INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer mortality among men in the United States (1). Once the tumor has metastasized, the long-term prognosis is poor because no curative therapy is available (2). Cancer development and metastasis are multistep processes that, among others, involve the inactivation of tumor suppressor genes. When the normal expression levels of these growth-inhibitory proteins are suppressed, uncontrolled cell cycling and growth can result. Identifying such specific molecular changes may contribute to prediction of the clinical state and aggressiveness of newly diagnosed prostate cancers.

DNA is methylated only at cytosines located 5’ to guanosine in the CpG dinucleotide, and DNA methylation of CpG sites in the promoter regions of genes is a frequent acquired epigenetic event in the pathogenesis of many human cancers (3, 4). This modification has important regulatory effects causing loss of gene expression, when there is involvement of CpG-rich areas known as CpG islands. Promoter region DNA methylation is acquired during tumorigenesis but is not normally present in nontumor tissue. DNA methylation may provide an alternate pathway to gene deletion or mutation for the loss of tumor suppressor gene function. Methylated DNA markers also represent a promising avenue for monitoring the onset and progression of cancer. Aberrant promoter methylation has been described for several genes in various malignancies, and the spectrum of genes involved suggests that specific tumors may have their own distinct pattern of methylation (4, 5).

For these reasons, we examined 10 genes the expression of which is frequently silenced by aberrant methylation in other cancers to develop a methylation profile for prostate cancer. We used the MSP1 method (6), analyzed the frequency and extent of methylation (by determining the MI) for the 10 selected genes in prostate cancer, and correlated our findings with clinicopathological considerations.

RESULTS

Methylation percentages of the genes tested in prostate cancers were: RARB, 53%; RASSF1A, 53%; GSTP1, 36%; CDH13, 51%; APC, 27%; CDH1, 27%; FHIT, 15%; p16INK4a, 5%; DAPK, 1%; and MGMT, 0%. Methylation percentages in nontumor tissues were much lower. For clinicopathological correlations, we divided the cancer cases into low (6 or less) or high (7 or more) Gleason score (GS) groups, and into low (8 ng/ml or less) or high (greater than 8 ng/ml) preoperative serum prostate-specific antigen (PSA) groups. Methylation of RASSF1A, GSTP1, RARB, and CDH13 genes was significantly more frequent in the high GS group than in the low GS group. Methylation of RASSF1A, CDH1, and GSTP1 genes was significantly more frequent in the high PSA group than in the low PSA group. The median MIs were significantly higher in the high GS and the high PSA groups. According to the Spearman rank-correlation test, there was significant correlation between MI and GS (coefficient = 0.43, $P < 0.0001$) and the preoperative serum PSA (coefficient = 0.37, $P = 0.0003$).

Conclusions: Our results indicate that the methylation profile of prostate cancers correlates with clinicopathological features of poor prognosis.
The clinical staging of prostate cancer could only be performed on patients undergoing radical prostatectomy.

 logical features known to be important for prognosis of prostate cancer patients.

**MATERIALS AND METHODS**

**Clinical Samples.** All prostate specimens were collected from 101 patients with prostate cancer, and adjacent nonmalignant tissue was available from 7 of these patients. In addition, we obtained nonmalignant tissues from 25 patients with benign prostatic hypertrophy or prostatitis. Patient operations were performed at Parkland Memorial or Zale-Lipsy University Hospitals, Dallas, TX, between 1994 and 2000, after Institutional Review Board-approved, signed consent was obtained. The patients underwent radical prostatectomy or transurethral resection. The tissue was maintained frozen in our urology tissue bank. The histological grading was according to the GS (7), and the stage of the disease was by the clinical TNM classification. The tissue was maintained frozen in our urology tissue bank. The histological grading was according to the GS (7), and the stage of the disease was by the clinical TNM classification of the American Joint Committee on Cancer (8). The clinical characteristics of cancer patients are detailed in Table 1.

**DNA Extraction and MSP.** Genomic DNA was isolated from frozen tissue by digestion with 100 μg/ml proteinase K followed by standard phenol/chloroform (1:1) extraction and ethanol precipitation.

For MSP assays, DNA was treated with sodium bisulfite as described previously (6). Briefly, 1 μg of genomic DNA was denatured by incubation with 0.2 M NaOH for 10 min at 37°C. Aliquots of 10 mM hydroquinone (30 μl; Sigma Chemical Co., St. Louis, MO) and 3 mM sodium bisulfite (pH 5.0, 520 μl; Sigma Chemical Co.) were added, and the solution (final volume 615.5 μl) was incubated at 50°C for 16 h. Treated DNA was purified by use of Wizard DNA Purification System (Promega Corp., Madison, WI), desulfonated with 0.3 M NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was stored at −70°C until used. Gene location and primer sequences of all genes for both the methylated and the unmethylated forms, annealing temperatures, cycle number, and reference for methodology are summarized in Table 2. Two sets of primers were used to amplify each region of interest: one pair recognized a sequence in which CpG sites are unmethylated (bisulfite modified to UpG), and the other recognized a sequence in which CpG sites are methylated (unmodified by bisulfite treatment). Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 2% agarose gels containing ethidium bromide.

The precise conditions for MSP were selected so as to (a) distinguish between tumors and control tissues (buccal smears, peripheral blood cells) from healthy individuals, and (b) correlate methylation and gene silencing in cell lines. These conditions select for specificity over sensitivity. Under these conditions, no methylation of any of the genes tested was detected in 10 buccal smears and 10 peripheral blood sample DNAs from healthy volunteers. Specifically, we raised the annealing temperature for GSTP1 from 59°C (9) to 64°C, because at 59°C some control buccal smears gave a MSP-positive band for GSTP1, whereas none were seen at 64°C. Because of the importance of GSTP1 methylation in prostate cancer pathogenesis, we reconfirmed that methylation correlated 100% with gene expression in eight cell lines.

**Data Analysis.** A comparison of the proportion was done using the χ² test or Fisher’s exact test. To compare the overall extent of methylation for the panel of genes examined, we calculated the MI for each case, as follows, and then determined the mean for the different groups.

\[
\text{Methylation Index (MI)} = \frac{\text{Total no. of genes methylated}}{\text{Total no. of genes analyzed}}
\]

Statistical analysis of MI between two variables was performed using the Mann-Whitney nonparametric U test. To examine the correlation between two variables, we used the Spearman rank-correlation test. A P less than 0.05 was defined as being statistically significant. All data were analyzed with the use of Survival Tools for StatView (Abacus Concepts Inc., Berkeley, CA).

**RESULTS**

**Frequency of Methylation in Prostatic Tissues.** The frequencies of methylation of the 10 genes in 101 prostate cancers and 32 nonmalignant prostate tissues (7 normal prostate tissues and 25 prostate gland tissues with benign disease) are detailed in Table 3. Methylation percentages in prostate cancers were: RARβ, 53%; RASSF1A, 53%; GSTP1, 36%; CDH13, 31%; APC, 27%; CDH1, 27%; FHIT, 15%; p16INK4A, 3%; DAPK, 1%; and MGMT, 0%. The percentages of methylation in nonmalignant tissues were similar for CDH1 and lower for the other genes. These differences were significant for RARβ, RASSF1A, GSTP1, APC, and FHIT. There was no methylation of FHIT, p16INK4A, or DAPK in nonmalignant tissues. Most nonmalignant tissue (18 of 32, 56%) lacked methylation of any gene, and none had methylation of four or more genes. By

<table>
<thead>
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<th>Characteristic</th>
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<tr>
<td>Total number</td>
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</tr>
<tr>
<td>Median age (range)</td>
<td>63 (43–81) yrs</td>
</tr>
<tr>
<td>Race</td>
<td></td>
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<td>White</td>
<td>69</td>
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<td>Black</td>
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<td>Hispanic</td>
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<td>Other</td>
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<td>11</td>
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<td>Preoperative serum PSA</td>
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<tr>
<td>4–8</td>
<td>39</td>
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<tr>
<td>8.1–12</td>
<td>17</td>
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<tr>
<td>&gt;12</td>
<td>28</td>
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</table>

Clinical characteristics of cancer patients are detailed in Table 1.
Methylation in Prostate Cancer

Table 2
Summary data of genes tested

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reverse primer</th>
<th>Forward primer</th>
<th>Gene location</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHIT</td>
<td>M: 5'-CTGAACGACGACACCCACACTA-3'</td>
<td>U: 5'-TTGGGGTGTGGGTTTGGGTTTTTATG-3'</td>
<td>3p21</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>M: 5'-CTGAACGACGACACCCACACTA-3'</td>
<td>U: 5'-TTGGGGTGTGGGTTTGGGTTTTTATG-3'</td>
<td>5q21</td>
</tr>
<tr>
<td>APC</td>
<td>M: 5'-CTGAACGACGACACCCACACTA-3'</td>
<td>U: 5'-TTGGGGTGTGGGTTTGGGTTTTTATG-3'</td>
<td>1p16/19</td>
</tr>
<tr>
<td>p16 INK4A</td>
<td>M: 5'-CTGAACGACGACACCCACACTA-3'</td>
<td>U: 5'-TTGGGGTGTGGGTTTGGGTTTTTATG-3'</td>
<td>9q34</td>
</tr>
<tr>
<td>CDH13</td>
<td>M: 5'-CTGAACGACGACACCCACACTA-3'</td>
<td>U: 5'-TTGGGGTGTGGGTTTGGGTTTTTATG-3'</td>
<td>11q13</td>
</tr>
</tbody>
</table>

M, methylated-specific primers; U, unmethylated-specific primers.

Methylation and Clinicopathological Correlations. The correlation of methylation frequencies and the MI are shown in Fig. 2; and these are related to GS (Fig. 2A), the preoperative serum PSA (Fig. 2B), and tumor stage (Fig. 2C), three known clinicopathological factors for prostate cancer patient survival. Prostatic tumors with GS values of 5–6 have a significantly better clinical course than those with values of 7 or greater (10). For this reason, we divided our tumors into those with values of 6 or lower (low GS group) and those with values of 7 or greater (high GS group). The median value of preoperative serum PSA in our patients was 7.5 ng/ml. To obtain approximately equal numbers in each group, we divided our patients into a “low PSA” group (8 ng/ml or less), and a “high PSA” group (greater than 8 ng/ml). The median values of PSA in the low and high PSA groups were 5.6 and 14.2 ng/ml, respectively. Staging information was available for 60 of the patients. Because of the relatively small numbers in each of the four stages, we pooled patients into low stage (stages I and II) or high stage (stages III and IV) groups.

The median MIs of the high GS, the high PSA, and the high stage groups were significantly greater than those of corresponding low groups. All three high value groups had significantly greater methylation frequencies of the GSTP1 gene, and contrast, most cancers (81 of 101, 80%) had methylation of one or more genes, and 32 (32%) had methylation of four to seven genes. Fig. 1 illustrates representative examples of the methylation patterns in tumors of the four most frequently methylated genes. The unmethylated form of p16INK4A (p16U) was used as a control for DNA integrity. T, tumor samples 1 through 10; N, negative control.
the high GS and PSA groups had a significantly greater frequency of methylation of RASSF1A, whereas the high GS group also had a significantly higher RARβ and CDH13 methylation frequency. According to the Spearman rank-correlation test, there was a significant correlation between MI and GS (coefficient = 0.43, n = 101, P < 0.0001), and between the MI and the preoperative serum PSA (coefficient = 0.37, n = 93, P = 0.0003).

Survival data were available for 44 patients with a median follow-up period of 27 months. Although methylation status did not correlate with survival, no deaths were noted until 48 months after surgery. Thus, a lengthy follow-up will be required to determine whether methylation is a predictive factor for survival.

DISCUSSION

Previous studies have described the importance of DNA methylation in human cancers and have focused on regions of the genome that may have functional significance resulting from the extinction of gene activity. Whereas most individual cancers have several, perhaps hundreds, of methylated genes (4), the methylation profiles of individual tumor types are characteristic (4, 5, 11).

Of the 10 genes studied, we found 6 genes (RARβ, RASSF1A, GSTP1, CDH13, APC, and CDH1) that were selectively methylated in prostate cancer tissues at frequencies greater than 25%. One gene (FHIT) was occasionally methylated, whereas three genes (p16INK4A, DAPK, and MGMT) were seldom if ever methylated. Of particular interest, the MI and the frequencies of methylation of three or four genes were significantly higher in tumors of patients with increased risk of death, namely high GS, high preoperative PSA, and high stage. Other studies have demonstrated that the preoperative serum PSA reflects both tumor grade (12) and tumor volume (13).

Several reports indicate that GSTP1 methylation is present in prostate cancers at very high frequencies (9, 14). Our finding of a methylation rate of 36% in prostate tumors is lower than most other reports and may reflect the use of a higher annealing temperature in our MSP assay, which provides for specificity over sensitivity. Using our conditions, several control tissues...
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from healthy individuals (data not shown) and all but one sample of nonmalignant prostate tissue were negative for methylation. In addition, there was a complete concordance between methylation and expression of the gene in eight tumor cell lines. Other reports have failed to find an association between GSTP1 methylation and markers of increased risk. In the high risk groups, the methylation frequencies of GSTP1 were between 44% and 53%. Our findings that frequent methylation of GSTP1 was correlated with markers of increased risk suggest that our stringent conditions may yield more clinically useful information.

Our finding of a low incidence of p16\(^{INK4A}\) methylation in prostate cancers is consistent with published data (15). There is little published information about the other genes we studied. Retinoic acid plays an important role in lung development and differentiation, acting primarily via nuclear receptors encoded by \(RAR\) gene. Because the isoforms \(RAR\beta2\) and \(RAR\beta4\) are frequently methylated in lung and breast cancers (16), we investigated methylation of their promoter, P2, and found frequent methylation in the prostate cancers (53%). Our gene panel included \(RASSF1\), a recently identified tumor suppressor gene. There are two major \(RASSF1\) gene products, \(RASSF1A\) and \(RASSF1C\). Selective promoter methylation of the \(RASSF1A\) promoter, but not of \(RASSF1C\), is frequent in many cancers including lung, breast, nasopharyngeal, ovarian, and renal carcinomas and mesotheliomas (17–21). The \(RASSF1A\) promoter was methylated frequently in prostate cancers (53%), and methylation was associated with high GS and preoperative serum PSA. These findings indicate a potential role of \(RASSF1A\) in the pathogenesis and spread of prostate cancers.

Inactivation of \(CDH13\) resulting from aberrant methylation and other mechanisms is frequent in some types of cancer (22, 23). We found methylation of \(CDH13\) in 31% of prostate cancers and 20% of benign diseases of prostate. Our findings suggest that methylation of \(CDH13\) is an early event during the pathogenesis of prostate cancer. Inactivation of the \(APC\) gene is frequent in colorectal and other gastrointestinal carcinomas, usually by truncating mutations (24). An alternative method of inactivation of the gene in some gastrointestinal cancers is by promoter methylation (25). Recently we reported that selective methylation and silencing of the \(APC\) 1A promoter and its specific products were frequent in lung and breast cancers (26). We found methylation of \(APC\) 1A in 27% of prostate cancers. It has been demonstrated that decreased expression of \(CDH1\) is associated with high grade, advanced stage prostate tumors (27), and methylation of a subset of prostate cancer cell lines has been described (28). We found methylation of \(CDH1\) in 27% of prostate cancers, indicating that aberrant methylation may be the major mechanism of silencing of this gene. Guo et al. (29) demonstrated down-regulation of \(FHIT\) protein in more than half of the primary prostate cancers examined by immunostaining. However, another study failed to identify the mechanism of inactivation (30). We found methylation of \(FHIT\) in 15% of prostate cancers, indicating that promoter methylation of the \(FHIT\) gene is one of the mechanisms involved in gene silencing.

Methylation of some genes (\(CDH1\), \(CDH13\), and \(RASSF1A\)) were occasionally methylated in nonmalignant prostatic tissues (benign prostatic hypertrophy and prostatitis). Of interest, \(GSTP1\) was methylated in only 3% of these tissues. Methylation has been described in various nonmalignant tissues as an aging change or following chronic inflammation (31, 32).

We have identified several new genes methylated at relatively high frequencies in prostate cancer. Our results indicate that the methylation profile of prostate cancers correlates with clinicopathological features of poor prognosis. Although our findings need to be extended to a larger series, our results suggest that the methylation profile of genes may be a potential new biomarker for determining the prognosis of prostate cancer patients.

REFERENCES


Aberrant Promoter Methylation Profile of Prostate Cancers and Its Relationship to Clinicopathological Features

Riichiroh Maruyama, Shinichi Toyooka, Kiyomi O. Toyooka, et al.


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