MINIMALLY INVASIVE PROSTATE CANCER DIAGNOSIS BY GLUTHATIONE S-TRANSFERASE P<sub>1</sub> (GSTP<sub>1</sub>) GENE METHYLATION ANALYSIS IN SERUM SPECIMENS

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ABSTRACT

Introduction: Prostate cancer (PCa) represents the most commonly diagnosed type of malignancy among men in Western countries, and the second cause of cancer-related deaths among men worldwide. Alterations in the methylation patterns of promoter CpG islands have been associated with the transcriptional inhibition of genes in many human cancers, including PCa. Objective: The aim of our study was to analyse the diagnostic value of aberrant promoter hypermethylation of gene glutathione S-transferase P<sub>1</sub> (GSTP<sub>1</sub>) in serum DNA to discriminate between prostate cancer (PCa) and benign prostatic hyperplasia (BPH) patients by minimally invasive methods. Material and methods: Aberrant promoter hypermethylation was investigated in DNA isolated from serum samples of 91 patients with diagnostic of PCa and 94 with BPH (control subjects). Extracted genomic DNA was bisulfite treated and analyzed using methylation-specific polymerase chain reaction (MS-PCR). Results: Promoter hypermethylation of GSTP<sub>1</sub> gene was detected in serum samples from 89 of 91 (92.86%) patients with PCa and in 10 (10.6%) of the 94 patients. Receiver operating curve (ROC) included clinico-pathological parameters such as: serum PSA levels, pathological stage, Gleason score, hypermethylation status of GSTP<sub>1</sub> gene, and gave a predictive accuracy of 96% with a sensitivity and specificity of 98% and 89%, respectively. Conclusions: In our study we have evaluated the ability of GSTP<sub>1</sub> gene to discriminate between PCa and BPH patients in serum samples by minimally invasive methods.

Key Words: prostate cancer, benign prostatic hyperplasia (BPH), glutathione S-transferase P<sub>1</sub>, methylation-specific polymerase chain reaction

INTRODUCTION

Prostate cancer (PCa) represents the most prevalent malignancy among men and the second cause of cancer related deaths worldwide. If diagnosed in its early stages, when the tumor is confined to the prostatic capsule, PCa can be treated. Diagnosis and clinical management of PCa are often confounded because of the lack of symptoms and the absence of minimally invasive diagnostic techniques that could be used to detect the early stages of the disease. The only molecular biomarker used for early detection and recurrence monitoring after radical prostatectomy is prostate-specific antigen (PSA). Measurement of the serum PSA levels alone is neither sensitive, nor specific for a definite diagnosis of prostate adenocarcinoma.
It has been demonstrated that most of the patients who had either an abnormal finding on digital rectal examination (DRE) or elevated serum PSA levels, require transrectal biopsies with ultrasound guidance (TRUS). Serum PSA levels are increased in benign prostatic hyperplasia (BPH), prostatitis, or prostatic ischemia. Approxiately one third from these men are found to have a negative biopsy result. Using these approaches, about 25% of men with false-negative prostate biopsy, are prone to develop PCa.

It is known that the process of carcinogenesis develops in time, because of multiple molecular events, which include changes in gene expression, through epigenetic mechanisms. The epigenetic alterations have been detected first in genomic DNA isolated from tissue samples from different types of tumors, especially DNA hypermethylation in the promoter region of tumor suppressor genes. Cytosine-phosphate guanine islands (CpG) are susceptible to hypermethylation in unknown growth conditions, and may develop some pathways leading to the development of certain types of tumors. Using molecular biotechnology, some types of cancers have been detected from several bodily fluids, including urine in urological cancers, saliva in head and neck cancer, sputum and broncho-alveolar lavage in lung cancer. Unlike RNA and proteins alterations, DNA methylation biomarkers are stable in bodily fluids and occur in definite regions, unlike DNA mutations. Therefore, DNA methylation biomarkers might be used as noninvasive biomarkers in early detection of cancer and in monitoring the disease outcome. The earliest and commonest alteration which occurs during prostate carcinogenesis is represented by the hypermethylation of the glutathione S-transferase \( \text{P}_1 \) (GSTP\(_1 \)) gene. GSTP\(_1 \) hypermethylation has been detected by methylation-specific polymerase chain reaction (MS-PCR) method, in various bodily fluids such as urine, ejaculates or blood serum or plasma.

GSTP\(_1 \) hypermethylation has been reported to be present in up to 100% in PCa tissue, in up to 2/3 of high grade prostatic intraepithelial neoplasia (HGPIN), rarely in benign prostatic hyperplasia (BPH) and is absent in normal prostatic tissues.

**AIM OF THE STUDY**

The main objective of our study was to determine the clinical utility of this new biomarker in serum samples to distinguish between PCa patients and BPH patients by minimally invasive methods.

**MATERIALS AND METHODS**

**Patients and samples collection**

In our study we included 91 cases with PCa histological confirmed and 94 BPH cases (cancer-free controls), hospitalized between January 2008 to February 2010 in the Department of Urology, Clinical County Emergency Hospital Timisoara. The study was conducted in accordance with The World Medical Association Declaration of Helsinki from 2008 statements and written informed consent was obtained from each patient.

The eligibility criteria for the PCa patients’ selection were:
1. Clinical tumor stage I or II;
2. No clinical evidence of lymph nodes or distant metastases;
3. No treatment with hormone or radiation therapy before blood samples collection

The average patient age ± SD in the BPH group was 61 ± 8 years and 63 ± 6 years in the PCa group. At the time of enrollment, the 91 patients presented tumors that were clinical stage I (n=54 [59%]) and stage II (n=37 [41%]). After the pathologic examination of radical prostatectomy specimens, enrolled patients were grouped according to tumor stage, as follows: \( pT_2, n=47 \) (52%), and \( pT_3, n=44(48%) \). Patients were grouped according to Gleason score (GS) intervals as follows: 3 through 4, \( n=17 \) (19%); 5 through 6, \( n=39 \) (43%); 7 through 10, \( n=35 \) (38%). Preoperative serum PSA levels were in range of 4.0 to 34 ng/ml.

**1. Blood collection and DNA isolation**

Five milliliters of blood were drawn and collected in a serum separator tube containing clot activator and gel (Vacutainer, Becton Dickinson, USA). Tubes were inverted 8 times and centrifuged within 2 hours of collection for 10 minutes at 1500 X g. Using ZR Serum DNA (Zymo Research, U.S.A) we extracted DNA from 1 ml serum following the manufacturer’s protocol, and stored it at – 80 °C until further analysis.

**2. Bisulfite treatment and methylation-specific PCR (MS-PCR) analysis**

Using EZ DNA Methylation Kit® (Zymo Research, Orange, CA) protocol, 2 μg of genomic DNA from each patient sample was treated with denaturation buffer, sodium bisulfite (converting unmethylated cytosine residues to uracil), and desulfonation buffer, with elution of the bisulfite-modified DNA into 10 μl of buffer. Two sets of primers were used to amplify each region of interest: one pair recognized a sequence in which CpG sites are unmethylated (bisulfite modification to UpG) and the other recognized a sequence in which CpG sites are methylated (modified
by bisulfite treatment). The reaction volume was of 50 μl containing: 10 μl of distilled water in which we added 25 μl Taq Polymerase mix, 2.5 μl from each forward and reverse primer (Eurogentec®, Belgium). To amplify the regulatory region of GSTP1 we used 2 μl of the bisulfite-modified DNA as a template for the MS-PCR reaction.

The PCR conditions were as follows: Hot start at 95°C for 5 minutes (to fully denaturate the bisulfite-modified genomic DNA), 35 amplification cycles (94°C for 30 seconds for denaturation, 58°C for 30 seconds for primer annealing and 72°C for 60 seconds for extension), and a final full extension at 75°C for 4 minutes.

The primers used for the amplification reaction were as follows:

Forward primers:
- 5’-TTTCGGGGTGTAGCGGTCGTC-3’ (methylated);
- 5’-GATGTTTTGGGGTGTAGTGGTTGTT-3’ (unmethylated);

Reverse primers:
- 5’-GCCCAATACTAAATCACGACG-3’ (methylated);
- 5’-CCACCCAATACTAAATCACAACA-3’ (unmethylated)

Sodium bisulfite-modified DNA from healthy donors’ lymphocytes served as unmethylated negative control. To detect the methylation levels of gene GSTP1 in patients with PCa and BPH we separated electrophoretically the MS-PCR products on a 2% Seakem agarose gel (Lonza, Switzerland) and visualized them under an ultraviolet (U.V) transluminator (Vilbert Lourmat®, France).

**Figure 1.** Serum molecular detection of GSTP1 hypermethylation by MS-PCR analysis.

**Statistical analysis**

Data were analyzed by SPSS statistical package version 12 (SPSS Inc, Chicago, USA), with χ² test, Spearman rank correlations and Mann-Whitney tests to comparison between groups and finding the correlations. A P-value < 0.05 was considered significant.

**RESULTS**

Correlations between the clinicopathological features and GSTP1 hypermethylation:

1. **Hypermethylation status of GSTP₁ gene in serum samples**

   We observed that PCa patients undergoing disease progression had significantly increased methylation levels of GSTP₁ gene when compared with BPH patients (Chi square test, p<0.001). In PCa patients, 89 (97.8%) of 91 presented hypermethylated levels of GSTP₁ gene, whereas 10 (10.6%) of the 94 BPH patients had hypermethylated GSTP1 gene.

   The sensitivity and specificity of GSTP₁ hypermethylation levels in discriminating PCa patients from BPH were determined by receiver operating curve (ROC) analysis. GSTP₁ gene had a sensitivity of 98%, a specificity of 89% and yielded an area under the curve (AUC) of 0.936 (95% CI; 0.895 to 0.977; p<0.001), as presented in Figure 2.

![ROC Curve](image)

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

2. **Hypermethylated levels of GSTP₁ gene correlates with serum PSA levels**

   According to Spearman rank-correlation, a significantly correlation has been found between serum PSA levels and GSTP1 hypermethylation in PCa patients (r=0.831; p<0.001).

3. **Correlation of pathologic tumor stage with GSTP₁ hypermethylation levels**

   In PCa patients with pT₁ stage we observed significantly increased methylation levels of gene GSTP1, when compared with pT₂ stage (p<0.001; Mann-Whitney test).

4. **Correlation of Gleason score (GS) with pathologic tumor stage**

   According to the Spearman rank-correlation test, a significant correlation between GS and the pathological stage exists (r=0.749; p<0.001). Increased GS score were significantly associated with pT₁ stage.
DISCUSSION

The evaluation of serum samples obtained from PCa patients, presents some advantages because, unlike tissue biopsy or imagistics, blood sampling is a minimally invasive method which does not present the risk of morbidity, and can be repeated to monitor the changes which occur during disease progression or to detect the recurrence of the disease.10,11

The main goal of our study was to determine whether serum detection of GSTP1 methylation levels can discriminate PCA men from those with BPH by minimally invasive methods, and the second aim of the study was to investigate possible correlations between GSTP1 methylation levels and different clinico-pathological parameters.

Sensitivity of 98% and specificity of 89% in distinguishing malignant cells, were determined by receiver operator curve (ROC) and the discriminatory power of the test was given by the area under the curve (AUC) which was 0.936 (95% CI:0.895 to 0.977; p<0.001).12 The results obtained by us, can be considered good for a minimally invasive serum diagnostic test. The different levels of GSTP1, hypermethylation between neoplastic and non-neoplastic prostatic tissue, suggests that measurement of GSTP1 hypermethylation levels could be more useful in distinguishing men at low risk for PCA from those with a clinically silent PCA, in comparison with the measurement of serum PSA levels.13

De Marzo et al. reported in their studies that methylation levels of GSTP1 gene are present in men with proliferative inflammatory atrophy (PIA) and high-grade prostatic intraepithelial neoplasia (HGPIN), indicating that GSTP1 methylation occurs at the beginning of prostate carcinogenesis.14 Detection of serum GSTP1 hypermethylation in patients with negative biopsy should be sufficient evidence to warrant the concern of the presence of an occult disease. The presence of methylation levels of GSTP1 gene in 10 (10.6%) men with previously BPH, indicates that they could harbor an occult microscopic foci of PCA in the context of BPH, which was omitted by prostatic biopsy. During the study, repeat biopsies have been performed to the 10 patients with BPH found with hypermethylated GSTP1 levels in serum samples. All of them (100%) have been found to have cancer on repeat biopsy. Abnormal GSTP1 methylation levels found in serum samples may help in identification of men who are at risk for harboring prostate cancer despite negative prostate biopsy.

Henrique et al. found in serum samples from PCA patients GSTP1 hypermethylation levels ranging between 15% and 70%.15 In our study we found the hypermethylation levels up to 90%. These results could be explained by the use of quantitative MS-PCR rather than a conventional MS-PCR method. MS-PCR seems to be more sensitive in serum and currently, no evidence showed that qMS-PCR could be more specific. Also, Maruyama et al. reported in one study the correlations between hypermethylation levels of GSTP1 gene from serum samples in PCA patients and Gleason score, serum PSA levels and pathologic stage.16

Patients with elevated serum PSA levels can have either PCA or BPH, and repeat biopsies can provide up to 20% detection rate after an initial negative biopsy. Because only 30-40% of patients with serum PSA levels between 4 and 10 ng/ml have PCA, there are a lot of patients with negative prostate biopsies who would benefit from improved ability to discriminate between cancer and benign lesions, by noninvasive diagnosis methods, as described in our study.17

The limitations of our study include the small number of patients and the lack of long-term follow-up.

Further studies in the area of noninvasive detection of PCA include: the detection of PCA in its early stages from voided urine samples using a panel of tumoral biomarkers by qMS-PCR method, and the detection of disease recurrence in men following radical prostatectomy from preoperative serum samples.

CONCLUSIONS

In our study we have demonstrated the feasibility of a novel clinical strategy, which is based on minimally invasive molecular test that can be used to aid to current investigation methods for prostate cancer detection.

The efficacy of this minimally invasive test for early molecular detection of PCA is important in developing future clinical management algorithms and in establishing indications regarding the surveillance or repeat biopsy.

REFERENCES