Unique DNA Methylation Patterns Distinguish Noninvasive and Invasive Urothelial Cancers and Establish an Epigenetic Field Defect in Premalignant Tissue

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Abstract

Urothelial cancer (UC) develops along two different genetic pathways, resulting in noninvasive or invasive tumors. However, it is unknown whether there are also different epigenetic pathways in UC. UC is also characterized by a high rate of recurrence, and the presence of a field defect has been postulated. In this study, we compared the DNA methylation patterns between noninvasive and invasive UC and the DNA methylation patterns between normal-appearing urothelium from bladders with cancer and urothelium from cancer-free bladders. We used the Illumina GoldenGate methylation assay at 1,370 loci in 49 noninvasive urothelial tumors, 38 invasive tumors with matched normal-appearing urothelium, and urothelium from 12 age-matched UC-free patients. We found distinct patterns of hypomethylation in the noninvasive tumors and widespread hypermethylation in the invasive tumors, confirming that the two pathways differ epigenetically in addition to genetically. We also found that 12% of the loci were hypermethylated in apparently normal urothelium from bladders with cancer, indicating an epigenetic field defect. X-chromosome inactivation analysis indicated that this field defect did not result in clonal expansion but occurred independently across the urothelium of bladders with cancer. The hypomethylation present in noninvasive tumors may counterintuitively provide a biological explanation for the failure of these tumors to become invasive. In addition, an epithelium-wide epigenetic defect in bladders with cancer might contribute to a loss of epithelial integrity and create a permissible environment for tumors to arise.

Introduction

Urothelial cancer (UC) is the fifth most commonly diagnosed cancer in the United States and has a high rate of recurrence, making it one of the most burdensome cancers to treat. UC is, however, a fascinating disease to study from the scientific standpoint because several details of its molecular pathology are now understood. There seem to be two pathways of UC formation in which distinct genetic alterations occur. Most patients who present with UC have low-stage tumors (T<sub>N</sub>–T<sub>1</sub>) that are normally treated by resection and rarely progress to form invasive tumors (T<sub>2</sub>–T<sub>4</sub>). The low-stage tumors often have FGFR3 mutations (1) together with chromosome-9 aberrations (2, 3). In contrast, flat carcinomas in situ frequently have TP53 mutations in addition to chromosome-9 deletions (4), and these tumors progress to become muscle invasive, acquiring RB1 aberrations (5) and DNA hypermethylation (6).

DNA methylation, nucleosome positioning, and histone modifications are epigenetic processes that heritably change gene expression without altering the DNA sequence (7). DNA methylation of cytosine residues occurs in the context of CpG dinucleotides. Most unmethylated CpGs are found in GC-rich sequences referred to as CpG islands. Approximately 40% to 50% of human genes are associated with CpG islands (8), and it has been shown repeatedly that methylation of the CpG sites within these promoters could effectively and heritably silence genes. The progressive increase in de novo methylation of CpG islands in urothelial carcinoma cells suggests that epigenetic gene silencing is involved in the development of UC (6, 9–13).

Successes in the clinic have opened up the era of epigenetic therapy in which the goal is to reactivate genes silenced inappropriately during carcinogenesis (14). The advantage of using epigenetic therapy to target defects in
DNA methylation is that, unlike mutations in the DNA sequence, these alterations are reversible. Although DNA methylation inhibitors are effective in treating cancers of the blood, their efficacy in treating solid tumors remains controversial. We have shown in mice that DNA methylation inhibitors can slow the growth of tumor xenografts (15) and prevent the formation of tumor precursors (16), as well as reduce DNA methylation levels in normal tissues. Therefore, it is possible that systemic treatment of humans with these same drugs will affect aberrant DNA methylation in a variety of tissues and will be useful for treating and preventing solid tumors such as those of the bladder. However, the epigenetic alterations in urothelial tumorigenesis must first be elucidated to determine whether they provide useful therapeutic targets for epigenetic therapy. An ideal therapy for UC would address many of its unique aspects, such as treating both noninvasive and invasive tumors even though they develop via two separate molecular pathways and reducing the high frequency of recurrences. The biological basis for the high rate of recurrence for urothelial tumors remains unknown, although the presence of a field defect has been postulated (9). A field defect is an area of tissue that is predisposed to undergo transformation and has been proposed as an underlying mechanism of tumor recurrences and multifocality. It remains unclear whether such premalignant changes in an affected organ are due to expansion of a single cell with a growth advantage or due to a generalized defect. DNA methylation has been shown to be involved in field defects in other cancers (17–20), highlighting the possible role of epigenetic alterations in the development of cancer. By conducting high-throughput DNA methylation analysis on urothelial tumors, corresponding normal-appearing tissues, and tissue from cancer-free bladders, we found DNA hypermethylation in normal-appearing tissues from bladders with cancer, supporting the presence of an epigenetic field defect. We also revealed that noninvasive tumors and invasive tumors arise via distinct epigenetic pathways. Noninvasive tumors display a unique hypomethylated phenotype, which may counterintuitively provide a biological explanation for the failure of these tumors to become invasive and life-threatening. Therefore, epigenetic therapy may be useful for targeting tumors and also help prevent future recurrences by reversing premalignant changes before a new tumor forms.

Materials and Methods

Tissue samples and DNA/RNA isolation

Tumor tissue samples were collected from patients undergoing cystectomy or transurethral resection of a bladder tumor for UC. Twelve samples of normal urothelium were obtained from five patients undergoing radical prostatectomy for prostate cancer (ages 50–80 years) and seven autopsy patients (ages 34–82 years; five from non–cancer-related deaths and the remaining from deaths due to cancers other than bladder). We also included 49 noninvasive urothelial tumors (T1–T4), 38 invasive tumors (T2–T4), and 34 normal-appearing tissues from patients that were having their entire bladders removed. See Table 1 for a summary of the clinicopathologic information on the patients from which tumors were collected and Supplementary Table S1 for more detailed information. All of these collections took place at the Norris Cancer Hospital at USC, and Tochigi Cancer Center Hospital, Nara, Japan, in compliance with Institutional Review Board–approved protocols with patients’ consent. H&E sections marked with the location of the corresponding normal urothelium or tumor were used as guide in microdissection. DNA was bisulfite treated as previously described (21). Total RNA extraction was done using an RNeasy Micro Kit (Qiagen). RNA was reverse-transcribed as previously described (11).

Illumina GoldenGate methylation assay

Bisulfite conversion of 2 to 4 μg of genomic DNA was achieved with the EZ DNA Methylation-Gold Kit (Zymo Research). The samples were interrogated using the Illumina GoldenGate Methylation Cancer Panel I, and measurements were computed using Illumina BeadStudio Software. A β value of 0 to 1.0 was reported for each CpG site signifying percent methylation from 0% to 100%, respectively. β values were calculated by subtracting background using negative controls on the array and taking the ratio of the methylated signal intensity to the sum of both methylated and unmethylated signals plus a constant of 100. Measurements with detection \( P > 0.05 \) were marked as missing.

Validation of GoldenGate DNA methylation results

Pyrosequencing was also performed as described previously (22). Briefly, bisulfite-converted DNA was PCR amplified

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using a biotin-labeled 3′ primer to enable purification and denaturation of the product by Streptavidin Sepharose beads. This was followed by annealing of a sequencing primer to the single-stranded PCR product. Pyrosequencing was performed using the PSQ HSR Pyrosequencing System, and the degree of DNA methylation was expressed for each DNA locus as percentage methylated cytosines over the sum of methylated and unmethylated cytosines.

**Bisulfite sequencing and X-chromosome inactivation analysis**

To analyze the DNA methylation status of individual DNA molecules, bisulfite PCR fragments were cloned into the pCR2.1 vector using the TOPO-TA cloning kit (Invitrogen). Individual colonies were screened for the insert, and the region of interest was sequenced using M13 primers. Bisulfite sequencing was performed on a region of the X-chromosome with an A/T single nucleotide polymorphism (rs4826507) and six CpG sites that were differentially methylated between the active and inactive X chromosomes (23), allowing for the simultaneous identification of which X-chromosome was being interrogated and its activation status.

**Statistical analysis**

Probes that were missing measurements in 10 or more samples were excluded from the statistical analysis. Average linkage hierarchical clustering using Euclidean distance was used to group the probes with similar DNA methylation patterns for all the samples.

The Wilcoxon rank sum test was used to evaluate the statistical significance of the separate comparisons between normal samples and invasive tumors, noninvasive tumors, and normal-appearing adjacent tissue. To account for testing three comparisons for each probe, we multiplied each P value by 3 (Bonferroni correction) before applying a false discovery rate (FDR) adjustment across all probes for each pairwise comparison. We used the Benjamini and Hochberg method (24) to control the FDR, using a threshold of 5% (two-sided) for multiple comparisons for each probe, we multiplied each P value with an A/T single nucleotide polymorphism (rs4826507) and six CpG sites that were differentially methylated between the active and inactive X chromosomes (23), allowing for the simultaneous identification of which X-chromosome was being interrogated and its activation status.

Gene expression data were obtained from GeneChiPs (Affymetrix) using U133A human Gene-Chips containing 22,283 probes representing known genes and expression sequence tag (Affymetrix; ref. 25). The differentially expressed genes were identified by comparing 105 urothelial tumors: 33 noninvasive (T\textsubscript{a}–T\textsubscript{1}), 72 invasive lesions (T\textsubscript{2}–T\textsubscript{4}), and 52 normal urothelium. Differential expression was evaluated using the signal as the main response measure, extracted from each gene on every sample, as determined by default setting of the MAS 5.0 (25). The gene expression and DNA methylation measurements of a gene were linked by the National Center for Biotechnology Information gene identifier. To carry out concordance analysis between DNA methylation and gene expression, we first selected the unique DNA methylation probe from each gene that is closest to the transcription start site based on the National Center for Biotechnology Information gene identifier. A total of 784 genes were selected. Genes with multiple expression probe sets were classified by the probe set with the minimum P value. We then categorized the genes into nine groups based on the DNA methylation and gene expression measurements: hypermethylation versus hypomethylation versus no significant DNA methylation changes; decreased expression versus increased expression versus no significant gene expression changes. We used χ\textsuperscript{2} statistics with 4 degrees of freedom to test for associations between DNA methylation and gene expression. To visualize these nine groups of genes, we used the starburst plot using the FDR-adjusted P values for differences in the DNA methylation and gene expression (26).

**Results**

Noninvasive urothelial tumors have a distinct pattern of hypomethylation

We used the Illumina GoldenGate platform to conduct a large-scale analysis of DNA methylation in tissues from cancer-free urothelial sample, corresponding normal-appearing tissues from bladders with cancer, and urothelial tumors at 1,370 autosomal loci (784 genes; Fig. 1; Supplementary Table S1). We included 49 noninvasive urothelial tumors (T\textsubscript{a}–T\textsubscript{1}) and 38 invasive tumors (T\textsubscript{2}–T\textsubscript{4}; Table 1). Progressive hypermethylation in invasive cancer has been widely documented. As expected, when we compared the two types of tumors to the 12 samples of urothelium obtained from patients without UC (Figs. 1 and 2A), more loci were hypermethylated in invasive tumors [526 of 1,370 (38%)] than in noninvasive tumors [132 of 1,370 (10%)], with the majority of those in the noninvasive tumors [117 of 132 (89%)] overlapping with those in the invasive tumors (P < 0.0001 by Fisher’s exact test). Most of the hypermethylated loci were located in the context of CpG islands (Figs. 1 and 2B). Interestingly, we also found a distinct pattern of hypomethylation in noninvasive (T\textsubscript{a}–T\textsubscript{1}) tumors compared with both normal urothelium and invasive tumors (Fig. 2A). A substantial number of probes [217 of 1,370 (16%)] were hypomethylated only in the noninvasive tumors compared with those only hypomethylated in invasive tumors [41 of 1,370 (3%); McNemar’s P < 0.001], in addition to those loci hypomethylated in both types [253 of 1,370 (18%)]. Most of the hypomethylated loci were located outside of CpG islands (Figs. 1 and 2B). The confirmation of five identified hypermethylated loci and five hypomethylated loci with Pyrosequencing is shown in Supplementary Fig. S1. This distinct pattern of hypomethylation in the noninvasive tumors suggests that the two separate molecular pathways to noninvasive or invasive urothelial tumors differ epigenetically as well as genetically.

**Gene promoter DNA methylation inversely correlates with expression in urothelial tumors**

To determine whether the aberrantly hypermethylated and hypomethylated loci might play a functional role in tumorigenesis, we compared our DNA methylation data to already available gene expression data (ref. 25; Fig. 2C). We found that differential DNA methylation was associated with differential gene expression for both invasive and superficial tumors (P < 0.0001 and P = 0.001, respectively). A substantial
number of hypermethylated genes correlated with decreased gene expression in the noninvasive tumors (37 observed compared with 30 expected, ratio = 1.24, \( P = 0.11 \)) and in the invasive tumors (117 observed compared with 92 expected, ratio = 1.28, \( P < 0.0001 \)) when compared with normal urothelium. We also found that similar ratios of observed to expected counts for hypomethylated genes showing overexpression relative to the normal tissues in both the noninvasive (101 observed compared with 83 expected, ratio = 1.21, \( P = 0.004 \)) and invasive (56 observed compared with 45 expected, ratio = 1.24, \( P = 0.04 \)) tumors, indicating that both DNA hypermethylation and DNA hypomethylation of gene promoters correlate with gene expression changes in tumors. It should be noted that although integrating DNA methylation and gene expression data from the same specimen is ideal, the correlations from these independent data sets are biologically intriguing in elucidating the role of aberrant DNA methylation in gene expression alterations in UC.

**Urothelial cancers have a widespread epigenetic field defect**

In addition to the widespread alterations of DNA methylation in urothelial tumors, we also discovered 169 probes spanning 155 unique gene regions that were aberrantly methylated in normal-appearing tissues taken at least 5 cm away from the site of their corresponding invasive tumor (Fig. 2A). The majority of these probes were also identified as DNA hypermethylated in invasive tumors [142 of 169 (84%); \( P < 0.0001 \) by Fisher’s exact test] and a smaller portion in noninvasive urothelial tumors [142 of 169 (84%); \( P < 0.0001 \) by Fisher’s exact test; Fig. 2A]. Only 18 genes (19 loci, 1%) were found to be hypomethylated, indicating that aberrant hypermethylation may constitute the majority of epigenetic defects present in the urothelium. To rule out potential contamination of corresponding normal tissues with tumor cells, we also performed unsupervised hierarchical clustering analysis (Supplementary Fig. S2). In general, corresponding normal
Figure 2. A, Venn diagram of the overlap of hypermethylated and hypomethylated loci. The hypermethylated and hypomethylated cancer-specific loci were determined using three separate comparisons of tissue from UC patients (CN, T1-3, and T4-4) to 12 N samples (5% FDR, Wilcoxon test). B, the majority of loci hypermethylated in corresponding normal-appearing tissues and tumor tissues are CpG islands, in contrast to the hypomethylated loci. C, starburst plots of transcriptome and epigenetic differences between normal urothelia and urothelial tumors (26). Each data point in the Starburst plot represents the FDR value calculated for gene expression and DNA methylation independently. Red vertical and horizontal lines indicate FDR level at 0.05. FDR values as log10 transformed data are plotted for normalized gene expression (y axis) and DNA methylation (x axis) for each gene. The sign (negative or positive log10(FDR)) depends on the direction of fold normalized gene expression and difference in DNA methylation β value. If the fold gene expression is higher in tumors compared with normal, –1 is multiplied to log10(FDR), providing positive values. If the fold gene expression levels are lower in tumors compared with normal, 1 is multiplied to log10(FDR), providing negative values. Concurrently, if the DNA methylation β value difference indicates hypermethylation in tumors compared with normal, –1 is multiplied to log10(FDR), providing positive values. If the DNA methylation β value difference indicates hypomethylation in tumors compared with normal, 1 is multiplied to log10(FDR), providing negative values. FDR values decrease (becoming more statistically significant) as the log10(FDR) proceeds in either direction from the P = 0.05 cutoff. Hypermethylated genes with decreased expression levels in noninvasive (T1-3, 39) and invasive (T4-4, 121) urothelial tumors are labeled with red data points, whereas hypomethylated genes having increased expression levels in T1-3 (103) and T4-4 (61) are labeled with green data points.
tissues cluster together, and tumors cluster together with no clustering of tissues from a single individual, thereby clearly ruling out potential contamination. These hypermethylated loci may provide markers for the identification of individuals at risk for developing UC, as they are only altered in bladders with cancer.

Further examination of DNA methylation changes at a few of these loci reveals a clear increase of methylation in corresponding normal-appearing tissues, including at ZO2 (zona occludens 2 or TJP2; Fig. 3A), a tight junction binding protein; MYOD1; and CDH13 (H-cadherin; Supplementary Fig. S3). In addition to serving as biomarkers, the DNA methylation changes found at these loci may have functional significance. Examining the expression of ZO2 was measured by reverse transcription-PCR. Horizontal lines represent the median value. Red arrows indicate the CpG sites queried. ***, *P* < 0.001; **, *P* < 0.01, as determined by unpaired *t* tests (N and CN, N and T) or paired *t* tests (CN and T). B, bisulfite sequencing of ZO2 was performed on one N, one CN, and the matched T using primers indicated by the black arrows to confirm the Illumina methylation results. C and D, tissue samples were taken from tumors of five patients (T; red) and at increasing distances from the tumor (0.5–2 cm) in the surrounding normal-appearing tissue in multiple directions (light blue; a–d). Additionally, distant normal-appearing samples were taken at least 5 cm from the tumor (C; dark blue). DNA methylation was measured by pyrosequencing. The green line represents the median methylation value of normal samples from cancer-free patients. White circles indicate unmethylated CpG sites while black circles indicate methylated CpG sites.
expression in the corresponding normal-appearing tissue and even less in tumors, indicating that DNA hypermethylation of ZO2 is associated with reduced expression (Fig. 3A). Bisulfite sequencing confirmed the absence of DNA methylation in normal urothelium from a bladder cancer–free patient, a low level of DNA methylation in corresponding normal-appearing tissue, and a high level of DNA methylation in the urothelial tumor from the same patient (Fig. 3B).

To determine the full extent of DNA hypermethylation in histologically normal tissue and to attempt to elucidate the mechanism driving such alterations, we collected a series of primary tissue samples taken from various distances away from tumors in five bladders (Fig. 3C). We found DNA hypermethylation of ZO2 across bladders with cancer when compared with the average level of ZO2 DNA methylation in normal urothelium from UC-free patients, indicating the presence of a widespread epigenetic field defect in bladders with cancer (Fig. 3D). ZO2 methylation was reduced by treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine in UC cell lines, leading to reexpression of the gene (data not shown). Taken together, these results suggest that epigenetic therapy, in addition to having the potential to treat urothelial tumors, may also be able to prevent cancer by reversing premalignant defects in bladders.

Figure 4. X-inactivation patterns reveal that the urothelium of two female patients with UC remains polyclonal. A, bisulfite sequencing was performed on a region located on the X-chromosome with an A/T single nucleotide polymorphism (rs4826507). This single nucleotide polymorphism is located within an exonic region that is methylated on the active X chromosome and unmethylated on the inactive X chromosome (23). White circles indicate unmethylated CpG sites while black circles indicate methylated CpG sites. Pink and blue boxes represent the portion of sequences from each clonal population. The tumors are monoclonal whereas the samples of corresponding normal-appearing urothelium taken at various distances from the site of the tumors are polyclonal, indicating that a single clone has not grown across the urothelium. B, two of the possible explanations for the presence of abnormal DNA methylation in normal-appearing urothelium from bladders with cancer are that either aberrant DNA methylation accumulates in one cell, followed by the clonal expansion of that cell population across the urothelium and subsequent transformation, or that there is a generalized epigenetic defect that occurs independently in many different cells, which does not result in a growth advantage but predisposes cells to undergo transformation. The X-inactivation results support the latter.
The epigenetic field defect in bladders is not due to clonal expansion

To address whether the widespread epigenetic field defect found in bladders with cancer is due to clonal expansion across the bladder or a generalized epigenetic alteration, we first sequenced DNA from tumors of these same patients for one of the earliest genetic alterations in UC, FGFR3 mutations (27, 28). FGFR3 was mutated only in the tumor samples and not in any of the corresponding normal samples (no. 6519 at S249C and no. 6671 at F384I), similar to recent results from Otto and colleagues (29). Because it is possible that clonal expansion could have occurred before the FGFR3 mutation was acquired, we also examined the pattern of X chromosome inactivation in a series of samples taken from two female patients, no. 6522 and no. 6671. X-inactivation patterns are permanent marks of clonality. By conducting bisulfite sequencing on a region that contains a single nucleotide polymorphism that is differentially methylated between the active and inactive X-chromosomes, we found that a random pattern of X-inactivation was maintained across these bladders (Fig. 4A). In addition, previous work with a different set of bladder specimens has shown that clonality is maintained across the urothelium of female patients with UC (30). These results suggest that the generalized epigenetic defect does not give rise to a clonal dominance of the histologically normal cells but may provide a permissive environment for either the emergence of tumor clones containing genetic mutations or the seeding or migration of tumor cells once they arise (Fig. 4B).

Genes hypermethylated in corresponding normal-appearing tissues may contribute to tumorigenesis

Because a large number of loci seem to become hypermethylated independently in both corresponding normal-appearing tissues and tumors, it is possible that these loci either are predisposed to undergo aberrant hypermethylation or contribute to tumorigenesis. It has been recently revealed that polycomb repressive complex 2 (PRC2) targets in embryonic stem cells are predisposed to undergo hypermethylation in cancer (31–33). As shown in Fig. 2B, hypermethylated loci show enrichment for CpG islands. Moreover, we found that 47% of loci that were hypermethylated in the corresponding normal-appearing tissues were PRC2 targets (Figs. 1 and 5). This is higher than the number of loci found in the noninvasive (40%) and invasive tumors (36%), indicating that PRC2 targets are predisposed to undergo DNA hypermethylation in premalignant tissues as well as tumors. These methylated loci may contribute to tumorigenesis, as 50 of the 155 genes (32%) that are hypermethylated in the corresponding normal-appearing tissues also show downregulated gene expression levels in tumors.

Discussion

DNA methylation changes are involved in both the initiation of carcinogenesis and progression. We have shown genome-scale alterations in DNA methylation patterns in urothelial tumors and normal-appearing tissues from bladders with cancer. We also found that hypomethylation of non-CpG island regions seems to be preferentially associated with low-stage (T1–T2) disease, providing additional molecular evidence that there are two distinct molecular pathways in the formation of carcinoma of the bladder (4). Interest in the field of DNA methylation and cancer was originally spurred by early observations that tumors were often hypomethylated relative to normal tissues (34), the majority of which was assumed to occur at repetitive elements. It has been suggested that such hypomethylation could lead to genomic instability and contribute to the tumor phenotype (35, 36).

A systematic analysis of DNA methylation of various types of repetitive elements in urothelial tumors revealed that both Sat-α and LINE-1 elements are significantly hypomethylated (37). We have recently revealed that one consequence of hypomethylation of LINE-1 elements in UC is the aberrant activation of transcripts of nearby genes (38). However, although LINE-1 promoters contain a small CpG island, the relationship between DNA methylation of non-CpG islands and expression remains controversial and underexplored even though approximately 45% of all human genes have promoters that are not located within CpG islands (39). DNA hypomethylation associated with increased gene expression has been reported in several studies (40, 41). We have recently confirmed a correlation between increased expression and hypomethylation at non-CpG island promoters in prostate cancer cell lines (32). In addition, it is possible that lower levels of DNA methylation may be related to a less malignant phenotype. For instance, when expression of the DNA methyltransferase Dnmt1 is reduced in ApcMin/+ mice, fewer intestinal tumors occur but more early intestinal lesions form, supporting the opposing effects of DNA hypomethylation of initiating lesions but suppressing further progression (42).

One of the most clinically relevant questions in tumor biology is why some tumors grow and progress while others remain quiescent (43). The high incidence of in situ tumors...
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discovered during autopsies indicates that there is a rarely occurring additional step required for a tumor to become life-threatening (43). Such a step has been postulated to include an angiogenic switch (43) or alterations in the stroma (44). Our results suggest that a switch to accumulating DNA hypermethylation may be involved, such as could be caused by overexpression of DNA methyltransferases (45).

We have also revealed that the urothelium in bladders with cancer is no longer “normal.” Instead, the urothelium in these diseased bladders has undergone widespread epigenetic alterations mainly consisting of aberrant hypermethylation. Our findings support a field cancerization model where independent events occur across the urothelium, resulting in a field defect that is polyclonal (Fig. 4B). The presence of an epithelial-wide defect in bladders with tumors is quite astounding. In contrast to work in other organs such as the colon (18), esophagus (17), breast (19), and stomach (20), we found no dependency on the distance from the tumor. This significant difference in the pathogenesis of UC is also supported by the unique characteristics of tumor multifocality and a tendency to recur. The most likely underlying cause is that the urothelium is uniformly exposed to carcinogenic compounds excreted in the urine, and once a tumor forms, it can be assumed that the entire urothelium has been exposed to critical levels of carcinogens (45). It is also possible that the presence of the tumor itself leads to epigenetic alterations across the bladder. We observed that 83% of the hypermethylated loci found in the corresponding urothelium were also found in invasive tumors. Given such a high degree of similarity between the two tissues, we believe that the most likely explanation is that these tissues are at two different steps in the tumorigenic pathway.

Although the alterations in DNA methylation that occur across the entire urothelium do not directly confer a growth advantage because the polyclonality of the urothelium is maintained, they may still have functional significance. The role of the tight junction binding protein ZO2 in cancer has not been studied in depth, but the expression of other components of tight junctions is frequently altered in cancer, with loss of expression leading to increased cell motility and invasiveness. Increased DNA methylation of MYOD1 has previously been shown to be associated with oncogenic transformation (46). CDH13 has previously been found to be hypermethylated in premalignant gastric tissue (47), and loss of expression is associated with increased invasion in melanocytes (48). We identified a total of 72 genes as hypermethylated in corresponding normal-appearing tissues and normal urothelium may contribute to a loss of epithelial integrity across the entire bladder. Such an epithelial-wide defect could allow for a more permissive environment for the growth of newly mutated cells.

One limitation of our study is that we are unable to determine whether the widespread field defect identified in bladders with invasive tumors is also present in bladders with noninvasive tumors. All of the corresponding normal-appearing tissues that we collected were from patients that were having their entire bladders removed. Treatment of noninvasive urothelial tumors usually involves surgical excision of the tumor and does not result in the collection of additional normal-appearing tissue. Therefore, because we have found that noninvasive and invasive urothelial tumors seem to undergo two separate epigenetic pathways to tumorigenesis, we cannot rule out that the generalized epigenetic defect we have uncovered is specific to bladders with invasive tumors.

In summary, urothelial tumors develop along two separate molecular pathways that differ both genetically and epigenetically. A generalized epigenetic defect exists across bladders with cancer that is not due to clonal expansion. Transurethral resection of urothelial tumors leaves behind large areas of epigenetically altered urothelium, possibly contributing to the high level of recurrence of UC. Fortunately, these hypermethylated loci may provide valuable biomarkers that have the potential to significantly affect the diagnosis and treatment of UC. Such alterations can be reversed by DNA methylation inhibitors in cell culture (49), mouse models (15), and WBC in humans (50). Therefore, it is possible that DNA methylation inhibitors may have an effect at all stages of bladder tumorigenesis: reversing premalignant epigenetic changes to prevent tumor formation or recurrence, preventing noninvasive tumors from becoming more aggressive, and inducing invasive tumors to become less malignant. In addition, the bladder may be an ideal organ for local treatment, as systemic side effects are avoided and the highest local efficacy is achieved.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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