Progressive Increases in de Novo Methylation of CpG Islands in Bladder Cancer

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ABSTRACT

We conducted a quantitative analysis of the extent of de novo methylation of four CpG islands in human urinary transitional cell carcinomas of different stages and grades to determine how frequently these CpG islands became methylated in transition cell carcinomas during progression. The CpG islands included exon 5 of PAX6, exon 2 of p16, the 5′ end of the deleted in bladder cancer gene, and the 5′ end of transmembrane protein containing epidermal growth factor and follistatin domains. These sequences were not methylated in normal urothelial tissues; however, 48 of the 54 tumors examined (89%) showed methylation levels in excess of 20% for at least one of the markers. The number of markers concurrently methylated in individual tumors increased with the stage of the tumor, with several of the more aggressive invasive cancers showing hypermethylation of all four markers compared with the less aggressive invasive cancers. However, considerable methylation defects were present in superficial, preinvasive, papillary tumors. These data demonstrate that 89% of bladder cancers have increased methylation of CpG islands relative to their normal counterparts and suggest the occurrence of a hypermethylation phenotype in which multiple independent CpG islands become concurrently methylated in individual tumors in a process associated with tumor progression.

INTRODUCTION

Alterations in the methylation patterns of the genome are among the most common genetic changes observed in human cancers (1, 2), though the true extent of these changes is not yet fully known. Analysis of the methylation patterns of candidate genes, particularly tumor suppressor genes such as p16, RB, and VHL, has convincingly demonstrated that the promoters of these genes become altered and contribute to carcinogenesis (3–7). However, the analysis of candidate genes does not give a comprehensive picture of the total of changes occurring in a given tumor class because these analyses are, by their nature, highly focused. Also, although many studies have demonstrated methylation changes in cancers, the methods used are not always quantitative, and therefore, it is difficult to appreciate the full extent of the changes from the semiquantitative data obtained.

Recently, several investigators have developed genome-scanning techniques sensitive to DNA methylation to gain an appreciation of the genome-wide changes occurring within various cancers. These approaches, which include methylation-sensitive arbitrarily primed PCR (8–10), methylated CpG islands amplification (11), and restriction landmark genomic scanning (12) among others, have shown that methylation changes are widespread, but it has not always been possible to quantify the extent of changes from these screening techniques. Our earlier analyses by genome scanning of bladder, prostate, and colon cancers suggested the possible occurrence of a hypermethylation phenotype (13). Toyota et al. (14) have recently described a hypermethylation phenotype in colorectal cancers that they called CIMP (CpG island methylator phenotype). Therefore, we have extended our earlier semiquantitative studies to use a quantitative technique developed in this laboratory for the assessment of methylation (15) to measure the levels of inappropriate methylation of CpG islands, which have been observed to become methylated in various cancers.

We focused our studies on human bladder cancer, the 5th most common cancer in the United States. TCCs are clinically managed according to their stage and grade. Currently, superficial (preinvasive) papillary bladder cancers and non-muscle-invasive (minimally invasive) bladder cancers are treated conservatively, whereas muscle-invasive bladder cancers are treated more aggressively. Furthermore, it has been shown previously that superficial (preinvasive) bladder tumors are genetically distinct from invasive bladder tumors with different aberrant molecular profiles (16). We have conducted a quantitative methylation analysis of four loci in bladder tumors of different grades and stages to determine whether methylation changes occur early in the carcinogenic process and/or become reinforced during tumor progression.

MATERIALS AND METHODS

Tissue Collection and DNA Isolation. Tissue specimens were obtained from patients at the University of Southern California/Norris Comprehensive Cancer Center and the Los Angeles County, University of Southern California Medical Center. Fifty-eight specimens were collected: 4 normal urothelial controls from individuals without bladder cancer, 5 minimally invasive bladder TCCs (pT1), 13 muscle-invasive bladder TCCs without associated lymphatic cancer (pT2N0), 16 muscle-invasive bladder TCCs with associated lymphatic cancer (pT2N+), 11 metastatic lymph nodes with associated muscle-invasive TCC (+ nodes), and 9 preinvasive bladder TCCs (papillary). Prior to DNA extraction, an H&E slide was prepared for each tumor specimen collected to verify the presence of cancer cells. DNA was isolated using standard procedures by treatment with proteinase K and phenol extraction (17).

Quantitation of Methylation by Ms-SNuPE Analysis. Methylation was quantitated using a Ms-SNuPE assay, described by Gonzalez and Jones in 1997 (15). Briefly, genomic DNA was treated with sodium bisulfite to convert unmethylated cytosines to uracil (which then converts to thymine after PCR) and leave 5-methylcytosines unchanged. Amplification of the desired target sequence was performed using PCR primers specific for bisulfite-converted DNA, and the PCR products then were isolated and used as a template for methylation analysis at the two or three CpG sites by two or three specific Ms-SNuPE primers (Table 1). The final PCR products were then resolved on a 15% polyacrylamide gel and a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) was used to quantitate the percent methylation averaged from the three CpG sites.

PCR Condition for Bisulfite-converted DNA. PAX6 exon 5: 95°C for 2 min, 95°C for 1 min, 50°C for 30 s, 72°C for 1 min, cycled 40 times, then 72°C for 4 min; p16 exon 2: 95°C for 2 min, 95°C for 1 min, 58°C for 45 s, 72°C for 90 s, cycled 38 times, then 72°C for 2 min; 5′ end region of DBC: 94°C for 3 min, 94°C for 1 min, 49°C for 45 s, 72°C for 45 s, cycled 44 times, then 72°C for 4 min; 5′ end region of TPEF: 95°C for 2 min, 95°C for 1 min, 51°C for 30 s, 72°C for 1 min, cycled 40 times, then 72°C for 4 min.

Ms-SNuPE Conditions. PAX6 exon 5: 95°C for 1 min, 46°C for 2 min, 72°C for 1 min, then 4°C; p16 exon 2: 95°C for 1 min, 50°C for 2 min, 72°C

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The abbreviations used are: TCC, transitional cell carcinoma; Ms-SNuPE, methylation-sensitive single nucleotide primer extension; DBC, deleted in bladder cancer; TPEF, transmembrane protein containing epidermal growth factor and follistatin domains.

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for 1 min, then 4°C; 5’ end region of DBC: 95°C for 1 min, 46°C for 2 min, 72°C for 1 min, then 4°C; 5’ end region of TPEF: 95°C for 1 min, 51°C for 2 min, 72°C for 1 min, then 4°C.

Statistical Analysis. Two-sided P values were generated for each tumor group and compared with normal controls using the Wilcoxon rank sum test. The significance of an increasing methylation trend between minimally invasive tumors and the muscle-invasive tumors was determined using a Mantel-Haenszel $\chi^2$ test (18).

RESULTS

We analyzed four CpG islands known to undergo de novo methylation during bladder carcinogenesis. The exon 5 region of PAX6 was selected because this small intronic CpG island was found to be methylated frequently in a genomic screen (13), although its methylation is not associated with down-regulation of the gene (19). The exon 2 region of p16 was selected because this region is frequently methylated in bladder cancer cell lines and tumors (3), and the 5’ end of the DBC gene was studied as a potential candidate suppressor gene in bladder cancer known to be methylated in cell lines (20). The fourth region studied was located in the 5’ end of a previously unknown gene, TPEF, which was also isolated from the arbitrarily primed-PCR screen previously conducted (13, 21). Both the latter two regions are associated with the 5’ region of their genes.

The methylation levels of these regions were analyzed by the quantitative Ms-SNuPE analysis (15), which was standardized in each case to ensure the linearity of the PCR amplification of the region after bisulfite treatment. Typical Ms-SNuPE results for the four regions investigated are shown in Fig. 1, in which extensive methylation of three of the four CpG islands is shown by the increased intensities of bands in the C lanes of the analysis. Therefore, this method gives a quantitative assessment of the methylation status of specific CpG sites within each CpG island region.

Figs. 2 and 3 summarize the data from the methylation analysis of the 58 tissue specimens collected. All of the loci for each of the four normal tissues were found to be methylated to a level less than 12%, averaging 5% overall (Fig. 2). This low amount of methylation was as expected because CpG islands on autosomal genes are generally unmethylated in normal tissues (22). We chose 20% as the cutoff for significant methylation to avoid false positive interpretations from our data.

Each of the tumor tissue groups were found to be significantly more methylated than the normal controls ($P = 0.002 – 0.0001$; Fig. 2). Increased methylation of some of the loci was seen in the minimally invasive tumors (pT1) with three of the five (60%) showing methylation levels greater than 20% of at least one of the markers examined. In contrast, the muscle-invasive tumors obtained from patients without lymphatic metastases (>pT2N0) showed methylation levels greater than 20% of at least one of the markers examined. Furthermore, the degree of CpG island methylation was also substantial in the muscle-invasive tumors with associated metastatic lymph nodes (>pT2N+), with 15 of the 16 tumors (94%) in this group showing methylation of greater than 20% of at least one of the markers. Interestingly, many of the tumors had multiple methylation changes with increased methylation greater than 20% seen at all four loci in a considerable proportion of the tumors (Fig. 3). These data clearly show that the

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**Table 1** Primer sequences for bisulfite PCR and Ms-SNuPE

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bisulfite PCR</strong></td>
<td></td>
</tr>
<tr>
<td>PAX6 exon 5</td>
<td></td>
</tr>
<tr>
<td>Sense 5’-GGG AGG ATT ATT TGT AG-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense 5’-CTT-TCC-TCA-AAT-CAC-3’</td>
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</tr>
<tr>
<td>p16 exon 2</td>
<td></td>
</tr>
<tr>
<td>Sense 5’-GTA GGT GGG GAG TTT AGT-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense 5’-TCT AAC CA ACAA CCT CC-3’</td>
<td></td>
</tr>
<tr>
<td>5’ Region of DBC</td>
<td></td>
</tr>
<tr>
<td>Sense 5’-GAA ATT TTT TAA(T/C) GGG TTA TTA GTT TAT-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense 5’-ACT AAC (AG)AA TAA AAA AAT TCC AAA TAC-3’</td>
<td></td>
</tr>
<tr>
<td>5’ Region of TPEF</td>
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<tr>
<td>Sense 5’-AAT TAG TTA TGG TGT GG-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense 5’-CTA AAA TAA ACT CTA TAC TAA ATA C-3’</td>
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<tr>
<td><strong>Ms-SNuPE</strong></td>
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<tr>
<td>PAX6 exon 5</td>
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<td>17 mer 5’-GTT GAT AAA GAT ATT AT-3’</td>
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<tr>
<td>19 mer 5’-GAG GAT TAG TTT TAG AAT T-3’</td>
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<tr>
<td>23 mer 5’-AGT TAG TTT TAG AT T-3’</td>
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</tr>
<tr>
<td>p16 exon 2</td>
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<td>15 mer 5’-GTT GGT GGT GTT GTA-3’</td>
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<td>18 mer 5’-AGG TTA TGA TGG GTA G-3’</td>
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</tr>
<tr>
<td>21 mer 5’-TAT TAG AGG TAG TAA TTA TGC-3’</td>
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<tr>
<td>5’ Region of DBC</td>
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</tr>
<tr>
<td>19 mer 5’-GTT TTA GTT TTA GTT TAT-3’</td>
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</tr>
<tr>
<td>21 mer 5’-AAA GGT GTA TAA AAT ATT TTT-3’</td>
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<tr>
<td>23 mer 5’-ATG GTA TGA TGG GTA TTA TAT-3’</td>
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</tr>
<tr>
<td>27 mer 5’-TAG GTT AGT AGG TTG ATAT C-3’</td>
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<tr>
<td>TPEF end</td>
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<tr>
<td>21 mer 5’-GGT GGT GGT GTT GTA-3’</td>
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</tr>
<tr>
<td>19 mer 5’-GTT GAT AAA GAT ATT AT-3’</td>
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<tr>
<td>23 mer 5’-AAT TAG TTA TGG TGT GG-3’</td>
<td></td>
</tr>
<tr>
<td>27 mer 5’-CTA AAA TAA ACT CTA TAC TAA ATA C-3’</td>
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</tbody>
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**Fig. 1.** Ms-SNuPE analysis (patient 487). Quantitative methylation analysis of two or three CpG sites in PAX6 exon 5, p16 exon 2, 5’ end of DBC, and 5’ end of TPEF using the Ms-SNuPE technique. The percent methylation represents the average of two or three sites using the following equation on PhosphoImager quantitation: (methylated)C × 100

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degree of aberrant methylation in bladder cancer increases substantially as the tumors progress from minimally invasive (pT1) to muscle-invasive with metastatic lymph nodes (pT2N+); $P = 0.047$. The levels of methylation of the four CpG islands examined in the primary tumors of patients 79, 11, 13, 19, 24, 15, and 23 were generally conserved or increased in the seven metastatic lymph nodes obtained from the same patients (Fig. 2). This suggests that further increases in cytosine methylation may accompany tumor metastasis.

Figs. 2 and 3 also show that substantial methylation changes were already present in the preinvasive (papillary) tumors with 8 of the 9 (88%) of these tumors showing methylation greater than 20% of at least one marker. Interestingly, the methylation changes were mainly confined to the PAX6 exon 5 and p16 exon 2 regions, and little increased methylation was seen in the two islands located in the 5’ regions of DBC or TPEF.

Most of the tumors examined in this study were grades 3 and 4 (Fig. 2), and there was no statistically significant relationship between grade and methylation level. Although many tumors had increased methylation of both the p16 and PAX6 markers, the methylation levels in each marker were not highly correlated with each other (correlation coefficient = 0.37).

**DISCUSSION**

The results of this quantitative analysis of the methylation levels of four independent CpG islands in human bladder cancer underscore the commonality with which these changes are observed in TCC. The four CpG islands selected for study were chosen because of their propensities to undergo de novo methylation as observed in earlier studies. The regions selected included two transcribed regions where methylation is not considered to be involved in gene silencing and two regions located in the 5’ regions of DBC or TPEF.
that CpG island methylation occurs more frequently in regions of DNA that are downstream of the transcriptional start site, making these changes one of the most common genomic alterations in human bladder cancer.

**REFERENCES**


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