Prostate cancer is a common malignancy and a leading cause of cancer death among the aging male population, but the mechanism underlying this association is unclear. Aberrant methylation of promoter CpG islands is associated with silencing of genes and age-dependent methylation of several genes has been proposed as a risk factor for sporadic cancer. We examined the extent of gene methylation in pathologically normal human prostate as a function of age.

**Purpose:** Prostate cancer is a leading cause of cancer death among the aging male population, but the mechanism underlying this association is unclear. Aberrant methylation of promoter CpG islands is associated with silencing of genes and age-dependent methylation of several genes has been proposed as a risk factor for sporadic cancer. We examined the extent of gene methylation in pathologically normal human prostate as a function of age.

**Experimental Design:** We used pyrosequencing to quantitatively analyze the methylation status of nine CpG islands in normal prostate tissue DNA from 45 organ donors and 45 patients who had undergone cystoprostatectomy for bladder cancer. We also analyzed 12 pairs of matched benign and prostate cancer tissue DNA from patients with prostate cancer.

**Results:** Linear regression analysis revealed a significant increase in promoter methylation levels correlating with age for CpG islands at RARβ2 (r = 0.4; P < 0.0001), RASSF1A (r = 0.27; P = 0.01), GSTP1 (r = 0.59; P < 0.0001), NKX2-5 (r = 0.27; P = 0.008), and ESR1 (r = 0.244; P = 0.023) in the normal prostate tissue samples studied. A calculated average methylation (z-score) at all nine CpG loci analyzed in the normal prostate tissues showed a strong correlation with age (r = 0.6; P < 0.001). Comparison of the methylation level for the matched benign and prostate cancer tissues from individual patients with prostate cancer showed significantly higher methylation in the prostate cancer tissue samples for RARβ2 (P < 0.001), RASSF1A (P = 0.005), GSTP1 (P < 0.001), NKX2-5 (P = 0.003), ESR1 (P = 0.016), and CLSTN1 (P = 0.01).

**Conclusions:** Our findings show aberrant hypermethylation as a function of age in the normal prostate tissues. Such age-related methylation may precede and predispose to full-blown malignancy.
whether such events in genes may predispose aging cells to neoplastic transformation. Second, such a methylation profile would be useful to distinguish benign prostate from cancerous prostate, and provide correlations between methylation and prostate tissue pathologic features such as stage, grade, and recurrence. Finally, differences in gene methylation profiles from individuals of the same age differing in the race or ethnicity may help in understanding how genetic factors or environmental exposures (or both) contribute to prostate cancer. In the present study, we used pyrosequencing to quantify the methylation status of nine CpG islands as a function of age in normal and cancerous human prostate tissues.

Materials and Methods

Human prostate tissue samples. We collected a total of 90 normal prostate (disease-free) peripheral zone tissue samples; of which 45 were from organ donors (age range, 17-68 years) and 45 samples from patients who underwent cystoprostatectomy for bladder cancer (age range, 39-84 years). In addition, we collected 12 pairs of matched normal and prostate cancer tissue samples from patients with prostate cancer (age range, 52-73 years). All samples were obtained from the Baylor Specialized Programs of Research Excellence (Baylor College of Medicine) Prostate Cancer Tissue Bank in accordance with institutional policies. Organ donor samples from accidental death victims were removed after obtaining consent from next of kin. Normal prostate tissues from bladder cancer patients without any evidence of prostate cancer were obtained at the time of cystoprostatectomy. Matched normal and prostate cancer tissues from patients with prostate cancer were obtained from the Baylor Specialized Programs of Research Excellence at the time of radical prostatectomy. In all cases, the normal tissues were free from prostate intraepithelial neoplasia and cancer on histologic examination.

Table 1. Primer sequences used in the pyrosequencing analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTP1</td>
<td>First step PCR: forward AGGAGGTTAGGGAAGGTTTAAGTTATA reverse CCAAAACCTCCCCCAATAC</td>
</tr>
<tr>
<td></td>
<td>Second step PCR: forward GAGGTAGGGAGAGGTATGT reverse biotin-CAAACCTCCCAATAC sequencing (F) GGGAGGAGGATTAAG</td>
</tr>
<tr>
<td>AR</td>
<td>First step PCR: forward TAGGAAGTAGGGGTTTTTATGTATG reverse ACCCAAACACCTCCCTTCACT</td>
</tr>
<tr>
<td></td>
<td>Second step PCR: forward GTAGGGGTATTGGTAGGATGT reverse GGGACCCCTGATCTGTTTTCTCTCTCTCC reverse biotin-UNIVR sequencing (F) TTGTGTTTTAAGGATTTAGGTA</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>Single-step PCR: forward GGGAGGATTGATTTTATTTGA reverse biotin-CTACCCCTTAACCTCCCTTCC sequencing GGGTAGTGTTTGGAG</td>
</tr>
<tr>
<td>MYOD1</td>
<td>First step: forward AATTAGGAGGAGGAGGTATTACG reverse ACAAACCTTAAACACTACCTAACCCTACCT</td>
</tr>
<tr>
<td></td>
<td>Second step: forward GAAGGTATTACGTTAGTTG reverse biotin-UNIVR sequencing GAGGTGTGGAAGGG</td>
</tr>
<tr>
<td>p16</td>
<td>Single step PCR: forward GTGGTTTTTGGTGTGGTGGTTT reverse biotin-ACCCCTTCTGCTAATCTCAAATAA sequencing TTTGTGGTGGAG</td>
</tr>
<tr>
<td>RAR/J2</td>
<td>First step: forward AGGTGGTATGTTAGTGA reverse TACCCAAACACCTACTC reverse universal UNIVR-CACTTTGCTAACCTACTACTACCT</td>
</tr>
<tr>
<td>ESR1</td>
<td>One step PCR: forward TGTTGTTTTTTTTTATTGTTTTTG reverse biotin-AACCTTTGTTTTATTTAATAA sequencing GGATGCAGTGTTTGC</td>
</tr>
<tr>
<td>CLSTN1</td>
<td>First step PCR: forward GAATTAGGTATGTTATTTTTATTTTGTAGAG reverse TACTACCCCTCTTTACCTTACCTTTTCAACA</td>
</tr>
<tr>
<td>NKX2-5</td>
<td>First step PCR: forward GAGGTAGGAGGAGGATTTTTGGTTTAAG reverse AACCCTCAACCTACCAAAACT</td>
</tr>
</tbody>
</table>

NOTE: The underlined sequence is the universal primer sequence also shown as 5'-UNIVR.
The methylation index (MtI) at each gene promoter and for each sample was calculated as the average value of mC/(mC+C) for all examined CpG sites in the gene and expressed as the percentage of methylation. The Spearman test was used to determine correlations, with significance set at $P < 0.05$. $r$ represents the measure of the relationship between two variables, and varies from -1 to +1.

**Results**

To investigate DNA methylation as a function of age in normal human prostate tissues, we used pyrosequencing to quantitatively measure DNA methylation of bisulfite-modified genomic DNA. We examined a total of nine CpG islands including GSTP1, RARβ2, the RASSF1A tumor suppressor gene, androgen receptor (AR), and p16, which has been previously shown to be hypermethylated in human prostate cancers (4), and myoblast determination protein 1 (MYOD1) and estrogen receptor 1 (ESR1), which has been shown to be affected by age-related methylation (9, 13). In addition, we analyzed two novel genes, i.e., calsyntenin-1 (CLSTN1) and a member of the homeobox gene family of transcription factors (NKX2-5) which we have identified to be differentially methylated in prostate cancer.4 Figure 1 shows CpG maps of the genes analyzed, along with the locations of the regions amplified. We investigated the methylation status of these CpG islands in normal prostate tissue DNA obtained from 45 organ donors (age range, 17-68 years) and 45 patients who underwent cystoprostatectomy for bladder cancer (age range, 39-84 years). In addition, we analyzed the methylation status of these CpG islands in DNA samples from 12 pairs of matched benign and prostate cancer tissues from patients with prostate cancer (age range, 52-73 years). Typical examples of bisulfite methylation profiles presented as pyrogram are shown for GSTP1 (Fig. 2). As shown in the pyrogram, the internal control (shown as a hatched box) in the pyrosequencing reaction checks for the adequacy of bisulfite treatment, that is, methylation of non-CpG cytosines, suggesting that 100% of the DNA samples used for the GSTP1 analysis were satisfactorily converted by bisulfite treatment. Samples with failed results were repeated, where possible, with freshly prepared bisulfite-modified genomic DNA from the original tissue samples, and the pyrosequencing reaction was repeated for all samples.

For each CpG island studied, the percentage of methylation at a specific promoter region was expressed as a function of age (Fig. 3). There was considerable variation in the percentage of CpG island methylation in the individual patient samples studied, presumably reflecting both random variability in tissue composition and variable methylation level per cell. The variable range of methylation could also reflect differences in genetic susceptibility to methylation, lifestyle or exposure factors (including diet), and the random nature of the methylation event. The methylation level was not significantly different (Mann-Whitney rank sum test; Fig. 4A) between the samples from organ donors and those from patients with bladder cancer for the same age group (age range, 38-68 years); therefore, we grouped these samples together and these are hereafter referred to as normal prostate tissues. Based on this criteria, we found a significant increase in the percentage of methylation at the GSTP1, RARβ2, RASSF1A, p16, and MYOD1 CpG islands in normal prostate tissues from organ donors as compared to those from patients with prostate cancer (Fig. 4B). The methylation level was not significantly different between the samples from organ donors and those from patients with prostate cancer for the same age group (age range, 52-73 years). Figure 5 shows the correlation between the percentage of methylation at the GSTP1 CpG island and age for the organ donor and patient samples. The correlation coefficient was $r = 0.56$ for the organ donor samples and $r = 0.45$ for the patient samples, indicating a strong and significant correlation between methylation and age. The correlation coefficient for the combined samples was $r = 0.50$, indicating a significant correlation between methylation and age across both organ donor and patient samples.

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4 Unpublished observation.
in promoter methylation to correlate with age for the CpG islands at RAR\(\beta\)2 (\(r = 0.4; P < 0.0001\)), RASSF1A (\(r = 0.27; P = 0.01\)), GSTP1 (\(r = 0.59; P < 0.0001\)), NKX2-5 (\(r = 0.27; P = 0.008\)), and ESR1 (\(r = 0.244; P = 0.023\)). We also observed methylation of the CLSTN1 gene but this did not significantly correlate with age. The MYOD1, AR, and p16 CpG islands were entirely unmethylated in the normal prostate tissue samples (data not shown). An unbiased analysis of all the data by z score normalization for the nine genes showed a strong correlation with age (\(r = 0.6; P < 0.001\)) in the normal prostate tissues (Fig. 4B). These results indicate that DNA methylation increases with age in normal human prostate.

Having established age-related methylation in the prostate, we next wanted to compare the level of the methylation in normal and prostate cancer tissues from men 50 years and older. We compared the methylation levels in normal prostate tissues from three different sources, i.e., normal prostate tissues from organ donors, cystoprostatectomy for patients with bladder cancer, and normal as well as prostate cancer tissues from patients with prostate cancer (Fig. 5A). Compared with the normal prostate tissues, we observed more extensive methylation in prostate cancer tissues. For the RAR\(\beta\)2 gene, the average methylation seen in cancer samples was at least 2.7-fold higher when compared with the normal prostate tissues, but there was no significant difference between the normal prostate tissues from different sources. Similarly, for the RASSF1A gene, the average methylation level in prostate cancer tissues was at least 2-fold higher compared with that in normal prostate tissues. Furthermore, the methylation level in the benign tissues from patients with prostate cancer was significantly higher (~2-fold; \(P < 0.001, t\) test) when compared with the normal prostate tissues from organ donors and the cystoprostatectomy for bladder cancer patient. Because RASSF1A methylation levels are low in normal prostate tissues and elevated in the prostate cancer and the surrounding benign tissues, quantitation of RASSF1A CpG islands could also be useful for distinguishing between normal and prostate cancer tissues. The methylation level of GSTP1 showed at least 5-fold higher methylation in the cancer samples when compared with normal tissues from organ donors and benign prostate tissues from patients with prostate cancer. However, the methylation level in the cancer samples was only 1.5-fold higher compared with that of the normal tissues from cystoprostatectomy for patients with bladder cancer. This result can be explained by the high methylation levels observed in normal aging prostate tissues. The average methylation level of NKX2-5 in prostate cancer samples was ~3-fold higher than in the normal samples, however, the methylation levels were virtually identical among the three different sources of normal prostate tissues analyzed, suggesting that NKX2-5 methylation levels could also be a good candidate for distinguishing between normal and prostate cancer tissues. Overall, the ESR1 and CLSTN1 CpG methylation levels were lower, however, the average methylation level of ESR1 and Clstn1 were each ~2-fold higher in the cancer tissues when compared with the normal prostate tissues.

Finally, we compared the methylation levels for benign and matched pair cancer tissue for each prostate cancer patient.
(Fig. 5B). The results showed significant hypermethylation for RARβ2 \( (P < 0.001, \ t \text{ test}) \), RASSF1A \( (P = 0.005, \ t \text{ test}) \), GSTP1 \( (P < 0.001, \ t \text{ test}) \), NKK2-5 \( (P = 0.003, \ t \text{ test}) \), ESR1 \( (P = 0.016, \ t \text{ test}) \), and CLSTN1 \( (P = 0.01, \ t \text{ test}) \) in the prostate cancer tissue samples when compared with the matched benign tissues. However, the frequency of hypermethylation in the prostate cancer tissues was low for the ESR1 gene. There was no methylation in the prostate cancer tissues for MYOD1, AR, and p16 CpG islands (data not shown) just as observed in the normal and benign prostate tissues. Because of the high frequency of hypermethylation of these five genes, i.e., RARβ2, RASSF1A, GSTP1, NKK2-5, and CLSTN1 in the prostate tumors, promoter methylation of these five genes could theoretically serve as useful tools to distinguish between benign and prostate cancer tissues. Also, these data suggest that hypermethylation occurring in normal prostate tissues is a prerequisite for hypermethylation in prostate cancer.

**Discussion**

In the present study, we used pyrosequencing to examine the methylation profile of nine CpG islands in 90 normal prostate tissue samples and 12 pairs of matched benign and prostate cancer tissues from patients with prostate cancer. Pyrosequencing offers a semiquantitative, high-throughput, and reliable method with an inbuilt internal control for adequacy of bisulfite treatment (12, 14).

DNA methylation is a common event in cancer, and in several genes, promoter methylation has been reported. For example, aberrant methylation of the GSTP1 gene is perhaps the most common genomic alteration in human prostate cancer and occurs in the earliest stages of prostate carcinogenesis (15). The RARβ2 gene is hypermethylated in the vast majority of prostate adenocarcinoma, high-grade prostatic intraepithelial neoplasia, and in a number of benign prostate hyperplasia lesions (8). RASSF1A promoter hypermethylation occurs at a high frequency in prostate tumor samples and is also detected in some nonmalignant prostate tissue samples (16), and a large percentage of prostatic intraepithelial neoplasia samples also exhibit RASSF1A promoter methylation (17). We have observed a significant increase in promoter methylation correlating with age in normal prostate tissues for the CpG islands at RARβ2, RASSF1A, and GSTP1. This observation indicates that not only are the RARβ2, RASSF1A, and GSTP1 genes hypermethylated in prostate cancer but promoter methylation of these genes actually starts in the normal prostate as a function of age, which markedly increases in cancer.

The use of epigenetic changes such as DNA methylation as noninvasive diagnostic tools in cancer has implications for the identification of high-risk subjects, patients with preinvasive or early stage lesions, and for monitoring residual disease. One such gene that holds promise as a diagnostic tool in patients with suspected prostate malignancy and a negative biopsy is

![Fig. 4](https://example.com/fig4.png)

Fig. 4. The age distribution and methylation levels of nine CpG islands in normal human prostate tissues. A, percentage of methylation at nine CpG islands for 90 normal prostate tissues consisting of 45 organ donor samples (left) and 45 samples from patients who underwent cystoprostatectomy for bladder cancer (right) are shown with the ages of the individual patients. B, normalized average methylation levels \( (z \text{ score}; \ z = [(\text{methylation} - \text{mean}) / \text{SD})] \) at CpG island were calculated for all the patient samples analyzed and expressed as a function of age. Y-axis, the average \( z \text{ scores} \) of nine CpG islands. X-axis, ages in years. The correlation coefficient \( (r) \) of 0.6 shows a significant increase in methylation as a function of age \( (P < 0.001) \).
GISTP1. Abnormal GISTP1 methylation found postbiopsy may be helpful for the identification of patients at risk for harboring malignancy despite a negative biopsy, and to determine whether or not a repeat biopsy in the event of negative initial result is necessary (18). However, our data indicates that not only is GISTP1 hypermethylated in prostate cancer but that it is also methylated in an age-related manner, suggesting that the use of GISTP1 as a diagnostic tool must be carried out with caution especially when using it as a tool for analyzing samples from older patients.

In contrast with RARα2, RASSF1A, and GISTP1, we found NKX2-5 to be specifically hypermethylated in the prostate tumors when compared with the normal prostate tissues. Furthermore, NKX2-5 does not show age-related methylation. NKX2-5 belongs to a family of homeobox genes that encodes a class of transcription factors regulating the expression of target genes in a time- and spatial-dependent manner. Loss of one member of this gene family, NKX3.1, has been shown to be an early event in the initiation of prostate cancer (19). NKX2-5 seems to be a novel frequent cancer-associated hypermethylated CpG island in prostate cancer, the hypermethylation of which is associated with neoplastic transformation unlike RARα2, RASSF1A, and GISTP1 which are hypermethylated in premalignant prostate tissues in an age-dependent manner. Therefore, the inclusion of NKX2-5 as a new marker in a panel of hypermethylated genes in prostate cancer can potentially increase the sensitivity and specificity of prostate cancer detection.

The incidence of methylation in the human prostate seems to be promoter-specific, because we did not detect methylation at CpG islands for AR, MYOD1, p16, and CLSTN1. Methylation-mediated inactivation for AR has been shown in primary prostate cancer tissues (20, 21) and in androgen-independent cancers (22). However, data on AR methylation remains discrepant. The MYOD1 has been shown to be hypermethylated in cervical cancer (23) and undergoes age-related methylation in colon cancer (9). We did not observe age-related methylation of MYOD1 in the human prostate, suggesting that MYOD1 methylation may be tissue-specific. The p16 gene has been reported to undergo methylation in primary prostate cancer and metastatic tumors, however, only partial methylation of the p16 gene was observed in this study (24–26). We did not see methylation of this gene in the normal or prostate cancer tissues examined in our study. On the other hand, aberrant methylation of p16 seems to occur more commonly in other solid tumors, including breast carcinomas, gliomas, and colorectal carcinomas (27, 28). The CLSTN1 gene has a cadherin domain and a calcium ion–binding domain, and it may be related to cell adhesion molecules (29). We have observed aberrant methylation of CLSTN1 in prostate cancer tissues as well as in normal prostate tissues, however, the methylation of CLSTN1 does not seem to be age-related.

In conclusion, we have used pyrosequencing technology to show the epigenetic profile of normal prostate tissues. Our data indicates that several genes which are hypermethylated in prostate cancer tissue may undergo methylation in normal prostate tissues in an age-dependent manner. To our knowledge, this is the first study to directly examine the relationship between methylation and age in human prostate tissues. Our studies clearly show that methylation starts in normal prostate tissues as a function of age and markedly increases in cancer.

Fig. 5. Comparison of percentage of methylation levels. A, the methylation levels for RARα2, RASSF1A, GISTP1, NKX2-5, ESRI, and CLSTN1 genes were compared between prostate samples as follows: normal prostate tissues from organ donors (Nl/org), normal cystoprostatectomy tissues from patients with bladder cancer (Nl/cyst), benign prostate tissues from patients with prostate cancer (Nl/PCa), and prostate cancer (Can) tissue samples (X-axis). Y-axis, the percentages of methylated cytosine in each patient sample as obtained from pyrosequencing. Each CpG island has a different scale range. Horizontal bars, average methylation levels. B, the methylation levels were compared between matched benign and prostate cancer tissues from 12 patients with prostate cancer. Y-axis, the percentages of methylated cytosine in each patient sample as obtained from pyrosequencing. Each CpG island has a different scale range. X-axis, benign (normal) or prostate cancer (cancerous) tissues.
A larger prostate cancer population study is needed to confirm our observations and to determine if methylation status can provide reliable information for the detection of prostate cancer.

References

Age-Related DNA Methylation Changes in Normal Human Prostate Tissues


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