

## MOLECULAR DETECTION OF PROSTATE CANCER USING A PANEL OF DNA METHYLATION BIOMARKERS

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### Abstract

**Introduction:** The diagnostic of prostate cancer (PCa) using serum-based prostate specific antigen (PSA) has some limitations due to false-positive and negative results. The purpose of our study was to analyse the hypermethylation of three genes from plasma samples and to determine the feasibility of these genes to aid as biomarkers in detecting PCa in plasma by noninvasive methods.

**Materials and Methods:** Genomic DNA was extracted from the peripheral blood plasma of 74 patients with localized PCa. All the samples were examined for aberrant hypermethylation in retinoic acid receptor  $\beta$  variant 2 (RAR $\beta$ 2), glutathione S-transferase P1 (GSTP1) and Ras association domain family 1 isoform A (RASSF1A) genes, using methylation-specific PCR (MSP), and the results were correlated with the clinicopathological parameters.

**Results:** The percent of methylation of the analyzed genes was as follows: RAR $\beta$ 2 was found methylated in 54 cases (73 %), GSTP1 in 58 cases (78.4%), and RASSF1A was found methylated in all 74 cases (100%).

**Conclusion:** Our study demonstrates that, using a panel of DNA methylated biomarkers aids the identification of PCa patients and with minimally invasive techniques, may yield information independent of serum PSA or the TNM stage.

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**Keywords:** Prostate cancer (PCa), methylation-specific polymerase chain reaction (MS-PCR), glutathione S-transferase P1 (GSTP1), Ras association domain family 1 isoform A (RASSF1A), retinoic acid receptor  $\beta$ 2 (RAR $\beta$ 2)

## Introduction

Prostate cancer (PCa) represents one of the most common malignancies among men worldwide, with an estimated 193.000 new cases and 27.500 PCa related deaths in the United States in 2009 (Jemal et al,2009). It is one of the most complicated human tumors and, like many other malignancies, arises from progressive genetic and epigenetic alterations. Since the introduction of the serum prostate-specific antigen (PSA) into clinical use, there has been a significant increase in the diagnosis of men at an early stages of the disease. Treatment decisions in patients with advanced tumors are based on older and known prognostic factors (Gleason score, pathological stage, serum PSA variables). Epigenetic changes represent changes in gene expression which are not caused by alterations in the primary sequence of the nucleotides that compose the gene. Epigenetic mechanisms such as DNA methylation and histone modification play an essential role in many molecular and cellular alterations associated with the development and progression of PCa (Li LC, 2005). Epigenetic events have been demonstrated to be an early event in cancer development, and thus can be used in assessing the risk of cancer developing (Alumkal JJ 2008). One possible approach to the detection of PCa is through analysis of the circulating DNA from cancer cells. In seeking more specific biomarkers, several studies have shown that DNA abnormalities may be detected in the plasma and the serum of cancer patients are those of the gene promoter hypermethylation. The presence of gene promoter hypermethylation in the serum and plasma DNA has also been demonstrated in patients with different types of cancers, also including prostate cancer (Schwarzenbach H et al, 2009).

The aim of our study was to evaluate the methylation status of the promoter regions of 3 cancer-related genes which are involved in DNA repair (GSTP1), cell cycle regulation (RASSF1A) and the mediator of the antiproliferative effect of retinoid (RARbeta2), in the prostate, according to the degree of malignancy .

## Materials and methods

The study was conducted with the participation of 74 men which had a prostate biopsy to establish their diagnosis by an urologist. They were hospitalized at the Urology Department of Clinical County Emergency Hospital, Timisoara, Romania. A transrectal ultrasound-guided prostate biopsy was made with a spin and in sextant. The exam was made with local anaesthesia on the recumbent patient. The clinicopathological characteristics of the patients are presented in **Table 1**. The identity of each patient was confidential and through the anatomopathological exam, the stage of cancer development was diagnosed and could be correlated. The study was conducted according to the Helsinki Statement, 2008.

<b>Table 1. Clinicopathological characteristics</b>	
	<b>n</b>
<b>Number of patients</b>	<b>74</b>
<b>TNM stage</b>	
<b>I/II</b>	<b>47</b>
<b>III</b>	<b>23</b>
<b>IV</b>	<b>4</b>
<b>Gleason score</b>	
<b>5</b>	<b>18</b>
<b>6</b>	<b>13</b>
<b>7</b>	<b>13</b>
<b>8</b>	<b>5</b>
<b>9</b>	<b>14</b>
<b>10</b>	<b>1</b>
<b>PSA, ng/ml</b>	
<b>≤4</b>	<b>29</b>
<b>&gt;4</b>	<b>45</b>
<b>Mean</b>	<b>25.4</b>
<b>Mean age, years</b>	<b>73.4</b>

#### Sample collection and genomic DNA isolation

Blood samples were collected from all the patients included in the study. To obtain the plasma for further DNA isolation, we centrifugated the blood samples for 10 minutes at 3500 X g. Genomic DNA was extracted from the plasma using the QIAmp® DNA Mini Kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). Each DNA sample was quantified using Nanodrop 8000 (LabTech®), measuring the optical density ratio at 260/280 nm.

#### Bisulfite treatment and MS-PCR analysis

We used 2 sets of primers, methylated and unmethylated, to amplify each region of interest: one pair of primers was used to recognize the sequence in which the CpG sites were unmethylated, and the other pair of primers was used to recognize the sequence in which the CpG sites were methylated. For each set of PCR we used negative control samples, without DNA. The procedure takes advantage of the bisulfite-mediated chemical conversion of cytosine to uracil followed by PCR using primers designed to distinguish methylated DNA from unmethylated DNA. The sequences of the primers were designed by MethPrimer software . Each modification was checked using a verification method prescribed by the manufacturer. PCR was carried out in a total volume of 30µL per reaction containing 0.5µL of AmpliTaq (Applied Biosystems®), 3µL of gene probes , 0.5µL of dNTP , 3 µL of PCR buffer (10X) , 19.3 µL of distilled water and 4µL of each DNA sample. Each PCR reaction was subjected under the following conditions: denaturation at 95 C for 10 min, and 45 cycles of the following profile: 30 s at 94, 30 s at 55C, and 30 s at 72C. The PCR products were then analyzed by

electrophoresis on a 2.5 % agarose gel and stained with ethidium bromide and visualized by a UV transilluminator. A 100 bp DNA Ladder (Zymo Research, CA,USA) was used. DNA was considered methylated if a PCR product using unmethylated specific primers was absent. When the PCR product was present using both unmethylated-specific primers and methylated-specific primers, it meant that between 25% and 75% of the gene promoter could be considered methylated.

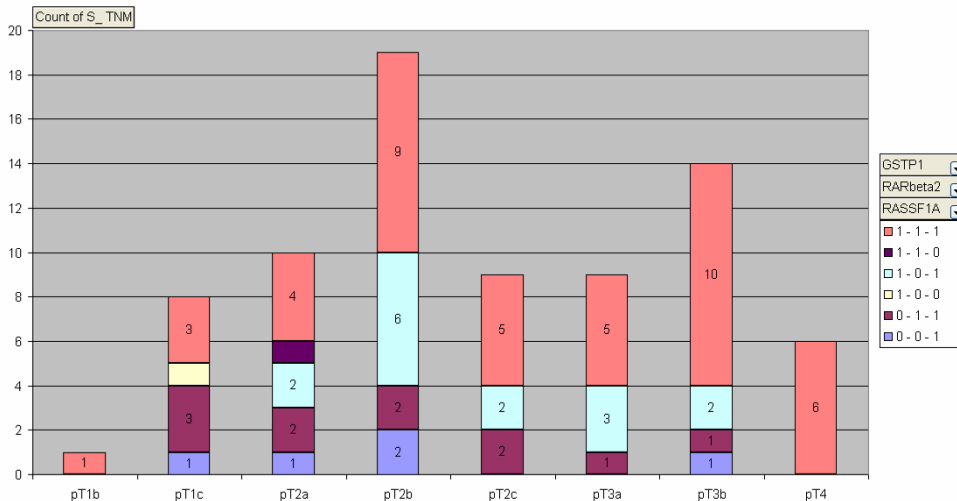
**Statistical Analysis**

To compare the clinical factors among the tumors demonstrating hypermethylation versus nonmethylation status in this study, we used ANOVA with the Dunnett method for the continuous variables and the  $\chi^2$  test for the categorical variables,a P value < 0.05 was considered significant.

**Results**

Frequency of hypermethylation of tumor-related genes in prostate samples:

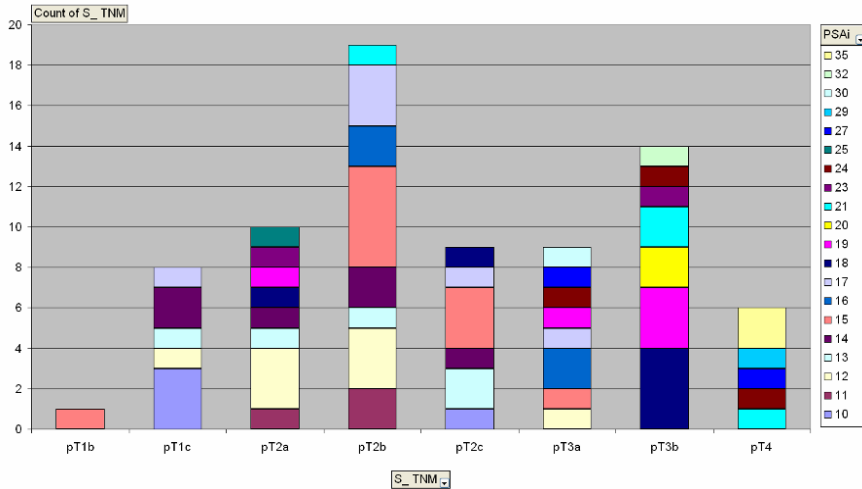
Methylation of the involved genes in PCa was as follows: RAR $\beta$ 2 in 54 cases (73 %), GSTP1 in 58 cases(78.4%), and RASSF1A was found methylated in all 74 cases (100%). In comparison, we found that the unmethylated genes in the serum samples were lower, for example:GSTP1 was unmethylated in only 16 cases (21.6%), RAR $\beta$ 2 in 20 cases (27%) and RASSF1A was not found unmethylated at all. Healthy males did not present these methylated circulating DNA biomarkers in their plasma samples under optimal assay conditions.



**Figure no.1.** Distribution of hypermethylated genes (RASSF1A,GSTP1,RAR $\beta$ 2)

**Correlation between the methylated DNA and serum prostate specific antigen (PSA):**

The mean serum PSA concentration in patients with hypermethylated tumor-related genes (GSTP1, RASSF1A, RARβ2) was significantly higher than in patients without hypermethylated genes (P=0.003, 0.021 and <0.005, respectively). Moreover, patients who had more than 1 tumor-related gene methylated presented serum PSA concentrations significantly higher than patients without circulating hypermethylated DNA (P=0.003).



**Figure no.2.** Correlation between hypermethylated genes and serum PSA values

is minimally invasive and can be repeated to monitor different changes which occur during the disease Correlation between Gleason score and circulating methylated DNA:

We correlated the Gleason score with the methylation status of the 3 genes. The mean PCa Gleason score in patients with hypermethylated GSTP1, RASSF1A and RARβ2 was significantly higher (P=0.019, 0.048 and 0.015, respectively) than in patients without hypermethylated DNA. Patients with ≥ 1 hypermethylated tumor-related gene presented a serum PSA concentration which was significantly greater than in patients without circulating DNA (P=0.003).

**Discussion**

Diagnosis of PCa by needle biopsy in small moderate-grade cancers can be difficult. Needle biopsies contain small samples of prostatic tissue and often only a few fragments of malignant gland( Maruyama et al, 2002). In this case, many patients are subjected to multiple biopsies in examinations before a correct diagnostic is made. The finding of tumor-derived DNA in the circulation of cancer patients has inspired further efforts to develop DNA based assays capable of detecting evidence of cancer using serum or plasma

samples (Altimari et al, 2008). Unlike tissue biopsy, blood sampling has a number of advantages because it progression and can also be used to detect recurrence. The only blood-based non-invasive screening method for PCa detection is serum PSA measurement which is of limited value because of the false positives or negatives results (Bock et al, 2004). In our study of circulating DNA from patients with the diagnosis of PCa, we examined the hypermethylation of the promoter regions of 3 tumor-related genes : RASSF1A,GSTP1 and RAR $\beta$ 2, which were reported to be hypermethylated in a percentage of 37%-99%,53%-75% and 55%-92% respectively in PCa samples(Enokida et al, 2005).It has been demonstrated in the past that, GSTP1 is found hypermethylated in about 30%-97% of PCa tissue (Ellinger et al, 2008), but not many reports are published of the hypermethylation of RASSF1A and RAR $\beta$ 2 in plasma samples. In our study, we found that RASSF1A, GSTP1 and RAR $\beta$ 2 were hypermethylated in 100% of RASSF1A (74 of 74), 78.4% of GSTP1(58 of 74) and 73% of RAR $\beta$ 2 (54 of 74). By combination, the 3 biomarkers identified 62% of patients with PCa. Other studies, used a panel of 9 biomarkers from urine sediments obtained from PCa patients, to detect the promoter region hypermethylation in urinary DNA (Hoque et al, 2005).

In our study, we found a significant correlation between serum PSA concentration and the methylation positive group (P=0.03; P=0.01 and P=0.025). We consider that these patients represent PCa cases which could be missed if the diagnosis were based only on serum PSA measurement in the absence of digital rectal examination (Hoque et al, 2009). We also noted that, the frequency of hypermethylation increased directly with the TNM stage of the disease. We observed that, RASSF1A and GSTP1 were also methylated in patients in stage IV of the disease, concluding that it is possible for these genes to be implicated in the processes of invasion and metastasis ( Phe et al,2010 and Gurban et al, 2012).

We have demonstrated here the potential of adding gene methylation tests to histologic examination for the precise diagnosis of PCa. Conventional methylation specific PCR assays of the key PCa genes should be incorporated into diagnostic trials to help the early detection of the disease. Validation of these studies could change the standard evaluation of tissue biopsies after PSA screening.

### **Conclusion**

Our study , tested the ability of a panel of three methylation biomarkers to aid the improve sensitivity of standard histology for PCa detection in needle biopsies. Conventional methylation specific PCR assays of the key PCa genes should be incorporated into diagnostic trials to help the early detection of the disease. Validation of these studies could change the standard evaluation of tissue biopsies after serum PSA screening.

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