

## Molecular Detection of Prostate Cancer by Methylation Analysis of Plasminogen Activator Inhibitor-1 in Serum DNA

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### Abstract

**Background:** Epigenetic modifications are common in malignant tissues. Here we analyzed the methylation degree of plasminogen activator inhibitor 1 (*PAI-1*) gene in comparison to the methylation of glutathione-s-transferase- $\pi$  (*GSTP1*) gene in prostate cancer (PCa).

**Methods:** PAI-1 hypermethylation was studied using methylation-sensitive high resolution melting (MS-HRM) analysis of bisulfite-modified DNA and methylation-sensitive restriction endonuclease based quantitative PCR (MSRE-qPCR) on unmodified genomic DNA.

**Results:** Data, obtained by these two methods, correlate closely. Methylation levels of PAI-1 analyzed in tissue specimens and serum samples were nearly similar. The diagnostic performance of the MSRE-qPCR assay characterized by AUC values was 0.944 and 0.937 for PAI-1 and GSTP1, respectively. Combination of both markers resulted in higher values of AUC, sensitivity and specificity.

**Conclusion:** MSRE-qPCR based methylation analysis of PAI-1 gene and especially - in combination with GSTP1 gene may have potential as epigenetic marker of PCa in biological fluids.

**Keywords:** Prostate cancer; PAI-1, epigenetics; Cell-free circulating DNA

### Introduction

Prostate cancer (PCa) remains the most common form of cancer affecting men in the western world today [1,2]. Diagnosis of PCa is settled by histological examination of prostate tissue obtained by ultrasound guided transrectal biopsy. An increased serum level of prostate specific antigen (PSA) and/or an aberrant digital rectal examination are major indications for biopsy [3]. The widespread use of PSA testing significantly increased the early diagnosis of prostate cancer [4]. However, an increased PSA level is observed not only in malignant, but also in benign prostatic disease resulting in a negative biopsy rate of 70–80% [5,6]. A precise characterization and risk assessment of the tumor remains difficult even when combining measures such as PSA value, Gleason score, clinical stage and tumor volume. Therefore, much attention is paid to the development of new noninvasive methods for early detection and better prognostic prediction of PCa that can complement the traditional PSA test [7,8]. It is expected that such biomarkers will have a higher diagnostic specificity in comparison to PSA measurement alone and the ability to differentiate between harmless and aggressive prostate disease progressing in the direction of castration resistance.

Alterations in gene expression due to aberrant DNA methylation are well documented in prostate and many other cancers. Hypermethylated genes in PCa include DNA damage repair genes, hormonal response genes, cell-cycle control genes, tumor-suppressor

genes, apoptosis genes, and invasion and metastasis genes [9,10]. The promoter hypermethylation of the glutathione-s-transferase- $\pi$  (*GSTP1*) gene is a common epigenetic alteration during prostate cancer development [11]. According to current data, tumor cells shed their DNA into the bloodstream and the circulating DNA shares molecular similarities with the primary tumor, including abnormal DNA hypermethylation [12-14]. Therefore, tumor specific DNA methylation in serum was suggested as useful biomarker for diagnosis and prognostic prediction of cancer disease [15]. The plasminogen activator inhibitor type-1 (*SERPINE1* gene hereafter referred to as PAI-1) is considered as a potential therapeutic target in cancer and its upregulation is associated with a poor prognosis in many human cancer types [16-18]. In some cancers however the overexpression of PAI-1 is related to a less aggressive phenotype probably due to the inhibition of uPA activity [17]. In a previous study we reported that the PAI-1 gene is significantly higher methylated in prostate cancer tissues in comparison to adjacent non-tumor tissues [19]. In this study, we addressed the question whether PCa-dependent PAI-1 hypermethylations can be detected in free circulating serum DNA samples.

### Materials and Methods

#### Patients

This study involving human material was approved by the Institutional Review Board. All patients gave their written informed consent. A total of twenty pairs of malignant and adjacent normal

prostate tissue specimens were analyzed thereby including 10 tissue pairs that were described in a previous study [19]. Samples were obtained from patients with primary prostate cancer who underwent radical prostatectomy. Median age of patients was 65 years (range 53-73) and tumor stage varied between 2 and 4. Gleason scores ranged between 6 and 9 (median 7.0) and pre-operative PSA levels between 2.9 and 46.7 ng/ml (median 8.1 ng/ml). Only tumor tissue samples with a percentage of >70% tumor cells in the epithelial cell population and normal adjacent tissue samples with less than 5% of tumor cells in the epithelial cell population were included. Genomic DNA from tissue samples was isolated using Invisorb Spin Tissue Mini Kit (Stratec, Berlin, Germany). Isolated DNA was then bisulfite-modified using the EpiTect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

In addition, a total of 29 serum samples of prostate cancer (PCa) patients with bone and lymph node metastases at the initial diagnosis were analyzed. Median age of PCa patients was 75 years (range 51-99) and tumor stage varied between 2 and 4. Gleason scores ranged between 6 and 10 (median 8.6) and pre-operative PSA levels between 518.7 and 3827.0 ng/ml (median 1032.5 ng/ml). Furthermore, 24 serum samples of healthy individuals (N) were analyzed. Median age was 73 years (range 61-85) and total PSA levels between 0.1 and 1.7 ng/ml (median 0.6 ng/ml).

The DNA from 600 µl serum was isolated using the charge switch<sup>®</sup> gDNA 1 ml Serum kit (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol.

### Cell culture and DNA isolation

Normal human prostate epithelial cells (PrEC; Cambrex Bio Science, Walkersville, MD, USA) and prostate cancer cell lines (PC-3, DU-145, and LNCaP; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured as described previously [20]. Genomic DNA samples from normal cells, HASMC (human aorta smooth muscle) and HCAEC (human coronary artery endothelial cell), as well as from cancer cell lines, Jurkat (human T lymphocyte acute leukemia), U937 (human histiocytic lymphoma) and HepG2 (human hepatocellular carcinoma), were also analyzed. Genomic DNA was isolated using the QIAamp<sup>®</sup> DNA Mini and Blood Mini Kit (Hilden, Germany) according to manufacturer's instructions. In addition, genomic DNA from Raji (human B-cell leukemia), MCF-7 (human mammary adenocarcinoma), HeLa (human cervical cancer), K-562 (human CML in blast crisis) and A431 (human epidermoid carcinoma) cell lines was purchased from BioCat GmbH (Heidelberg, Germany).

### Methylation-specific high resolution melting (MS-HRM) analysis of bisulfite-modified DNA

To measure the methylation degree of PAI-1 in tissue samples 7 CpG sites located in the PAI-1 gene spanning from position +2 to +152 (CpG sites #1-7) [21] were analyzed. MS-HRM analysis was carried out on Rotor-Gene Q (Qiagen GmbH) as previously described [19]. The used primers were 5'-AGT TGT GTT TGG TTG TAG GGT TAA G-3' and 5'-CTT TTC TCC TAC CTA AAA TTC TCA AAA A-3' amplifying products with a length of 151 bp. Methylation percentage, representing the methylated proportion of the seven analyzed CpG sites, was calculated using calibrators with known

methylation degrees. For this purpose, bisulfite modified non-methylated and 100% methylated standard DNA samples (Qiagen GmbH) were combined giving the indicated methylation degrees.

### DNA digestion

To investigate CpG hypermethylation in samples of serum DNA an assay based on the methylation-sensitive restriction endonuclease (MSRE) HhaI was established. HhaI cuts the DNA sequence GCGC and is sensitive to DNA methylation. Enzyme activity is blocked if the recognition site contains a 5-methylcytosine (C5 mGCG). In 1xNE Buffer supplemented with 100 µg/ml BSA 2.5 µl of total serum DNA were incubated with 20 U HhaI giving a total reaction volume of 11 µl. Incubation was performed at 37°C for 24 hours. Restriction endonuclease was inactivated at 65°C for 20 min. A universal methylated human DNA standard (Zymo Research, Freiburg, Germany) was used as a positive control. An unmethylated human DNA standard (EpiTect Qiagen, Hilden, Germany) served as negative control. Additionally, a water blank containing no DNA was included. In order to quantify the amount of input DNA, a control reaction containing no restriction endonuclease was set up for each sample.

### MSRE-based quantitative PCR (MSRE-qPCR)

MSRE-qPCR was carried out in triplicate on a Rotor Gene Q (Qiagen GmbH) using primer sets that bracket a recognition site of HhaI. The applied primer pairs were 5'- TTC CTG CCC ACA TCT GGT AT-3' and 5'-AGG TTG GAG GGA GTT TGC TT-3' for PAI-1, giving PCR products with a length of 218 bp, and 5'-GGG ACC CTC CAG AAG AGC-3' and 5'-ACT CAC TGG TGG CGA AGA CT-3' for GSTP1 amplifying products with a length of 134 bp. MSRE-qPCR primers for GSTP1 were described previously (1), primers for PAI-1 were designed using Primer3 software. Cycling conditions for PAI-1 were set as follows: 95°C for 5 minutes followed by 45 cycles of 95°C for 5 seconds and 55°C for 10 seconds. PCR conditions for GSTP1 involved 95°C for 5 min followed by 45 cycles of 95°C for 15 seconds and 59 °C for 60 seconds. Cycle 1-10 were run in a touch down mode decreasing temperature by 1°C each cycle. Each PCR reaction mix consisted of 4 µl template DNA from restriction, forward and reverse primer in a final concentration of 110 nM (PAI-1) or 300 nM (GSTP1) respectively and 12.5 µl 2xSybr<sup>®</sup> Green PCR Master Mix (Qiagen, Hilden, Germany) giving a total volume of 25.0 µl. Melting curve analysis and comparative quantification analysis were performed after each run using Corbett Rotor-gene series software v1.7 (Corbett life sciences, Mortlake, Australia). For melting curve analysis a heating ramp from 59°C to 95°C rising by one degree each step was applied. In order to verify the length of the obtained amplicons the PCR products were analyzed by 2.0% agarose gel electrophoresis and ethidium bromide staining followed by visualization with ultraviolet illumination.

The methylation index (MI) of the PAI-1 gene was calculated using the comparative quantification results of qPCR with a 10 ng/µl standard of methylated DNA as calibrator:  $MI [\%] = \frac{HhaI \text{ treated DNA}}{\text{input DNA}} \times 100$ . In case of GSTP1, qPCR gave unspecific DNA products resulting from primer dimerization that would falsify the result of comparative quantification. Therefore, the methylation index of the GSTP1 gene was calculated using the peak height obtained for the specific product in the melt curve analysis. In both cases, the methylation index corresponds to the fraction of methylated DNA in

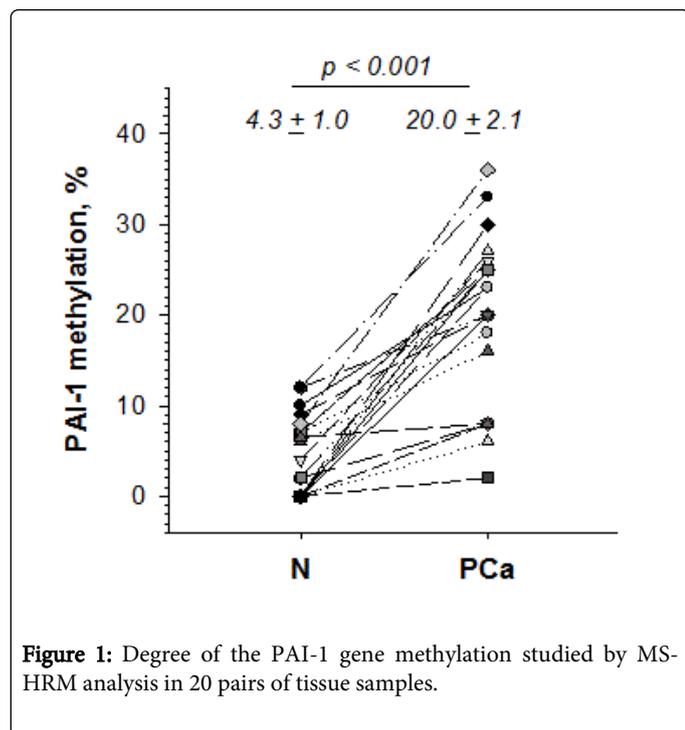
the total amount of serum DNA taken as 100%. The MSRE-qPCR assay was validated by analyzing the methylation index of DNA samples from prostate cancer cell lines and comparing the results with those obtained by MS-HRM method.

### Statistics

The difference between studied groups was settled using Mann-Whitney Rank Sum test. Differences were considered significant at  $p < 0.05$ . The correlation of variable pairs was studied with Pearson correlation test. The diagnostic performance including sensitivity and specificity of the MSRE-qPCR assays for PAI-1 and GSTP1 was assessed with receiver operating characteristic (ROC) curves. All statistical analyses were based on the statistic module integrated in the SigmaPlot11.2 software (Systat Software GmbH, Erkrath, Germany).

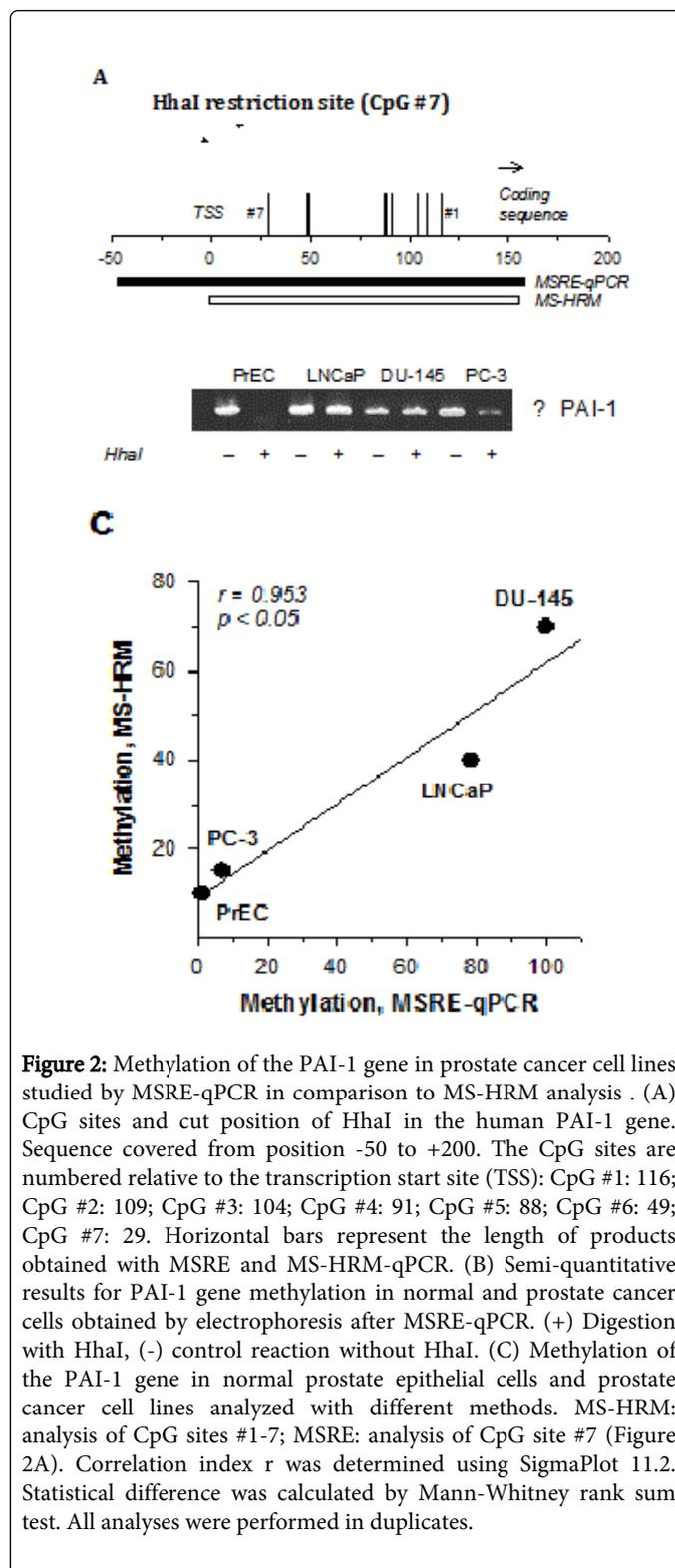
### Results

Using MS-HRM assay, we analyzed the PAI-1 gene methylation in twenty pairs of malignant and adjacent normal prostate tissue specimens. Normal adjacent tissue samples averaged  $4.3\% \pm 1.0\%$  (range: 0-12.0%) in methylation of the PAI-1 gene whereas tumor tissue samples showed an average methylation rate of  $20.0\% \pm 2.1\%$  (range: 2.0-36.0%). The difference in PAI-1 methylation between malignant and adjacent normal tissue was statistically significant (Figure 1).



**Figure 1:** Degree of the PAI-1 gene methylation studied by MS-HRM analysis in 20 pairs of tissue samples.

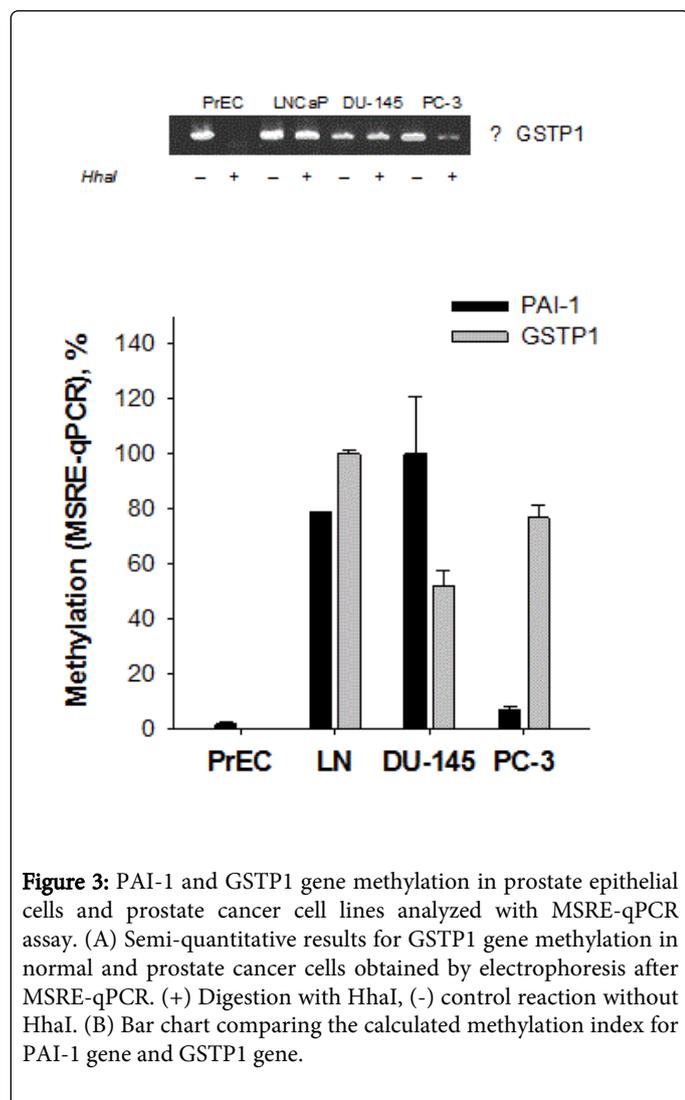
Further experiments were designed to detect the above described cancer-associated PAI-1 gene methylation in circulating DNA as a non- or semi-invasive approach. In relation to this, a method based on the methylation-sensitive restriction endonuclease HhaI was established.



**Figure 2:** Methylation of the PAI-1 gene in prostate cancer cell lines studied by MSRE-qPCR in comparison to MS-HRM analysis. (A) CpG sites and cut position of HhaI in the human PAI-1 gene. Sequence covered from position -50 to +200. The CpG sites are numbered relative to the transcription start site (TSS): CpG #1: 116; CpG #2: 109; CpG #3: 104; CpG #4: 91; CpG #5: 88; CpG #6: 49; CpG #7: 29. Horizontal bars represent the length of products obtained with MSRE and MS-HRM-qPCR. (B) Semi-quantitative results for PAI-1 gene methylation in normal and prostate cancer cells obtained by electrophoresis after MSRE-qPCR. (+) Digestion with HhaI, (-) control reaction without HhaI. (C) Methylation of the PAI-1 gene in normal prostate epithelial cells and prostate cancer cell lines analyzed with different methods. MS-HRM: analysis of CpG sites #1-7; MSRE: analysis of CpG site #7 (Figure 2A). Correlation index  $r$  was determined using SigmaPlot 11.2. Statistical difference was calculated by Mann-Whitney rank sum test. All analyses were performed in duplicates.

The results obtained by this MSRE-qPCR assay on prostate cancer cells were comparable with those of MS-HRM analysis that was used as a validated reference method. Analyzing CpG site #7 with MSRE-qPCR the highest methylation rate was detected in DU-145 (100%)

and LNCaP cells (78%), whereas PAI-1 methylation rates in PC3 and PrEC cells were negligible (7% and 1%, respectively).



**Figure 3:** PAI-1 and GSTP1 gene methylation in prostate epithelial cells and prostate cancer cell lines analyzed with MSRE-qPCR assay. (A) Semi-quantitative results for GSTP1 gene methylation in normal and prostate cancer cells obtained by electrophoresis after MSRE-qPCR. (+) Digestion with HhaI, (-) control reaction without HhaI. (B) Bar chart comparing the calculated methylation index for PAI-1 gene and GSTP1 gene.

The differential methylation of PAI-1 gene in studied cells is clearly visible on electrophoresis of the MSRE-qPCR product (Figure 2B). In case of CpG sites #1-7 analyzed using MS-HRM similar results were obtained. DU-145 cells (70%) and LNCaP cells (40%) showed the highest PAI-1 methylation rates. PC3 cells yielded a methylation of PAI-1 gene of 15% and PrEC cells displayed 10%. The correlation between both methods was statistically significant ( $p < 0.05$ ) with a correlation index  $r$  of 0.953 (Figure 2C).

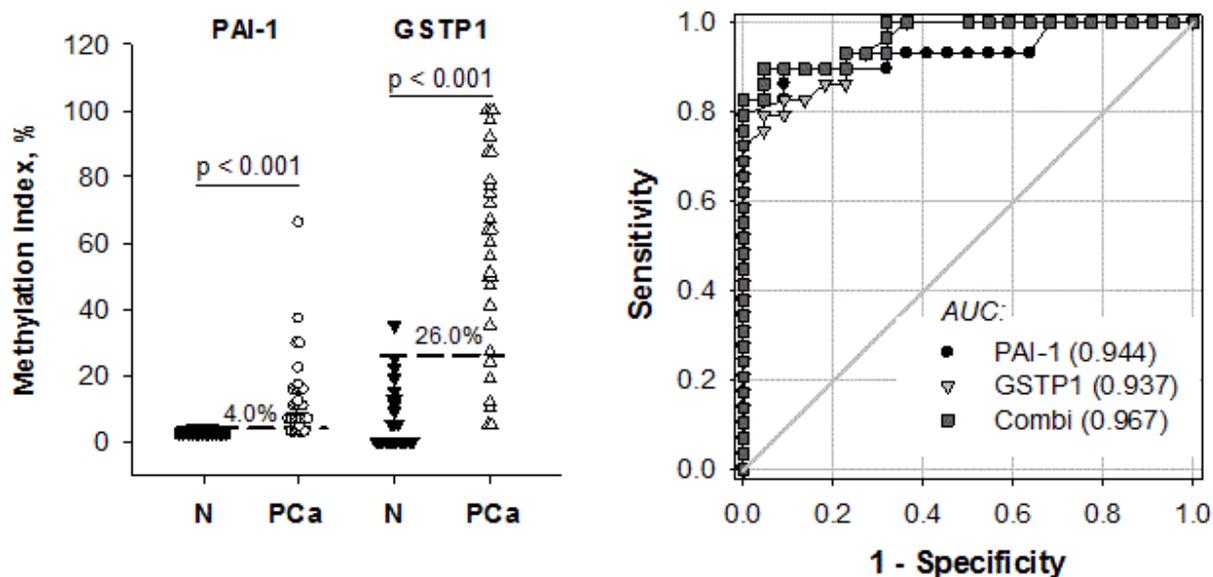
Using MSRE-qPCR we analyzed further the GSTP1 gene methylation in these cells. In normal PrEC cells GSTP1 gene was unmethylated (Figure 3). In LNCaP and PC3 cells, the GSTP1 gene was highly methylated (100% and 77%, respectively) whereas it was moderately methylated in DU-145 cells (52%). The results obtained by electrophoresis of the PCR products of GSTP1 are in accordance with the calculated methylation values (Figure 3).

Comparison of PAI-1 gene methylation with that of GSTP1 gene methylation in other human primary cells and cell lines revealed that in mammary MCF-7 adenocarcinoma cell line promoters of PAI-1 and GSTP1 were highly methylated - 73% and 99% respectively (Table 1). In human HepG2 hepatocellular carcinoma cells, the GSTP1 gene was largely methylated (99%), in contrast to PAI-1 which was unmethylated. Both genes showed an aberrant methylation in Raji B cell leukemia cells (31% for PAI-1 and 81% for GSTP1) but no methylation was found in monocytic (U-937) or T cell leukemia (Jurkat). K-562 CML cells in blast crisis displayed an aberrant methylation for PAI-1 gene (27%). Neither A431 epidermoid carcinoma cells nor HeLa cervical cancer cells and none of the normal, non-tumor cells (HCAEC, HASMC) showed a methylation of the investigated gene promoters (Table 1).

Cell lines	classification	PAI-1 MI [%] (M ±SD)	GSTP1 MI [%] (M ±SD)
HCAEC	human coronary endothelium	0.8 ± 0.4	0.0 ± 0.1
HASMC	human smooth muscle	1.0 ± 0.1	0.0 ± 0.2
PrEC	human prostate epithelium	1.5 ± 0.6	0.0 ± 0.1
LNCaP	human prostate adenocarcinoma	78.5 ± 0.1	100.0 ± 1.6
DU-145	human prostate cancer	100.0 ± 21.0	52.1 ± 5.3
PC-3	human prostate cancer	7.0 ± 0.8	76.6 ± 4.5
Jurkat	human T cell leukemia	1.4 ± 0.1	0.0 ± 0.1
U-937	human monocytic leukemia	9.1 ± 1.7	0.0 ± 0.3
Raji	human B cell leukemia	31.0 ± 0.1	81.0 ± 7.0
K-562	human CML in blast crisis	26.5 ± 3.0	0.0 ± 0.2
HepG2	human hepatocellular carcinoma	1.3 ± 0.3	99.4 ± 19.3
MCF-7	human mammary adenocarcinoma	72.8 ± 5.6	98.9 ± 12.4
A431	human epidermoid carcinoma	2.6 ± 0.5	0.0 ± 0.1
HeLa	human cervical cancer	2.2 ± 0.7	0.0 ± 0.3

**Table 1:** PAI-1 and GSTP1 genes methylation in different human cells and cell lines.

Further, we studied the PAI-1 and GSTP1 gene methylation in serum DNA samples of prostate cancer patients and healthy individuals. Obtained results shows that the methylation of the PAI-1 gene averaged  $13.3\% \pm 2.5\%$  (range: 1.7-78.9%) in patients with prostate cancer (PCa) and  $2.3\% \pm 0.2\%$  (range: 0.8-3.9%) in healthy individuals (N) using MSRE-qPCR assay, respectively. In case of GSTP1, methylation values were  $57.0\% \pm 5.7\%$  (range: 5.0-100.0%) in PCa and  $7.4\% \pm 2.0\%$  (range: 0-35.0%) in N group. The differences in methylations between PCa and N were significant for both PAI-1 and GSTP1 ( $p < 0.001$ , Figure 4A).



**Figure 4:** Methylation degree of PAI-1 and GSTP1 analyzed using MSRE-qPCR in serum DNA of patients with prostate carcinoma (PCa, n=29) and healthy individuals (N, n=22). (A) Histogram: Dotted lines show the cutoff values. Statistical difference was calculated by Mann-Whitney rank sum test. All analyses were performed in triplicates. (B) Receiver-Operating Characteristic (ROC) curves comparing sensitivity and specificity of MSRE-qPCR assay applied for PAI-1 gene methylation, GSTP1 gene methylation and the combination of both markers. The AUC values (in brackets) were calculated with SigmaPlot 11.2. All analyses were performed in triplicates.

The diagnostic performance of the MSRE-qPCR assay was assessed using ROC analysis (Figure 4B). The obtained AUC values for the PAI-1 and GSTP1 assay were 0.944 (CI, 95% confidence interval 0.89-1.00) and 0.937 (CI, 0.8-1.00), respectively. The sensitivity of the assay for PAI-1 gene was 82.8%, the specificity – 100% and for GSTP1 79.3% and 95.8%, respectively. The cut-off values for assessment of diagnostic sensitivity and specificity were determined by maximum likelihood estimation and gave 4.0% for PAI-1 and 26.0% for GSTP1 gene methylation (Figure 4A). Importantly, that PAI-1-specific MSRE-qPCR analysis detected three cases of patients with prostate cancer that were missed using GSTP1-specific MSRE-qPCR. When compared to the single markers, a combination of both markers resulted in higher AUC value - 0.967 (CI, 0.89-1.00) in the ROC analysis (Figure 4B). The sensitivity of the combined assay was 86.2%, the specificity – 100%.

## Discussion

In this study we confirm that the methylation degree of the PAI-1 gene is significantly elevated in tissue samples of PCa patients when compared to those of non-malignant adjacent tissues. An aberrant methylation of the PAI-1 gene was found for the first time in free circulating DNA of serum samples obtained from PCa patients. The PAI-1 methylation levels in tissue specimens and in serum samples are nearly similar. Furthermore it is shown that PAI-1 gene hypermethylation occurs not only in prostate cancers, but also in a breast cancer cell line and in part in leukemia cells whereas in normal non-tumor cells the PAI-1 gene was completely unmethylated (Table 2).

There are different methods established to detect DNA methylation. The most commonly used method is MS-HRM [8]. However, this technique requires bisulfite conversion of DNA which may remain incomplete [22]. Moreover, up to 90% of DNA is degraded during bisulfite treatment thus limiting the detection of very few copies of methylated DNA [23]. Thus, for analysis of serum DNA a method based on the methylation-sensitive restriction endonuclease HhaI was established in this study which does not require a previous bisulfite treatment and gets along with 25 ng of genomic DNA for methylation analysis. Such method has already been applied for the analyses of other genes including GSTP1 [22-25].

In this study we analyzed the methylation of the PAI-1 gene in comparison to GSTP1 gene methylation that is considered as a new epigenetic marker for early PCa detection. According to ROC analyses, the ability to distinguish between healthy individuals and prostate cancer patients is high for both PAI-1 and GSTP1 MSRE-qPCR assays with AUC values of 0.944 and 0.937, respectively. In order to compare sensitivity and specificity of both assays the specificity (sensitivity) was set to a fixed value (90%) and the corresponding values for sensitivity (specificity) were calculated. The PAI-1 assay was more sensitive and more specific in comparison to the GSTP1 assay. A combination of the two markers improved the diagnostic performance being superior to that of each marker alone. A combined score of several epigenetic cancer markers was reported also in other studies. For example, Hoque et al. [26] analyzed the methylation status of four genes (p16, ARF, MGMT and GSTP1) in urine samples yielding a sensitivity of 87% and a specificity of 100% for prostate cancer detection. According to our findings, a combined analysis of PAI-1 gene and GSTP1 gene methylation in serum DNA allowed to detect 86% of prostate cancers with 100% specificity.

An impairment of this study is the low analytical sensitivity of the MSRE-qPCR assay with about 5% of methylated DNA strands in a background of unmethylated DNA. The fraction of tumor DNA in blood samples of cancer patients was found to vary inter-individually from 0.1% to more than 90.0% of total serum DNA depending on tumor stage [27,28]. In plasma samples of patients with early cancer the tumor derived DNA fraction ranges from 0.01% to 2.00% [29]. Therefore, an increase in analytic sensitivity is necessary to improve the diagnostic performance of the DNA methylation assays. Currently performed optimization of the DNA methylation analysis allows to detect even <0.1% of methylated DNA in plasma or tissue samples [30,31] *Further studies* will be *needed* to elucidate whether such approaches can help to improve the analytical detection of methylated PAI-1 gene in serum samples of patients with prostate cancer.

The investigated CpG sites of PAI-1 are located in the proximal part of the gene, downstream to the transcription start site. The methylation of such CpG sites may effectively decrease the promoter activity due to the blockage of the binding of RNA polymerase II and TATA-box-binding protein to the promoter region [32]. Indeed, a decreased PAI-1 expression was found in prostate cancer cell lines [33]. Nevertheless, a general inverse correlation between PAI-1 gene methylation and PAI-1 mRNA levels remains questionable. For example, in oral cancers the CpG methylation was not related to the PAI-1 expression [34]. However, in prostate cancer cells a down-regulation of PAI-1 expression was shown to be associated with epigenetic modifications [19]. Whether or not PAI-1 gene methylation plays a causal role in development and progression of prostate cancer is not yet known. However this question is gaining interest since an increasing number of epigenetic drugs is clinically tested for prostate cancer, thus opening new curative options [35].

## Conclusions

In the current study, PAI-1 gene hypermethylation was detected in serum DNA of prostate cancer patients. The MSRE-qPCR based methylation analysis of PAI-1 gene alone and in combination with GSTP1 gene may have potential as epigenetic marker in biological fluids of patients with prostate cancer. The degree of methylation was measured using MSRE-qPCR. MI, methylation index; M, mean value; SD, standard deviation. All analyses were performed in duplicate.

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