accepted 12/28/06.

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© 2007 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-06-2467

Human Cancer Biology
extended panel of methylation markers on urine sediment for the detection of prostate cancer (19, 20).

In the present study, we examined 10 genes that are either silenced or activated by aberrant methylation mechanisms and/or that play an important role in human neoplasia (12, 19, 21) to establish a small, reliable set of prostate cancer markers, which could be assessed from urine obtained after prostate massage.

Materials and Methods

Patients and urine collection. We analyzed urine samples collected prospectively (October 2005-February 2006) from patients with localized prostate cancer who underwent laparoscopic radical prostatectomy at the Institut Mutualiste Montsouris (Paris, France). Urine sample collection was approved by the Ethics Committee of the Institut Mutualiste Montsouris (Paris, France). Urine samples were obtained from 95 consecutive radical prostatectomy patients and from 38 age-matched males (controls) with no history of genitourinary malignancy, negative prostate biopsies, and with or without benign prostate hypertrophy (Table 1). Median number of prostate biopsies was 10 (range, 0-12) in both groups according to our local protocol. Radical prostatectomy patients underwent prostate massage by digital rectal examination (at least 1 min) in the operating room to obtain prostate secretions at the external urethral meatus. The first urine stream was then collected by urethral catheterization of the bladder. Control patients also underwent prostate massage to collect urine sediments either before a set of biopsies or an endoscopy or a medical visit.

DNA extraction from urine. Urine samples (40-120 mL) were immediately placed in Carboxv-PEG. They were centrifuged at 4°C (800 g × 10 min). The pelleted material, including exfoliated cells, was resuspended in 3 mL PBS and recentrifuged (800 g × 10 min). DNA was extracted from the washed urine suspension using Qiagen Tissue and Blood kits (Qiagen, Valencia, CA). Its concentration was measured on an aliquot with Hoechst 33258 as DNA-binding fluorochrome (DyNA Quant 200, Hoefer Pharmacia Biotech, San Francisco, CA). The DNA extracts were frozen at −20°C until use.

Methylation analysis. For methylation analysis, 1 to 2 µg DNA was treated with sodium bisulphite using the CpGenome kit (Chemicon, Hampshire, United Kingdom) according to the manufacturer's instructions as described previously (23, 24). The bisulphite-treated DNA was then used as a template for the quantitative fluorescence-based real-time methylation-specific PCR (QMSP) as described previously (19, 20). Ten gene promoter regions [GSTPI, Ras association domain family 1 isoform A (RASSF1a), CDH1 (E-cadherin), adenomatosis polyposis coli (APC), DAPK, MGMT, p14, p16, retinoic acid receptor β (RARβ), and TIMP3] were investigated for aberrant methylation by QMSP. These genes have been examined previously and shown to be of interest in early prostate cancer detection (12, 19, 21). For methyl quantification, PCR was done using bisulphite-specific primers to β-actin (as the internal reference gene). Universally methylated DNA (Chemicon) was used as a positive control.

QMSP Accuprime Taq polymerase (Invitrogen, Paisley, United Kingdom) using the ABI Prism 7000 Sequences Detection System. Primers and fluorescence-labeled probes were obtained from MWG Biotech (London, United Kingdom). A 10 µL PCR volume was used, comprising 1 µL Accuprime buffer, 0.25 µL Accuprime Taq polymerase, 5 pmol/L forward and reverse primers, 5 pmol/L probe, 0.2 µL Rox reference dye, and water. Sixty cycles of denaturation (95°C for 45 s), annealing (specific primer temperature for 2 min), and extension (72°C for 1 min) were used (Fig. 1).

Statistical analysis. For quantification, the relative methylation levels for each promoter were calculated from the ratio of fluorescence released by the QMSP reaction and that from the β-actin reaction of same sample (gene/β-actin ratio). For analysis with respect to the presence of methylation, we used a threshold of 0.2 ΔRn. We have found previously that this correlates well with the absolute presence of methylation within sample using bisulphite sequencing (data not shown; Fig. 1). Correlations between methylation levels and clinicopathologic variables were evaluated by the χ² test and Fisher's exact test. The odds ratio [95% confidence interval (95% CI)] for each of the 10 gene loci was calculated. The sensitivity and specificity of methylation in discriminating malignant cells were determined by receiver operator curve (ROC) analysis. The discriminatory power of the test was given by the area under the ROC modified for censored data (1 = a perfect test; 0.5 = a worthless test). ROC analysis was also used to determine the methylation cutoff levels yielding optimal sensitivity and specificity for the “best” set of gene loci. Continuous QMSP data were converted into
Results

In our patients, PSA (with a threshold of 4 ng/mL) had a sensitivity of 94.7% and a specificity of 18.4% for prostate cancer. Methylation analysis of urine DNA samples successfully diagnosed 88 of 95 cases of early prostate cancer (sensitivity, 0.93), and 74 of 95 (78%) of these samples were positive for at least three genes. Of the 38 patients with no evidence of disease, 28 showed no aberrant methylation at any promoter site (specificity, 0.74). The frequency of aberrant methylation at CpG islands ranged from 6.3% (p14) to 79% (GSTP1; Table 2). Methylation at all 10 gene loci was more frequent in prostate cancer patients than in age-matched controls. The difference reached statistical significance ($P < 0.05$) for eight loci (RASSF1a, CDH1, APC, DAPK, MGMT, GSTP1, RAR$eta_2$, and TIMP3; Table 2). There was no correlation between hypermethylation and either tumor stage or grade in the current study. Hypermethylation of no single gene locus was significantly related to a clinicopathologic variable (preoperative serum PSA level, pathologic stage, or Gleason score; Table 3).

We plotted the methylation levels for the four gene loci (GSTP1, APC, RASSF1a, and RAR$eta_2$), which showed the greatest statistical difference ($P < 0.0001$) in aberrant methylation between prostate carcinoma and controls (Fig. 2). As shown, the relative amount of methylated promoter was much higher in urine sediment from cancer patients than from controls. The estimated odds ratios for these four genes ranged from 18.4 to 60.6 (Table 2). All four ROC curves are plotted in Fig. 3. The area under the ROC was 0.86 (95% CI, 0.79-0.93).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Methylation-positive urine*</th>
<th>Cancer patients (%)</th>
<th>Controls (%)</th>
<th>P*</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RASSF1a</td>
<td>74 (77.9)</td>
<td>3 (7.9)</td>
<td>&lt;0.0001</td>
<td>41.1 (11.5-147.1)</td>
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<td>CDH1</td>
<td>29 (30.5)</td>
<td>2 (5.3)</td>
<td>0.002</td>
<td>7.9 (1.8-35.1)</td>
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<tr>
<td>APC</td>
<td>48 (50.5)</td>
<td>2 (5.3)</td>
<td>&lt;0.0001</td>
<td>18.4 (4.2-80.7)</td>
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<td>DAPK</td>
<td>27 (28.4)</td>
<td>2 (5.3)</td>
<td>0.003</td>
<td>7.1 (1.6-31.8)</td>
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<td>MGMT</td>
<td>14 (14.7)</td>
<td>1 (2.6)</td>
<td>0.046</td>
<td>6.4 (0.8-50.5)</td>
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<td>p16</td>
<td>11 (11.6)</td>
<td>2 (5.3)</td>
<td>0.268</td>
<td>2.3 (0.5-11.2)</td>
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<td>p14</td>
<td>6 (6.3)</td>
<td>1 (2.6)</td>
<td>0.39</td>
<td>2.5 (0.3-21.4)</td>
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<td>GSTP1</td>
<td>79 (83.2)</td>
<td>5 (13.2)</td>
<td>&lt;0.0001</td>
<td>32.6 (11-96.3)</td>
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<td>RAR$eta_2$</td>
<td>59 (62.1)</td>
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<td>&lt;0.0001</td>
<td>60.6 (7.9-461.3)</td>
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<td>TIMP3</td>
<td>41 (43.2)</td>
<td>0 (0)</td>
<td>0.001</td>
<td>2.1 (1.6-2.7)</td>
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*Methylation-positive: methylation level above the empirical cutoff level obtained by comparing patients and controls and by maximizing the likelihood ratio for a positive test. Cancer patients ($n = 95$), controls ($n = 38$).

$^*$P value according to the $\chi^2$ test.

Table 3. Cross-table between positive promoter methylation in urine DNA and clinical variables of prostate cancer patients

<table>
<thead>
<tr>
<th>Precursor PSA (ng/mL)</th>
<th>RASSF1a</th>
<th>CDH1</th>
<th>APC</th>
<th>DAPK</th>
<th>MGMT</th>
<th>P16</th>
<th>P14</th>
<th>GSTP1</th>
<th>RAR$eta_2$</th>
<th>TIMP3</th>
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<td>2-8</td>
<td>5</td>
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<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>4</td>
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<tr>
<td>4-8</td>
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<td>39</td>
<td>17</td>
<td>28</td>
<td>14</td>
<td>47</td>
<td>6</td>
<td>2</td>
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<td>32</td>
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<td>16</td>
<td>10</td>
<td>3</td>
<td>4</td>
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<td>7</td>
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<td>1</td>
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<td>4</td>
<td>1</td>
<td>1</td>
<td>6</td>
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<tr>
<td>Preoperative PSA (ng/mL)</td>
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<td>3</td>
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*Methylation-positive: methylation level above the empirical cutoff level obtained by comparing patients and controls and by maximizing the likelihood ratio for a positive test.
for GSTP1, 0.85 (95% CI, 0.77-0.92) for RASSF1a, 0.8 (95% CI, 0.73-0.87) for RARβ2, and 0.74 (95% CI, 0.65-0.82) for APC. Threshold values for distinguishing benign and malignant cells with 95% specificity were calculated (1.0, 1.0, 0.34, and 0.91 ΔRn for GSTP1, RASSF1a, RARβ2, and APC, respectively). Combining these four genes cause the greatest discriminatory power. The theoretical sensitivity of the four-gene set was 86% and its diagnostic accuracy was 89%.

**Discussion**

Our study has confirmed that aberrant methylation can be detected in cells from post-prostate massage voided urine specimens from radical prostatectomy patients with early prostate cancer (8, 10, 17, 18). The sensitivity of the 10 gene loci in diagnosing cancer on these specimens was 93% (i.e., slightly higher than for preoperative voided or catheterized urine specimens; refs. 15–18). Prostate tissue and serum samples have given similar results but they yield larger amounts of DNA for extraction (12, 14, 25, 26). Prostate massage could be a first step in increasing the shedding of prostate cells in urine (10) and collecting enough material for QMSP.

The most common somatic genome alteration during prostate cancer development seems to be hypermethylation in the regulatory region of the promoter of the π-class glutathione S-transferase (GSTP1) gene (20, 21, 27). Its prevalence in body fluids (urine or serum) can reach 76% (15, 17); it was 83% in this study. No urine sediment DNA sample has yet given a positive GSTP1 methylation result in the absence of methylation in the corresponding tumor (20, 28). Although rare in normal prostate tissue (29, 30), GSTP1 methylation did occur in 5 (13.2%) of our controls. It may be age related as promoter hypermethylation of certain genes in apparently normal tissues seems to be associated with aging (29). Further follow-up is needed to determine whether our subjects with methylated markers but no evidence of cancer actually have disease.

The use of a single gene locus to discriminate malignant from benign cells has 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. Furthermore, methylation of a single gene locus may occur in cancers other than prostate cancer or even in benign disease. Investigating several genes may have more discriminatory power because the number of hypermethylated genes rises as prostate cancer progresses (8, 19, 21, 25). A panel of carefully selected methylation markers for use on urine sediments might help both detect and discriminate among a variety of urologic tumors. Cancers other than prostate cancer (e.g., bladder and kidney cancer) can contribute cellular DNA to urine sediment (23, 31–33).

In our panel of 10 genes, 3 genes besides GSTP1 proved to be of special interest: APC, RASSF1a, and RARβ2. APC is a tumor suppressor involved in down-regulating WNT/β-catenin signaling but it also plays a role in cell-adhesion, stability of the
microtubular skeleton, and possibly apoptosis. APC was methylated in the urine sediment DNA of 50.5% of our patients. This is within the reported 27% to 95% range for APC hypermethylation in primary prostate tumors (11, 34). RASSF1a is a tumor suppressor that interacts with Cdc20, an activator of the anaphase-promoting complex, to inhibit complex activity and prevent mitotic progression. RASSF1a was methylated in 77.9% of our patients. Epigenetic inactivation of RASSF1a is observed in 53% to 71% of solid tumors and epithelial cancers, including prostate cancer (35, 36). RARβ2 functions as a tumor suppressor in lung, breast, and gynecologic neoplasia. It is hypermethylated in 62.1% of prostate adenocarcinomas (37, 38). In the present study, RARβ2 clearly discriminated between neoplastic and nonneoplastic cells. Three of these genes (APC, RARβ2, and GSTP1) are the same as those found to be most discriminatory between cancer tissue and benign prostate hypertrophy in a recent study (39).

For most genes, we observed higher frequencies of promoter methylation than reported previously (19, 21, 40), although our results seem similar to those found by Hoque et al. (20) for many genes. In particular, we found less methylation of p16 and MGMT and more of GSTP1 and RARβ2 than Hoque et al. (20). The reasons for differences in reported methyl frequency are unclear and may reflect patient selection [European versus North American patients (different PSA screening prevalences)], different materials examined (primary tumor tissue versus urine), or laboratory methodology (QMSP versus MSP). Our data once again confirm the importance of GSTP1 in the biology of prostate cancer and that for most loci levels of methylation are reproducible in different laboratories. With regards to tissue examined, most previous reports focus on prostate tissue rather than bodily fluids (19, 21, 40) and authors have reported results in line with our findings (26, 37, 41). With respect to methodology, quantitative method (QMSP) uses PCR conditions that differ from those of conventional MSP and seems more sensitive to the latter (15, 20) and enables tissue differentiation by quantitative rather than just qualitative methyl analysis (20, 41).

There is currently no consensus for managing high-risk men with an abnormal digital rectal examination or persistently elevated PSA levels and a negative initial biopsy (5, 6). Surveillance includes measuring the percentage of free PSA or elevated PSA levels and a negative initial biopsy (5, 6). There is currently no consensus for managing high-risk men with an abnormal digital rectal examination or persistently elevated PSA levels and a negative initial biopsy (5, 6). Surveillance includes measuring the percentage of free PSA or elevated PSA levels and a negative initial biopsy (5, 6).

Fig. 3. ROC curves for RASSF1a, APC, GSTP1, and RARβ2 used to determine the methylation cutoff level for optimal sensitivity and specificity for each gene locus.

Acknowledgments

Morgan Roupért was awarded a European Urological Scholarship Programme from the European Association of Urology for performing the laboratory aspect of this work at the Academic Urology Unit and Institute for Cancer Studies, University of Sheffield.

References


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