



# ASSESSMENT OF GLUTATHIONE-S-TRANSFERASE (GSTP1) METHYLATION STATUS IS A RELIABLE MOLECULAR BIOMARKER FOR EARLY DIAGNOSIS OF PROSTATIC INTRAEPITHELIAL NEOPLASIA

E. VARRIALE<sup>1</sup>, A. FERRANTE<sup>1</sup>, F. CROCETTO<sup>2</sup>, M. R. CIANCIULLI<sup>3</sup>, E. PALERMO<sup>3</sup>, A. VITALE<sup>4</sup>, G. BENINCASA<sup>4</sup>

<sup>1</sup>UOS Oncologia, Ospedale Fatebenefratelli, Naples, Italy

<sup>2</sup>Department of Neuroscience and Reproductive Sciences and Dentistry, University "Federico II" Naples, Naples, Italy

<sup>3</sup>Department of Clinical Pathology Laboratory, A.O. "San Giuseppe Moscati", Avellino, Italy

<sup>4</sup>Pineta Grande Hospital Castel Volturno, Caserta, Italy

**Abstract – Objective:** Prostate Specific Antigen (PSA) is a commonly used marker for the diagnosis and follow-up of Prostate Cancer (PC) and Prostatic Intraepithelial Neoplasia (PIN). Furthermore, in order to ensure early detection of the patients at risk of PC and PIN, there is a growing need for new tools able to early identify this subject. Molecular analysis of neoplastic prostate tissues showed the inactivation of the Glutathione-S-Transferase gene (GSTP1) due to the hypermethylation. The aim of this study is the validation of the specific and sensitive detection of the methylation status of the GSTP1 gene for potential biomarker to assess early detection of PIN.

**Patients and Methods:** The methylation status of 5' promoter region of the GSTP1 gene was obtained by Methylation Sensitivity-PCR (MS-PCR). The test was optimized in terms of the specificity and sensitivity. The cost-efficacy of the test was tested on the DNA from 20 donors healthy subject, 57 benign prostatic hypertrophy (BPH), and 57 PC patients.

**Results:** GSTP1 promoter gene methylation was detected in 0% of healthy subjects (20/20, median age 32,7 years), in 43,9% of patients with BPH (25/57 mean age 60,5 years) and in 57,6% of patients with PC (34/57 mean age 67,8 years). Significantly, the 81,8% of patients with PC, age >65 years and total PSA ≤ 4 ng/ml were positive for the methylation status of GSTP1 gene.

**Conclusions:** In this way, specific evaluation of the methylation status of the GSTP1 gene may be a useful tool for the prediction of patients at risk of PC. In addition, the test is cost-effectiveness and could be used extensively for cancer prevention.

**KEYWORDS:** Methylation sensitivity, Analytical validations, Molecular diagnostics, GSTP1, Prostate cancer.

## INTRODUCTION

Improvements of the molecular diagnostic methods have been ameliorating the early diagnosis of prostate cancer<sup>1,2</sup>. Many studies have been investigating a genetic test for the screening, revelation and treating of the prostate cancer. The results of these researches have already been commercialized. These assays, so far, have not shown any clinical usefulness as far as the diagnosis, prognosis or the

risk evaluation are concerned. The National Comprehensive Cancer Network® (NCCN) and National Cancer Institute (NCI) don't impose any genetic or epigenetic test for the prediction and treating of the prostate cancer. Actually the prostate-specific antigen (PSA) has been used in combination with the digital rectal examination (DRE) for diagnosing and treating of prostate cancer. The association of a gene methylation marker, *GSTP1*, with prostate cancer has been investigated. Several studies of *GSTP1*



hypermethylation, using tissue samples, showed significant results for the prostate cancer identification with a sensitivity of 92%, specificity of 85% and an area underneath the AUC curve of 0,9<sup>3,4</sup>. The *PCA3* test, instead, has been used as a possible additional tool for the prostate cancer diagnosis. The *PCA3* gene (previously known as DD3) is extremely over-regulated in prostatic tumor cells and it is not expressed, or poorly expressed or it is hyper-plastic in prostatic tissue<sup>5</sup>. Many studies showed the results of the *PCA3* gene testing in men examined with a prostate biopsy having a specific prostatic antigen (PSA) exceeded 4ng/ml<sup>6</sup>. Mainly, the diagnostic companions of the *PCA3* test, accounting 67% analytical sensitivity and the negative predictive value (NPV), is about 90%. Many authors suggested that, thanks to its NPV, the use of the *PCA3* test could make the biopsy useless if the PSA levels are slightly high. Recently, a useful correlation between the epigenetic profile of two genes *HOXD3* and *GSTP1*, in 408 patients at risk to prostate cancer, shown a non-invasive tool for early diagnosis<sup>7</sup>. The aim of this study is to demonstrate that *GSTP1* methylation status should be used as a “liquid biopsy biomarker” and it could be detected with early and simply in urinary cells DNA.

## PATIENTS AND METHODS

120 urine samples have been collected after Digital Rectal Examination (DRE). Samples belonged to men showing a suspect prostatic pathology and, therefore, a prostatic biopsy was suggested. All 120 patients were enrolled in this study. Men with a history of urothelial cancer were not included. Samples collection: venous blood was collected from each patient for the PSA measurement; the DNA of prostatic cells was obtained by urine centrifugation (1300 rpm). The urine sediment (1 ml) was collected through prostatic massage by DRE. The sediments were frozen at -80°C until DNA and RNA isolation. A total of 120 patients (average age: 65,5) with a not well defined diagnosis, with PSA >4 ng/mL (average value: 9,33 ng/ml, SD 4-10 ng/ml), were evaluated by Siemens Immulite 2000 system UK. They have been examined *PCA3* gene expression through RNA extracted from prostatic liquid cells. *PCA3* RNA was isolated according to Hologenic *PCA3* assay Gene Probe USA protocol. In order to evaluate the *GSTP1* methylation status, the DNA was isolated by “DNA-extract kit” in accordance with Dia-Chem (Naples, Italy) protocol.

The *PCA3*/PSA score mRNA values have been established using transcription-mediated amplification. The *PCA3*/score is considered positive when the value is >30 and negative when it is <30, as

shown by the manufacturer’s protocols.

Both bisulfite modification and methylation Sensitivity- PCR (MS-PCR) were performed in accordance with the manufacturer’s protocol (Dia-Chem, Naples, Italy). The antigen prostate-specific measurements of *PCA3* and *GSTP1* were carried out from December 2014 to October 2016 in the Clinical Pathology and Molecular Biology Department of the “San Giuseppe Moscato” Hospital (Avellino, Italy).

## STATISTICAL ANALYSIS

Mean  $\pm$  SD (standard deviation) values were calculated in all investigated parameters. Within the RA patients and healthy controls, frequencies of different polymorphisms investigated were calculated using the  $\chi^2$ -test, using Software: Stata 13.1 Copyright 1985-2013 StataCorp (College Station, TX, USA). The association between clinical laboratory markers and SNPs screened were determined by analysis of variance (ANOVA).

## RESULTS

The *PCA3*/PSA mRNA values were established using transcription-mediated amplification. The results were correlated with prostatic pathologies diagnosed through samples biopsy. 76 of 120 patients (63,3%) showed a *PCA3* greater than 30, and 94.0% of these patients had serum PSA value greater than 4,0 ng/ml.

*PCA3*/PSA score lower than 30<30 showed an hypo-expression in 44 cases. Of them, 20 BPH have PSA 8,7 ng/mL (median value) and *GSTP1* methylated in 4/20; 10 PIA have PSA 7,4 ng/mL and *GSTP1* methylated in 6/10; 8 PIN have PSA 5,0 ng/mL and *GSTP1* methylated in 6/8; 6 PC have PSA 6.0 ng/mL and *GSTP1* methylated in 2/6 (**Table 1**).

Over-expression of *PCA3* was recorded in 76 samples (with a score higher than 30). Of them, 14 BPH have PSA 12.1 ng/mL (median value) and *GSTP1* methylated in 2/14; 11 PIA have PSA 9,6 ng/mL and *GSTP1* methylated in 8/11; 19 PIN have PSA 9.5 ng/mL and *GSTP1* methylated in 18/19; 32 PC have PSA 16.3 ng/mL and *GSTP1* methylated in 4/32 (**Table 2**).

Unlikely, this results is not discriminatory between patients with PC and BPH, PIA, PIN. The epigenetic status of *GSTP1* samples is statistically significant in PC patients who have a *PCA3* score >30: methylated 12.5% and unmethylated 87.5% ( $p=0.00043$ ). Similarly, in BPH subjects methylated were 14.3% - 20.0% and unmethylated 85.7% - 80.0% ( $p=0.00083$  and  $0.00043$ ) in *PCA3* score >30 and

**TABLE 1.** Correlation between GSTP1 methylation status and PSA values on 44 cases with “PCA3 score” lower than 30.

PCA3 Score <30  N=44	PSA average value ng/mL	GSTP1 methylation status		
		Methylated (%)	Unmethylated (%)	p
BPH (20)	8.7	4 (20.0)	16 (80.0)	<b>0.00083</b>
PIA (10)	7.4	6 (60.0)	4 (40.0)	>0.05
PIN (8)	5.0	6 (75.0)	2 (25.0)	>0.05
PC (6)	6.0	2 (33.3)	4 (66.7)	>0.05
	Total	18 (40.9)	26 (59.1)	>0.05

*Abbreviations:* BPH: Benign Prostatic Hyperplasia; PIA: Prostatic Inflammation Atrophy; PIN: Prostatic Intraepithelial Neoplasia.

<30, respectively. In PIA GSTP1 is methylated at 72.7%, but this cohort of sample is few. In PIN we detect GSTP1 hypermethylation in 18/19 cases; this feature is a promise biomarker for discrimination between BPH, PIA and PC in patients with PCA3 score higher than 30. The PSA value is not significant for PC, PIN and PIA while in BPH is significant at 80%. The PCA3 score in the prostatic cells has a confirmed over-expression resulting in a positive biopsy result. GSTP1's methylation is correlated with carcinogenesis process and, more specifically with PC, due to its role in cellular cycle regulation.

## DISCUSSION

Evaluation of the methylation status of the GSTP1 gene may be cost-effectiveness and could be used extensively for cancer prevention. Recent progress has provided extraordinary opportunities to identify prognostic and predictive markers of efficacy to chemotherapy. Genetic markers can be used to identify patients who will benefit from therapy, exclude patients at high risk to develop severe toxicity, and adjust dosing<sup>8</sup>.

Furthermore, trials evaluating the economic impact of epigenetic testing in cancer prevention are still low<sup>9</sup>. Furthermore, the major issues to consider

for the clinical laboratories (who are responsible for providing epigenetic services) are: i) the availability of FDA-cleared guidelines; ii) the current absence of public reimbursement; iii) the need for genotyping accuracy and choice of eligible methods; and iv) the need to find clinical expertise to interpret laboratory data results<sup>10</sup>. However, there exist a persistent derisory known in of education of both the physicians regarding epigenetics test. The current knowledge of healthcare professionals regarding epigenetics is still low, and school curricula are only slowly including the teaching of this subject in their courses. Epigenetic knowledge is rapidly developing and changing, and it is imperative that healthcare professionals keep abreast of the advances and clinical indications, especially in so-called frail patients<sup>11</sup>. Moreover, epigenetics testing, currently available in several assay kits, may support clinicians to identify patients who are less likely to benefit from expensive drugs, which are susceptible to severe treatment-related toxicities at standard doses, and also reduce the delay of the patient receiving the correct antioxidant supplements<sup>12</sup>. Finally, several issues to assess the quality of cost-effectiveness in cancer therapy managements have become available. An important example is the National Institute for Health and Clinical Excellence (NICE). NICE forms a diverse clinical advisory committee, which stimulates Phar-

**TABLE 2.** Correlation between GSTP1 methylation status and PSA values on 44 cases with “PCA3 score” higher than 30.

PCA3 Score <30  N=44	PSA average value ng/mL	GSTP1 methylation status		
		Methylated (%)	Unmethylated (%)	p
BPH (14)	12.1	2 (14.3)	12 (85.7)	0.0032
PIA (11)	9.6	8 (72.7)	3 (27.3)	0.055
PIN (19)	9.5	18 (94.7)	1 (5.3%)	<b>0.00012</b>
PC (32)	16.3	4 (12.5)	28 (87.5)	0.00043
	Total	32 (42.1)	44 (57.9)	>0.05

*Abbreviations:* BPH: Benign Prostatic Hyperplasia; PIA: Prostatic Inflammation Atrophy; PIN: Prostatic Intraepithelial Neoplasia.



ma and Academic communities to produce a robust set of data, including the design and data source, for economic models of personalized healthcare<sup>13</sup>. It is well known that molecular genetics counseling performed before selected cancer treatment, provide lower overall medical costs and higher quality of life. NICE, also provides a method for measuring Quality-Adjusted Life-Years (QALY); methods that combine heterogenic information on outcomes, analytical, and cost-effectiveness for each treatment<sup>13</sup>.

There is an increasing interest in the urinary biomarkers' research with specific PCR techniques for prostatic pathologies<sup>14</sup>. The *PCA3* score has been the first test to be commercialized; it has been suggested for patients with high PSA or with an increasing PSA. Anyhow it has still to be evaluated as active surveillance as well as described above<sup>15</sup>. Our study shows that the prostatic urinary cells of the *PCA3* have a higher specificity to detect early PC than the serum value of PSA. The results were correlated with prostatic pathologies diagnosed through samples biopsy. In the routine diagnostic activity we recorded 76 of 120 patients (63,3%) showed a *PCA3* greater than 30; 24.3% of them in PC at diagnosis and had serum PSA value greater than 16,3 ng/mL. Noteworthy, the PC patients with lower score <30 have 1 fold the unmethylated case related to methylated *GSTPI*. Instead, in PC patients carrying higher *PCA3* score the unmethylated case is 7-fold higher than methylated. The *GSTPI* methylation is extremely expressed in the basal cell layer, in the luminal cells and prostatic intraepithelial neoplasm (PIN). In our study we detect *GSTPI* hypermethylation in 18/19 PIN cases; this feature is a promise biomarker for discrimination between BPH, PIA and PC in patients with *PCA3* score higher than 30. In conclusion, *GSTPI*'s methylation is correlated with carcinogenesis process and, more specifically with PC, due to its role in cellular cycle regulation.

## CONCLUSIONS

*GSTPI* represents a typical example of an ideal epigenetic biomarker, as its methylation is involved in a number of diseases, especially in PCa. Finally, we assert that *GSTPI* can be used as a "liquid biopsy biomarker" and could be detected with good results in circulating cell-free DNA and urinary DNA. For PCa early diagnosis, it has many good features that make it suitable for clinical application. However, further multicentric, large, prospective studies are needed to validate this assay and apply it in clinical practice.

We believe that the right way to face these challenges is based on a multidisciplinary treatment approach and to rationalize the costs of these treatments due to aimed-interventions.

## AUTHOR CONTRIBUTIONS:

The research design, E. V. and G. B. have designed this study. A. F. and F.C. have product the samples. Methodology, and analytical test were performed by M. R. C.; software, X.X.; validation, G:B and A.V., X.X.; supervision, by F.F.

## FUNDING:

This research received no external funding.

## ACKNOWLEDGMENTS:

We are grateful to Dr Fausto Ferrini (www.andrologoferrini.com) European Academy of Andrology, Ancona, Italy, for his expert supervision of the manuscript.

## CONFLICT OF INTEREST:

The authors declare no conflict of interest

## REFERENCES

1. Narayan VM. A critical appraisal of biomarkers in prostate cancer. *World J Urol* 2019. doi: 10.1007/s00345-019-02759-x. [Epub ahead of print].
2. Hadavand Siri F, Salehiniya H. Prostate cancer in Iran: an epidemiological review. *WCRJ* 2019; 6: e1268.
3. Eilers T, Machtens S, Tezval H, Blaue C, Lichtinghagen R, Hagemann J, Jonas U, Serth J. Prospective diagnostic efficiency of biopsy washing DNA *GSTP1* island hypermethylation for detection of adenocarcinoma of the prostate. *Prostate* 2007; 67: 757-763.
4. Hauptstock V, Kuriakose S, Schmidt D, Düster R, Müller SC, von Ruecker A, Ellinger J. Glutathione-S-transferase pi 1(*GSTP1*) gene silencing in prostate cancer cells is reversed by the histone deacetylase inhibitor depsipeptide. *Biochem Biophys Res Commun* 2011; 412: 606-611.
5. Rodon N, Trias I, Verdú M, Calvo M, Banus JM, Puig X. Correlation of mRNA-*PCA3* urine levels with the new grading system in prostate cancer. *Rev Esp Patol* 2019; 52: 20-26.
6. Wei JT, Feng Z, Partin AW, Brown E, Thompson I, Sokoll L, Chan DW, Lotan Y, Kibel AS, Busby JE, Bidair M, Lin DW, Taneja SS, Viterbo R, Joon AY, Dahlgren J, Kagan J, Srivastava S, Sanda MG. Can urinary *PCA3* supplement PSA in the early detection of prostate cancer? *J Clin Oncol* 2014; 32: 4066-4072.
7. Zhao F, Olkhov-Mitsel E, Kamdar S, Jeyapala R, Garcia J, Hurst R, Hanna MY, Mills R, Tuzova AV, O'Reilly E, Kelly S, Cooper C, Movember Urine Biomarker Consortium, Brewer D, Perry AS, Clark J, Fleshner N, Bapat B. Urine-based DNA methylation assay, ProCURE, to identify clinically significant prostate cancer. *Clin Epigenetics* 2018; 10: 147.
8. Di Francia R, Siesto RS, Valente D, Spart D, Berretta M. Pharmacogenomics panel test for prevention toxicity in patient who receive fluoropyrimidine/oxaliplatin-based therapy. *Eur Rev Med Pharmacol Sci* 2012; 16: 1211-1217.
9. Crescente G, Di Iorio C, Di Paolo M, La Campora MG, Pugliese S, Troisi A, Muto T, Licito A, De Monaco A. Loop-mediated isothermal amplification (LAMP) and its variants as simple and cost effective for genotyping method. *WCRJ* 2018; 5: e1116.

10. Di Francia R, Valente D, Pugliese S, Del Buono A, Berretta M. What health professions in oncology needs to know about pharmacogenomics? *WCRJ* 2014; 1: e90.
11. Facciola A, Ceccarelli M, Venanzi Rullo E, d'Aleo F, Condorelli F, Visalli G, Cacopardo B, Pinzone MR, di Rosa M, Nunnari G, Pellicanò GF. Prostate cancer in HIV-positive patients: a review of the literature. *WCRJ* 2018; 5: e1136.
12. Khazaei M, Pazhouhi M. Induction of apoptosis and inhibition of autophagy cell death in the human prostate cancer cell lines by *Trifolium Pratens L.* hydroalcoholic extract. *WCRJ* 2019; 6: e1232.
13. Dhalla IA, Garner S, Chalkidou K, Littlejohns P. Perspectives on the National Institute for Health and Clinical Excellence's recommendations to use health technologies only in research. *Int J Technol Assess Health Care* 2009; 25: 272-280.
14. Gurioli G, Martignano F, Salvi S, Costantini M, Gunelli R, Casadio V. GSTP1 methylation in cancer: a liquid biopsy biomarker? *Clin Chem Lab Med* 2018; 56: 702-717.
15. Rossi FW, Napolitano F, Pesapane A, Mascolo M, Stai-bano S, Matucci-Cerinic M, Guiducci S, Ragno P, di Spigna G, Postiglione L, Marone G, Montuori N, de Paulis A. Upregulation of the N-formyl peptide receptors in scleroderma fibroblasts fosters the switch to myofibroblasts. *J Immunol* 2015; 194: 5161-5173.