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PROSTATE CANCER IN SOUTHWEST PAKISTAN: MOLECULAR DETECTION BY METHYLATION-SPECIFIC PCR FROM URINE SPECIMENS

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Abstract

Introduction: 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 non-invasive methods.

Methodology: Voided urine specimens were collected from 78 patients with PCa and 57 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 hyper methylation of the GSTP1 gene in voided urine samples was found in 71 of 78 (91%) males with PCa and in 7 of 57 (12%) males with BPH. The sensitivity and specificity of GSTP1 in discriminating between PCa and BPH males were 97% and 88%, respectively.

Conclusion: Gene analysis of GSTP1 using conventional MSP in urine specimens can be used as a non-invasive 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

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 non-malignant 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 hyper methylation 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 hyper methylation in the promoter region of the glutathione S-transferase P1 (GSTP1) gene, localized on chromosome 11q.3. Hyper methylation 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 in South-western region of Pakistan.

Methodology

The cross-sectional study was conducted at Bolan Medical University of Health Sciences in Quetta, Khuzdar, Loralai, and Turbat in Balochistan province, Pakistan. It focused on male inpatient and outpatient adults from September 2019 to August 2021.A total of one hundred and thirty-five patients with clinically localized prostate adenocarcinoma, primarily treated with radical prostatectomy at the Urology units of Bolan Medical University of Health Sciences Quetta, Pakistan. 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 trans-rectally under ultrasound guidance. In addition, 57 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 subject's patients with BPH, because previous studies have demonstrated that GSTP1 promoter hyper methylation 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).

Table 1: The eligibility criteria for selection of PCa and BPH patients.

Criteria for the PCa patients	Criteria for the BPH patients
Clinical tumor stage I or II	Serum PSA level between 4.0 ng/mL and 10.0 ng/mL
No clinical evidence of lymph node or distant metastases	Negative prostate biopsy result.
No treatment with hormone or radiation therapy before urine sample collection	

Collection of the samples

Urine samples (20–30 mL) were collected immediately following a 15-second digital rectal examination (DRE) performed by a 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.

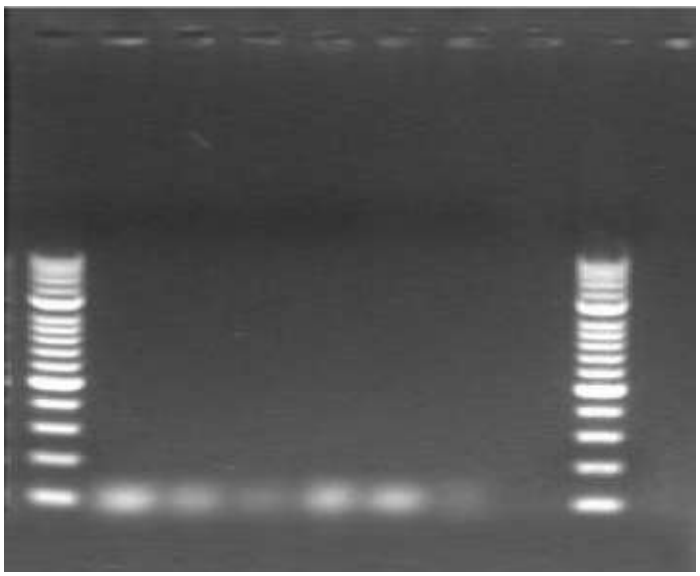

Figure 1 Analysis of glutathione S-transferase P1(GSTP1) gene on 2% agarose gel.

a) Genomic DNA extraction

The micGeno DNA was extracted from the urine pellet using the ZR Urine DNA Isolation KitTM (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/mL (range 65 to 150) with an average volume of 70 mL.

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

Genomic DNA was subjected to sodiumbisulfite 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 mL of bisulfite modified DNA template in a 50 mL reaction mixture containing: 25 mL Taq Polymerase mix (Fermentas, Lithuania), 2.5 mL of each primer (Euro gentec®, Seraing, Belgium), and 10 mL distilled water.

PCR conditions:

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.

Table 2: The details of primers used for amplification.

Primers for amplification			
1	Forward primer	5’-TTCGGGGTGTAGCGGTCG TC-3’	Methylated;
		5’-GATGTTTGGGGTGTAGTGGTTGTT-3’	Unmethylated
2	Reverse primer	5’- GCCCCAATACTAAATCACGACG-3’	Methylated
		5’-CCACCCCAATACTAAATCACAACA-3’	Unmethylated

The reaction was set up on a Master cycler Gra dient thermal cycler (Eppendorf, Hamburg, Germany). Leukocytes’ DNA collected from healthy indivi duals 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 methyl ation bands, presenting signals equivalent or greater than the size marker (20 bp DNA Ladder, Fer mentas, Lithuania), as presented in Figure 1. All urine samples were processed and analyzed in a blinded manner. Methylation status was then correla ted with biopsy results and clinical information.

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 Balochistan Quetta approved the study design.

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. 23.0 (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 3. Correlation between preoperative serum PSA levels and GSTP1 gene (Table 4). 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 hyper methylation in PCa patients (Spearman coefficient =0.827; p<0.001).

Table 3: Characteristics of Prostate cancer and BPH patients at time of diagnosis.

Patients Age (Years) at diagnosis			
No	Total samples=135	Median	Range
1	Ca prostate percentage % (n= 78)	65	17 (51-68)
2	BPH patients % (n=57)	67	20 (51-71)

Table 4: Details of preoperative serum PSA levels.

Preoperative Serum PSA							
No	Total samples=135	Median (ng/mL)	Range				
			Range	≤ 4 ng/mL	4–8 ng/mL	8.1–10 ng/mL	> 10 ng/mL
1	Ca Prostate Percentage % (n= 78)	7.28	3.5-26.3	6 (7.6)	32 (41.1)	30 (38.5)	10 (12.8)
2	BPH Percentage % (n=57)	4.9	1.1-10.2	12 (20.6)	27 (47.9)	18 (31.5)	

GSTP1 methylation in prostate samples Hyper-methylation of GSTP1 was significantly increased in PCa patients compared to BPH (Chi square test; p<0.001). GSTP1 was methylated in 71 of 78 (91.3%) and 7 of 57 (12.2%) 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 88%, respectively.

The area under the curve (AUC) of the GSTP1 methylation level reached 0.928 (95% confidence interval [CI], 0.891 to 0.981). In our study, hyper methylation of GSTP1 was detected by MSP in 7 patients diagnosed with BPH. These patients were followed-up at 3 and 6 month intervals. Their serum PSA levels were monitored, and they 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 T3 tumor stage compared with T2 ($p<0.001$; Mann-Whitney test) presented in Table 5.

Table 5: Clinico-pathological characteristics of study samples.

Clinico-pathological characteristics of study samples							
No	Total samples=135	Pathologic Stage			Gleason score		
		pT2a & b	pT2c	pT3a & b	6	7	8-9
1	Ca Prostate Percentage % (n= 78)	31 (40.0)	35 (44.8)	12 (15.2)	30 (38.5)	42 (54.0)	6 (7.5)

Discussion

In the present study, we investigated the usefulness of GSTP1 methylation in voided urine samples by MSP, as a non-invasive biomarker in distinguishing between malignant and non-malignant 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 hyper methylation of GSTP1 has been detected in more than 90% of prostate tumors, whereas no hyper methylation has been observed in BPH and normal prostate tissues (16). In one of their studies, Goessl et al. (17) determined hyper methylation 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, hyper-methylation 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 harbouring PCa and the determination of GSTP1 methylation 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 non-invasive diagnosis of PCa. These advantages would mean fewer unnecessary prostate biopsies, non-invasive detection of early PCa, accurate distinguishing between PCa men and those with BPH, and monitoring for disease recurrence and therapeutic response.

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