Hypermethylation of the CpG islands in the promoter region of the GSTP1 gene in prostate cancer: a useful diagnostic and prognostic marker?

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Abstract

Background: Recently, many studies have focused on the potential diagnostic value of the promoter hypermethylation of the GSTP1 gene in prostate cancer.

Method: A total of 144 patients, undergoing eight-core prostatic biopsies for a clinically suspected prostate cancer, was analyzed. Two different tissue samples were collected from the same area of the prostate and then divided for both genomic DNA extraction and pathological examination. In order to perform molecular analysis, prostatic tissue samples were digested with the methylation-sensitive restriction enzyme HpaII and then amplified by conventional polymerase chain reaction (PCR).

Results: Prostate cancer was diagnosed in 42/144 patients, and promoter hypermethylation of GSTP1 gene was detected in 31/42 of prostate cancer (sensitivity=74%) and in 2/102 of negative specimens (specificity=98%). A significant association between GSTP1 promoter hypermethylation both with a Gleason score ≥7 (Fisher’s exact P=0.01) and the presence of Gleason grade 4 and/or grade 5 (Fisher’s exact P=0.03) was found.

Conclusion: Promoter hypermethylation of the GSTP1 gene is a highly specific—but not a very sensitive—marker of prostate cancer. Our data showed a significant association between the methylation status of the GSTP1 gene and Gleason score and grade, suggesting a potential prognostic value of this epigenetic DNA alteration.

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1. Introduction

It is estimated that 230,110 new cases of prostate cancer will be diagnosed and 29,900 men will die of the disease in 2004 [1].

The risk of developing prostate cancer is strongly influenced by environmental exposures; therefore, the activity of many enzymes involved in cellular detoxification might be critical to determine individual susceptibility to neoplastic transformation and progression. In particular, glutathione transferase class P1-1 (GSTP1-1) could play a pivotal role, being a “caretaker” enzyme for prostatic cells [2]. GSTP1-1 (EC 2.5.1.18) belongs to a large family of functionally different enzymes, which catalyzes the $S$-conjugation of glutathione (GSH) with a wide variety of electrophilic compounds including carcinogens, anticancer drugs, reactive oxygen species, and products of cellular metabolism [3,4]. The soluble GSTs in human tissues are classified in eight classes: alpha, kappa, mu, pi, sigma, theta, zeta, and omega, some of which are present in multiple isoforms. GSTs are enzymes functionally active as homodimers or heterodimers formed between subunits of the same class. Even though the $S$-conjugation reaction protects the cell from cytotoxic and carcinogenic agents, much evidence shows that some classes might also be involved in anticancer drug resistance and even be considered as tumor markers [5,6]. Indeed, elevated levels of GSTP1-1 have been found in stomach, colon, bladder, testicular, breast, skin, acute myeloid and lymphoid leukemia, and lung tumors, compared with corresponding normal tissues [7–10].

It has been shown that the methylation of the 5′ region of the GSTP1 gene is the most common epigenetic alteration described in prostate adenocarcinoma [11–13]. Methylation of cytosines at the CpG islands within the 5′ region of the GSTP1 gene plays a decisive role in the control of gene expression, being associated with the loss of GSTP1 expression and, consequently, with a defi-

Table 1
Previous reports on the diagnostic value of GSTP1 promoter methylation

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ciency of GSTP1-1 activity. In human tumors, hypermethylation of normally unmethylated CpG islands is found in the promoter region of several tumor-suppressor genes, leading to the loss of their expression [14,15]. Prostatic epithelial cells with defective GSTP1 gene expression may be vulnerable to neoplastic transformation mediated by dietary components and other environmental factors, normally detoxified by GSTP1-1 enzymatic activity [2].

Methylation of the 5' region of GSTP1 has also been described in prostatic intraepithelial neoplasia (PIN) lesions, whereas GSTP1 is usually overexpressed in the prostate lesions termed “proliferative inflammatory atrophy” (PIA) [16].

Besides the interest about the pathogenetic role of GSTP1 methylation, this epigenetic modification could have a role as a diagnostic and prognostic molecular marker of prostate cancer. The diagnostic role of the methylation of GSTP1 gene 5' region has been the object of many studies (Table 1). Many methods of methylation-specific polymerase chain reaction (PCR) have been developed using both conventional and real-time PCR; however, almost all the studies have been performed on prostatic tissues obtained from surgical specimens that need to be confirmed on tissues obtained from diagnostic needle biopsies.

The aim of our study was to correlate the presence of CpG island hypermethylation of GSTP1 gene with the histological diagnosis of prostatic carcinoma and with the level of differentiation of the tumor (Gleason score and grade) using tissues obtained from diagnostic transperineal needle biopsies in order to determine the clinical relevance of this epigenetic modification and its possible role as a diagnostic and prognostic marker.

2. Patients and methods

2.1. Patients

A total of 144 patients (mean age 68 years, range 52–80 years) undergoing prostatic biopsy for a clinically suspected prostate cancer was analyzed. Indication for biopsy was a positive digital rectal examination, a serum PSA level >4 ng/ml, or a PSA ratio <0.10 in the presence of a PSA value between 2.5 and 4 ng/ml. Biopsies were performed via a transperineal approach using an eight-core technique. Two different tissue samples were collected from the same area of the prostate and then divided for both genomic DNA extraction and pathological examination. The study was approved by the local Ethical Committee. Informed consent was obtained from patients prior to the procedure.

2.2. Histology

Two pathologists reviewed all of the histological slides containing formalin-fixed, paraffin-embedded tissue fragments obtained from the prostatic biopsy specimens and graded each prostate tumor according to Gleason grading system [17]. The Gleason grade is based on histological criteria showing the level of differentiation of the prostatic cells. A Gleason grade ranges from 1 to 5, and the presence of the highest grades (grades 4 and 5) is associated with a poor prognosis and a more aggressive phenotype. The Gleason score is the sum of the primary (most predominant) Gleason grade and the secondary (second most predominant) Gleason grade. Where no secondary Gleason grade exists, the primary Gleason grade is doubled to arrive at a Gleason score. The Gleason score ranges from 2 to 10.

2.3. DNA isolation from tissue samples and neoplastic cell line

Genomic DNA was extracted from tissue samples, LNCaP, and DU145 human prostatic carcinoma cells using DNAeasy Tissue Kit Qiagen (Qiagen, Hilden, Germany) following the standard protocol provided by the manufacturer. LNCaP cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and have been reported to contain hypermethylated CpG islands in the GSTP1 gene [11]. DU145 cells were purchased from the American Type Culture Collection and have been reported to contain unmethylated CpG islands in the GSTP1 gene [18]. The cells were grown in vitro in RPMI 1640 with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate supplemented with 10 mM HEPES, 1 mM sodium pyruvate, and 10% FBS (Invitrogen, Paisley, UK). Cells were incubated at 37 °C with 5%
2.4. Enzymatic restriction

To detect the hypermethylation of CpG islands in the 5' promoter region flanking the GSTP1 gene, samples were digested with the restriction enzymes HpaII and MspI (New England Biolaboratories, Beverly, MA, USA) that recognize a DNA sequence CCGG.

The enzyme HpaII is methylation-sensitive and digests the sequence CCGG but not CC^5meGG, while MspI digests both the unmethylated and methylated sequences and was used to check the results.

Digestion was performed in a total volume of 10 μl containing 200 ng of genomic DNA, buffer 1×, and 10 U of enzyme. The mixture was incubated at 37 °C for 5 h. Digested DNA obtained from LNCaP was used as positive control for the presence of methylation, while DNA obtained from the DU145 cell line was used as negative control, being the 5' promoter region flanking the GSTP1 gene unmethylated in this prostatic adenocarcinoma cell line.

2.5. PCR amplification

Digested DNA was used as template for PCR amplification using specific primers for the 5' promoter region flanking the GSTP1 gene [19]. Digested DNA was incubated at 65°C for 20 min to obtain heat inactivation of the restriction enzyme before PCR. Primers sequences: fw 5' Cgg TCC TCT TCC TgC TgT CT 3'; rev 5' CgT ACT CAC Tgg Tgg CgA Ag 3'. The PCR was performed with a GeneAmp PCR system 9700 (Perkin Elmer Applied Biosystems, Foster City, CA, USA). PCR condition: reaction buffer 1×, 200 μM dNTPs, 10 pmol of each primer, 2.5 U of DNA Taq polymerase, 4% DMSO, and 200 ng of DNA/HpaII in a total volume of 25 μl. The cycling conditions were: preheating at 94 °C×5 min, 25 cycles (denaturation 94 °C×1 min, annealing 62 °C×1 min, extension 72 °C×1 min) and a final extension at 72 °C×7 min. All PCR reagents were purchased from Amersham Pharmacia Biotech UK (Buckinghamshire, England, UK).

2.6. Total prostate-specific antigen (PSA)

PSA serum levels were determined with an ordinary quantitative immunoassay (Elecsys System; Roche Diagnostics, Mannheim, Germany).

2.7. Statistics

Comparison of categorical variables (methylation and Gleason score and grade) was made using chi-square or Fisher’s exact tests, and the level of statistical significance was defined as p<0.05.

Comparison of logarithmically transformed total PSA serum levels of patients affected by prostate cancer with and without hypermethylation of the CpG islands in the 5' promoter region flanking the GSTP1 gene was made using unpaired Student’s t test.

The association between total PSA serum levels and Gleason score was studied by the mean of ANOVA test.

The sensitivity was calculated as true positive (TP)/TP+false negative (FN); specificity as true negative (TN)/TN+FP; and accuracy as TP+TN/all samples analyzed.

3. Results

3.1. Analysis of GSTP1 promoter hypermethylation

In order to investigate the hypermethylation of CpG islands in the 5' promoter flanking the GSTP1 gene, 144 DNA samples obtained from prostatic biopsies were analyzed. Prior to PCR amplification, each sample was digested with a methylation-sensitive restriction enzyme (HpaII) and an insensitive one (MspI). Both HpaII and MspI recognize six restriction sites in the 303-bp region amplified afterwards. The LNCaP cell line was used as a positive control, whereas the DU145 cell line was used as a negative control. In the presence of a hypermethylation in the considered region, HpaII digestion did not occur and therefore a 303-bp band was found, as shown by the positive control in Fig. 1 (panel A). In the absence of methylation, HpaII digestion did occur and therefore no PCR amplicon was generated, as shown by the negative control in Fig. 1 (panel A). To ensure that the results did not arise from mutations in this region, a
digestion with MspI was performed, considering that this enzyme recognizes and cleaves both methylated and unmethylated samples. Five of the 144 samples analyzed were shown in Fig. 1 (panel B).

Overall, prostate cancer was diagnosed in 66/144 (46%) patients. Considering only the tissue samples collected for molecular studies, prostate cancer was diagnosed in 42/144 patients and promoter hypermethylation of the GSTP1 gene was detected on 31/42 of prostate cancer (sensitivity=74%) and 2/102 of negative specimens (specificity=98%). Accuracy was 91% (131/144).

It should be pointed out that both the false-positive samples in the single biopsy fragments analyzed for methylation were diagnosed as prostate cancer in the overall histological analysis of all eight cores.

The methylation state of the 42 tumor samples was compared with Gleason score and grade was assigned by means of pathological diagnosis (Table 2).

We found a significant association between Gleason score ≥7 and the hypermethylation of the CpG islands in the 5' promoter flanking the GSTP1 gene ($\chi^2=0.008$, Fisher’s exact $P=0.01$). Moreover, we found a significant association between the presence of the single Gleason grade 4 and/or grade 5 and hypermethylation ($\chi^2=0.02$ Fisher’s exact $P=0.03$).

We found a significant association between PSA serum levels and Gleason score ($p=0.0017$), whereas
we did not find any significant differences between PSA serum levels of patients with prostate cancer with and without hypermethylation (mean F S.D. 19.7 F 31.8 and 10.7 F 15.0, respectively, p=0.1; median 9.8 and 5.8 ng/ml, respectively).

4. Discussion

Many studies have been performed to assess the presence of hypermethylation of the CpG islands in the 5′ promoter region flanking the GSTP1 gene in patients diagnosed with prostate cancer; however, almost all of them have been performed in tissues obtained from surgical specimens (Table 1).

The aim of our study was to correlate the presence of CpG island hypermethylation of the GSTP1 gene with the histological diagnosis of prostatic carcinoma and with the level of tumor differentiation using tissues obtained from diagnostic transperineal needle biopsies, in order to determine the diagnostic relevance of this epigenetic modification and its possible role as prognostic marker.

In our study, the percentage of positive GSTP1 gene methylation in patients with prostate carcinoma was 74%, with the percentage of positive methylation reported in the literature ranging from 36% to 100%. Eleven of 42 prostatic carcinoma DNA specimens analyzed failed to demonstrate methylation changes involving the GSTP1 promoter region. According to Chu et al. [19], we amplified a region including six methylation-sensitive HpaII restriction sites in the 5′ promoter region of the GSTP1 gene; thus, we cannot exclude the presence of other methylation sites in different portions of the GSTP1 regulatory region. On the other hand, in the presence of HpaII-digested samples, the method employed in our study does not permit us to know how many of the six HpaII sites are unmethylated, with one unmethylated site being sufficient to cleave the target sequence into fragments and to prevent its amplification. It has been suggested that the presence of false-negative results might arise from an incomplete inactivation of the restriction enzyme used before performing the PCR on the digested DNA [12]; to ensure that this did not happen in our samples, we heat-inactivated HpaII before PCR and we checked it at each digestion round using methylated LNCaP prostatic carcinoma cell lines as control (LNCaP’s DNA was used at the same concentration of the samples). Moreover, we used an annealing temperature of 62 °C, which has an intermediate stringency compared with those reported in studies with very low methylation rate (36%) [20] and very high methylation rate (90–100%) [12,19,21]. On the other hand, using our conditions, only 2/102 patients (2%) with negative biopsy showed methylated GSTP1 sequences; nevertheless, they were diagnosed with prostate cancer considering the overall histological analysis of all eight cores, showing a specificity of 100%. Finally, we cannot exclude the possibility that a greater sensitivity could be obtained using real-time PCR methods [19,22,23], even if, in this case, the methylation rate is highly influenced by the stringency of the threshold value.

Approaching the study of gene methylation and analyzing the different data reported in literature, it is clear that many variables have to be considered: the source of the sample (surgical specimen or needle biopsy), the preanalytical treatment of the DNA sample (digestion with methylation-sensitive enzyme or bisulfite treatment), the PCR conditions (especially the stringency of annealing temperature and the choice of the primers), and the type of PCR method (conventional or real-time).

In order to correlate clinical and pathological features, we divided the prostate cancer cases in two different groups on the basis of Gleason score (<7 and ≥7), according to a previous study [20]. We found a significant association between a total Gleason score ≥7 and the hypermethylation of the GSTP1 gene’s promoter region, as well as a significant association between the presence of a single Gleason grade 4 and/or grade 5 and the hypermethylation. Our data seem to

| Table 2 |
| Analysis of the hypermethylation of the CpG islands in the 5′ promoter region flanking the GSTP1 gene in 144 samples and association between the hypermethylation of the CpG islands in the 5′ promoter region flanking the GSTP1 gene and the Gleason score and grade in 42 prostate cancer samples from prostatic biopsy |
| Met+ | Met- |
| Histological diagnosis | Prostate cancer | 31 | 11 |
| Nonmalignant Prostatic tissue | 2 | 100 |
| Gleason score | ≥7 | 17 | 1 |
| <7 | 14 | 10 |
| Gleason grade | ≥4 | 18 | 2 |
| <4 | 13 | 9 |
suggest a role for GSTP1 gene methylation as a potential biological marker for aggressiveness in prostate cancer, as already reported [20]. Nevertheless, other authors, looking for a possible methylation index, reported an association with the Gleason score for other genes involved in cell cycle regulation (p16, signal transduction (APC, RassFIA), apoptosis (RUNX3), and other cancer-related genes (Timp-3), but not for GSTP1 [24,25].

To better understand the clinical usefulness of GSTP1 gene methylation, its role as a molecular marker should not be distinguished from the pathogenetic one. What triggers DNA methylation changes in the cancer cell is still unanswered, even if an interesting model has recently been proposed. This model, named “the seeding and silencing model,” suggests that a low-level CpG sporadic methylation is in constant flux and might promote the GSTP1 gene hypermethylation only if the gene is inactivated, either transiently or via an independent oncogenic process [18]. If this is the case, the “density of CpG methylation” might be critical to reach the threshold of CpG methylation necessary to silence the GSTP1 gene and this event has to be considered by looking at the transcriptional activity of the gene. In addition, it should be considered the role of stromal expression of GSTP1 gene that seems to characterize the microenvironment of the tumor during progression to hormone independence [26].

Recent findings about the study of GSTP1 gene methylation in prostatic secretion from surgical specimens [27] and in urine after prostatic massage [28] suggest that the study of bodily fluids might be an interesting development in prostatic cancer prevention strategies. In this context, prostatic secretions obtained by means of prostatic massage could be the future step.

In conclusion, promoter hypermethylation of the GSTP1 gene is a highly specific—but not a very sensitive—marker of prostate cancer. We found a significant association between the methylation status of the GSTP1 gene and Gleason score as well as the presence of a single Gleason grade 4 and/or grade 5, suggesting its potential prognostic value in patients affected by prostate cancer. Nevertheless, in our opinion, the study of the hypermethylation of the promoter region of the GSTP1 gene should still be interpreted with caution, and the overall biological context of prostate carcinoma, as well as the efficiency of each laboratory technique employed, should be better understood before being utilized in the current clinical practice.

References


