A Quantitative Promoter Methylation Profile of Prostate Cancer

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ABSTRACT

Purpose: Promoter hypermethylation is an alternative pathway for gene silencing in neoplastic cells and a promising cancer detection marker. Although quantitative methylation-specific PCR (QMSP) of the \textit{GSTP1} promoter has demonstrated near perfect specificity for cancer detection in prostate biopsies, we postulated that identification and characterization of additional methylation markers might further improve its high (80–90%) sensitivity.

Experimental Design: We surveyed nine gene promoters (\textit{GSTP1}, \textit{MGMT}, \textit{p14/ARF}, \textit{p16/CDKN2A}, \textit{RASSF1A}, \textit{APC}, \textit{TIMP3}, \textit{S100A2}, and \textit{CRBP1}) by QMSP in tissue DNA from 118 prostate carcinomas, 38 paired high-grade prostatic intraepithelial neoplasias (HGPIN), and 30 benign prostatic hyperplasias (BPH). The methylation levels were calculated and were correlated with clinical and pathologic indicators.

Results: Only the methylation frequencies of \textit{GSTP1} and \textit{APC} were significantly higher in prostate carcinoma compared with BPH ($P < 0.001$). Methylation levels of \textit{GSTP1}, \textit{APC}, \textit{RASSF1A}, and \textit{CRBP1}, differed significantly between prostate carcinoma and HGPIN, and/or HGPIN or BPH ($P < 0.0001$). With QMSP and empirically defined cutoff values, the combined use of \textit{GSTP1} and \textit{APC} demonstrated a theoretical sensitivity of 98.3\% for prostate carcinoma, with 100\% specificity. Methylation levels were found to correlate with tumor grade (\textit{GSTP1} and \textit{APC}) and stage (\textit{GSTP1}, \textit{RASSF1A}, and \textit{APC}).

Conclusions: Our data demonstrate the existence of a progressive increase of promoter methylation levels of several cancer-related genes in prostate carcinogenesis, providing additional markers to augment molecular detection of prostate carcinoma. Because methylation levels of \textit{GSTP1}, \textit{APC}, and \textit{RASSF1A} are associated with advanced grade and stage, QMSP might augment the pathologic indicators currently used to predict tumor aggressiveness.

INTRODUCTION

Prostate cancer is a leading healthcare concern in North America and Europe (1). This malignant disease is associated with considerable morbidity and mortality, but curative treatment (radical prostatectomy or radiotherapy) is feasible for patients with the earliest-stage disease (2, 3). However, locally advanced or metastatic disease carries a poor long-term prognosis because of the notable lack of curative therapy (4). Despite growing research efforts, the genetic mechanisms underlying prostate cancer development and progression are still largely unknown. A better understanding of the molecular alterations associated with prostate cancer is likely to contribute to improved diagnosis, clinical management, and outcome prediction.

Silencing of cancer-associated genes by hypermethylation of CpG islands within the promoter and/or 5'-regions is a common feature of human cancer and is often associated with partial or complete transcriptional block (5). This epigenetic alteration provides an alternative pathway to gene silencing in addition to gene mutation or deletion. Moreover, the finding of promoter methylation of several genes in small biopsies and bodily fluids of cancer patients has proven to be useful as a molecular tool for cancer detection (6).

In prostate cancer, a number of gene promoters were found to be hypermethylated by using conventional methylation-specific PCR (MSP; refs. 7, 8). Indeed, the methylation index, defined as ratio between the number of methylated genes and the total number of genes analyzed, was found to correlate with clinicopathologic indicators of poor prognosis, although this association was not established for any individual gene (7). However, conventional MSP is also of limited usefulness for specific cancer detection because benign lesions can be weakly positive and cannot be distinguished from cancer cases. This distinction has become possible by the development of quantitative assays (quantitative MSP, QMSP; ref. 6).
In previous studies we have shown that quantitation of GSTP1 promoter methylation accurately discriminated between normal or hyperplastic prostate tissue and prostatic adenocarcinoma (9–11), resulting in the detection of 80 to 90% of prostate adenocarcinomas with perfect specificity. A more extensive quantitative characterization of genes hypermethylated in prostate cancer could provide additional molecular markers that might further improve the quantitative GSTP1 assay and also add relevant information for pathologic assessment and clinical management.

We thus sought to characterize quantitatively the promoter methylation status of several genes previously known to be epigenetically silenced in several human cancers. For that purpose, QMSP was used to analyze the promoter of two genes involved in DNA repair (GSTP1 and MGMT), three cell cycle regulators (p16/CDKN2A, p14/ARF, and RASSF1A), and three genes involved in tumor growth and progression (APC, TIMP-3, and S100A2). Moreover, a gene previously found to be frequently methylated in a number of tumor types with a putative role in the retinoic acid pathway (CRBP1) was also studied. Tissue specimens from primary prostate carcinoma and paired high-grade prostatic intraepithelial neoplasia (HGPIN) lesions, as well as benign prostatic hyperplasia (BPH) were used. Relationships between methylation levels and clinicopathologic indicators were further assessed.

PATIENTS AND METHODS

Patients, Sample Collection, and DNA Extraction. Primary tumors and paired HGPIN lesions from 118 patients who had clinically localized prostate adenocarcinoma [stages T1c and T2, according to the TNM staging system (12)] and who were consecutively diagnosed and primarily treated with radical prostatectomy at the Portuguese Oncology Institute-Porto, Porto, Portugal, were prospectively collected. In addition, prostate tissue samples were obtained from 30 randomly selected patients with BPH who underwent transurethral resection of the prostate (TURP). All of the tissue specimens obtained immediately after surgery were promptly frozen and stored at −80°C. All of the tissue specimens obtained by TURP. Then, the tissue block was sectioned to identify representative areas of HGPIN in the radical prostatectomy specimens, and BPH in the normal or hyperplastic prostate tissue and prostatic adenocarcinomas with perfect specificity. A more extensive quantitative characterization of genes hypermethylated in prostate cancer could provide additional molecular markers that might further improve the quantitative GSTP1 assay and also add relevant information for pathologic assessment and clinical management.

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Histologic slides from formalin-fixed, paraffin-embedded tissue fragments were obtained from the same surgical specimens and assessed for Gleason grade (14) and TNM stage (12) by two pathologists (R. H. and C. L.) who were blinded to clinical and methylation analysis data. Relevant clinical data were collected from patients’ clinical records. These studies were approved by the institutional review board of Portuguese Oncology Institute-Porto. Permission to test these samples without identifiers was also granted by exemption from the institutional review board of The Johns Hopkins University.

Bisulfite Treatment and Quantitative Methylation-Specific PCR. Sodium bisulfite conversion of unmethylated (but not methylated) cytosine residues to uracil of genomic DNA obtained from patient tissue samples was done as described previously (15). Four micrograms of DNA were used for the chemical treatment. DNA samples were then purified with the Wizard purification resin (Promega, Madison, WI), treated again with sodium hydroxide, precipitated with EtOH, and resuspended in 200 μL of water and stored at −80°C.

The modified DNA was used as a template for real-time fluorogenic MSP. The primers and probes used for GSTP1, MGMT, p14, p16, RASSF1, APC, and TIMP3, are described elsewhere (9, 16–19). The primers and probes used for S100A2 and CRBP1 were, respectively: (sense) 5′-TGG TTT CGA TTT TTT GAT TTC G-3′, (antisense) 5′-TCA AAA TTC TTT TTA CAA CAA CGG C-3′, (probe) 6FAM-5′- CGA CGG CAC GGG ATA ACT TAC TCC-3′, (antisense) 5′-CGG CAT CAT AGC CAT CAG CAA CAA A-3′, (probe) 6FAM-5′-GAG CCG AAA ATA AAC GCC CTC CGA AAA CA-3′-TAMRA. In addition, primers and a probe were used to amplify areas without CpG nucleotides of ACTB (β actin), an internal reference gene (10). To determine the relative levels of methylated promoter DNA in each sample, we compared the values of each gene of interest with the values of the internal reference gene to obtain a ratio that was then multiplied by 1,000 for easier tabulation (target gene/reference gene × 1,000).

Fluorogenic quantitative MSP assays were carried out in a reaction volume of 20 μL in 384-well plates in an Applied Biosystems 7900 Sequence Detector (Perkin-Elmer, Foster City, CA). PCR was done in separate wells for each primer/probe set and each sample was run in triplicate. The final reaction mixture consisted of 600 nmol/L of each primer (Invitrogen, Carlsbad, CA); 200 nmol/L probe (Applied Biosystems, Foster City, CA); 0.75 unit of platinum Taq polymerase (Invitrogen); 200 μmol/L concentration each of dATP, dCTP, dGTP, and dTTP; 16.6 mmol/L ammonium sulfate; 67 mmol/L Trizma; 6.7 mmol/L magnesium chloride (2.5 mmol/L for p16); 10 mmol/L mercaptoethanol; 0.1% DMSO, and 3 μL of bisulfite-converted genomic DNA. PCR was done with the following conditions: 95°C for 2 minutes, followed by 50 cycles at 95°C for 15 seconds and 60°C for 1 minute. Each plate included multiple water blanks, a negative control, and serial dilutions of a positive control for constructing the calibration curve on each plate. Leukocyte DNA collected from healthy individuals was used as negative control. The same leukocyte DNA was methylated in vitro with Sss1 bacterial methyltransferase (New England Biolabs Inc., Beverly, MA) and was used as positive control for all studied genes.

Statistical Analysis. For each gene, the frequency of methylated and unmethylated cases, as well as the median and interquartile range of the methylation ratios for each group of tissue samples, was determined. The Shapiro-Wilk’s W test allowed for the examination of the appropriateness of a normal distribution assumption for each of the parameters (data not shown). Then, values were analyzed with nonparametric tests, i.e., the Kruskal-Wallis one-way ANOVA, followed by the
RESULTS

Clinical and Pathologic Data. We tested tissue samples from 118 patients with clinically localized prostate adenocarcinoma and 30 patients with BPH. Thirty-eight HGPIN lesions were further identified from the cancerous prostate samples and were carefully microdissected for separate analysis. The clinical and pathologic characteristics of these patients are depicted in Table 1. As expected, PSA levels were higher in patients with cancer, but there was considerable overlap with BPH cases.

Quantitative Methylation-Specific PCR in Prostatic Tissues. The frequency of methylation and median (interquartile range) values are listed in Table 2. Statistically significant differences among the three groups of tissue samples were noted only for GSTP1, APC, and CRBP1 (P < 0.00001, P < 0.00002, and P = 0.02, respectively). For GSTP1, the methylation frequency observed in prostate carcinoma differed from that of HGPIN (P = 0.002) and of BPH (P < 0.00001), whereas the frequency in HGPIN also differed from that in BPH (P < 0.00001). For APC, prostate carcinoma, and HGPIN, methylation frequencies differed significantly from those in BPH (P < 0.001 and P = 0.034, respectively), but this was not true between prostate carcinoma and HGPIN. For CRBP1, differences in methylation frequencies were detected only between prostate carcinoma and HGPIN (P = 0.016).

Regarding methylation levels (Table 2), statistically significant differences among the three groups of lesions were found for all of the genes, except for MGMT, p14, and S100A2. Generally, prostate carcinoma displayed the highest methylation ratios, and these statistically differed from those of HGPIN and BPH for GSTP1 (Fig. 1A), APC (Fig. 1B), RASSF1A, and CRBP1 (P < 0.0001 for all of these genes). Except for CRBP1, methylation levels of HGPIN and BPH also differed (P < 0.0001 for GSTP1 and APC, and P = 0.0117 for RASSF1A). For TIMP3, prostate carcinoma methylation levels were significantly different only from HGPIN (P = 0.009), and the latter also differed from BPH (P = 0.015). For p16, statistically significant differences were found only between HGPIN and BPH (P = 0.011).

We further analyzed the methylation levels of GSTP1, APC, RASSF1A, and CRBP1 in the paired prostate carcinoma and HGPIN samples (n = 38). The Wilcoxon Matched Pairs test demonstrated that methylation levels were significantly higher

Table 1. Frequency of positive cases and distribution of methylation levels in prostate carcinoma (PCa), prostatic intraepithelial neoplasia (HGPIN), and BPH

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCa</th>
<th>HGPIN</th>
<th>BPH</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>Median (IQR)</td>
<td>n (%)</td>
<td>Median (IQR)</td>
</tr>
<tr>
<td>GSTP1</td>
<td>112 (94.9)</td>
<td>178.67 (20.84–353.95)</td>
<td>29 (76.3)</td>
<td>1.14 (0–1.177)</td>
</tr>
<tr>
<td>MGMT</td>
<td>22 (18.6)</td>
<td>0 (0–0)</td>
<td>11 (28.9)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>p16</td>
<td>91 (77.1)</td>
<td>0.71 (0.05–6.47)</td>
<td>31 (81.6)</td>
<td>0.21 (0.04–1.33)</td>
</tr>
<tr>
<td>p14</td>
<td>5 (4.2)</td>
<td>0 (0–0)</td>
<td>2 (5.6)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>117 (99.2)</td>
<td>370.95 (130.21–844.66)</td>
<td>38 (100)</td>
<td>53.04 (36.84–135.81)</td>
</tr>
<tr>
<td>APC</td>
<td>118 (100)</td>
<td>85.50 (35.55–310.74)</td>
<td>38 (100)</td>
<td>22.39 (9.98–63.48)</td>
</tr>
<tr>
<td>TIMP3</td>
<td>114 (96.6)</td>
<td>1.44 (0.45–4.15)</td>
<td>35 (92.1)</td>
<td>0.59 (0.24–1.34)</td>
</tr>
<tr>
<td>S100A2</td>
<td>117 (99.2)</td>
<td>1049.13 (825.88–1190.26)</td>
<td>38 (100)</td>
<td>1334.05 (666.71–1177.23)</td>
</tr>
<tr>
<td>CRBP1</td>
<td>96 (81.4)</td>
<td>10.48 (1.27–75.74)</td>
<td>23 (60.5)</td>
<td>1.18 (0–10.24)</td>
</tr>
</tbody>
</table>

Note. Frequency of positive cases is expressed as number and (%). Distribution of methylation levels is the ratio of the methylation of the gene to the methylation of the ACTB gene × 1.000 and, in the Table, is expressed as the median (IQR).

Abbreviations: IQR, interquartile range; NS, not significant.

* Kruskall-Wallis One-Way ANOVA test.
in prostate carcinoma compared with the respective HGPIN lesions for all of the aforementioned genes \( (P < 0.001) \).

After analyzing the methylation levels of the genes that were differentially methylated in BPH, HGPIN lesions, and prostate carcinoma, cutoff values that would allow the distinction between benign and malignant tissue with 100% specificity were empirically established. The cutoff values were 1.0, 140.0, 10.0, and 1.0, for \( \text{GSTP1} \), \( \text{RASSF1A} \), \( \text{APC} \), and \( \text{CRBP1} \), respectively. With the use of these cutoff values, the combined use of \( \text{GSTP1} \) and \( \text{APC} \) methylation levels provided a theoretical sensitivity of 98.3% (116 of 118 prostate adenocarcinomas), and estimated positive and negative predictive values of 100% and 93.8%, respectively. Addition of the other two gene markers did not increase the theoretical detection rate.

**Methylation Levels and Clinicopathologic Correlations.**

In tumor samples, no correlation was found between age or PSA levels and methylation ratios of any of the nine target genes. Strikingly, \( \text{GSTP1} \) (Fig. 2) and \( \text{APC} \) methylation levels correlated positively with Gleason score \( (r = 0.26, P = 0.019; \text{and} \ r = 0.25, P = 0.02, \text{respectively}) \). Moreover, methylation levels of \( \text{GSTP1}, \text{RASSF1A}, \text{and} \text{APC} \) also correlated with pathologic tumor stage \( (r = 0.37, P = 0.00004; \text{and} \ r = 0.28, P = 0.0025; \text{and} \ r = 0.28, P = 0.0020, \text{respectively}) \). For BPH, correlations were found only between age, on the one hand, and \( \text{S100A2} \) and \( \text{TIMP3} \) methylation levels, on the other \( (r = 0.47, P = 0.016; \text{and} \ r = 0.40, P = 0.04, \text{respectively}) \).

**DISCUSSION**

To identify and characterize emerging markers in prostate cancer, we used QMSP to evaluate a panel of genes among benign (BPH), premalignant (HGPIN), and malignant (prostate carcinoma) prostate lesions. We found that methylation levels of \( \text{GSTP1}, \text{RASSF1A}, \text{APC}, \text{and} \text{CRBP1} \) were significantly higher in prostate carcinoma compared with HGPIN or BPH. Moreover, we were able to demonstrate associations between methylation levels and clinicopathologic indicators, namely tumor grade \( (\text{GSTP1} \text{ and} \text{APC}) \) and pathologic stage \( (\text{GSTP1}, \text{APC}, \text{and} \text{RASSF1A}) \).

In our study, the frequency of promoter methylation for the majority of genes was higher than previously reported \( (7, 8, 20–22) \). Besides possible differences in the patient population, it is noteworthy that conventional MSP was used in those studies, whereas we used a quantitative methodology with different PCR conditions that might be more sensitive than conventional MSP. Interestingly, divergent results were also obtained by QMSP and conventional MSP for the frequency of \( \text{APC} \) methylation in the same small-cell lung cancer cell lines: 58% by QMSP versus
We also demonstrated a correlation between methylation levels of individual genes (GSTP1, APC, and RASSF1A) and standard clinicopathologic conditions (tumor grade and stage). A correlation between the methylation index (i.e., the proportion of methylated genes) and markers of poor outcome in prostate carcinoma has been reported (7). However, the use of conventional MSP did not disclose any statistically significant association between the methylation status of a single gene and markers of clinical outcome. In our previous study on quantitative GSTP1 methylation in prostate cancer (9), no association was confirmed with any of these conditions, probably because of the smaller sample size. Moreover, in the present study, a wider spectrum of pathologic tumor stages (ranging from pT2a to pT4) was analyzed, thus enabling a more powerful statistical analysis. Although prostate cancer is widely recognized for its heterogeneity and multifocality, we chose to analyze the methylation profile of the index (or dominant) tumor in a given prostate. This strategy was based on the reported association of the characteristics of the index tumor with prognostic factors and the lack of clinical significance of secondary tumors (27). These findings may have clinical implications for the management of prostate cancer. Because methylation levels were found to correlate with tumor aggressiveness, these molecular assays could be included in predictive models for preoperative prostate cancer staging. If proven, carcinomas found to harbor high levels of promoter methylation in prostate biopsy may be more likely to benefit from appropriate therapeutic intervention. Finally, because several cancer-related genes are epigenetically silenced in prostate cancer, the role of therapy with demethylating agents that may restore gene expression remains to be explored.

Remarkably, with the combined use of GSTP1 and APC methylation levels, we increased the theoretical detection rate of prostate adenocarcinoma to 98.3%, with estimated positive and negative predictive values of 100 and 93.8%, respectively, when compared with GSTP1 alone, which displayed a theoretical sensitivity of 94.9%, a positive predictive value of 100%, and a negative predictive value of 83.3%. This result may thus improve the QMSP assay for GSTP1 alone (9–11). It is noteworthy that this finding was attained without compromising specificity (100%), because the definition of the cutoff values took into consideration the highest methylation level detected in benign lesions for each gene. Indeed, absolute specificity is a key issue because of the need for definitive diagnosis after imperfect PSA screening for prostate cancer in the general population. Although none of the 30 BPH lesions analyzed showed GSTP1 methylation, a cutoff value of 1.0 was chosen to exclude very low-level background readings that may occur in other benign conditions such as proliferative inflammatory atrophy (28). Recently, we reported that a QMSP assay for retinoic acid receptor β2 (RARβ2) promoter displays high sensitivity and specificity for prostate cancer detection (29). Moreover, a correlation between RARβ2 methylation levels and pathologic stage was found. Thus, a combined assay With GSTP1, APC, and RARβ2 may augment the detection rate of prostate cancer in tissue biopsies and may also provide clinically relevant prognostic information. Clearly, these promising results need to be validated in a large series of prospectively collected samples, as demonstrated for GSTP1 (11).
Another interesting finding from our study was the small set of prostate adenocarcinomas with undetectable levels of GSTP1 methylation. These tumors were all confined within the capsule (stage pT2a and b) and were scored Gleason 6 or 7 (data not shown). Notably, most of these tumors also showed low or absent methylation levels for other genes, such as RASSF1A, APC, and CRBP1. Because promoter hypermethylation effectively turns off gene expression, the lack or low level of methylation of these important genes might not affect their transcription in most neoplastic cells. This event may justify, at least partially, the relatively less aggressive pathologic features of this small set of prostate carcinomas. This finding is further supported by the reported association between increased methylation index and poor prognosis in prostate cancer (7) as well as our own observations correlating increased GSTP1, APC, and RASSF1A methylation levels with higher tumor grade and stage. The analysis of these markers in larger series of prostate carcinomas with long-term follow-up and outcome remains to be explored.

The intermediate level of promoter methylation found in HGPIN, compared with prostate carcinoma and BPH, is consistent with its role as precursor of prostate cancer (30–32). Moreover, HGPIN lesions displayed lower methylation levels for GSTP1, APC, RASSF1A, and CRBP1, compared with the matched prostate carcinoma procured from the same radical prostatectomy specimen. This finding suggests that the emergence of epigenetic alterations at several gene promoters is an early event in prostate carcinogenesis. The progressive accumulation of cells that carry these alterations (conceivably resulting in a growth or survival advantage) might be involved in the acquisition of invasive and metastatic abilities that characterize prostate adenocarcinoma. This model could explain why cells harboring these epigenetic alterations become the dominant population in the invasive carcinoma.

The p16 gene was frequently methylated in all three groups of lesions, although at low levels. Surprisingly, the slightly higher levels found in BPH differed substantially from those in HGPIN but not from those in prostate carcinoma. Interestingly, one study reported that p16 protein was elevated in prostate cancers and its relationship to clinical-pathologic features. Clin Cancer Res 2002;8:514–9.

The analysis of these markers in larger series of prostate carcinomas with long-term follow-up and outcome remains to be explored.

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