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Alterations of DNA methylome in human bladder cancer

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Abbreviations: 3'-UTR, untranslated region; 5-Aza-CR, 5-Azacytidine; 5-Aza-CdR, 5-aza-2'-deoxycytidine; FDA, Food and Drug Administration; HDACs, histone deacetylases; HDACi, histone deacetylase inhibitors; LNAs, locked nucleic acids; MCAM, methylated CpG island amplification and microarray; MI, methylation index; miRNA, microRNA; MSP, methylation-specific polymerase chain reaction; MS.AP.PCR, methylation-sensitive arbitrarily primed polymerase chain reaction; ROC, Receiver Operating Characteristic; TCC, transitional cell carcinomas

Bladder cancer is the fourth most common cancer in men in the United States, and its recurrence rate is highest among all malignancies. The unmet need for improved strategies for early detection, treatment, and monitoring of the progression of this disease continues to translate into high mortality and morbidity. The quest for advanced diagnostic, therapeutic, and prognostic approaches for bladder cancer is a high priority, which can be achieved by understanding the molecular mechanisms of the initiation and progression of this malignancy. Aberrant DNA methylation in single or multiple cancer-related genes/loci has been found in human bladder tumors and cancer cell lines, and urine sediments, and correlated with many clinicopathological features of this disease, including tumor relapse, muscle-invasiveness, and survival. The present review summarizes the published research on aberrant DNA methylation in connection with human bladder cancer. Representative studies are highlighted to set forth the current state of knowledge, gaps in the knowledgebase, and future directions in this prime epigenetic field of research. Identifying the potentially reversible and "drugable" aberrant DNA methylation events that initiate and promote bladder cancer development can highlight biological markers for early diagnosis, effective therapy and accurate prognosis of this malignancy.

Introduction

Bladder cancer is a major public health problem, causing significant mortality and morbidity worldwide.[1-3] In the US, bladder cancer is the fourth most common cancer in men, with an estimated 72 570 new cases and 15 210 deaths in 2013.[2] The vast majority (90%) of bladder cancer cases are transitional cell carcinomas (TCC), of which 75–85% present as non-muscle invasive tumors at the time of first diagnosis (Tis/CIS, Ta, and T1).[4-6] The prognosis of these non-invasive tumors is favorable although up to 80% of cases will recur after complete transurethral resection, and up to 45% of cases will progress to invasive cancer (T2–T4) within 5 years (**Fig. 1**).[5,7-10] The gold standard method for bladder cancer diagnosis is cystoscopy followed by biopsy of suspicious lesions. However, this approach is highly invasive and costly, and can miss up to 30% of malignant cases.[10-15] The non-invasive tests for bladder cancer diagnosis include voided urine cytology,[16] cytogenetic analysis by fluorescence in situ hybridization, and detection of genetic mutations in urine.[17-22] However, these tests have reported sensitivities of 54–86%, and specificities of 61–90%.[17-19,21] Thus, there is a need to improve the non-invasive tests of bladder cancer detection and progression.[23] The quest for sensitive, specific, and non-invasive diagnostic, therapeutic, and prognostic tools for bladder cancer is a high priority research area, and can be achieved by understanding the molecular mechanisms underlying the initiation and progression of this malignancy.[24]

Epigenetics is a fast growing field in cancer biology, and has enormous potential for clinical and translational research.[25-27] Cancer epigenetics refers to inheritable, yet reversible, changes associated with gene dysregulation, which manifest in a (pre) malignant phenotype where sequence of the genome is not altered[28-30]. Aberrant DNA methylation, dynamic changes in chromatin structure through post-translational modification of histones, nucleosome positioning, and microRNA-mediated modulation of gene expression are known epigenetic changes associated with carcinogenesis.[25,28,31-33] The occurrence of epigenetic changes prior to malignant transformation, which frequently manifests in target and non-invasively obtainable surrogate organs, and the reversibility of these changes through pharmacologic and genetic interventions[30,33-40] provide a unique opportunity for cancer research, ultimately leading to the discovery of non-intrusive diagnostic, therapeutic, and prognostic approaches for human malignancies. Characterizing the epigenetic changes that initiate and promote bladder cancer development can help identify biological markers that can be used for early detection, treatment, and monitoring of the progression of this malignancy. The continuous shedding of bladder lining cells into the urine is highly advantageous for non-invasive surveillance of the epigenetic changes occurring during bladder carcinogenesis.[39-41] From a

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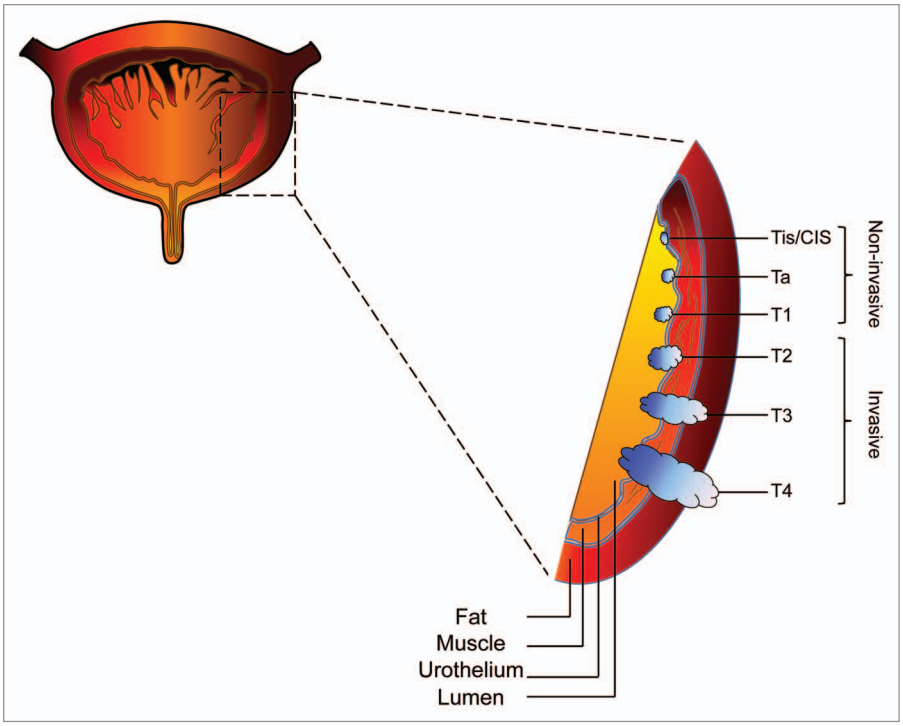

Figure 1. Diagram of progressive stages of transitional cell carcinoma of the bladder. Non-muscle invasive transitional cell carcinoma is staged at Tis/CIS, Ta, and T1, whereas muscle invasive transitional cell carcinoma is staged at T2, T3, and T4. Tis/CIS, carcinoma in situ.

therapeutic standpoint, the anatomical confinement of the bladder makes this organ amenable to targeted epigenetic therapies whose complications and side effects should be fewer and less severe than those of systemic treatments.[29,33,37,42,43]

Aberrant DNA methylation is the most extensively studied epigenetic change associated with all types of human cancer, including bladder cancer.[25,32,33,44] Aberrant DNA methylation was initially found in single genes/loci of relevance to carcinogenesis in human bladder tumors and cancer cell lines. Subsequent advances in genome-scale technologies for DNA methylation analysis and bioinformatics approaches have enabled a comprehensive characterization of DNA methylation patterns in human bladder cancer.[39-41,44-51] The present review summarizes the published research on aberrant DNA methylation in connection with human bladder cancer. Representative studies are highlighted to set forth the current state of knowledge, gaps in the knowledgebase, and future directions in this prime epigenetic field of research. The utility of the in vivo animal models of induced-bladder tumorigenesis, and the need for population-based studies in "at risk" individuals for bladder cancer development are also discussed. A synopsis of research on microRNA (miRNA) methylation and human bladder cancer, and an overview of the promises and pitfalls of epigenetic therapy for solid tumors are also provided.

Aberrant DNA Methylation and Carcinogenesis

In mammalian genomes, DNA methylation occurs almost exclusively in the context of 5'-CpG dinucleotides (CpGs).[31,52] A family of DNA methyl transferases (DNMTs) comprised of DNMT1, DNMT3A, and DNMT3B catalyzes this reaction by transferring a methyl group from the donor *S*-adenosyl methionine to the fifth carbon position of the cytosine pyrimidine ring.[27,53-55] In humans, the vast majority (80–90%) of CpGs in the genome are normally methylated.[52,53,56] The remaining methylation-free CpGs are found in sequence stretches, termed CpG islands, that are >500 base pairs long, and have a GC content of >55% and an observed/expected CpG ratio of ≥0.65.[57]

Aberrant DNA methylation has been found in almost all types of human cancer.[25,31-33] Aberrant DNA methylation is characterized by global loss of DNA methylation (hypomethylation) and locus-specific gain/loss of DNA methylation (hyper/hypomethylation).[25,31,58-61] Whereas DNA hypomethylation is thought to contribute to oncogenesis by reactivation of latent retrotransposons, induction of genomic instability, and activation of proto-oncogenes,[62,63] DNA hypermethylation is believed to elicit tumorigenesis by dysregulation of gene expression, e.g., through transcriptional silencing of tumor suppressor genes.[26,54,56,64] Hypermethylation of CpG islands, clustered at the promoter, untranslated 5'-region and exon 1 of known genes (promoter CpG islands) or localized within gene bodies (intragenic CpG islands) is a common event in human carcinogenesis (**Fig. 2**).[25,32,33,56,65,66] Global DNA hypomethylation at repetitive DNA elements, such as long- and short interspersed nuclear elements (*LINE* and *SINE*, respectively), and long-terminal repeat retrotransposons (*LTR*) is also a frequent occurrence in human cancers.[63,67,68]

Overview of Research on DNA Methylation and Human Bladder Cancer

The evolution of classic single-gene DNA methylation detection assays to genome-wide microarray based technologies, and most recently, next-generation sequencing platforms coupled with cutting-edge bioinformatics approaches has provided an unprecedented opportunity to investigate the role of aberrant DNA methylation in the genesis and progression of human cancers.[32,65,69,70] Thus far, single- and multi-locus aberrant DNA methylation has been demonstrated in human bladder tumors and cancer cell lines, and urine sediments.[39-41,44-51] In the following, we provide a synopsis of representative research on aberrant DNA methylation in connection with human bladder carcinogenesis.

Markl et al.[45] used a semi-quantitative methylation-sensitive arbitrarily primed polymerase chain reaction (MS.AP.PCR)

technique to investigate the methylation status of GC-rich regions in the genome of metachronous tumors and their derived cell lines from two patients with TCC of the bladder as compared with normal urothelium from five disease-free individuals. The authors detected 17% changes in the methylation status of the 214 evaluable sequences between tumors and normal urothelia from disease-free subjects. These methylation changes were cancer-specific (3%: common in all tumors from both patients), patient-specific (13%: present in all tumors from one patient only), and tumor-specific (1%: present in one tumor from one patient only). When compared with the normal urothelia and tumors concurrently, there were also 3% cell-line specific methylation changes.[45]

Maruyama et al.[46] used the methylation-specific PCR (MSP) assay to investigate the promoter methylation profile of 10 different cancer-related genes, including *CDH1*, *RASSF1A*, *APC*, *CDH13*, *FHIT*, *RAR*β, *GSTP1*, *p16*^{INK4a}, *DAPK*, and *MGMT*, in 98 bladder tumors in connection to clinicopathological features of the aggressiveness of the disease. Except for one squamous cell carcinoma, all the tumors were TCC of the bladder of varying grades and stages. High methylation frequency, expressed as median methylation index (MI), was detected for four genes, including *RASSF1A* (35%), *APC* (35%), and two members of the cadherin family, *CDH1* (36%), and *CDH13* (29%), which significantly correlated with various parameters of poor prognosis, such as tumor grade, growth pattern, muscle invasion, tumor stage, and ploidy status. In Kaplan-Meier analysis, the methylation of *CDH1* and *FHIT* significantly associated with shortened survival. In a multivariate analysis adjusted for tumor grade, papillary or non-papillary histology, muscle invasion, and MI index of other genes, *CDH1* methylation was independently associated with poor prognosis.[46]

Sathyanarayana et al.[47] used the MSP assay to analyze the promoter methylation of three invasion and metastasis-related genes (*LAMA3*, *LAMB3*, and *LAMC2*) in bladder tumors (n = 128) and exfoliated cells (bladder washes and voided urine; n = 128), and correlated the results to clinicopathological findings. All the tumors were TCC except for two squamous cell carcinomas of the bladder. The methylation frequencies of these three genes in bladder tumors and urinary exfoliated cells, respectively, were as follows: 45% and 39% for *LAMA3*, 21% and 19% for *LAMB3*, and 23% and 15% for *LAMC2*. There was excellent concordance in methylation between tumors and corresponding exfoliated cell samples, including 88% for *LAMA3* (P = 0.0006), 92% for *LAMB3* (P = 0.002), and 83% for *LAMC2* (P = 0.003). The *LAMA3* and *LAMB3* methylation significantly correlated with several parameters of poor prognosis, whereas the *LAMC2* methylation was independently associated with shortened survival (P = 0.03; 95% CI = 1.14–10.84). Of significance, the frequency of *LAMA3* methylation was significantly higher in invasive tumors than in non-invasive tumors (80% vs. 10%; P = 0.0001).[47]

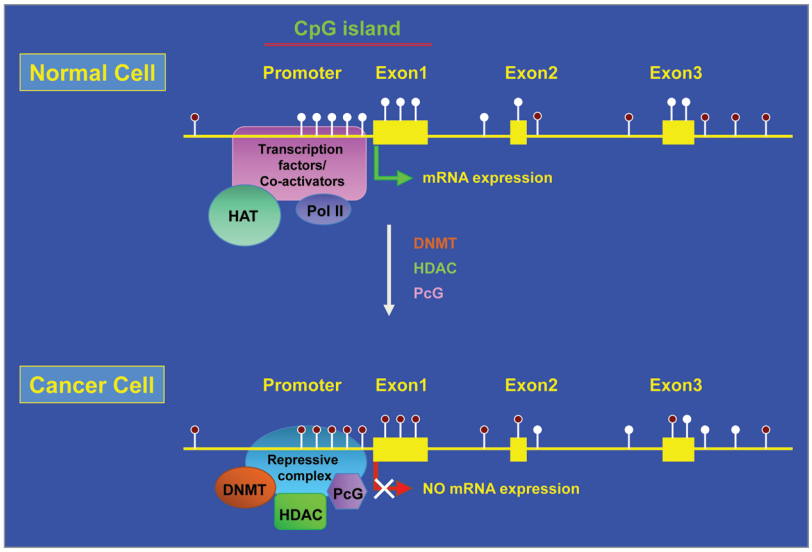

Figure 2. Schematic representation of promoter CpG island hypermethylation in cancer. Filled and unfilled lollypops represent methylated and unmethylated CpGs, respectively. HAT, histone acetyltransferase; Pol II, DNA polymerase II; DNMT, DNA methyl transferase; HDAC, histone deacetylase; PcG, polycomb group proteins.

Christoph et al.[48] used quantitative real-time MSP analysis to establish promoter CpG island methylation in four pro-apoptotic *TP53* target/effector genes, including the *APAF-1*, *CASP-8*, *DAPK-1*, and *IGFBP-3*, in non-invasive and invasive TCC of the bladder (n = 110) as compared with normal urothelium from patients without urological malignancy (n = 20). Hypermethylation of the promoter regions of the *APAF-1*, *CASP-8*, and *DAPK-1* was detected in 100%, 74%, and 66%, respectively, of all tumors, with no or very low levels of methylation being detectable in normal urothelia. The *APAF-1* methylation significantly correlated with both tumor stage (P < 0.01) and tumor grade (P = 0.04). The *APAF-1* and *IGFBP-3* methylation were predictors of tumor relapse by differentiating tumors at higher recurrence risk from those at low-risk of recurrence. In multivariate analysis adjusted for tumor stage, tumor grade, and MI index of other genes, *APAF-1* and *IGFBP-3* methylation were independent prognostic factors for recurrence in superficial bladder tumors.[48]

Hoque et al.[49] used quantitative real-time MSP analysis to investigate hypermethylation of promoter regions of nine cancer-related genes, including *APC*, *ARF*, *CDH1*, *GSTP1*, *MGMT*, *CDKN2A*, *RARβ2*, *RASSF1A*, and *TIMP3*, in primary tumors and urine sediments from 15 bladder cancer patients and 25 control urine sediments from subjects with no history of genitourinary malignancy. For all paired tumors and urine samples from bladder cancer patients, there was a perfect match in promoter methylation of all the tested genes. Four genes (*ARF*, *GSTP1*, *MGMT*, and *CDKN2A*) displayed promoter hypermethylation in all the samples from bladder cancer patients and no methylation in samples from controls (100% specificity). The methylation status of this four-gene panel was further investigated in the urine sediments of an additional 160 bladder cancer patients with tumors of varying stages and grades (90% with TCC histology) as

compared with 69 urine samples from age-matched control subjects. Promoter hypermethylation was detectable for *CDKN2A* in 45% (95% CI = 38–53%), *GSTP1* in 43% (95% CI = 35–51%), *MGMT* in 35% (95% CI = 28–42%), and *ARF* in 28% (95% CI = 21–35%) of all samples from bladder cancer patients, with no detectable level of methylation in the respective gene promoters in control samples. A two-stage prediction model based on the methylation of the four-gene panel followed by logistic regression analysis of the methylation of the remaining five genes was developed, which produced an internally validated Receiver Operating Characteristic (ROC) with an overall sensitivity of 82% (95% CI = 75–87%) and specificity of 96% (95% CI = 90–99%). The ROC Curve represents the trade-off between the false negative and false positive rates for every possible cut off points of a diagnostic test.[49]

Chung et al.[39] used bisulfite pyrosequencing to investigate promoter hypermethylation of ten cancer-related genes, including *A2BP1*, *NPTX2*, *SOX11*, *PENK*, *NKX6-2*, *DBC1*, *MYO3A*, *HSPB9*, *NPY2R*, and *CA10*, in 26 primary bladder tumors and 6 bladder cancer cell lines. This panel of ten genes was previously identified by methylated CpG island amplification and microarray (MCAM) analysis of 85 primary bladder tumors and 12 bladder cancer cell lines.[71,72] Eight genes were highly methylated in bladder tumors, with very low levels of methylation detectable in normal controls (bladder and leukocyte DNA from three individuals). The methylation frequencies in these genes in declining order were 92% (*PENK*), 88% (*NPTX2*), 85% (*CA10*), 77% (*SOX11*), 69% (*DBC1* and *NKX6-2*), 65% (*MYO3A*), and 62% (*A2BP1*). Subsequently, a quantitative real-time MSP assay was used to analyze the 8-gene set in urine sediments from 128 bladder cancer patients (86% TCC histology) and 110 age-matched control subjects with no history of bladder malignancy. Using combinatorial analysis of methylation of these 8 genes, a predictive model was developed for bladder cancer detection, which showed 81% sensitivity and 97% specificity based on a panel of four genes (*MYO3A*, *CA10*, *NKX6-2*, and *DBC1* or *SOX11*), and 85% sensitivity and 95% specificity based on a panel of five genes (*MYO3A*, *CA10*, *NKX6-2*, *DBC1*, and *SOX11* or *PENK*). Analyzing the data by cancer invasiveness, predictive models of ≥3-gene panel showed 81% sensitivity and 95% specificity for non-muscle invasive tumors (Ta, Tis, and T1), and 90% sensitivity and 95% specificity for muscle invasive tumors (T2, T3, and T4).[39]

Wolff et al.[40] used the Illumina GoldenGate methylation assay to interrogate 1370 autosomal loci (784 genes) in 49 non-invasive urothelial tumors (Ta–T1), 38 invasive tumors (T2–T4) and their matched normal-appearing urothelia, and control urothelia from 12 age-matched individuals with no history of urothelial cancer. There were distinct patterns of aberrant DNA methylation in non-invasive tumors vs. controls and invasive tumors vs. controls; whereas hypermethylation was more pronounced in the invasive tumors, hypomethylation occurred more frequently in the non-invasive tumors. Relative to control samples from urothelial cancer-free patients, invasive tumors had 526 hypermethylated loci (38%), whereas non-invasive tumors had 132 hypermethylated loci (10%) of which 117 (89%) overlapped with those found in the invasive tumors ($P < 0.0001$). Normal-appearing urothelia taken at least 5 cm away from their corresponding invasive tumors had 169 hypermethylated loci (12%) of which 142 (89%) were common to those found in the invasive tumors ($P < 0.0001$), indicating an epigenetic field defect. Most of the identified hypermethylated loci were located in the context of CpG islands. Conversely, non-invasive tumors had more unique hypomethylated loci (217 = 16%) than invasive tumors (41 = 3%) as compared with control urothelial cancer-free tissues ($P < 0.001$), in addition to the 253 hypomethylated loci (18%) that were common to both tumor types. The majority of the identified hypomethylated loci were located outside of CpG islands.[40]

Investigating the Mechanistic Involvement of Aberrant DNA Methylation in Human Bladder Carcinogenesis

The salient findings of the studies highlighted in the preceding section are: (1) aberrant DNA methylation is a common event in human bladder carcinogenesis; (2) aberrant DNA methylation is likely to occur in the early stages of human bladder carcinogenesis; and (3) detection of aberrant DNA methylation in the urine sediments can be potentially used as a diagnostic tool for human bladder cancer detection, and a prognostic tool for monitoring of the progression of this malignancy. However, the above findings are limited to clinically diagnosed bladder cancer patients only. Ideally, bladder cancer detection should be achievable in the general population before clinical manifestation of the disease.[10,41,73,74] In addition, prognosis of bladder cancer and prediction of the recurrence of tumors should be attainable in apparently disease-free individuals who have undergone therapeutic regimens.[10,41,73,74] Thus, a major goal of future research should be to find the role of aberrant DNA methylation in the initiation and progression of bladder cancer before clinical symptoms of the disease become manifest.

To achieve this goal, three questions need to be answered:

1) Timing: How early in the process of bladder carcinogenesis does aberrant DNA methylation occur, and when is it manifest at a detectable level?

2) Scale: What is the genomic distribution of aberrant DNA methylation in bladder carcinogenesis (genome-wide and locus-specifically)?

3) Origin: Is aberrant DNA methylation a cause or a consequence of bladder carcinogenesis?

The answers to these questions should be sought in proof-of-principal experiments in in vivo models of induced-bladder tumorigenesis, where different stages of carcinogenesis can be intercepted. The existing animal models of bladder tumorigenesis include rodents, rabbits, and dogs in which bladder tumors can be induced in vivo by administering known bladder carcinogens, e.g., aromatic amines[75-78] and arsenic.[78,79] The advent of high throughput genome-wide technologies for DNA methylation detection will be instrumental in establishing the genomic distribution of aberrant DNA methylation at various stages of bladder carcinogenesis.[32,44,65,69] Such an approach will verify the

mechanistic involvement of aberrant DNA methylation in the evolution of bladder cancer from an early stage non-invasive neoplasia to late and aggressive malignancy.

Obviously, the use of in vivo animal models of induced-bladder tumorigenesis can best serve the above purpose because experimental exposure of humans to known bladder carcinogens is unethical and simply out of the question.[80] This said, however, one should remain mindful of the need to validate the in vivo findings in animal models in follow up studies in human populations. Decades of experimental studies in animal models of human cancer have provided invaluable information on various aspects of human carcinogenesis.[81-83] Nonetheless, the "incomplete" comparability of these models to humans underscores the need to validate the in vivo data in confirmatory research in humans.[80] Thus, a requisite for animal studies of induced-bladder tumorigenesis is to recapitulate the in vivo findings in disease-free human populations, e.g., in case-control studies in populations with known susceptibility to bladder cancer development, e.g., smokers.[84-86] For illustrative purposes, the utility of animal models of aromatic amine-induced bladder tumorigenesis, and the need for validation studies in smokers at high risk of developing bladder cancer are discussed below.

Smoking-Related Bladder Cancer and Tobacco Smoke-Derived Aromatic Amines

Bladder cancer represents a unique model of chemical carcinogenesis in humans.[87] Unlike other types of human cancer with unknown or less well-defined etiologic agent(s), bladder cancer is primarily linked to tobacco smoking.[84-86] The smoking-attributable bladder cancer is widely believed to originate from exposure to aromatic amines, a family of proven bladder carcinogens present in tobacco smoke.[87-90] The elevated risk of bladder cancer in smokers of black (air-cured) tobacco relative to blond (flue-cured) tobacco is ascribed to the richer content of aromatic amines in the former tobacco products.[89-91] Although the bladder-specific carcinogenicity of aromatic amines is well-established, the underlying mechanisms of action of these chemicals in the genesis and progression of bladder cancer are not fully delineated.[24]

A genotoxic mode of action for aromatic amines has been demonstrated that involves the induction of DNA damage and mutations.[92-95] However, the genotoxicity of aromatic amines is not exclusive to the target organ of tumorigenesis.[24,94-98] This suggests that an alternative mode of action (epigenetics) may also exist that singly or in combination with genotoxicity can explain the bladder-specific tumorigenicity of aromatic amines.[24] The concept of a chemical carcinogen exerting its biological effects through epigenetic changes is very novel, and has never been investigated comprehensively. Historically, investigations of the mode of action of chemical carcinogens have been dominated by genotoxicity studies.[93,99-101] Exploring the ability of aromatic amines to induce epigenetic changes, such as aberrant DNA methylation, can emerge as a paradigm shifting research in chemical carcinogenesis. Establishing the alterations of DNA methylome in vivo in animal models of aromatic amine-induced bladder tumorigenesis followed by confirmatory research in "at risk" populations, such as smokers, can help elucidate the underlying mechanisms of the initiation and progression of human bladder cancer.

Although smoking is the main source of exposure to aromatic amines in the general population,[84-86] occupational exposure to these chemicals also occurs in a wide range of industries, including rubber, cable, and textile manufacturing, aluminum transformation, and gas, coal, pesticide, and cosmetics production.[102-104] Furthermore, other sources of exposure to aromatic amines also exist, including dietary (e.g., pesticides in food), life style (e.g., hair dye use), and environmental (e.g., engine exhaust) sources.[102-104] Thus, the widespread exposure of human populations to carcinogenic aromatic amines constitutes a major public health problem.[102,103] The unmet need for biomonitoring of humans exposed to aromatic amines[86] can be addressed by the use of mechanistic markers that can chronicle the initiation and progression of bladder cancer.

Earlier research on aberrant DNA methylation in smoking-attributable bladder cancer has provided initial clues on the underlying mechanisms of the initiation and progression of this malignancy. For example, Wolff et al.[50] conducted an elegant study in which smoking proved to be independently associated with aberrant DNA methylation in a unique genomic locus in bladder cancer patients. The authors used quantitative real-time MSP analysis to investigate the promoter methylation of nine cancer-related genes, including *RUNX3*, *BCL2*, *PTGS2* (*COX2*), *DAPK*, *CDH1* (*ECAD*), *EDNRB*, *RASSF1A*, *TERT*, and *TIMP3*, in 342 matched bladder tumors (TCC) and corresponding normal-appearing mucosa in comparison to urolthelia from age-matched cancer-free patients undergoing prostatectomy. In matched sets of bladder tumors and control mucosa, the methylation frequencies were 65% vs. 15% for *EDNRB*, 60% vs. 11% for *RASSF1A*, 39% vs. 9% for *BCL2,* and 30% vs. 4% for %*TERT,* although for *RUNX3* the respective values were 56% vs. 0%, which was of most specificity (two normal-appearing mucosa showed *RUNX3* methylation patterns that were nearly identical to their matched high-grade and invasive tumors, suggesting that these bladders had epigenetic aberrancies throughout or that the invasive tumor had spread across the bladder; the two samples were excluded from the above analysis). No or very low levels of methylation were detectable in normal urothelia of control subjects for all the nine genes tested. Of significance, methylation of *RUNX3* preceded methylation of the other eight genes ($P < 0.001$), and its prevalence increased as a function of age at diagnosis ($P = 0.031$; adjusted for sex, smoking history, tumor stage and tumor grade), and history of smoking ($P = 0.015$; adjusted for age, sex, tumor stage, and tumor grade). It was suggested that because *RUNX3* methylation increases with age, is absent in normal urothelium, and occurs early in tumorigenesis, it can be used as a molecular clock to determine the age of a bladder tumor. Doing so, tumors from smokers appear to be "older" than tumors from nonsmokers $P = 0.009$) due to either initiating earlier or undergoing faster cell divisions. Since *RUNX3* methylation is acquired early on in tumorigenesis, its detection in biopsy or urine specimens may provide a marker to screen smokers long before any symptoms of bladder cancer become manifest.[50]

miRNA Methylation and Human Bladder Cancer

miRNAs are a class of short noncoding RNAs (~22 nt) that negatively regulate gene expression at the post-transcriptional level through sequence-specific targeting of mRNA.[105-107] The recognition of the target mRNA is based on complementarity of the seed sequence of the miRNA (i.e., 7–8 nt at the 5'-end) to a specific sequence motif within the 3'-untranslated region (3'-UTR) of the mRNA.[108,109] Whereas partial base pairing between the seed region of the miRNA and the 3'-UTR of the target mRNA results in translational inhibition, (near) perfect complementarity causes degradation of the mRNA by endonuclease cleavage.[107,110] Approximately 30% of human genes are putative targets of miRNAs.[111-114] A single miRNA can regulate up to 200 different target mRNAs, whereas one individual mRNA can be controlled by multiple miRNAs.[111,115,116] Thus, the influence of miRNAs in crucial biological processes, such as cell cycle regulation, differentiation, proliferation, apoptosis, development, metabolism, and aging, is unassailable.[117,118] Accumulating evidence suggests that dysregulation of miRNA expression contributes to a wide range of human diseases, including cancer.[106,118-121] A growing body of literature supports the involvement of miRNAs in the pathogenesis of human bladder cancer.[122-125] For example, aberrant expression of various miRNAs has been observed in human bladder tumors and cancer cell lines, and urine sediments, and correlated with various clinicopathological characteristics of this disease.[126-130] Although the mechanisms underlying miRNA dysregulation in human bladder cancer are not fully determined, proof-of-principle experiments have established a tight link between epigenetic changes, such as aberrant DNA methylation and histone modifications, and altered miRNA expression in bladder carcinogenesis.[131-133] For instance, Saito et al.[134] have shown that simultaneous treatment of T24 human bladder cancer cells with 5-aza-2'-deoxycytidine (5-Aza-CdR) and 4-phenylbutyric acid, which inhibit DNA methylation and histone deacetylase, respectively, resulted in upregulation (>3-fold) of 17 out of 313 (5.4%) human miRNAs examined. Epigenomic studies in various types of human cancer, including bladder cancer, have shown that the number of dysregulated miRNA genes with aberrant DNA methylation at their promoter CpG islands and/or the flanking CpG "shores" (regions with less CpG density) is rapidly rising.[135-137] Also, an increasing body of evidence suggests that detection of aberrantly methylated miRNA genes in human bladder cancer can serve as biomarkers for disease detection, evaluation of prognosis, and prediction of response to therapy.[122-125] Furthermore, emerging research supports the notion that restoration of epigenetically dysregulated miRNAs in human bladder cancer, e.g., through drugs that mitigate aberrant DNA methylation and histone modifications, can constitute an effective therapeutic strategy for this disease.[138,139] For more detailed information, we refer the readers to recent comprehensive reviews.[118,120,140,141]

Promises and Pitfalls of Epigenetic Therapy for Solid Tumors

Tumors display widespread epigenetic aberrancies, including alterations in DNA methylation patterns, histone modifications and variants, miRNAs dysregulation, chromatin remodeling, and nucleosome positioning.[25,28,31-33] These epigenetic abnormalities are potentially reversible as the enzymes maintaining the epigenetic state are regulatable through pharmacologic interventions, i.e., epigenetic therapy.[28,29,142,143] To date, the best-characterized and only Food and Drug Administration (FDA) approved drugs for epigenetic therapy are DNA demethylating agents and histone deacetylase inhibitors (HDACi).[33,144,145] As cytosine analogs, Decitabine (5-Aza-CdR) and Vidaza (5-Azacytidine; 5-Aza-CR) target aberrant DNA methylation by getting incorporated into DNA and sequestering DNMTs, thus, resulting in depletion of these enzymes and global hypomethylation upon cell divisions.[37,42,146] Targeting histone deacetylases (HDACs) for epigenetic therapy is challenging because this group of enzymes has multiple subclasses with mechanisms of action still under contention.[147-149] More than a dozen HDACi are currently undergoing preclinical and clinical investigations for the treatment of both hematological malignancies and solid tumors, including bladder cancer.[150-153] The common mechanism of action of HDACi is the chelation of Zn^{2+} ions, which are critical to the activity of these enzymes.[149,154] As single agents, two HDACi, including Vorinostat and Romidepsin have FDA approval for the treatment of cutaneous T-cell lymphoma.[155] More recently, the utility of synthetic anti-miRNA oligonucleotides with tumor suppressive function as epigenetic regulators has also been investigated.[156-160] For instance, locked nucleic acids (LNAs)-anti-miRNA-based therapy has been explored in nonhuman primates for inhibition of oncogenic miRNAs.[161]

Thus far, epigenetic therapy has shown encouraging results for the treatment of hematological malignancies; however, the promise of targeting epigenetic aberrancies in solid tumors has not been realized yet.[143,162,163] Major challenges include the delivery of epigenetic drugs, maintenance of a pharmacodynamics response, and achievement of a therapeutic index.[164,165] Obviously, a better understanding of the mechanisms of action of the epigenetic drugs and greater insights into tumor biology will help improve the efficacy of epigenetic therapy for solid tumors. The goal is to combine epigenetic therapy with other chemotherapeutic approaches, such as hormonal therapies, immunomodulatory therapies, and standard chemotherapy, to help sensitize tumor cells to cytotoxic effects of targeted/systemic therapies or to durably slow or reverse resistance to these therapies. For more detailed information, we refer the readers to recent comprehensive reviews.[121,166-169]

Concluding Remarks: Potential Challenges and Future Directions

Bladder cancer continues to be a major public health problem in the United States and throughout the world.[1-3] The ineffective strategies for early detection, treatment, and monitoring of the progression of this disease have translated into significant mortality and morbidity.[23] A requisite for alleviating the global burden of this disease is to understand the underlying mechanisms of the initiation and progression of this disease.[24] As the most

extensively studied epigenetic change associated with human cancers, aberrant DNA methylation in single or multiple genes/loci of relevance to carcinogenesis has been found in human bladder tumors and cancer cell lines, and urine sediments. In addition, detection of aberrant DNA methylation in cancer-related genes/loci in the above specimens has prognosticated many clinicopathological features of this disease, including tumor relapse, muscle-invasiveness, and survival.[39-41,44-51]

The advent of high throughput genome-wide technologies for epigenomics and genomics studies has provided a unique opportunity to identify global changes in DNA methylation with functional impact on gene expression, which can best predict bladder cancer initiation and progression.[32,44,65,69,70] Future exploratory research should focus on characterization of aberrant DNA methylation, on a genome-wide scale, in experimental animals wherein bladder tumors can be induced by administering known bladder cancer-causing agents. Confirmatory research in humans at high risk for bladder cancer development should validate the exploratory research in animal models. The ultimate goal will be to construct the whole DNA methylome of bladder cancer at various stages of bladder carcinogenesis, including the initiation and progression stages. Identifying the potentially reversible and "drugable" aberrant DNA methylation events that initiate and promote bladder cancer development can serve as biological markers for early detection, effective treatment and accurate prognosis of this malignancy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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