II05V Polymorphism and Promoter Methylation of the GSTP1 Gene in Prostate Adenocarcinoma

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Abstract

The GSTP1 gene encodes for an enzyme, glutathione S-transferase π (GSTπ), involved in detoxification of carcinogens. An amino acid substitution (II05V) in GSTP1 produces a variant enzyme with lower activity and less capability of effective detoxification. This variant GSTP1*B allele has been associated with a propensity to develop several neoplasms. Because GSTP1 promoter hypermethylation and inactivation of GSTP1 expression is a frequent alteration in prostate carcinoma, we hypothesized that this somatic epigenetic modification could obviate any reduced enzyme activity caused by the germ-line polymorphism. We tested for the GSTP1 genotype in a population of prostate cancer patients, and in a control group composed of patients with benign prostatic hyperplasia (BPH) and healthy blood donors. Tissue samples from the 105 prostate cancer cases (105 adenocarcinomas and 34 prostatic intraepithelial neoplasia lesions), and from 43 BPH patients were tested for GSTP1 hypermethylation by methylation-specific PCR. GSTπ protein expression was assessed by immunohistochemistry. No significant effect on prostate cancer risk was detectable for GSTP1 genotype compared with the control population (odds ratio, 1.02; 95% confidence interval, 0.59–1.75). Moreover, no association was found between this genotype and tumor or BPH methylation status. Patients with unmethylated carcinomas did not disclose significant differences in genotypic distribution compared with the control population. In adenocarcinoma, a strong association (P < 0.00001) between GSTP1 promoter hypermethylation and loss of GSTπ expression was observed; however, this trend was not retained in prostatic intraepithelial neoplasia or BPH lesions. Although the GSTP1 polymorphism is not associated with altered susceptibility to prostate cancer, somatic promoter hypermethylation is an effective, but not the only, cause of decreased GSTπ function.

Introduction

Prostate adenocarcinoma is the most frequently diagnosed cancer among men in the Western world, and the second leading cause of cancer death among men in the United States (1). Etiologically, prostate cancer is a multifactorial disease in which several environmental and genetic factors may be involved, yet little is known about the interaction between these factors (2). Moreover, the role of epigenetic phenomena, such as DNA de novo methylation, in the modulation of gene expression, has become a major focus of investigation in the field of prostate cancer research (3).

GSTP1, located at 11q13, belongs to a supergene family of enzymes, the GSTs, involved in the detoxification of electrophilic compounds (such as carcinogens and cytotoxic drugs) by glutathione conjugation (4, 5). In addition, these enzymes are believed to play a role in the protection of DNA from oxidative damage (6). GSTP1 has a polymorphic site at codon 105 (exon 5), where an adenosine-to-guanosine (A-G) transition causes an iso-to-Val substitution (II05V), giving rise to the GSTP1*B allele (4–7). Moreover, recent studies have found that individuals with the valine allele display a significantly lower enzyme activity and less effective capability of detoxification (8).

Hence, an association between GSTP1*B allele and lung, bladder, and testicular neoplasms has been reported (6, 7). A significant decrease in the frequency of GSTP1*A has been reported in prostate cancer patients compared with controls (7), but these results have been challenged by other studies, using larger series of patients (9–12).

Over the last few years, several studies revealed that GSTP1 (usually expressed in normal human epithelial tissues, including prostate) could be somatically inactivated by hypermethylation of the promoter region (13–15). This alteration, which is often associated with a loss of GSTπ expression, is the most common event (~90%) described thus far in prostate adenocarcinoma (15, 16). Furthermore, GSTP1 inactivation may lead to an increased cell vulnerability to oxidative DNA damage and to the accumulation of DNA base adducts, which...
can make a tumor cell move to acquire other relevant genetic alterations in prostatic carcinogenesis (17, 18). Thus, we hypothesized that this somatic epigenetic modification could obviate any effect on the differential enzyme activity caused by allelic variants and could perhaps explain the conflicting reports on the effect of the GSTP1 polymorphism in prostate cancer risk.

We first investigated the association between the I105V GSTP1 polymorphism and the risk for developing prostate cancer, to investigate a primary genotypic effect on prostate cancer susceptibility. Then, we searched for a possible association between this polymorphism and de novo methylation in a relatively large series of early-stage (clinically localized) prostate cancer patients (19, 20). Finally, immunohistochemical analysis was done to determine whether GSTP1 hypermethylation affects gene expression.

Materials and Methods

GSTP1 Polymorphism

Blood Samples and DNA Extraction. For this study, two populations of male subjects were enrolled at the Portuguese Institute of Oncology, Porto, Portugal. One population consisted of 105 patients with histologically confirmed adenocarcinoma. The control population comprised 43 patients with BPH, and 98 healthy male volunteer blood donors from the same institution. Blood was collected from all of the individuals, and genomic DNA was extracted from fresh peripheral leukocytes as described previously (21). Briefly, DNA was digested overnight at 48°C in 1% SDS/proteinase K (0.5 mg/ml), extracted with phenol-chloroform, and ethanol precipitated.

GSTP1 Genotype Analysis. The exon 5 polymorphic site in GSTP1 locus (Ile-105→Val) was detected by restriction fragment length polymorphism of PCR-amplified fragments. The primers used were: P105 forward, 5′-ACC CCA GGG CTC TAT GGG AA-3′, and P105 reverse 5′-ACC CCA GGG CAC AAG AAC CCC CT-3′ (7). PCR reactions were carried out in a 30-μl volume containing about 50 ng of genomic DNA template, 200 μM each dNTP, 200 ng each primer, 1.5 mM MgCl₂, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), and 0.01% H₂O₂ in PBS, respectively. The PCR products were directly loaded onto a nondenaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination.

Methylation Analysis

Patients and Tissue Sample Collection. All of the patients from the prostate cancer group harbored clinically localized prostate adenocarcinoma (T₁c, according to the TNM staging system (22)), and were consecutively diagnosed and treated with radical prostatectomy. The 43 patients with BPH were submitted to TURP and harbored no histological evidence of malignancy. Two pathologists (R. H., C. L.) reviewed all of the histological slides, and each tumor was graded according to the Gleason grading system (23). Fresh tissue, snap-frozen in isopentane and stored at −80°C, or paraffin-embedded prostatic tissue was collected from each surgical specimen. Sections were cut for the identification of areas of high-grade PIN and adenocarcinoma (radical prostatectomy specimens), and BPH (TURP tissue). These areas were then carefully microdissected from 12-μm-thick sections for enrichment of PIN, adenocarcinoma, and hyperplastic tissue. On average, 50 sections for each area with enrichment (>70%) in neoplastic cells were used for DNA extraction of PIN or cancer. Paraffin-embedded tissue was similarly microdissected but was placed in xylene for 3 h at 48°C to remove the paraffin. DNA was extracted using the method described by Ahrendt et al. (21).

Bisulfite Treatment. Sodium bisulfite conversion of 2 μg of genomic DNA was performed by a modification of a previously described method (24). Briefly, NaOH was added to denature DNA (final concentration, 0.2 M) and incubated for 20 min at 50°C. A volume of 500 μl freshly made bisulfite solution [2.5 M sodium metabisulfite and 125 mM hydroquinone (pH 5.0)] was added to each sample, and incubation was continued at 50°C for 3 h in the dark. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer (Promega Corp., Madison, WI) and was eluted in 45 μl of water at 80°C. After treatment with NaOH (final concentration, 0.3 M) for 10 min at 37°C, isolation was continued with 75 μl of 7.5 M ammonium acetate, followed by an incubation step of 5 min at room temperature. Finally, the modified DNA was precipitated by adding 2.5 volumes of 100% ethanol and 2 μl of glycogen (5 mg/ml). The pellet was washed with 70% ethanol, dried, and eluted in 30 μl of 5 mM Tris (pH 8.0).

MSP Analysis. For PCR amplification, 2 μl of bisulfite-modified DNA was added in a final volume of 25 μl of PCR mix containing 1× PCR buffer [16.6 mM ammonium sulfate/67 mM Tris (pH 8.8) and 6.7 mM MgCl₂/10 mM 2-mercaptoethanol], dNTPs (each at 1.25 mM), 1 unit Platinum Taq DNA polymerase (Life Technologies, Inc., Rockville, MD) and primers (300 ng each per reaction). Primer sequences for either methylated or modified unmethylated GSTP1 have been described previously (25). MSP was carried out using the following conditions: 1 cycle at 95°C for 1 min; 35 cycles of 1 min 94°C for 1 min 62°C for 1 min 72°C for 1 min; 35 cycles of 1 min 95°C, 1 min 62°C, and 1 min 72°C; and a final extension for 5 min at 72°C. In each performed PCR, treated DNA extracted from two prostate cancer cell lines, the LNCaP and DU145 were used as positive and negative controls, respectively. The PCR products were directly loaded onto a nondenaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination.

Immunohistochemical Analysis

Four-μm sections were cut and placed in aminopropyltriethoxysilane (Sigma) coated slides. After dewaxing the sections, endogenous peroxidase activity was inhibited with freshly prepared 0.5% hydrogen peroxide in distilled water for 20 min. Then, they were processed in a 600-W microwave oven at maximum power, three times for 2 min, each time in citrate buffer (pH 6). Immunostaining was performed using an immunoperoxidase method according to the manufacturer’s instructions (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). The incubation of the primary anti-GST-π antibody (clone 3 BD; Transduction Laboratories, Lexington, KY) was performed overnight at 4°C, at a dilution of 1:250 in 1% BSA in PBS. Sections were developed with a peroxidase substrate solution (0.05% 3,3-diaminobenzidine tetrahydrochloride, 0.01% H₂O₂ in PBS), counterstained with hematoxylin, dehydrated, and mounted. Appropriate positive controls were used for each antibody, and negative controls consisted of the replacement of the primary antibody for 1% BSA in PBS.

Assessment of GST-π expression was performed by light microscopy at ×400. The presence or absence of immuno-
staining was evaluated in morphologically normal areas, PIN lesions, and tumor, as well as in BPH samples.

Statistical Analysis
The odds ratio and 95% confidence intervals were calculated as a measure of the association between GSTP1 genotype and the risk of development of prostate cancer. Associations between GSTP1 genotype and methylation status, as well as any correlation between GSTP1 methylation and GSTP1 expression were examined using the $\chi^2$ test, and Fisher’s exact test, when appropriate. Analyses were conducted using a computer-assisted program, Epi Info, version 6 (Centers for Disease Control and Prevention, Atlanta, GA). Statistical significance was reached when $P < 0.05$.

Results
Population Characteristics and Distribution of GSTP1 Genotypes
One hundred five early-stage prostate cancer patients and 141 controls (43 patients with BPH and 98 healthy male volunteers) were recruited for this study. The median age was 63 years (range, 48–74) and 56 years (range, 45–82), for prostate cancer cases and controls, respectively. The age distributions differed significantly between the two groups ($P < 0.00001$).

Table 1 depicts the frequency distribution of each GSTP1 genotype (Fig. 1) among the prostate cancer cases and controls considered for this study, and no statistically significant difference was found ($P = 0.32$). Concerning the control group, the frequency distribution of GSTP1*A/A, GSTP1*A/B, and GSTP1*B/B among the BPH patients was 16 (37.2%), 24 (55.8%), and 3 (7%), respectively. For the blood donor population, the frequency distribution for the same genotypes was, respectively, 45 (45.9%), 43 (43.9%), and 10 (10.2%). Statistical analysis did not disclose a significant difference in genotype distribution between the BPH patients and blood donors ($P = 0.42$). No significant effect on prostate cancer risk was detectable for the GSTP1 genotype (odds ratio, 1.02; 95% confidence interval, 0.59–1.75) compared with the control population.

Methylation Analysis
The results of the methylation analysis are displayed in Table 2. MSP PCR identified 89 (84.8%) of 105 adenocarcinomas and 17 (50%) of 34 paired high-grade PIN lesions as positive for GSTP1 methylation (Fig. 2). Moreover, 9 (20.9%) of 43 patients with BPH also displayed GSTP1 methylation.

No statistically significant association was found between GSTP1 genotype and tumor methylation status ($P = 0.64$). When the genotype distribution of the patients with unmethylated tumors (16 cases) was compared with the control population, no statistically significant association was found either ($P = 0.80$). The same trend was observed when GSTP1 genotype and BPH methylation status were analyzed ($P = 0.71$).

Immunohistochemical Analysis
In normal and hyperplastic tissue, GSTP1 mRNA expression was always present in basal cells (Fig. 3A). This staining was mainly cytoplasmic but nuclear staining was also a frequent finding. Luminal secretory cells displayed much weaker and inconsistent staining than basal cells. No difference in immunostaining was observed between BPH cases with or without GSTP1 hypermethylation.

Table 3 shows the immunohistochemical findings in prostate adenocarcinoma and PIN lesions. Of the 99 tumors that lacked GSTP1 expression, 89 (89.9%) displayed GSTP1 promoter hypermethylation (Fig. 3A), whereas none of the six tumors that expressed GSTP1 were methylated at the GSTP1 locus (Fisher’s exact test, $P < 0.00001$). Tumors expressing GSTP1 showed cytoplasmic, but no nuclear, staining, except in one case in which there was strong nuclear immunoreactivity (Fig. 3B). In PIN lesions, cytoplasmic staining was observed in 7 (20.6%) of 34 cases, and 5 of these cases showed GSTP1 hypermethylation (Fig. 3D). In the remaining PIN lesions, immunostaining was observed in basal cells but not in the dysplastic luminal cells (Fig. 3C).

Discussion
Although there is an increasing body of evidence suggesting an association between the 1105V polymorphism in GSTP1 and cancer susceptibility in bladder, testicle, breast, and lung (6, 19, 26), the influence of this same polymorphism in prostate cancer risk remains controversial (7, 9–12).

In the present study, we found no evidence of a differential risk for prostate adenocarcinoma among men possessing the Iso or Val variants of codon 105 of GSTP1. This result is in accordance with most previously published studies (9–11), but is in disagreement with the recent report of Gsur et al. (12). However, a major limitation of our work and the latter study is the control population. Gsur et al. used 166 age-matched control patients with BPH, and we used both a group of BPH patients and a group of healthy blood donors. In this study, the observed difference in age distribution between the blood do-

![Image](https://example.com/image.png)
Fig. 2. Illustrative example of MSP for GSTP1 promoter region in prostate tissues from nonneoplastic areas (MN), and clinically localized prostate adenocarcinoma (T) of patients 54 and 90. Lanes U and M correspond to unmethylated (97 bp) and methylated (93 bp) reactions, respectively. In each case, DNA from normal lymphocytes was used as negative control for methylation (U+), DNA from LNCaP cell line was used as positive control for methylation (M+), and water was used as negative PCR control (H2O). On the right side, the 100-bp DNA ladder.

Fig. 3. GSTπ immunoeexpression in the prostate. A, a case with GSTP1 promoter methylation and absence of GSTπ expression. on the left side, a normal gland showing strong immunoreactivity in basal cells. B, an adenocarcinoma without GSTP1 promoter methylation and with strong immunoreactivity for GSTπ, both cytoplasmic and nuclear. C, a nonmethylated PIN lesion with no immunoreactivity for GSTπ in the dysplastic columnar cells, although the basal cells are negative. D, a GSTP1-methylated PIN lesion shows immunoreactivity for GSTπ in all cell types (basal and columnar epithelial).
nors and the cancer patients could be potentially problematic because of the latency of prostate cancer. The inclusion of a group of BPH patients partially overcomes this problem, and no difference in GSTP1 genotype frequencies between the control group and the prostate cancer patients was noted.

Another potential limitation of our study would be the exclusion of patients with advanced-stage prostate cancer, because these patients are not candidates for radical prostatectomy procedures. In this study, only patients with clinically localized disease, submitted to radical prostatectomy were included, and thus, this group would not reflect the true incident population at risk for prostate cancer. However, because no association between GSTP1 genotype and stage in prostate cancer patients has been found (11), this potential limitation is obviated.

To the best of our knowledge, this study is the first ever to report the frequency distribution of GSTP1 genotypes in a Portuguese population. Furthermore, it is noteworthy that the genotype frequencies found in our series of patients and controls fall in the expected range for a Western European population (7, 27, 28). Although the control populations in some of these studies included women (27, 28), no gender influence in GSTP1 genotype distribution has been reported thus far.

GSTP1 promoter hypermethylation is a frequent alteration in prostate cancer cells and is associated with gene silencing and decreased GSTπ expression (15, 16). Thus, we hypothesized that this epigenetic modification could obviate the difference in enzyme activity caused by the I105V polymorphism, unless the polymorphism itself would influence GSTP1 promoter methylation status. MSP analysis of GSTP1 promoter hypermethylation in prostate adenocarcinoma tissue samples obtained from the PA group disclosed a high percentage of methylated tumors (84.9%), which is in accordance with previously published results (13, 14, 19, 20). Moreover, no association was found between GSTP1 hypermethylation and the GSTP1 genotype. The A–G substitution (1105V polymorphism) occurs at position 1578 (exon 5), and GSTP1 hypermethylation associated with prostate cancer takes place at the gene promoter region (5–8, 16). In fact, exon 5 is enriched in methylated CpG sites even in normal tissue (16), and, thus, it would be unlikely that an A–G substitution so far downstream would influence methylation in the gene promoter.

If, hypothetically, a low GSTπ activity caused by allelic variation (e.g., I105V polymorphism), were linked to prostate cancer pathogenesis, then the patients with unmethylated tumors would be expected to show a higher frequency of the I105V polymorphism. Our results do not sustain this hypothesis because the genotype distribution of the patients with unmethylated tumors (16 cases) did not differ significantly from the control population. Yet, this finding does not exclude a role for GSTP1 in prostate carcinogenesis because other mechanisms responsible for a decrease in GSTπ activity may exist, as demonstrated by the lack of immunoreactivity for GSTπ in 10 of these 16 unmethylated carcinomas.

To confirm the regulation of GSTP1 promoter hypermethylation in GSTπ expression, we performed an immunohistochemical analysis in the radical prostatectomy and TURP specimens from our patients. The immunohistochemical findings confirm that GSTP1 promoter hypermethylation is related to the loss of GSTπ expression in prostate cancer, because all methylated tumors lacked GSTπ. Previous studies reached the same conclusion (13, 16), and similar findings were also reported in breast cancer (25). However, a novel finding from our study is the lack of GSTπ expression in 10 primary tumors not displaying GSTP1 methylation. This result suggests that alternative mechanisms for GSTP1 transcription inactivation may occur in addition to promoter hypermethylation. Interestingly, some prostate cancer cells displaying GSTπ immunoreactivity have been found to lack enzyme activity (16). Moreover, loss of expression of GSTπ associated with GSTP1 promoter methylation has been identified in potential precursor lesions such as PIN (15). Hence, a loss of GSTπ function appears to play a critical role in the early steps of prostate carcinogenesis.

For this reason, we also analyzed 34 PIN lesions from the radical prostatectomy specimens. GSTP1 hypermethylation has been reported in 50–70% of PIN lesions (15, 20), and other researchers were unable to detect GSTπ expression in this preneoplastic condition (29). However, we found immunoreactivity for GSTπ in 7 of 34 cases, 5 of which were methylated at the GSTP1 promoter region. This result may be related to the difference in GSTP1 methylation levels found between PIN and adenocarcinoma (20). In this respect, it is noteworthy that all of the methylated BPH lesions herein analyzed, expressed GSTπ, and these lesions also displayed a significantly lower level of GSTP1 methylation (20). These findings favor the existence of a critical level of methylation for the silencing of GSTP1.

Different patterns of GSTπ expression in PIN lesions from the transition and the peripheral zone of the prostate have been previously reported (30). In the present study only PIN lesions from the peripheral zone were analyzed, but even lesions therein located were found to differentially express GSTπ in that report (30). In our study, the tissue samples microdissected for methylation analysis and those used for immunohistochemical analysis in adenocarcinoma, PIN, or BPH lesions) were very closely colocated. Thus, it is unlikely that methylation results and immunohistochemical findings in each case reflect different foci of a given type of lesion. Although genetic heterogeneity of PIN lesions is acknowledged (31), no data concerning variation in GSTP1 promoter methylation status across the same prostate specimen has been reported to date.

Our results confirm that GSTP1 promoter hypermethylation is a highly prevalent event in prostate cancer; it is already present in potential precursor lesions and is tightly linked to GSTπ loss of expression. Moreover, the I105V GSTP1 polymorphism is not associated with hypermethylation in the promoter region nor related with altered susceptibility to prostate cancer. These findings suggest that common somatic GSTP1 inactivation obviates any major effects from inherited genotypic variants in prostate cancer progression.

**Acknowledgments**

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### Table 3

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>GSTπ immunoexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Present</td>
</tr>
<tr>
<td>Prostate adenocarcinoma</td>
<td>105</td>
<td>89 (0%)</td>
</tr>
<tr>
<td>GSTP1 methylated</td>
<td>89</td>
<td>6 (37.5%)</td>
</tr>
<tr>
<td>GSTP1 unmethylated</td>
<td>16</td>
<td>6 (37.5%)</td>
</tr>
<tr>
<td>PIN lesions</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>GSTP1 methylated</td>
<td>17</td>
<td>5 (29.4%)</td>
</tr>
<tr>
<td>GSTP1 unmethylated</td>
<td>17</td>
<td>2 (11.8%)</td>
</tr>
</tbody>
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References

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