

**MOLECULAR DETECTION OF PROSTATE CANCER BY METHYLATION-SPECIFIC POLYMERASE CHAIN REACTION FROM URINE SPECIMENS**

## MOLEKULARNA DETEKCIJA KANČERA PROSTATE IZ UZORAKA URINA POMOĆU METILACIJA SPECIFIČNE LANČANE REAKCIJE POLIMERAZE

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**Background:** Prostate cancer (PCa) represents the second most prevalent malignancy among males, which is characterized by a high mortality rate. The aim of our study was to evaluate the methylation status of glutathione S-transferase P1 (GSTP1) in urine specimens from males with PCa and benign prostatic hyperplasia (BPH) and its usefulness in distinguishing between males with PCa and BPH by noninvasive methods.

**Methods:** Voided urine specimens were collected from 65 patients with PCa and 45 patients with BPH. Genomic DNA was isolated and subjected to bisulfite modification. Methylation status of the GSTP1 gene was determined by conventional methylation-specific polymerase chain reaction (MSP) analysis.

**Results:** Promoter hypermethylation of the GSTP1 gene in voided urine samples was found in 63 of 65 (97%) males with PCa and in 5 of 45 (11%) males with BPH. The sensitivity and specificity of GSTP1 in discriminating between PCa and BPH males were 98% and 89%, respectively.

**Conclusions:** Gene analysis of GSTP1 using conventional MSP in urine specimens can be used as a noninvasive biomarker to distinguish between men with malignant and benign prostatic diseases.

**Keywords:** prostate cancer, benign prostatic hyperplasia, methylation-specific polymerase chain reaction, glutathione S-transferase P1

**Kratak sadržaj**

**Uvod:** Kancer prostate (KP) predstavlja drugi najčešći malignitet kod muškaraca, koji se odlikuje visokom stopom mortaliteta. Cilj studije bio je da se odredi status metilacije glutathione S-transferaze P1 (GSTP1) u uzorcima urina uzetim od muškaraca sa KP i benignom hiperplazijom prostate (BHP), kao i njegova korisnost u razlikovanju muškaraca sa KP i muškaraca sa BHP pomoću neinvazivnih metoda.

**Metode:** Čisti uzorci urina uzeti su od 65 pacijenata sa KP i 45 pacijenata sa BHP. Genomska DNK je izolovana i podvrgnuta bisulfitnoj modifikaciji. Status metilacije gena GSTP1 određen je konvencionalnom analizom lančane reakcije polimeraze specifične za metilaciju (MSP).

**Rezultati:** Hipermetilacija promotera gena GSTP1 u čistim uzorcima urina otkrivena je kod 63 od 65 (97%) muškaraca sa KP i kod 5 od 45 (11%) muškaraca sa BHP. Osetljivost i specifičnost GSTP1 za diskriminisanje između KP i BHP bile su 98% i 89%.

**Zaključak:** Genska analiza GSTP1 u uzorcima urina pomoću konvencionalne metode MSP može se koristiti kao neinvazivan biomarker za razlikovanje muškaraca sa malignim i benignim oboljenjima prostate.

**Ključne reči:** kancer prostate, benigna hiperplazija prostate, lančana reakcija polimeraze specifične za metilaciju, glutathione S-transferaza P1

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List of abbreviations: PCa, Prostate Cancer; BPH, Benign Prostatic Hyperplasia; MSP, Methylation-Specific Polymerase Chain Reaction; GSTP1, Glutathione S-Transferase P1; PSA, Prostate-Specific Antigen; TRUS, Transrectal ultrasound guided prostate biopsy.

## Introduction

In Western countries, prostate cancer (PCa) represents the most commonly diagnosed malignancy among males, and is the second related cause of cancer. Each year, about 230,000 men from the USA and 90,000 from Western Europe are newly diagnosed with this malignancy (1). Prostate-specific antigen (PSA) is the molecular biomarker which is used currently for PCa detection and disease recurrence monitoring (2). PSA is a protein secreted by the prostatic epithelial cells and represents an important component of the ejaculate. It appears in the blood circulation only in case of epithelial damage or dysfunction of the prostate. In patients with serum PSA levels in the range from 4.0 ng/mL to 10.0 ng/mL, known as the »gray zone«, prostatic biopsy is recommended. Serum PSA levels are increased in nonmalignant prostate diseases, such as benign prostatic hyperplasia (BPH). Because these inflammatory conditions affect men in the 5th decade of life, the significance of increased serum PSA as a screening biomarker is not very clear. Greene et al. (3) reported that only 22% of the men with an elevated serum PSA value in the range of 4.0 ng/mL to 10.0 ng/mL were found to have PCa upon prostate biopsy. Because of this, to avoid unnecessary biopsies, physicians need new, minimally invasive tests to determine which men with a slightly to moderately raised serum PSA concentration require further investigation.

It has been recognized that prostatic manipulation from needle biopsy, TRUS probe or DRE, causes prostatic DNA to appear in urine by shedding the neoplastic cells or debris into prostatic ducts and the urethra (4). Epigenetic alterations, like hypermethylation of tumor-suppressor genes (TSG), have been previously described in different bodily fluids, such as: whole blood, serum, plasma, urine, ejaculate in patients with PCa (5). The most common epigenetic alteration described in prostate carcinogenesis is hypermethylation in the promoter region of the glutathione S-transferase P1 (GSTP1) gene, localized on chromosome 11q.3. Hypermethylation of GSTP1 has been reported in 90% of prostate cancer lesions, 70% of the prostatic intraepithelial neoplasia (PIN) lesions, in about 7% of the proliferative inflammatory atrophy (PIA) lesions, but occurs rarely in BPH lesions (6, 7). Most investigations of the epigenetic changes which occur in PCa have focused on prostate tissue and blood, while only a limited number of studies have investigated the GSTP1 methylation in voided urine samples (8, 9). The aim of our study was to determine whether detection of aberrant promoter methylation of GSTP1 from urine specimens can distinguish between men with localized PCa and those with benign conditions by non-invasive methods.

## Materials and Methods

### *Selection of the patients*

A total of sixty-five patients with clinically localized prostate adenocarcinoma, primarily treated with radical prostatectomy at the Urology Clinic, Timisoara, Romania, were selected for our study. The cases were identified by an increased serum prostate specific antigen (PSA) in routine analysis, and confirmed by sextant prostate biopsy. All the biopsies were performed transrectally under ultrasound guidance. In addition, 45 men with BPH, confirmed by serum PSA levels in the range of 4.0 ng/mL to 10.0 ng/mL and a negative biopsy result, and submitted to transurethral resection of the prostate (TURP), were included in the study as control subjects. We have included as control subjects patients with BPH, because previous studies have demonstrated that GSTP1 promoter hypermethylation is the most common somatic genome alteration during PCa development, being absent in healthy prostatic tissue, but present in benign or malignant prostatic tissue. All histological slides were staged and graded according to the TNM staging system (10) and the Gleason grading system (11).

The eligibility criteria for the PCa patients' selection were as follows:

1. clinical tumor stage I or II
2. no clinical evidence of lymph node or distant metastases
3. no treatment with hormone or radiation therapy before urine sample collection.

For the BPH patients the eligibility criteria for study inclusion were as follows:

1. serum PSA level between 4.0 ng/mL and 10.0 ng/mL
2. negative prostate biopsy result.

### *Collection of the samples*

Urine samples (20–30 mL) were collected immediately following a 15-second digital rectal examination (DRE) performed by an urologist. Urine samples were held at temperatures between 2° to 8 °C and processed within 4 hours. Voided urine specimens were centrifuged for 10 minutes at 1000× gravity to isolate cellular material and sediment. Serum prostate specific antigen (PSA) levels were measured by the enzyme-linked immunosorbent assay (ELISA) technique.

## Methods

### a) Genomic DNA extraction

Genomic DNA was extracted from the urine pellet using the ZR Urine DNA Isolation Kit™ (Zymo Research, CA, USA). Extracted DNA was measured using a Nano Drop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The average DNA concentration was approximately 100 ng/μL (range 65 to 150) with an average volume of 70 μL.

### b) Bisulfite modification and methylation-specific polymerase chain reaction (MSP)

Genomic DNA was subjected to sodium bisulfite modification using the EZ-Methylation Kit (Zymo Research Corp., USA) following the instructions suggested by the manufacturer, and stored at -80 °C until analysis. PCR was performed with 5 μL of bisulfite modified DNA template in a 50 μL reaction mixture containing: 25 μL Taq Polymerase mix (Fermentas, Lithuania), 2.5 μL of each primer (Eurogentec®, Seraing, Belgium), and 10 μL distilled water.

PCR conditions were: initial denaturation at 95 °C for 5 minutes, followed by 35 to 40 cycles of denaturation at 94 °C for 30 seconds, annealing at the corresponding temperature for 30 seconds, extension at 72 °C for 1 minute, and a final full extension at 75 °C for 5 minutes.

The primers used for amplification were:

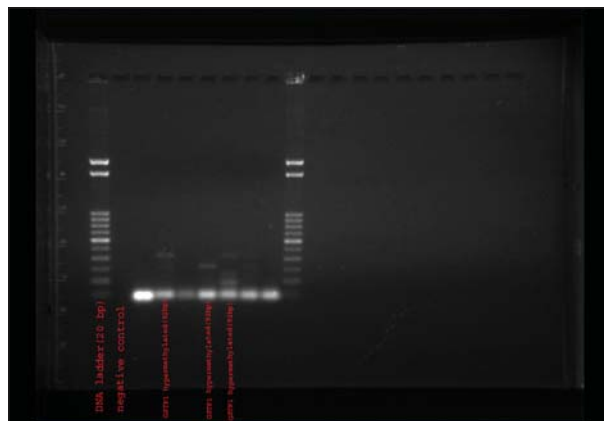
Forward primer: 5'-TTCGGGGTGTAGCGGTCGTC-3' (methylated);

5'-GATGTTTGGGGTGTAGTGGTTGTT-3' (unmethylated)

Reverse primer: 5'-GCCCAATACTAAATCACGACG-3' (methylated)

5'-CCACCCAATACTAAATCACAACA-3' (unmethylated).

The reaction was set up on a Master cycler Gradient thermal cycler (Eppendorf, Hamburg, Germany). Leukocytes' DNA collected from healthy individuals was used as negative control. The MSP products were loaded on 2% agarose gel (Lonza, Basel, Switzerland) and prestained with ethidium bromide, separated electrophoretically and visualized under an ultraviolet (U.V) transilluminator (Vilbert Lourmat®, France). Hypermethylation of the GSTP1 gene was defined as the presence of positive methylation bands, presenting signals equivalent or greater than the size marker (20 bp DNA Ladder, Fermentas, Lithuania), as presented in *Figure 1*. All urine samples were processed and analyzed in a blinded manner. Methylation status was then correlated with biopsy results and clinical information.



**Figure 1** Analysis of glutathione S-transferase P1(GSTP1) gene on 2% agarose gel.

## Ethics

The study was conducted in accordance with the World Medical Association Declaration of Helsinki statements from 2008, and written informed consent was obtained from each patient. The Institutional Ethics Committee of the University of Medicine and Pharmacy »Victor Babes« Timisoara, Romania, approved the study design.

## Statistical analysis

We compared the GSTP1 methylation level and its association with clinico-pathological characteristics in BPH and PCa patients. The optimal sensitivity and specificity with DNA methylation of GSTP1 for discriminating between PCa and BPH were determined by receiver operating characteristic (ROC) analysis. Pearson's correlation was used to evaluate the relation between GSTP1 methylation level and clinico-pathological parameters. Statistical analysis was performed by using SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA), and a p-value <0.05 was considered statistically significant.

## Results

The baseline characteristics of the patients included in this study are presented in *Table 1*.

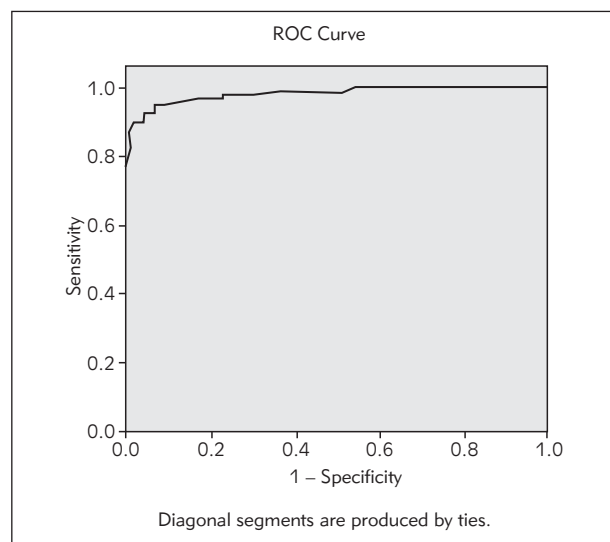
**Correlation between preoperative serum PSA levels and GSTP1 gene.** We observed that serum PSA level was significantly increased in PCa patients in relation to BPH patients (Mann-Whitney test;  $p < 0.001$ ). According to the Spearman rank-correlation test, there was a significant correlation between serum PSA levels and GSTP1 hypermethylation in PCa patients (Spearman coefficient = 0.831;  $p < 0.001$ ).

### GSTP1 methylation in prostate samples

Hypermethylation of GSTP1 was significantly increased in PCa patients compared to BPH (Chi

**Table 1** Clinicopathological characteristics of prostate cancer and control patients.

|                           | Cancer patients<br>(%), n = 65 | Control patients<br>(%), n = 45 |
|---------------------------|--------------------------------|---------------------------------|
| Age at diagnosis<br>(y)   |                                |                                 |
| Median                    | 63                             | 60                              |
| Range                     | 51–82                          | 57–79                           |
| Preoperative<br>serum PSA |                                |                                 |
| Median (ng/mL)            | 7.28                           | 4.9                             |
| Range                     | 3.5–26.3                       | 1.1–10.2                        |
| ≤ 4 ng/mL                 | 5 (7.7)                        | 9 (20)                          |
| 4–8 ng/mL                 | 27 (41.6)                      | 22 (48.9)                       |
| 8.1–10 ng/mL              | 25 (38.4)                      | 14 (31.1)                       |
| > 10 ng/mL                | 8 (12.3)                       |                                 |
| Pathologic stage          |                                |                                 |
| pT2a and b                | 26 (40.0)                      | –                               |
| pT2c                      | 29 (44.6)                      | –                               |
| pT3a and b                | 10 (15.4)                      | –                               |
| Gleason score             |                                |                                 |
| 6                         | 25 (38.5)                      | –                               |
| 7                         | 35 (54.0)                      | –                               |
| 8–9                       | 5 (7.5)                        | –                               |

**Figure 2** (A) Receiver operating curve (ROC) analysis of GSTP1 gene hypermethylation levels in prostate cancer (PCa) patients.

square test;  $p < 0.001$ ). GSTP1 was methylated in 63 of 65 (97.8%) and 5 of 45 (11.1%) of the PCa and BPH patients, respectively. The sensitivity and specificity of methylation in discriminating malignant from benign lesions were determined by the receiver operating curve (ROC) analysis (12). GSTP1 presented a sensitivity of 97% and a specificity of 89%, respective-

ly. The area under the curve (AUC) of the GSTP1 methylation level reached 0.936 (95% confidence interval [CI], 0.895 to 0.977).

In our study, hypermethylation of GSTP1 was detected by MSP in 5 patients diagnosed with BPH. These patients were followed-up at 3 and 6 month intervals. Their serum PSA levels were monitored, and all of them underwent a second prostate biopsy. They were diagnosed with adenocarcinoma of the prostate on the second biopsy.

#### *Pathologic tumor stage correlates with GSTP1 methylation.*

Methylation of GSTP1 was found significantly increased in voided urine DNA from PCa patients with pathologic T<sub>3</sub> tumor stage compared with T<sub>2</sub> ( $p < 0.001$ ; Mann-Whitney test).

## Discussion

In the present study, we investigated the usefulness of GSTP1 methylation in voided urine samples by MSP, as a noninvasive biomarker in distinguishing between malignant and nonmalignant prostatic lesions. It is known that GSTP1 is involved in the detoxification, metabolism, and elimination of genotoxic compounds, thus being involved in cell protection from DNA damage (13). However, some studies have demonstrated that suppression of GSTP1 activity might result in an increased susceptibility to DNA damage and an increased cancer incidence (14). Unlike tissue biopsy or imagistic test, cancer detection from urine samples is a minimally invasive method which does not present the risk of morbidity, and can be repeated to monitor the changes which occur during the disease progression and to detect disease recurrence (15).

Previous studies have demonstrated that hypermethylation of GSTP1 has been detected in more than 90% of prostate tumors, whereas no hypermethylation has been observed in BPH and normal prostate tissues (16). In one of their studies, Goessl et al. (17) determined hypermethylation of GSTP1 in urinary sediments of PCa men after 1 minute of prostate massage and found PCa in 68% of patients with early confined disease, 78% of patients with locally advanced disease, 30% of patients with PIN and in 2% patients with BPH, obtaining a specificity of 98% and a sensitivity of 73%. Also, Woodson et al. (18) using the MSP method investigated the methylation of GSTP1 in voided urine specimens from 100 males with PCa, after prostatic massage. GSTP1 had a sensitivity of 75% and 98% sensitivity, respectively. Their research group found a higher frequency of GSTP1 methylation in urine specimens from men with pathologic stage III vs. II (100% vs. 20%;  $p = 0.05$ ) (18).

Our results demonstrated that GSTP1 methylation was significantly higher in PCa patients than in

BPH patients, confirming the results obtained by Woodson et al. (18). Moreover, hypermethylation of GSTP1 was associated with an increased incidence of PCa, and was positively related to increases in serum PSA levels and in pathologic tumor stage T3. In our opinion, the presence of malignant lesions at the second biopsy is linked to the disease evolution during the process of carcinogenesis (19).

## Conclusion

Our study suggests that detection of GSTP1 methylation in voided urine specimens may complement serum PSA testing. Thus, the serum PSA test might be used to screen for potential patients at risk of harboring PCa and the determination of GSTP1 methy-

lation shall be provided to those found with increased serum PSA levels. Only patients found with methylated GSTP1 will undergo further prostate biopsy.

Our approach may represent a complement to serum PSA in the noninvasive diagnosis of PCa. These advantages would mean fewer unnecessary prostate biopsies, noninvasive detection of early PCa, accurate distinguishing between PCa men and those with BPH, and monitoring for disease recurrence and therapeutic response.

## Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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Received: October 7, 2012

Accepted: December 1, 2012