

# Molecular Detection of Prostate Cancer in Urine by GSTP1 Hypermethylation<sup>1</sup>

Paul Cairns, Manel Esteller, James G. Herman, Mark Schoenberg, Carmen Jeronimo, Montserrat Sanchez-Cespedes, Nan-Haw Chow, Marc Grasso, Li Wu, William B. Westra, and David Sidransky<sup>2</sup>

Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 [P. C.], and Tumor Biology, Oncology Center [M. E., J. G. H., D. S.], Departments of Urology [M. S., D. S.], Otolaryngology, Head and Neck Surgery, Division of Head and Neck Cancer Research [C. J., M. S.-C., N.-H. C., M. G., L. W., D. S.], and Pathology [W. B. W., D. S.], Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2195

## ABSTRACT

Novel approaches for the early detection and management of prostate cancer are urgently needed. Clonal genetic alterations have been used as targets for the detection of neoplastic cells in bodily fluids from many cancer types. A similar strategy for molecular diagnosis of prostate cancer requires a common and/or early genetic alteration as a specific target for neoplastic prostate cells. Hypermethylation of regulatory sequences at the glutathione *S*-transferase pi (*GSTP1*) gene locus is found in the majority (>90%) of primary prostate carcinomas, but not in normal prostatic tissue or other normal tissues. We hypothesized that urine from prostate cancer patients might contain shed neoplastic cells or debris amenable to DNA analysis. Matched specimens of primary tumor, peripheral blood lymphocytes (normal control), and simple voided urine were collected from 28 patients with prostate cancer of a clinical stage amenable to cure. Genomic DNA was isolated from the samples, and the methylation status of *GSTP1* was examined in a blinded manner using methylation-specific PCR. Decoding of the results revealed that 22 of 28 (79%) prostate tumors were positive for *GSTP1* methylation. In 6 of 22 (27%) cases, the corresponding urine-sediment DNA was positive for *GSTP1* methylation, indicating the presence of neoplastic DNA in the urine. Furthermore, there was no case where urine-sediment DNA harbored methylation when the correspond-

ing tumor was negative. Although we only detected *GSTP1* methylation in under one-third of voided urine samples, we have demonstrated that molecular diagnosis of prostate neoplasia in urine is feasible. Larger studies focusing on carcinoma size, location in the prostate, and urine collection techniques, as well as more sensitive technology, may lead to the useful application of *GSTP1* hypermethylation in prostate cancer diagnosis and management.

## INTRODUCTION

Prostate cancer is the most commonly detected male cancer and the second leading cause of male cancer deaths in the United States (1). Diagnosis and management are confounded by the lack of symptoms and the lack of cancer-specific diagnostic techniques to be used during early stages of the disease. Prostate cancer is indeed curable if detected early, while still localized within the capsule (2). Novel approaches for the detection and control of this cancer are therefore extremely important. Adult sporadic cancers are known to arise through the accumulation of multiple genetic events (3), and these clonal genetic alterations can be used as targets for the detection of neoplastic cells in clinical samples (4). To develop such targets, a common and early genetic event unique to neoplastic cells must be identified and combined with a sensitive molecular assay that is able to detect this genetic event among a high background of normal wild-type cells. Several specific genetic alterations have been identified in prostate cancer (5), including *ras* oncogenic activation and inactivation of the tumor suppressor genes *Rb*, *p53*, *CDKN2a*, and *PTEN*. However, *RAS* or *p53* mutations are infrequent (5), and *PTEN* inactivation generally occurs relatively late in prostate cancer progression (6). Loss of heterozygosity at critical suppressor loci, such as *8p* and *16q*, occurs frequently (5), but successful loss of heterozygosity detection requires a high proportion of tumor cells for robust analysis of a diagnostic sample.

Hypermethylation of normally unmethylated CpG islands in the promoter regions of tumor suppressor genes correlates with loss of gene expression in human tumors (7–9). Hypermethylation of regulatory sequences at the detoxifying *GSTP1*<sup>3</sup> gene locus is found in the majority (>90%) of primary prostate carcinomas but not in normal prostatic tissue or other normal tissues nor in benign hyperplasia of the prostate (10). *GSTP1* methylation is thus the most common genetic alteration thus far described in prostate cancer. The initial studies of *GSTP1* methylation status in prostate tumors and cell lines were performed using Southern blot analysis (10). A new method, MSP, which is more sensitive and requires less DNA, has been described since (11). MSP uses a DNA modification step before PCR to

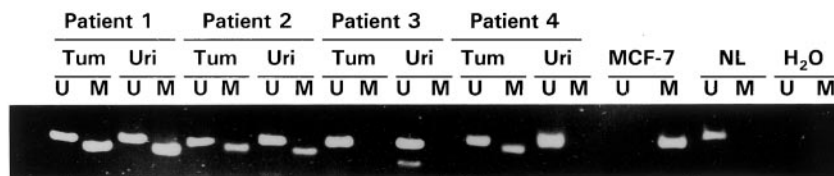
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<sup>2</sup> To whom requests for reprints should be addressed, at Head and Neck Cancer Research, 820 Ross Research Building, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Baltimore, MD 21205-2195. Phone: (410) 502-5153; Fax: (410) 614-1411.

<sup>3</sup> The abbreviations used are: *GSTP1*, glutathione *S*-transferase pi; MSP, methylation-specific PCR.



**Fig. 1** MSP of GSTP1 in prostate carcinoma and urine DNAs. The presence of a visible PCR product in the methylated lane (M) of the tumor DNA from patients 1, 2, and 4 indicates the presence of methylated alleles of GSTP1. A PCR product is also present in the methylated lane (M) of the urine DNA from patients 1 and 2, indicating the presence of neoplastic cell DNA in the urine. The absence of a visible PCR product in the methylated lane (M) of urine-sediment DNA from patient 4 indicates that neoplastic cell DNA is absent or undetectable in the urine. Patient 3's tumor DNA is not methylated, and the corresponding urine DNA also had no PCR product in the methylated lane (M); whereas a product can be clearly seen in the unmethylated lane (U). The PCR product from the tumor DNA of patients 1, 2, and 4, in the unmethylated lane (U), most likely arises from normal cell contamination of the tumor specimen. Tumor cell line MCF-7 DNA as a positive control for GSTP1 methylation, normal lymphocyte DNA (NL) as a negative control, a water control for contamination in the PCR reaction (right), and *MspI*-digested pBR322 as a molecular weight marker (far left) are also shown.

determine the presence or absence of methylation of a gene locus at a sensitive level of up to 1 methylated allele in 1000 unmethylated alleles.

Bodily fluids from several types of cancer have been successfully used for the molecular detection of neoplasia, including stool in colon and pancreatic cancer, urine in bladder cancer, and sputum and bronchial lavage fluid in lung cancer (4). Recently promoter hypermethylation has been used successfully to detect neoplastic DNA in sputum (12), bronchial lavage fluid (13), and serum (14) from lung cancer patients and in serum from liver cancer (15), head and neck cancer (16), and breast cancer patients (17). Most prostate tumors occur in the peripheral zone that contains three-quarters of the glands, the minilobes of which form secretory ducts that empty their contents into the urethra. We hypothesized that urine from prostate cancer patients might therefore contain shed neoplastic cells or debris amenable to DNA analysis. We therefore examined the potential of GSTP1 hypermethylation as a cancer-specific marker in simple voided urine specimens from 28 prostate cancer patients about to undergo radical prostatectomy for clinically curable disease.

## MATERIALS AND METHODS

**Specimen Collection and DNA Isolation.** Samples were obtained from patients undergoing radical prostatectomy. Urine was collected from each patient immediately before surgery. Tumor samples were obtained after pathological review, and areas rich in neoplastic cells were selected and microdissected from formalin-fixed blocks. A peripheral blood sample in EDTA was also obtained for isolation of leukocyte DNA as a normal control. Genomic DNA was isolated as described previously (18).

**Bisulfite Treatment.** One  $\mu$ g of each DNA sample was denatured by sodium hydroxide and modified by sodium bisulfite. Then DNA samples were purified using Wizard DNA purification resin (Promega, Madison WI), treated again with sodium hydroxide, precipitated with ethanol, and resuspended in water.

**MSP.** MSP was performed separately with GSTP1 primers specific for the methylated reaction and the unmethylated reaction (19) for each DNA sample as follows: unmethylated reaction: 5'-GATGTTTGGGGTGTAGTGGTTGTT-3' (sense),

5'-CCACCCCAATACTAAATCACAACA-3' (antisense); methylated reaction: 5'-TTCGGGGTGTAGCGCTCGTC-3' (sense), 5'-GCCCAATACTAAATCACGACG-3' (antisense). Thirty-five cycles of PCR were performed with an annealing temperature of 59°C. A water control without DNA for contamination and controls for unmethylated and methylated reactions were performed for each set of PCR. PCR reactions were analyzed on nondenaturing 6% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination.

## RESULTS AND DISCUSSION

We collected 28 primary resectable prostate tumors of pathological grade and stage amenable to surgical cure [7 T<sub>2A</sub> (Gleason 5–8), 5 T<sub>2B</sub> (Gleason 6–7), 15 T<sub>3A</sub> (Gleason 5–7), and 1 T<sub>3B</sub> (Gleason 7)] and extracted genomic DNA from tumor, peripheral blood lymphocytes (normal control), and urine sediment (from a simple voided urine obtained preoperatively). The DNA samples were coded, and the methylation status of GSTP1 was assessed in a blinded manner. Decoding of the results revealed that 22 of 28 (79%) prostate tumors were positive for GSTP1 methylation. In 6 of 22 (27%) cases, the corresponding urine-sediment DNA was positive for GSTP1 methylation, indicating the presence of neoplastic DNA in the urine (Fig. 1; tumors 1 and 2). There was no case where a urine-sediment DNA gave a positive methylation result in the absence of methylation in the corresponding tumor (potential false positive; Fig. 1, tumor 3). The six tumors with positive urine results were Gleason 5–7 and stages T<sub>2A</sub> (1), T<sub>2B</sub> (1), and T<sub>3A</sub> (4).

Although we only detected GSTP1 methylation in under a third of voided urine samples, we have clearly demonstrated that molecular diagnosis of prostate neoplasia in urine is feasible. Moreover, albeit in a limited study, we observed absolute specificity, because we did not find any GSTP1 hypermethylation in the urine DNA from the six patients with unmethylated GSTP1 tumor DNA. We detected GSTP1 hypermethylation in a minority of paired urine, and this level of sensitivity can likely be improved upon. Goessl *et al.* (20) reported a higher percentage of positive cases with a fluorescence-based conventional PCR technique. It is possible that prostatic massage and the higher number of cycles used in their study yielded more positive urine DNAs, but specificity is known to decrease in MSP, as in other PCR protocols, with increased cycle number (21). Indeed, in the

study by Goessl *et al.* (20), some urine samples were positive, yet the primary tumor was not found to harbor GSTP1 hypermethylation. Additional work needs to focus on understanding factors such as tumor size and localization within the prostate, urine collection techniques, *e.g.*, the potential benefits of a prostatic massage before urine collection, and continuing improvements in molecular technology to increase the detection rate (12).

Six tumors of 28 did not have GSTP1 hypermethylation, preventing assessment of neoplasia in the urine. However, screening for methylation of other loci, such as the *endothelin B receptor* (methylated in ~70% of prostate tumors; Ref. 22) or *CD44* (methylated in 77% of tumors; Ref. 23), is likely to increase further the number of primary tumors with methylation (amenable for screening) to allow 100% diagnostic coverage. GSTP1 hypermethylation has not been reported in bladder cancer and is found infrequently in renal tumors (19). Inadvertent detection of a renal cell carcinoma in urine is therefore possible. Even so, GSTP1 hypermethylation is cancer-specific; unlike PSA it is not found in normal prostatic tissue or benign prostatic hyperplasia.

An additional consideration is that of our choice of optimal negative controls. For a study of the type presented here, normal age-matched controls would present problems of ethics and interpretation of results. The high frequency of incidental prostate cancer in men over 50 years of age (estimated at 30–50%), the hypothesized early timing of GSTP1 hypermethylation in prostate tumorigenesis, and the ability of MSP to detect 1 cancer cell in a background of 1000 normal cells argues against initial inclusion of a cohort of age-matched men with no evidence of prostate cancer as controls. Whether a positive MSP test arose from a false-positive result or from asymptomatic prostate cancer would be difficult to ascertain. In our exploratory study, control urine samples from the 6 of 28 (21%) of patients whose prostate cancer did not show GSTP1 hypermethylation were negative for urine methylation.

Thus, we envision the possibility of a sensitive, noninvasive molecular test that may indicate the presence of prostate cancer in individuals with lesions undetectable by currently existing methods and, theoretically, more specific for neoplasia than serum PSA. Only 80% of the patients in our study of clinically early cancer (mostly T<sub>1a</sub>) had elevated PSA. Two subgroups of men in whom GSTP1 hypermethylation has clinical utility would be the 20% of men with prostate cancer with a near-normal PSA value and men with a high PSA value but negative biopsies. If our results are confirmed in larger studies, GSTP1 hypermethylation could be used to augment PSA and other current diagnostic procedures for the detection of prostate cancer in the general population.

This technique could also be used to identify neoplastic disease in other diagnostic clinical material, such as needle biopsies or serum. Similarly, in prostate cancer patients, hypermethylation may be a marker of neoplastic cell burden or minimal residual disease after removal of the primary tumor. Finally, it has been shown previously that nearly all bladder cancers (24) and many kidney cancers (25) can be detected by molecular analysis of urine, raising the possibility of simultaneous molecular screening for three common adult cancer types in one simple voided urine specimen.

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