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HYPERMETHYLATION OF GLUTATIONE-S-TRANSFERASI (GSTP1) AS PLASMATIC MOLECULAR BIOMARKER FOR PROSTATE CANCER

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ABSTRACT: Background: *Currently, a widely used marker for the diagnosis and follow-up of Prostate cancer (PC) is the Prostate Specific Antigen (PSA). Furthermore, in order to ensure an efficient monitoring of the patients at risk of PC, there is a growing need of new tools able to early identify these subject. Molecular analysis of neoplastic prostate tissues shown the inactivation of the Glutatione-S-Transferase gene (GSTP1), due to the hypermethylation. This features could be a potential biomarker for PC. The aim of this study is the specific and sensitive detection of the methylation status of GSTP1 gene in plasma.*

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

Results: *GSTP1 promoter gene hypermethylation 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 hyper-methylation status of GSTP1 gene.*

Conclusions: *By this means, specific evaluation of methylation status of GSTP1 gene may be an 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.*

KEY WORDS: *Methylation Sensitivity methods, Analytical validations, Molecular diagnostics, GSTP1, Prostate Cancer.*

INTRODUCTION

Prostate cancer (PC) is the most common serious cancer in men. Actually, a widely used marker for the diagnosis and follow-up of this cancer is the PSA (Prostate Specific Antigen), but there is a growing need for new tools able to identify subject at risk of PC, in order to ensure an efficient monitoring of these patients[1]. Research is looking for the comprehension of the molecular pathogenesis of PC. Molecular analysis of neoplastic prostate tissues has shown the inactivation of the Glutatione-S-Transferase gene (GSTP1), due to the hypermethylation[2]. This feature could be a po-

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tential biomarker for PC. The aim of this study was the specific and sensitive detection of the methylation status of GSTP1 gene in plasma (through a simple blood test). In addition, the diagnostic efficacy of the test was evaluated on 20 donors healthy subject, 57 benign prostatic hypertrophy (BPH), and 57 PC patients. In this way, the specific evaluation of methylation status of GSTP1 gene may represent an useful tool for the prediction of patients at risk of PC.

MATERIALS AND METHODS

A case-control epidemiological study has been performed on peripheral blood samples of 3 groups of patients: 57 patients with PC, 57 with benign prostatic hypertrophy (BPH) and 20 healthy subjects. All subject have been recruited in retrospective only with informed consent.

Sample collection (important considerations)

It was reported that both a delay in blood processing and storage temperature can influence the amount of DNA extracted from plasma[3]. Anticoagulants did not influence the quantity of the recovered DNA from plasma, but EDTA showed a stabilizing effect on blood during the time between sample drawn and processing, both at room temperature and at 4°C.

In order to get rid of contaminating DNA deriving from cells, both filtration[4] and repeated centrifugations at low and high speed were reported[5], demonstrating that no release of circulating nucleic acid was induced from blood cells even at maximum centrifugation speed[6]. As regard to the stability of C-DNAs in the frozen samples, some authors showed that plasma can be conserved frozen for years (at least 2 for RNA and 6 for DNA)[7] at -70 or -20°C without affecting C-DNA concentration, while other authors reported a decay of 30% in DNA from stored plasma[8].

Plasma sample collection procedure in our lab was adopted as following:

a) Samples were collected in EDTA-containing tubes ;
b) The tubes arrived within 1 hour from blood draw. They were submitted to a first centrifugation step at 1600 g, 4°C for 10 minutes for plasma recovering.
c) A second centrifugation was performed at maximum speed, at 4°C, for 10 minutes. Pellets eventually formed in this step were discarded.

Plasma was divided in one- extraction-volume aliquots (500 l) in 1,5mL tubes and stored at -80°C until extracted[9].

Circulating DNA extraction

Extraction method is an important issue to be addressed in the field of C-DNA, for which there is no agreement in literature and several protocols have been reported [10].

The isolation of C-DNA from plasma was obtained by using a *Cartridge based-DNA isolation* Kit NucliSpin Circulating DNA (Diachem, Naples, Italy).

PCR Based amplification of circulating DNA

After sodium-bisulfite treatment, extracted DNA was analyzed for GSTP1 promoter hyper-methylation. Real-Time PCR was carried out using specific primer sets for methylated and un-methylated sequences (MSP: methylated Specific PCR). The experiments were performed following the protcol of "Ampli GSTP1 kits®" manufactured by DiaChem Italy

Statistical Data

Analysis of coefficient of variation (CV), calculated on the slope values from daily standard curves, were used to evaluate intra-assay and inter-assay reproducibility. The slope of standard curves was employed to determine the efficiency of target amplification using the equation E=10e(-1/slope). In theory, this slope should not be lower than –3.3 because this implies a PCR efficiency of more than 1 (> 100%) and indicates that more than twice as many amplicons are being made per PCR cycle.

Analysis of variance (ANOVA) was calculated to study variability within the CV% data and expressed as Fisher's exact test

RESULTS

GSTP1 promoter gene hypermethylation 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

TABLE 1: REPORT ON GSTP1 METHYLATION STATUS

Subjects	No	Methylated GSTP1 (%)	Median age (years)
Healthy donors	20	0 (0)	32.7
BPH	57	25 (43,9)	60.5
PC Patients	57	34 (57.6)	67.8

Legend: BPH Benign prostate hypertrophy ; PC prostate cancer

hyper-methylation status within promoter of GSTP1 gene (Table 1).

The results were blinded in terms of sensitivity and specificity.

Calibration curve were adopted to allow the analytical sensitivity assay (A). The analytic detection limit was 0.1 pg of input DNA.

Specificity assay were performed by Melting assay on the specific amplicon (B). As visualized either no alien amplicons and/or primers dimerizzation were found.

DISCUSSION

Somatic silencing of GSTP1 gene is an early epigenetic event in the carcinogenesis of prostate. The total absence of hypermethylation of this gene in blood of healthy subjects and the presence in 57,6% of samples of PC patients were in accord with literature. Our results, confirmed a good sensitivity and specificity of the test based on circulating cell-free DNA (isolated by plasma), in comparison of data regarding commercial tests performed on biological fluids. It should be emphasized that the mean age of healthy subjects was significantly lower than patients with BPH or PC. This seems to indicate a correlation between age and carcinogenesis process of prostate tissue, establishing the basis of the criteria assessment in subjects potentially at risk of PC.

At the present time Methylation testing is a small and specialized sector, in the context of global diagnostics industry, comprising less than 3% of molecular diagnostics segment[11]. Over the next few years, the emergence of molecular/genetic involvement in the new therapies as results

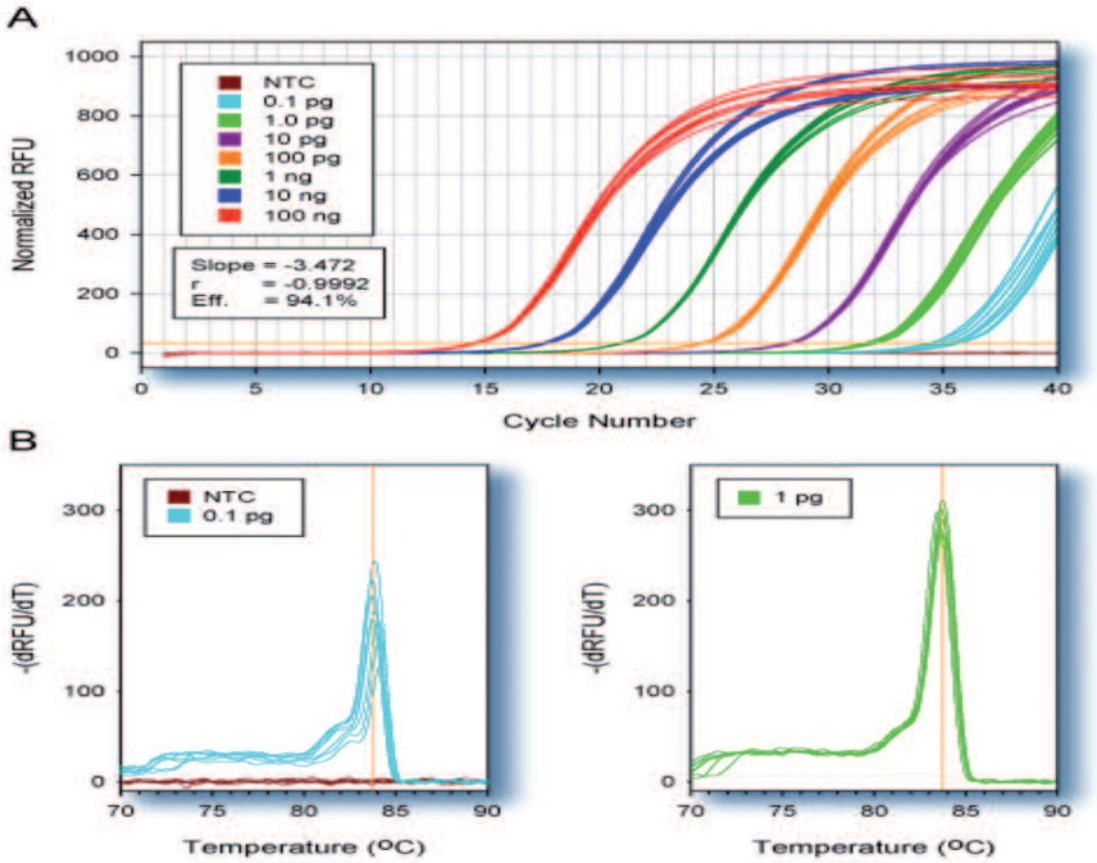

Figure 1. Real Time PCR results. Calibration curve were adopted to allow the analytical sensitivity assay (**A**). The analytic detection Limit was 0.1 pg of input DNA. Specificity assay were performed by Melting assay on the specific amplicon (**B**). As visualized either no alien amplicons and/or primers dimerization were found.

of genomic alteration, will drive diagnostics company to develop new test able to produce results indicative for tailoring patient's treatment. Hopefully, the future implementation of the methods for methylation detection, will result in personalized treatments and eventually, in shifting the balance from disease relapse towards disease eradication[12]. Therefore, pharmaceutical and biotechnology companies should join together, in order to develop a commercial test suitable for routine diagnostics in pharmacogenomics.

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