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DNA Methylation of *GSTP1* in Human Prostate Tissues: Pyrosequencing Analysis

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Purpose: DNA methylation is an important epigenetic mechanism of gene regulation and plays essential roles in tumor initiation and progression. Differences in methylation patterns between neoplastic and normal cells can be used to detect the presence of cancer. The aim of the present study was to evaluate the usefulness of *glutathione-S-transferase-Pi (GSTP1)* hypermethylation in discriminating between normal and prostate cancer (PCa) cells and in predicting tumor characteristics by use of quantitative pyrosequencing analysis.

Materials and Methods: A total of 100 human prostate tissues obtained from our institute were used in this study: 45 for benign prostatic hyperplasia (BPH) and 55 for PCa. The methylation level of *GSTP1* was examined by a quantitative pyrosequencing analysis. The associations between *GSTP1* methylation level and clinico-pathological parameter were also compared.

Results: The level of *GSTP1* methylation was significantly higher in PCa samples than in BPH samples (56.7±32.7% vs. 1.6±2.2%, $p < 0.001$). The sensitivity and specificity of *GSTP1* methylation status in discriminating between PCa and BPH reached 85.5% and 100%, respectively. Even after stratification by stage, Gleason score, and prostate-specific antigen (PSA) level, similar results were obtained. A positive correlation between *GSTP1* methylation level and serum PSA level was observed (r=0.303, p=0.002). There were no associations between *GSTP1* methylation level and age, Gleason score, and staging.

Conclusions: Our study demonstrates that *GSTP1* methylation is associated with the presence of PCa and PSA levels. This methylation marker is a potentially useful indicator for the detection and monitoring of PCa.

Key Words: *GSTP1; Methylation; Neoplasms; Prostate*

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INTRODUCTION

Prostate cancer (PCa) remains a major medical health issue representing one of the frequent cancers among men. The discovery and widespread utilization of serum prostate-specific antigen (PSA) monitoring for early detection has greatly changed the way PCa is diagnosed and has resulted in the downstaging of the disease [1-5]. Active PCa screening with the use of serum PSA level monitoring and digital rectal examination is now widely prevalent in many countries [1,2]. The gold standard for PCa diagnosis is histopathologically reviewed prostatic biopsy cores. This technique, although generally effective, is subject to sampling errors and a significant false-negative rate [1,2]. Thus, the development of new tumor markers with better sensitivity and specificity to detect early cancer remains vitally important.

DNA hypermethylation-induced silencing of tumor suppressor and DNA repair genes is a frequent phenomenon affecting the hallmarks of cancer [6-9]. The genes under-

going methylation during the early phases of tumorigenesis could potentially be used as markers for identifying individuals at increased risk of developing malignancy or for aiding in the diagnosis of early malignancy, whereas those genes undergoing methylation during the progression of malignancy could potentially be used as prognostic markers [6-9]. These hypermethylation markers are promising tools for detecting cancer cells in tissue and body fluids, and various approaches for the detection of aberrant DNA methylation regions have been developed in recent years [7-10]. Most previous studies used methylation-specific polymerase chain reaction (MSP) or quantitative methylation-specific polymerase chain reaction (QMSP) to assess methylation status and often artificially categorized the data into simplistic hypermethylated or hypomethylated categories [7-9]. Despite common use, these methods have known disadvantages such as false-positive and false-negative results as well as the use of relative comparisons instead of absolute quantification [11,12]. To quantify DNA methylation, pyrosequencing (PSQ) is one of the most accurate techniques available [7,11,12].

CpG island methylation of *glutathione-S-transferase-Pi* (*GSTP1*) has been detected in several cancer types, including breast and hepatocellular cancer, but only in PCa is this abnormality consistently detected with a high specificity and rarely found in patients without disease [13-18]. The aim of the present study was to evaluate the relevance of *GSTP1* methylation status in discriminating between benign prostatic hyperplasia (BPH) and PCa as well as its association with clinico-pathological parameters by use of a quantitative PSQ analysis.

MATERIALS AND METHODS

1. Human tissue samples

A total of 100 human prostate tissue samples obtained from our institute were used in this study: 45 for BPH and 55 for PCa. Patients with PCa underwent radical prostatectomy or palliative transurethral resection (TUR), and patients with BPH underwent TUR. All tissues were macro-dissected within 15 minutes of surgical resection. Each prostate specimen was confirmed by pathological analysis of fresh-frozen sections, and the rest of the tissue was frozen in liquid nitrogen and stored at -80°C until use. The collection and analysis of all samples were approved by the local institutional review board, and informed consent was obtained from each subject.

2. DNA extraction and pyrosequencing analysis

Genomic DNA was extracted by standard methods by use of the Wizard Genomic DNA Purification System (Promega, Madison, WI, USA). Bisulfite modification of genomic DNA (500 ng) was performed with an EZ DNA methylation kit (Zymo Research, Orange, CA, USA) according to the manufacturer's recommendation. *GSTP1* methylation was examined by PSQ. Polymerase chain reaction (PCR) and sequencing primers were designed with PyroMark Assay design ver. 2.0.1.15 (Qiagen, Valencia, CA, USA). The PSQ assay was designed to evaluate the methylation status of seven CpG sites. The total length of the amplicon was 199 bp and the following primers were used: forward 5'-GAGTTAGAGGGATTTTTTAGAAGAG-3' and reverse biotinylated 5'-CAATTAACCCCATACTAAAAACTCT-3'. The PCR thermal cycling conditions were as follows: denaturation at 94°C for 5 minutes; 45 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 5 minutes. PCR reactions were carried out with 1 μl of bisulfite-converted genomic DNA for all prostate tissues. A biotin-labeled primer (reverse primer) was used to purify PCR product by use of streptavidin-coated Sepharose beads (GE Healthcare, Buckinghamshire, UK). PCR product was bound to Sepharose beads, purified, washed, denatured by using a 0.2 mol/l NaOH solution, and washed again. Then 0.3 μmol/l pyrosequencing primer (5'-GGGAGGGATTATTTTTATAAGG-3') was annealed to the purified single-stranded PCR product and the pyrosequencing was performed on a PyroMark Q96 ID (Qiagen) according to the manufacturer's instructions. To provide the internal control for total bisulfite conversion, a non-CG cytosine in the region for PSQ was included where possible. Target CpG sites were evaluated by use of instrument software (PSQ96MA 2.1, Qiagen) that converts the pyrograms to numerical values for peak heights and calculates the proportion of methylation at each base as a C/T ratio. Data analysis was performed by using PyroMark Q96 ID ver. 1.0 (Qiagen).

3. Statistical analysis

We compared the quantitative *GSTP1* methylation level and its association with clinico-pathological characteristics in BPH and PCa patients. The methylation level of each sample was expressed as the mean value (the sum of the methylation level of each CpG site, n=7). The differences in continuous variables between the groups were assessed by use of two-sample t-tests. Categorical variables were compared by using the chi-square test. The optimal sensitivity and specificity with DNA methylation of *GSTP1* for discriminating between PCa and BPH was determined by receiver operator characteristic (ROC) curve analysis by use of MedCalc software (MedCalc Software, Mariakerke, Belgium). The area under the ROC curve and the methylation threshold yielding the optimal sensitivity and specificity for the prediction of PCa were calculated. By use of the same thresholds, the sensitivity, specificity, positive predictive value, and negative predictive value were also calculated. Pearson's correlation was used to evaluate the relation between the *GSTP1* methylation level and clinico-pathological parameters. Tests for trend were performed by analysis of variance trend analyses by using polynomial contrasts. For statistical purposes, PCa patients were divided into subgroups according to the following clinico-pathological parameters: 1) Gleason score ($GS \leq 6$, GS=7, and $GS \geq 8$), 2) stage (T2, T3, and T4), and 3) PSA level at diagnosis ($PSA < 10$, 10 to 20, and ≥ 20 ng/ml).

Statistical analysis was performed by using SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA), and a p-value of <0.05 was considered statistically significant.

RESULTS

1. Baseline characteristics

The baseline characteristics of the patients are presented in Table 1. The mean age and total prostate volume were not significantly different between the BPH and PCa groups. PCa patients had elevated levels of PSA compared with BPH patients.

2. Methylation level in prostate tissues

Fig. 1 shows an example of the pyrograms in BPH and PCa patients. The methylation level of *GSTP1* was significantly higher in PCa samples than in BPH samples (56.7±32.7% vs. 1.6±2.2%, $p<0.001$) (Fig. 2A). In the ROC analysis, the area under the curve of the *GSTP1* methylation level for the prediction of PCa reached 0.908 (95% confidence interval [CI], 0.834 to 0.957) (Fig. 2B). For well-balanced sensitivity and specificity, the *GSTP1* methylation value corresponded to a cutoff of 13.3%. That cutoff showed 85.5% sensitivity (95% CI, 73.3 to 93.5%), 100% specificity (95% CI, 92.1 to 100%), 100% positive predictive value (95% CI, 92.5 to 100%), and 84.9% negative predictive value (95% CI, 72.3 to 93.3%), respectively. Patients were divided into hypermethylated and unmethylated groups by use of a cutoff level. As presented in Table 2, *GSTP1* was methylated in 85.5% (47/55) and 0% (0/45) of PCa and BPH samples, respectively. Similar results were obtained even after stratification by PSA level, stage, and Gleason score.

TABLE 1. Baseline characteristics of the patients included in the study

	BPH (n=45)	PCa (n=55)	p-value
Age			
Range	68.6±8.2	70.1±6.8	0.333
PSA (ng/ml)	4.9±5.7	149.1±325.0	0.002
Total prostate volume (ml)	36.7±17.3	39.5±17.1	0.430
Source of tissue (%)			
TUR	45 (100)	33 (60.0)	
Prostatectomy		22 (40.0)	

Values are presented as mean±standard deviation or number (%).
BPH, benign prostatic hyperplasia; PCa, prostate cancer; PSA, prostate-specific antigen; TUR, transurethral resection.

3. Association between methylation level and clinico-pathological parameters in PCa patients

To evaluate the relation between *GSTP1* methylation level and clinico-pathological parameters, we performed a correlation analysis. No correlation was found between *GSTP1* methylation level and age (r=0.077, p=0.445) or Gleason score (r=-0.025, p=0.859). In contrast, we found a positive correlation between *GSTP1* methylation level and serum PSA level (r=0.303, p=0.002). We also compared the *GSTP1* methylation levels on the basis of the corresponding clinico-pathological parameters (Gleason score [GS≤6, GS=7, and GS≥8], stage [T2, T3, T4], and PSA level [PSA<10, 10 to 20, ≥10 ng/ml]). *GSTP1* methylation levels increased similarly with increasing PSA level (p trend<0.001) (Fig. 3), whereas no trend was found between *GSTP1* methylation level and Gleason score or stage (p trend>0.05, respectively).

DISCUSSION

Our results demonstrated that the methylation level of *GSTP1* was significantly higher in PCa patients than in

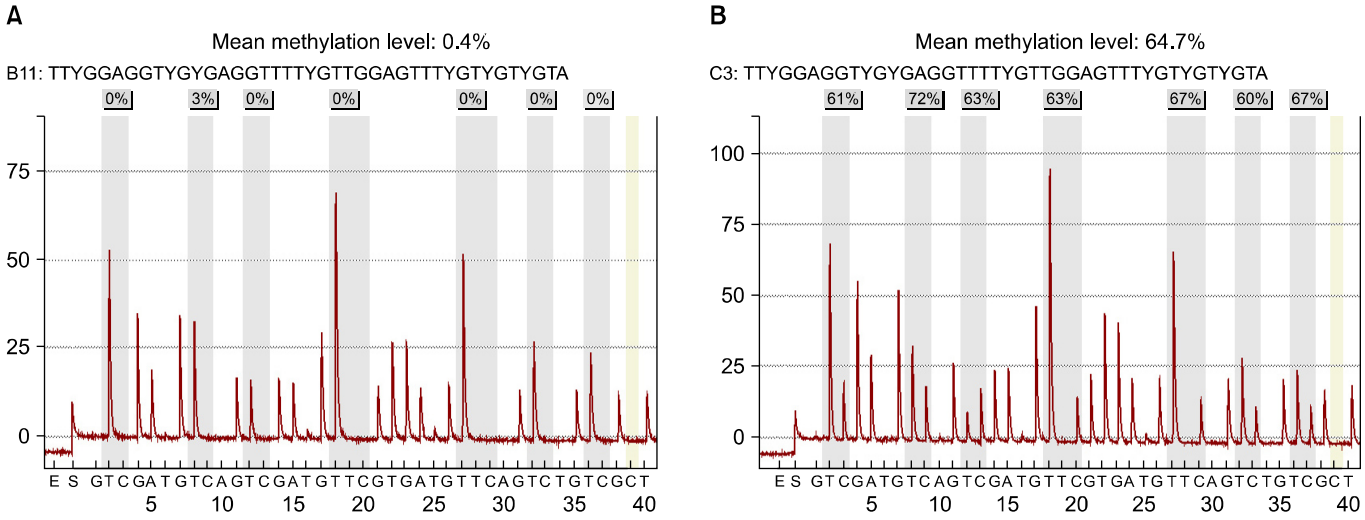

FIG. 1. An example of *GSTP1* pyrograms in benign prostatic hyperplasia (A) and prostate cancer (B) patients. Pyrogram includes 7 CpG sites and methylation rates. The methylation level of *GSTP1* is expressed as a mean.

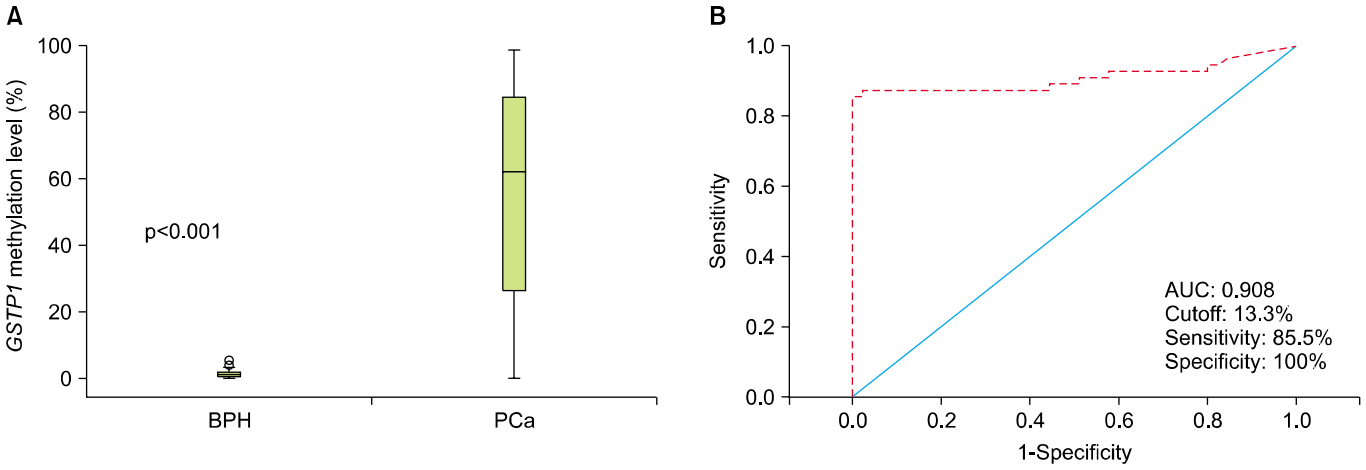

FIG. 2. (A) *GSTP1* methylation level in benign prostatic hyperplasia (BPH) and prostate cancer (PCa) patients. Differences (p-value) between BPH and PCa patients were obtained by two-sample t-test. The y axis indicates the absolute methylation expression level of *GSTP1*. (B) Receiver operator characteristic (ROC) curve analysis of *GSTP1* methylation for PCa prediction.

TABLE 2. Frequency of *GSTP1* methylation in human prostate tissues

	BPH (n=45)	PCa (n=55)
Methylation rate[a]	0 (0/45)	85.5 (47/55)
PSA level (ng/ml)		
<10	0 (0/40)	62.5 (5/8)
10–20	0 (0/3)	66.7 (4/6)
≥20	0 (0/2)	92.7 (38/41)
T staging		
T2	-	81.5 (22/27)
T3	-	83.3 (15/18)
T4	-	100 (10/10)
Gleason score		
≤6	-	100 (4/4)
7	-	84.2 (16/19)
≥8	-	84.4 (27/32)

BPH, benign prostatic hyperplasia; PCa, prostate cancer; PSA, prostate-specific antigen.
[a]: Methylation rate is expressed as percentage (no. of methylated samples/total number of samples).

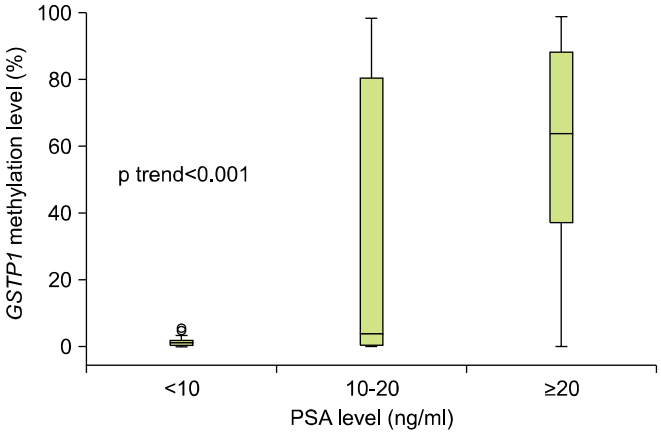

FIG. 3. *GSTP1* methylation level according the prostate-specific antigen (PSA) level. Differences (p-value) among groups were calculated by analysis of variance trend analyses. The y axis indicates the absolute methylation expression level of *GSTP1*.

BPH patients. Moreover, *GSTP1* hypermethylation was not only associated with an increased incidence of PCa, but was positively related to increases in the PSA level. In contrast, no significant relationships between *GSTP1* methylation and age, stage, or Gleason score were established.

Methylation of a promoter region has been shown to be associated with suppression of gene transcription, and hence gene expression. CpG island methylation is thought to influence transcription by allowing the attachment of methyl-binding domain proteins, which indirectly block the binding of transcription factors [7-9,14]. Abnormal DNA methylation patterns are associated with many human tumor types, and differences in methylation patterns have also emerged as markers for cancer risk assessment, cancer diagnosis, and therapy monitoring in several different types of cancer [7-9]. Moreover, DNA methylation for cancer detection is quite powerful owing to the inherent stability of DNA compared with RNA or protein [8]. To date, aberrant methylation of CpG island-containing promoters of numerous hypermethylated loci including *GSTP1* has been identified in PCa [7,10,14]. However, even in the same gene, the inconsistency of methylation levels reported in PCa could be the result of several factors, not the least of which is the different sample types used [7,13,14]. The variation could also be a result of different analytical methods and how they are applied, including the pretreatment of the DNA, the PCR conditions, and the PCR method. Recently, with technical developments, various methods have become available for the detection of the methylation status of certain genes in clinical samples [7,8]. Conventional MSP has been shown to have limited usefulness for specific cancer detection because benign lesions can be weakly pos-

itive and cannot be distinguished from cancer cases. Moreover, the results of MSP at a particular DNA region are simply reported as methylated or unmethylated, which may be also subjective, thus not allowing quantitation or identification of partial methylation. For improvement of sensitivity and quantitation, various techniques, such as nested two-stage PCR and QMSP, have been developed. However, these methods also have known drawbacks, such as false-positive and false-negative results, as well as the use of relative comparisons instead of absolute quantification [7,8,11,12]. In these regards, PSQ might be a best alternative tool, because it provides absolute quantitative information on bases at each interrogated CG site. Additionally, the assay design readily allows the interrogation of different parts of the gene(s) of interest as well as the inclusion of internal controls to address inaccuracies resulting from incomplete bisulfite conversion [7,11,12]. In the present study, we utilized PSQ to evaluate methylation status, and provided an optimal cutoff level to discriminate between PCa and normal controls. Our provided cutoff level was relatively low; thus, it might be misleading with the use of other techniques [7,11,12]. One of the major advantages of PSQ is the ability to quantitatively compare samples and to more accurately segregate varied pathologic covariates on the basis of methylation levels. If we did not use PSQ, we would not have found the significant correlation between *GSTP1* methylation and PSA level.

GSTP1 is involved in the metabolism, detoxification, and elimination of potentially genotoxic foreign compounds, and thus acts to protect cells from DNA damage and cancer initiation. Suppression of *GSTP1* activity can result in enhanced susceptibility to DNA damage and increased cancer incidence [13,14]. Many works have been reported on the development of *GSTP1* methylation as a biomarker that could be used to detect PCa [13-18]. Most studies have reported a consistent link between *GSTP1* methylation and PCa. Although the strength of the association has varied, in tissue samples taken by biopsy or during surgery, *GSTP1* methylation has been detected in at least 70% of cases and is only rarely present in benign prostate tissue [13-18]. As previously mentioned, various reasons exist for this inconsistency in *GSTP1* methylation levels, including the detection methods and different tissue types used. Our findings are consistent with these previous results and indicate that *GSTP1* hypermethylation is a reliable indicator for the prediction of PCa.

Although histologically confirmed prostate tissues were used, the possibility of unrevealed PCa in BPH patients and a small fraction of undetected methylated DNA molecules might have affected our results for sensitivity and specificity. Nonetheless, in terms of methylation frequency, our results are promising. The sensitivity and specificity of *GSTP1* methylation in distinguishing PCa and benign tissue reached 85.5% and 100%, respectively. Moreover, the frequency of *GSTP1* methylation in PCa was independent of serum PSA level, Gleason score, and stage. Clearly, biopsy has no potential as a routine screening test because of its highly invasive nature. Although these findings are promising, multicenter, large-scale clinical validation studies with primary human cancer tissue and body fluids are currently underway at our institute to confirm *GSTP1* as a diagnostic PCa methylation marker. These kinds of efforts will improve our understanding of the role of *GSTP1* methylation in tumorigenesis and its clinical relevance.

CONCLUSIONS

The results of our study show that *GSTP1* methylation is associated with the presence of PCa and PSA levels. This methylation marker represents a potentially useful indicator for the detection and monitoring of PCa.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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