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Promoter methylation profile of *GSTP1* and *RASSF1A* in prostate cancer and benign hyperplasia in Vietnamese men

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Background/aim: The *GSTP1* and *RASSF1A* methylations that were considered as prostate cancer-specific molecular biomarkers have been extensively reported in Western/American patients with prostate cancer but are rarely reported in Southeast Asian patients. In the present study, the methylation status of the *GSTP1* and *RASSF1A* promoters was evaluated in prostate cancer (PCa) and benign prostate hyperplasia (BPH) tissues from Vietnamese men.

Materials and methods: The accuracy of methylation-specific polymerase chain reaction (MSP) was validated to analyze the methylation pattern of *GSTP1* and *RASSF1A* in 59 PCa and 37 BPH patients, respectively. The methylation status was confirmed by the sequencing of cloned MSP products. The association between methylation status and the clinical and pathological parameters of tumors was statistically analyzed.

Results: The methylation of *GSTP1* and *RASSF1A* was detected in 39/59 and 19/59 PCa patients and in 4/37 and 10/37 BPH patients, respectively. The methylation frequency of *GSTP1* was significantly associated with PCa ($P < 0.01$). The *RASSF1A* methylation frequency (32.2%) observed in the study was lower relative to that detected in other populations.

Conclusions: *GSTP1* and *RASSF1A* methylation was accurately detected using the validated MSP method and can be used as a biomarker to diagnose prostate cancer.

Key words: *GSTP1*, methylation-specific polymerase chain reaction, *RASSF1A*

1. Introduction

Prostate cancer (PCa) is the second most frequently diagnosed cancer and the sixth leading cause of death in males with cancer worldwide (1). The most current method for diagnosis for PCa is prostate-specific antigen (PSA) assays (2). The wide utilization of PSA tests has reduced the death rates of PCa but it has been associated with a high risk of overdiagnosis and overtreatment (3). Up to 60% of PSA-detected prostate cancer was overdiagnosed (4). This disadvantage leads to trouble for healthy men and is expensive for patients with benign prostate hyperplasia (BPH) because PSA is a prostate-specific marker only (5). Thus, the development of additional biomarkers with high sensitivity and specificity for early detection of prostate cancer is vitally necessary.

Aberrant methylation of deoxycytidine nucleotides distributed on CpG islands in promoter sequences is considered as the earliest somatic genome change in cancer; thus, it is a promising marker for cancer diagnosis (6). In prostate cancer, aberrant DNA methylation frequently occurs at *GSTP1* (glutathione S-transferase P1) and *RASSF1A* (RAS association domain family member 1) genes (7,8). GSTP1 protects cells from DNA damage and contributes to cancer initiation (9). The metaanalysis of *GSTP1* methylation in prostate cancer confirmed that *GSTP1* methylation is a cancer-specific molecular biomarker for diagnosing prostate cancer with a sensitivity of 82% and a specificity of 95% (10). The sensitivity and specificity of *GSTP1* methylation status in discriminating between PCa and BPH reached 85.5% and 100%, respectively (11). The measurement of *GSTP1* promoter methylation in body fluids showed an excellent specificity, which was much higher than that of PSA for prostate cancer diagnosis (12). In addition to *GSTP1*, *RASSF1A* is a tumor suppressor involved in DNA repair and apoptotic effects (13). Similar to *GSTP1*, the metaanalysis indicated that *RASSF1A* methylation was a potential biomarker in PCa diagnosis and therapy (14). Aberrant promoter

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methylation of *RASSF1A* has been frequently detected (>70%) in prostate cancer, while it was rarely detected in normal tissues (15). Additionally, *RASSF1A* is often used in combination with *GSTP1* in making a panel of methylation markers to improve sensitivity and specificity of prostate cancer detection (16). Currently, the methylation status of *GSTP1* and *RASSF1A* is being examined in clinical trials as a promising diagnostic marker of prostate carcinoma (15,17).

The highest incidence rates of prostate cancer are in developed countries and the lowest ones are in developing countries (1). In prostate cancer, DNA methylation of individual genes is also highly divergent between populations (18). DNA methylation in the promoters of *GSTP1* and *RASSF1A* has extensively been reported in prostate cancer patients in Europe and the United States, but it was rarely reported in patients in Southeast Asian countries. In the present study, we used methylation-specific polymerase chain reaction (MSP) to investigate DNA methylation status of *GSTP1* and *RASSF1A* in benign prostatic hyperplasia and cancerous prostate tissues from Vietnamese patients. Through assessing the methylation status of these two epigenetic markers, the goal of our study was to evaluate their potential as diagnosis biomarkers of prostate cancer in Vietnamese men.

2. Materials and methods

2.1. Prostate tissue samples

Ninety-six formalin-fixed, paraffin-embedded radical prostatectomy patient specimens, including 59 specimens of primary PCa and 37 specimens of BPH, were collected during 2011 and 2012 at the Department of Pathology of National Cancer Hospital K in Hanoi, the largest cancer hospital in Vietnam. The blocks with more than 70% cancerous tissue were selected after histological examination. Clinicopathological characteristics of the patients were obtained from surgical and pathological records. Each tumor was graded according to the Gleason grading system. Informed consent was obtained from the patients via a written form and the study was approved by the guidelines of a local ethics committee in Vietnam.

2.2. Genomic DNA extraction and bisulfite modification

Genomic DNA was extracted using a QIAamp DNA FFPE Tissue Kit (QIAGEN) for formalin-fixed paraffin embedded specimens and then treated with sodium bisulfite using an EpiTect Bisulfite Kit (QIAGEN). During the modification, the unmethylated cytosines of the genomic DNA were converted to uracils, but the methylated cytosines remained unchanged (19). Polymerase chain reaction (PCR) that used globin F/R primers for the native DNA and Un-globin F, R, and R1 for treated DNA (Table 1) was performed to

Table 1. MSP primers for analysis of *β-globin*, *GSTP1*, and *RASSF1A* methylation. The lower letters "t" and "a" indicate the unmethylated cytosines that were changed to "t" in the forward primers and to "a" in the reversed primers.

Gene	Primers	Sequence (5'–3')	Size (bp)	MSP conditions	References
β-globin U01317.1	Globin F	CAACTTCATCCACGTTCACC	268	94 °C 5 min, 40 cycles of (94 °C 30 s, 62 °C 10 s, 72 °C 10 s), 72 °C 5 min	(20)
	Globin R	GAAGAGCCAAGGACAGGTAC			
	Un-globin F	AGAAGAGttAAGGAtAGGTAtGGtTGT	Round 1: 250	94 °C 5 min, 40 cycles of (94 °C 30 s, 62 °C 10 s, 72 °C 10 s), 72 °C 5 min	Present study
	Un-globin R	CTTaCCCCACAaaaCAaTAACaaCAaA			
	Un-globin F	AGAAGAGttAAGGAtAGGTAtGGtTGT	Round 2: 224	94 °C 5 min, 40 cycles of (94 °C 30 s, 65 °C 10 s, 72 °C 10 s), 72 °C 5 min	Present study
	Un-globin R1	ACTTCTCCTCAaaAaTCAaATaCACCA			
GSTP1 NC_000011.9	GS Me-F1	ttCGGttAGtTGCGCGGCGAtTtC	Round 1: 210	94 °C 5 min, 40 cycles of (94 °C 30 s, 65 °C 20 s, 72 °C 20 s), 72 °C 5 min	(21,22)
	GS Me-R	CGaCGAAACTCCAaCGAAaaC			
	GS Me-F2	ttCGGGGTGtAGCGGtCGtC	Round 2: 155	94 °C 5 min, 40 cycles of (94 °C 30 s, 65 °C 20 s, 72 °C 15 s), 72 °C 5 min	(23,24,25)
	GS Me-R	CGaCGAAACTCCAaCGAAaaC			Present study
	GS Un-F1	AGtTGtGtGGtGAtTttGGGGAtA	Round 1: 194	94 °C 5 min, 40 cycles of (94 °C 30 s, 62 °C 20 s, 72 °C 20 s), 72 °C 5 min	Present study
	GS Un-R	CCAaCaAAaaCCTCaCaaCCTCCa			
	GS Un-F2	GAtGtttGGGGTGtAGtGGttGttG	Round 2: 149	94 °C 5 min, 40 cycles of (94 °C 30 s, 66 °C 10 s, 72 °C 15 s), 72 °C 5 min	Present study
	GS Un-R	CCAaCaAAaaCCTCaCaaCCTCCa			
RASSF1A NG_023270.1	RM F	GGtTtTGCGAGAGCGCGtttA	170	94 °C 5 min, 40 cycles of (94 °C 30 s, 68 °C 20 s, 72 °C 20 s), 72 °C 5 min	Present study
	RM R	CaaCGCTAaCAAaCGCGaaCCGa			
	UM240 F	GGGGtTtTGtGAGAGtGtGtttAG	Round 1: 175	94 °C 5 min, 40 cycles of (94 °C 30 s, 60 °C 20 s, 72 °C 10 s), 72 °C 5 min	(26)
	UM241 R	TaaaCaCTAaCAAaCaCaaaCCaaaC			
	Un F	GAGAGtGtGtttAGttttGttT	Round 2: 135	94 °C 5 min, 40 cycles of (94 °C 30 s, 65 °C 10 s, 72 °C 10 s), 72 °C 5 min	Present study
	Un R	CCACAaaaCaaaCCCCaACTT			

determine the efficiency of bisulfite conversion, and PCR that used MSP primers for the native DNA was performed to confirm the primer's specificity only to methylated targets.

2.3. Methylation-specific PCR (MSP)

The methylation status of *GSTP1* and *RASSF1A* was evaluated by using MSP for amplification of bisulfite-treated DNA with primers that distinguish methylated (M) from unmethylated (U) DNAs. Based on the primer designing tool for the MSP method (http://www.urogene.org/methprimer/index1.html), the primers for *GSTP1* and *RASSF1A* were designed, and some of these primers were used in combination with the published ones (20–26). The primer sequences and amplicon lengths are shown in Table 1. Bisulfite-treated DNAs were subjected to single or nested PCR based on particular targeted genes. The PCR products were then subjected to electrophoresis on 12% acrylamide gel. All the PCR reactions were replicated at least three times.

DNA that was extracted from the lymphocytes of the healthy volunteers and then treated with bisulfite was used as a positive control for *GSTP1* and *RASSF1A* unmethylation. DNA that was extracted from the PC3 cell line and then treated with bisulfite was used as a positive control for *GSTP1* and *RASSF1A* methylation (27). Water without a DNA template was included in each PCR reaction as a control for any contamination. The methylation status was confirmed by sequencing the cloned MSP products for a subset of samples from each assay.

2.4. Statistical analysis

Associations between clinicopathological characteristics and individual promoter methylation status were examined by using the chi-square test (SPSS Inc., Chicago, IL, USA). For all statistical analyses, $P \leq 0.05$ was considered significant.

3. Results

The population in this study consisted of 59 patients with PCa and 37 patients with BPH, all of whom underwent radical prostatectomy. The clinicopathologic characteristics of all 96 patients are shown in Table 2. The median age of the cases was 71.65 years (range: 42–91), and most of the cases had tumors with Gleason grade IV or V (41/59 PCa, 69.4%).

3.1. Verification of the specificity of MSP primers

Validating the precision of the MSP primers specific only to the methylated target has been recommended in order to avoid false positive results due to coamplification of incompletely converted sequences (28). Thus, the bisulfite-untreated DNA and the bisulfite-treated DNA were separately subjected to MSP with the *GSTP1* and *RASSF1A* primer sets that were specifically designed for the methylated targets. Efficient amounts of the DNA templates were checked by PCR with the globin primer sets that were designed from the native and unmethylated DNA targets (Figure 1A). No MSP products corresponding to the methylated *GSTP1* and *RASSF1A* were amplified from untreated DNA extracted either from PC-3 cells or from the lymphocytes. Similarly, no MSP products corresponding to the methylated targets were amplified from treated DNA extracted from the lymphocytes, which was used as the positive control for unmethylated DNA. The methylated *GSTP1* and *RASSF1A* were detected from only the treated DNA extracted from PC-3 cells (Figure 1B). The results confirmed the accuracy of the designed primer sets specific only to the methylated targets. These primers were subsequently subjected to analysis of the methylation status of *GSTP1* and *RASSF1A* in prostate patients.

Table 2. Methylation frequencies of *GSTP1* and *RASSF1A* in the samples of benign hyperplasia (BPH) and prostate cancer (Pca) patients.

Characteristics	Overall, n = 96	*GSTP1*			*RASSF1A*		
		Un, n (%)	Me, n (%)	P-value	Un, n (%)	Me, n (%)	P-value
Age (years) Median Range	71.65 42–91	92	43		94	29	
Histological type Pca BPH	 59 37	 55 (93.2) 37 (100.0)	 39 (66.1) 4 (10.8)	 <0.01	 58 (98.3) 36 (97.3)	 19 (32.2) 10 (27.0)	 0.59
Histological grade (Gleason) I+II III IV+V	 5 13 41	 5 (100) 10 (76.9) 40 (97.6)	 4 (80.0) 10 (76.9) 25 (61.0)	 0.45	 5 (100) 12 (92.3) 41 (100)	 1 (20.0) 5 (38.5) 13 (41.5)	 0.75

P-value: statistical analysis of the associations between clinicopathological characteristics and methylation status.

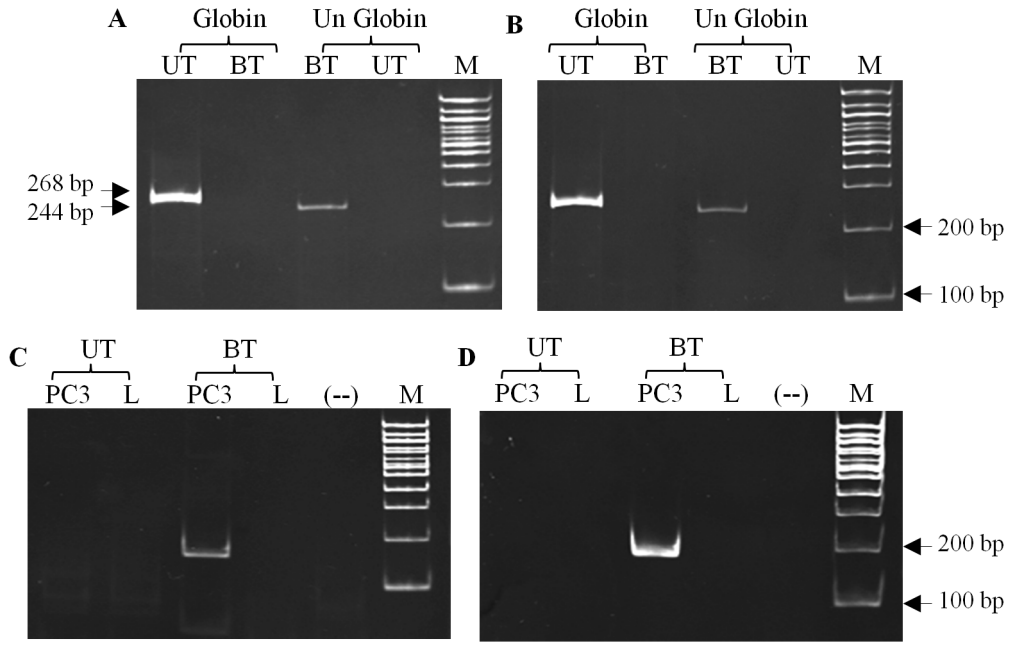

Figure 1. A–B) The efficiency of bisulfite conversion of genomic DNA that was extracted from PC3 cell line (A) and from the lymphocytes (L) of the healthy volunteers (B). A band of 268 bp amplified from only the untreated DNAs (UT) by globin primers and a band of 244 bp amplified from only the treated DNAs (BT) by nested Un globin primer sets. C–D) Specificity of the *GSTP1* and *RASSF1A* primer sets to only the methylated DNAs. A band of 155 bp and 170 bp amplified from only the treated genomic DNAs by the *GSTP1* (C) and *RASSF1A* (D) primer sets specifically designed for methylated sequences. M: 100-bp DNA ladder. (–): Negative control without DNA template.

3.2. Methylation status of GSTP1 and RASSF1A in PCa and BPH tissues

The genomic DNAs extracted from 59 PCa and 37 BPH specimens were treated with bisulfite and subjected directly to MSP. Representative results of the MSP products for methylation status of *GSTP1* and *RASSF1A* are shown in Figures 2 and 3, respectively. Three patterns of M/M, M/U, and U/U signals of *RASSF1A* were observed in both PCa and BPH cases, but these patterns of *GSTP1* were observed in cases of PCa only (Table 3). Biallelic unmethylation (U/U) and monoallelic methylation (M/U) signals of *GSTP1* were detected from BPH. Monoallelic M/M and biallelic M/U were count as the methylated status. MSP analysis revealed that the number of the methylated *GSTP1* and *RASSF1A* was 39/59 (66.1%) and 19/59 (32.2%) patients with PCa, respectively (Table 2). MSP analysis also revealed that the methylation of *GSTP1* and *RASSF1A* was detected in 4/37 (10.8%) and 10/37 (27%) patients with BPH, respectively. Forty-three out of 59 PCa (72.9%) samples showed methylation status of one or two genes.

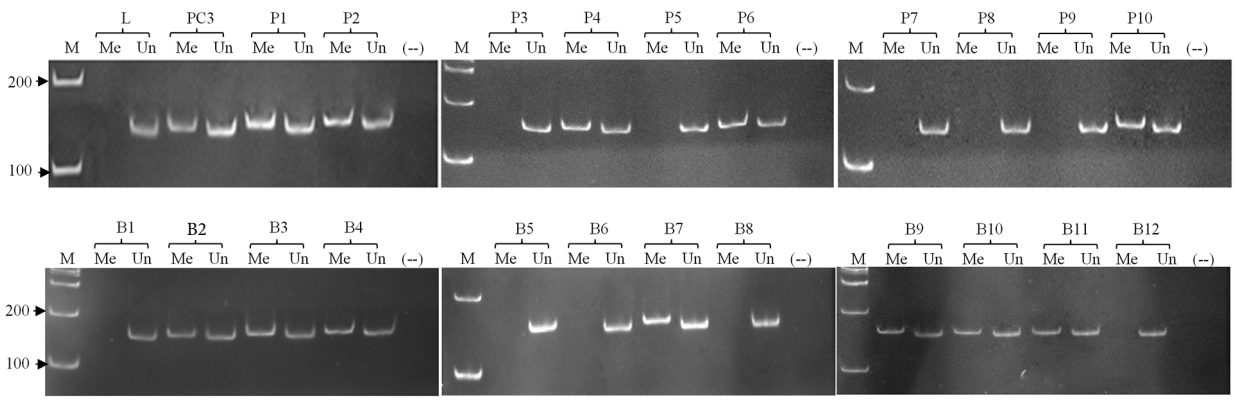

Figure 2. Representative results of the methylation analysis of *GSTP1* in the prostate cancer (P1–P10) and benign hyperplasia (B1–B12) samples. The PCR products in lanes Me and Un indicate the presence of methylated (155 bp) and unmethylated (149 bp)*GSTP1*. L: lymphocytes of the healthy volunteers. PC3: prostate cell line. (–): Negative control without DNA template. M: 100-bp DNA ladder.

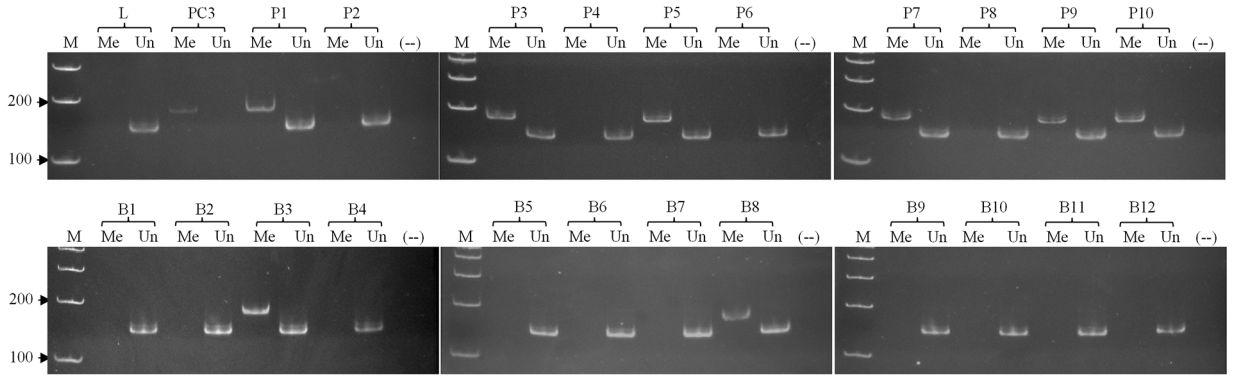

Figure 3. Representative results of the methylation analysis of *RASSF1A* in the prostate cancer (P1–P10) and the benign hyperplasia (B1–B12) samples. The PCR products in lanes Me and Un indicate the presence of methylated (170 bp) and unmethylated (135 bp) *RASSF1A*. L: lymphocytes of the healthy volunteers. PC3: prostate cell line. (–): Negative control without DNA template. M: 100-bp DNA ladder.

Table 3. Status and frequency of methylation of *GSTP1* and *RASSF1A* in PCa and BPH. Three patterns of M/M, M/U, and U/U.

Genes	PCa		BPH	
	GSTP1	*RASSF1A*	*GSTP1*	*RASSF1A*
M/M	4	1	0	1
M/U	35	18	4	9
U/U	20	40	33	27
Methylation ratio	39/59 (66.1%)	19/59 (32.2%)	4/37 (10.8%)	10/37 (27%)

The DNA methylation frequencies and clinical characteristics corresponding to surgical and pathological records of the cases were compared. There was a significant difference in the methylation rate between PCa and BPH for only *GSTP1* ($P < 0.01$) (Table 2). No significant differences in the methylation frequencies of *GSTP1* and *RASSF1A* were observed in terms of age and histological grade (Gleason) of the PCa patients (Table 2).

The methylation and unmethylation of *GSTP1* and *RASSF1A* were confirmed by cloning and sequencing MSP products that were amplified from the treated DNAs extracted from the prostate cancer samples (Figure 4). The nucleotide sequences showed that all cytosine residues were converted to thymidines in the *GSTP1* and *RASSF1A* unmethylated products, and that all cytosines in the CpG sites remained as cytosines and the cytosines that were not in the CpG sites were converted to thymidines in the *GSTP1* and *RASSF1A* methylated products.

4. Discussion

Among several DNA methylation markers associated with prostate cancer, *GSTP1* and *RASSF1A* methylations captured the most interest because they were strongly associated with and considered as specific molecular biomarkers of prostate cancer (5). Highly significant *GSTP1* and *RASSF1A* methylation rates have been extensively reported from tissue biopsies and body fluids (plasma, serum, whole blood, urine, semen) from patients with prostate cancer (12,24,28) This evidence makes them the most promising commercial DNA methylation markers for early detection of this cancer (5).

DNA methylation profiles of thousands of genes or of a particular gene can be quantitatively assessed by technological approaches such as DNA microarrays or methylation-sensitive high-resolution melting (MS-HRM), which may not be accessible to many institutions in developing countries (29,30). The MSP method was chosen in the present study because of its sensitivity, specificity, and suitability in most moderately equipped laboratories (31). However, false positive results due to MSP primers unspecific to the methylated target have been reported (28). Thus, the standard controls were set up to test the accuracy of the primers specific to the methylated *GSTP1* and *RASSF1A* through PCR, in which MSP products were amplified neither from the native DNA that was extracted from the lymphocytes or from PC3 cells, nor from the bisulfite-treated DNA that was extracted from the lymphocytes (Figure 1). This finding

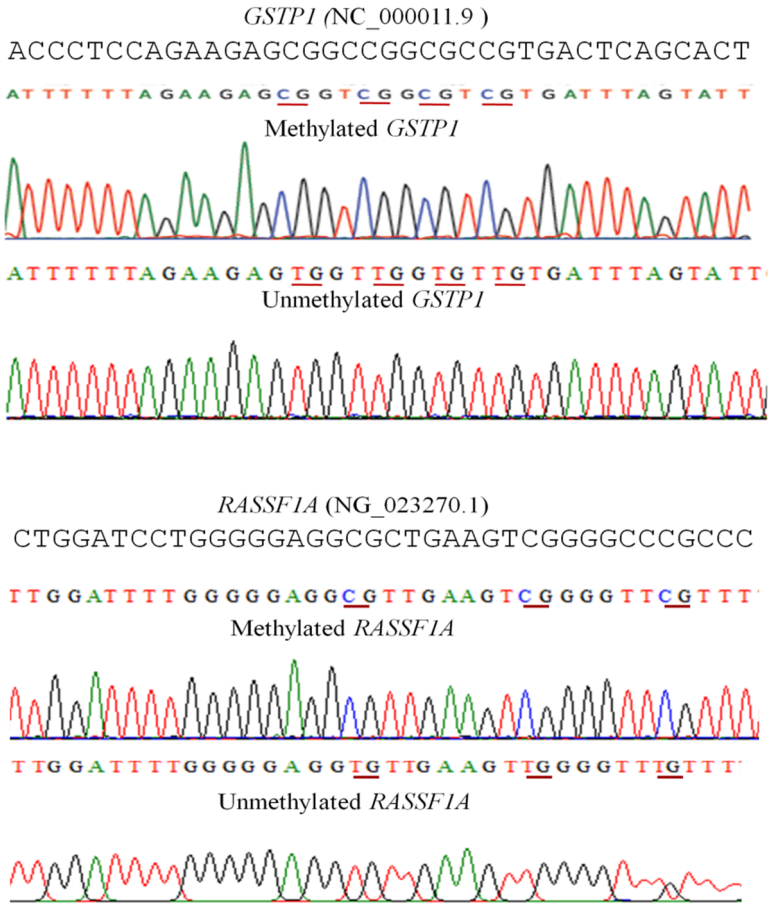

Figure 4. The native sequences of *GSTP1* (NC_000011.9) and *RASSF1A* (NG_023270.1) in comparison with their methylated (upper) and unmethylated (lower) sequences. Cytosines in the CpG sites (underlined) remained cytosines in the methylated sequences but they converted to thymidines in the unmethylated ones. All cytosines alone were converted to thymidines in both the methylated and unmethylated sequences.

confirmed that the unmethylated targets, as well as a trace of incompletely treated DNA, had not interfered with the MSP results; thus, false positive results were avoided.

The MSP result for one gene is dependent on the analyzed sequence of the 5' region; thus, the same nucleotide regions of *GSTP1* and *RASSF1A* promoters that have been previously analyzed by the MSP method were also chosen in the present study (21–24,26,28). *GSTP1* and *RASSF1A* methylation status of malignant (PCa) and benign (BPH) prostate lesions was elucidated by performing the MSP with the validated primer sets. In a total of 96 patients, 39/59 (66.1%) and 19/59 (32.2%) of PCa cases and 4/37 (10.8%) and 10/37 (27%) of BPH cases were methylated at the *GSTP1* and *RASSF1A* promoters, respectively. A significant difference in *GSTP1* methylation rates was only observed between PCa and BPH ($P < 0.01$) (Table 3). This result was consistent with previous reports in which PCa was sensitively and specifically discriminated from BPH based on *GSTP1* methylation (11,21). The *GSTP1* methylation frequency of 66.1% detected in the present study was comparable to that detected in different racial groups. Indeed, the *GSTP1* methylation frequency in the same nucleotide region that was analyzed in this study was 90.9%, 73.2%, and 58% from American, Korean, and Indian patients with PCa, respectively (22,33,34). On the contrary, no significant association was observed between *RASSF1A* methylation status and the clinicopathological parameters from the Vietnamese men who suffered from prostate lesions. In addition, the *RASSF1A* methylation frequency of 32.2% observed in this study was relatively lower than that detected in other populations, although a similar frequency was found in one other study (34). The methylation frequency of *RASSF1A* in Japanese and Pakistan patients, which was analyzed using the MSP method, was 74.0% and 100%, respectively (15,35). In a Portuguese population the methylation frequency of *RASSF1A* was more than 90% as analyzed by the quantitative MSP method (36). Alternatively, the lower frequency of *RASSF1A* methylation

in this study might be due to the MSP primer's specificity that was validated (Figure 1). Indeed, false positive results gave rise to an increase of 4 and 2 times the DNA methylation frequency (28).

A similar frequency of *RASSF1A* methylation in PCa (32.2%) and in BPH (27%) cases was observed in this study and in previous reports (25,34). The occurrence of methylation of *RASSF1A* in tumor and nontumor tissues from various cancers suggested that it is an early and premalignant sign (37). Thus, *RASSF1A* methylation in BPH has been considered as a sign of tumor progression. Indeed, a metaanalysis from 19 published studies on the association between *RASSF1A* promoter methylation and prostate cancer indicated that *RASSF1A* methylation was significantly associated with an increased risk of PCa (38).

Currently, the *GSTP1* and *RASSF1A* methylation in body fluids is extensively studied because of its noninvasive character and its ability to monitor prostate cancer (24,39). A high specificity of *GSTP1* and *RASSF1A* methylation was found in these studies, regardless of methylation methods (12,14). Thus, the MSP method, which was supported by previous studies and was standardized in this study, is advantageous for further analyzing *GSTP1* and *RASSF1A* methylation in body fluid specimens. Our study emphasized the authentic value of the MSP method that will allow the use of DNA methylation marker to quickly progress toward clinical application, especially in developing countries.

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