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GSTP1 Hypermethylation as a Molecular Marker in the Diagnosis of Prostatic Cancer: Is There a Correlation with Clinical Stage, Gleason Grade, PSA Value or Age?

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Abstract
Objectives: Epigenetic events such as promoter hypermethylation have been implicated in prostate carcinogenesis. We present a real-time, methylation specific protocol to detect hypermethylation in the promoter region of the *GSTP1* gene in benign hyperplasia and adenocarcinoma of the prostate.
Methods: In our preliminary study, 31 prostate cancer and 5 benign prostatic hyperplasia (BPH) tissue samples were analyzed. Genomic DNA was isolated from formalin-fixed and paraffin-embedded specimens and subjected to sodium bisulfite modification, followed by real-time, methylation specific PCR. Patients with prostatic cancer were also subdivided according to their Gleason score, PSA, age and TNM Staging. Prostate cancer cell lines (LNCaP, DU145, PC3) and a BPH cell line (BPH-1) were also tested as controls.
Results: GSTP1 promotor hypermethylation was detected in 28 of the 31 prostate cancer cases (90.3%) and none of the five (0%) BPH cases. Statistical analysis did not reveal a significant correlation between *GSTP1* hypermethylation and Gleason score, PSA, age or TNM staging. All prostate cancer cell lines were testes positive for *GSTP1* promotor hypermethylation, whereas the BPH cell line (BPH-1) was tested negative.
Conclusion: GSTP1 promotor hypermethylation occurs during carcinogenesis and is considered to be a major event of prostate carcinogenesis. Our data support this thesis and shows that *GSTP1* hypermethylation reliably distinguishes between prostate cancer and BPH . Although it is not yet clear at what time during carcinogenesis hypermethylation of the *GSTP1* promotor occurs it seems to provide valuable information for prostate cancer screening and diagnosis. Larger studies are underway to determine the potential role for *GSTP1* hypermethylation in clinical settings.

Key words: prostate cancer, benign prostatic hyperplasia, DNA methylation, CpG island hypermethylation, GSTP1

Introduction

Prostatic adenocarcinoma is the most commonly diagnosed cancer in men over the age of 40 years and the second leading cause of cancer related deaths in Western countries. According to the German tumor registry about 38,000 patients were newly diagnosed with prostate cancer in Germany in 2002. It is estimated that ~220,900 Americans will be diagnosed with prostatic cancer, and ~28,900 men will die from the disease in 2003 [1]. The most common diagnostic tool for detecting prostate cancer is the digital rectal examination. Due to test subjectivity and examiner`s experience the detection rate has been reported to be between 0.8% to 25.2% with a positive predictive value of 6.3% to 50% [2-5]. The use of serum prostate specific antigen (PSA) during the last two decades has changed the diagnosis of prostate cancer. However, the utility of PSA and digital rectal examination for harboring patients at risk for prostatic carcinoma is limited in specifity and may result in repeated biopsies [6]. Elevated levels of PSA have also been found in serum of patients with benign prostatic hyperplasia (BPH).

Methylation at the 5´-position of cytosine in CpG islands regions of genes is a common modification of DNA in vertebrate genomes and has been recognised as an important epigenetic alteration that is associated with the gene`s inactivation [7, 8].

Glutathione S-transferases are part of the intracellular detoxification by conjugating chemically electrophiles to glutathione [9]. Human glutathione S-transferases are encoded in four classes of genes, namely α, μ, π and τ [9]. It has been reported that many human prostatic cancers fail to express π-class glutathione S-transferase and hypermethylation was observed in the regulatory sequence of the *GSTP1* gene, which encodes for the π-class glutathione S-transferase [10].

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Autopsy studies have shown a prevalence of organ confined prostate cancer in 64% in men between 60 and 70 years of age [11]. Not all of these men will progress to clinical significant prostate cancer and die because of symptomatic or systemic disease. In the PSA-era there may be a chance of over-detection and over-treatment since many men seem to die of other causes than prostatic cancer [12]. Therefore, the development of an additional, independent test of a molecular marker to detect prostate cancer early in carcinogenesis could improve the accurate diagnosis and identify those patients that might progress to clinical significant prostate cancer. In this study we present a real-time methylation specific protocol to detect hypermethylation in the promoter region of the *GSTP1* gene in benign prostatic hyperplasia and adenocarcinoma of the prostate.

Material and Methods

Patients and Sample Collection

A total of 31 patients with prostate cancer and 5 with BPH were recruited into this study. All tissue samples were obtained by standard urological procedures performed at the Department of Urology, University of Bonn, Germany. All patients had given informed consent for the collection of tissue according to the institutional guidelines. Three prostate cancer cell lines (LNCaP, DU145, PC3) and one BPH cell line (BPH-1) were also tested. The cell lines were obtained from the "Deutsche Sammlung für Mikroorganismen und Zellkultur", Braunschweig, Germany, and cultured according to the vendor`s protocol (www.dmsz.de).

A series of 5µm sections were cut from each tissue block and hematoxylin and eosin (H&E) stained for pathological evaluation. Gleason score, pathological stage, age and serum PSA values at the time of surgery were collected for each of these specimens (Table 1). Microdissection was performed for enrichment of epithelial cells. Genomic DNA was isolated from formalin-fixed, and paraffin-embedded specimens according to the manufactures instructions using the Qiagen DNA Mini Kit (Qiagen GmbH, Hilden, Germany). The diagnosis for prostate cancer or BPH was confirmed by a pathologist.

Table 1. Clinical characteristics of the patients.

	BPH	PCA
Age (years) (n = 36)		
Mean	62	64
Range	57-75	51-74
Pathological stage (n = 31) (%)		
pT2		18 (58)
pT3		12 (39)
pT4		1 (3)
Gleason score (n = 31) (%)		
2-3		3 (10)
4-6		13 (42)
7-10		15 (48)
PSA (ng/ml) (n = 31) (%)		
0-4		4 (13)
4-10		10 (32)
10-20		9 (29)
<20		5 (16)
unknown		3 (10)

BPH: benign prostatic hyperplasia; PCA: prostate cancer

Bisulfite Treatment

Bisulfite treatment was performed using the following protocol [13]: 2µg of DNA and 2µg tRNA-Carrier (Sigma) were resolved in 100µl distilled water and denatured by NaOH (final concentration 0.3M) for 20 minutes at 37°C, sodium bisulfite solution (520 µl) was added and incubated at 55°C for 18 hours. The modified DNA was desalted using QIAex II (Qiagen, Hilden, Germany), resolved in 100µl 1mM Tris-Cl pH 8.0 and treated with NaOH (final concentration 0.3M) for 20 minutes at 37°C. 47 µl of ammonium acetate and 1µg tRNA were added, followed by ethanol precipitation over night, washing in 70% ethanol, drying and resuspending in 35µl 1mM Tris (pH 8.0).

Real-time Methylation Specific PCR

We performed real-time PCR amplification of the promotor region flanking the *GSTP1* gene. The principal of real-time quantitative PCR is achieved by continuous optical monitoring the progress of a fluorescent PCR reaction. All PCR reactions were carried out on an ABIPrism 7700 Sequence Detection system (Applied Biosystems, Foster City, USA). The primer sequences to amplify sodium-bisulfite converted CpG islands located at the *GSTP1* gene were designed at our institution to detect methylated and unmethylated alleles in prostate cancer and BPH. *GSTP1* methylated specific primer: 5`-TTC GGG GTG TAG CGG TCG C-3`(forward), 5`-GCC CCA ATA CTA AAT CAC GAC G-3` (reverse); *GSTP1* unmethylated specific primer: 5`-GAT GTT TGG GGT GTA GTG GTT GTT-3` (forward), 5`-CCA CCC CAA TAC TAA ATC ACA ACA-3` (reverse). The fluorescence signal was generated by the inclusion of a double strand DNA SYBR Green dye (SYBR Green PCR Master Mix, Applied Biosystems, Foster City, USA). The PCR was performed using the following conditions: one cycle at 95°C for 15 minutes, followed by 45 cycles of 95°C for 30 seconds, 62°C for 30 seconds, 1 minute at 72°C, 15 seconds at 75°C and a final extension step for 10 minutes at 72°C. The PCR reaction was done in a total reaction volume of 25µl containing 12.5µl SYBR Green dye, 2.5µl of forward and reverse primer each (1pmol/µl each), 5.0µl template and 2.5µl distilled water. Each reaction was performed in triplicate.

A positive control (CpGenome universal methylated DNA, Serological Cooperation, Temecula, USA) and two negative controls (bisulfite converted WBC DNA from normal volunteers and distilled water) were included in each amplification. Each reaction was performed in triplicate.

Statistical Analysis

Statistical analysis was done using the two sided Fisher`s exact test. For all tests, significance was concluded at $p<0.05$.

Results

The study was designed to identify the status of *GSTP1* hypermethylation in patients with benign prostatic hyperplasia and prostate cancer. Samples with signs of DNA hypermethylation at a cycle threshold of 35 or less were considered positive. *GSTP1* promotor hypermethylation was found in 28 of 31 patients (90.3%) with prostatic cancer and in 0 of 5 patients (0%) with benign prostatic hyperplasia ($p<0.0001$) (Table 2). Despite that, *GSTP1* promotor hypermethylation was found in all three (100%) prostate cancer cell lines (LNCaP, DU145, PC3) and was not detected in the BPH cell line (BPH-1) (Table 3). Thus, based on the limited number of patients tested, the specificity of our essay was 100%. No significant correlation ($p<0.05$) between the status of methylation and PSA, Gleason score, age or TNM staging was detected.

Table 2. Hypermethylation in *GSTP1* CpG island in benign prostatic hyperplasia (BPH) and prostatic cancer (PCA).

	n (patients)	patients with *GSTP1* hypermethylation
BPH	5	0 (0%)
PCA	31	28 (90.3%)
BPH cell line	1	0 (0%)
PCA cell line	3	3 (100%)

Discussion

We were able to find CpG-island hypermethylation at *GSTP1* in the majority of the study patients (90.3%) who had histological confirmed prostate cancer and in all three prostate cancer cell lines. Other studies that have used either non-quantitative or quantitative, real time PCR have also reported *GSTP1* hypermethylation in more than 90% of the cases analysed [14, 15, 16, 18, 19, 32]. A limitation of the conventional methylation specific PCR (MSP) for detection of hypermethylation is that prostate cancer and benign prostatic hyperplasia may score as positive for *GSTP1* hypermethylation.

Another goal of our study was to determine whether detection of CpG-island hypermethylation of the promotor of *GSTP1* can distinguish between neoplastic and non-neoplastic prostatic tissue. We were not able to detect *GSTP1* hypermethylation in tissue samples of BPH patients or the BPH 1 cell line.

Despite the small sample size of our study, we were able to distinguish between prostate cancer and BPH to a statistically significant extent. Another study using quantitative, real time PCR demonstrated *GSTP1* hypermethylation in 91% of the prostate cancer patients and in 29% of the BPH patients [19]. Laser capture microdissection (LCM) was not performed because we wanted to test whether the real-time MSP was able to detect small neoplastic lesions and serve as an additional test to histology to ease the diagnosis in cases when definite diagnosis of prostate cancer can not be made easily. LCM is very time consuming procedure, but is inevitable to further distinguish between normal epithial cells, BPH, prostate cancer and precursor lesions and is part of an ongoing study. However, microdissection for cellular enrichment was performed.

Regional hypermethylation of CpG islands have been reported to participate in carcinogenesis [16]. Although, the mechanism by which genes acquire somatic CpG island hypermethylation during carcinogenesis have not been established, it has been suggested that hypermethylation of the the promotor region of the *GSTP1* gene occurs early during the carcinogenesis of prostatic cancer. A recent study using laser-capture-microdissection to evaluate *GSTP1* hypermethylation in precursor lesions of prostatic cancer, namely the proliferative inflammatory atrophy (PIA) and high-grade prostatic intraepithelial neoplasia (HGPIN) [17], detected hypermethylation in 6.3% and 68.8%, respectively [18]. Although *GSTP1* island hypermethylation only occurred in a subset of PIA lesions, at least some PIA lesions may lead to high-grade PIN and/or may progress to adenocarcinoma of the prostate [18].

The different levels of *GSTP1* hypermethylation in neoplastic and non-neoplastic prostatic tissue suggest that *GSTP1* hypermethylation may be more useful than measurement of serum PSA in distinguishing men at low risk for prostate cancer from those with clinically silent prostatic cancer. As others, we could not show a correlation between PSA levels and methylation level of *GSTP1* in prostate cancer patients [19]. These findings add further support to the notion that the latter can potentially serve as an independent marker for prostatic cancer. A high specificity of any test that may lead to invasive surgery, that is potentially quality of life limiting (urinary incontinence, erectile dysfunction), is inevitable. The test showed a specificity of 100%, which supports the usefulness that *GSTP1* hypermethylation as an independent test for diagnosis of prostate cancer. Larger studies, however, are necessary to proof the value of this test. Interestingly, Zhou et al. found a correlation between CpG island hypermethyltion and Gleason grade and cancer volume [33]. Although not shown in any of the other studies, including our data, this seems to be a promising marker for prostate cancer [14, 15, 16, 18, 19, 32]. Furthermore, the role of tumor volume in prostate cancer specimens as a independent prognostic marker remains controversial [34].

Recent reports suggest that CpG island hypermethylation in promotor regions of certain genes may be associated with aging [20, 21]. In our study we could not observe a age-related difference (<65 compared to > 65 years of age) in the methylation levels in the analysed samples.

Environmental factors such as diet, hormones, arsenic, and selenium have been reported to affect DNA methylation in experimental models [22]. Our series of

German patients with prostate cancer, however, show the same rates of *GSTP1* promotor hypermethylation as patients from other studies from the US or Asia [14, 15, 18, 19, 22, 24], although prostate cancer is not as common in Asia as it is in Western countries [25]. No correlation of CpG hypermethylation of the *GSTP1* gene between Blacks and Whites were detected in the US in a recent study [26]. This may indicate that, despite the different environmental settings in Germany, Asia and the United States, the hypermethylation of the *GSTP1* promotor does play an important role during prostate cancer development independent of the environmental setting.

The results of others studies using quantitative, real time PCR have shown that *GSTP1* hypermethylation can be helpful in the evaluation of prostate biopsy specimens [27, 31, 33]. Findings of *GSTP1* CpG island hypermethylation from urine and other bodily fluids have been reported with sensitivities approaching 75%. [28, 29].

Conclusion

GSTP1 hypermethylation distinguishes between benign prostatic hyperplasia and prostatic carcinoma. It demonstrates that quantitative real-time, methylation specific PCR detecting *GSTP1* hypermethylation may be a useful tool to develop future clinical management algorithms for the screening, diagnosis and treatment of prostate cancer. Larger studies are underway to determine the future role for *GSTP1* hypermethylation in clinical settings. Additionally, tumor specific methylation patterns, as shown for other types of human cancer, may contribute to current diagnostic tools and the clinical management of prostatic cancer [30, 32].

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