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Hypermethylation of tumor-suppressor gene CpG islands in small-cell carcinoma of the urinary bladder

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Small-cell carcinoma of the urinary bladder (SCBC) is a rare tumor, which shows a common clonal origin with urothelial carcinoma. It bears a high metastatic potential, even when discovered in a localized state. Identifying the molecular underpinnings of this disease may elucidate useful clinical information regarding prevention, diagnosis, prognosis, treatment, and surveillance. As DNA methylation is widely recognized as having a pivotal role in the process of carcinogenesis, we analyzed the DNA methylation status of four frequently hypermethylated tumor suppressors in small-cell and transitional-cell carcinoma (TCC) arising concomitantly in 13 patients. Fourteen cases of pure TCC were also included in the analysis. We identified frequent methylation of *RASSF1* and *MGMT* and infrequent methylation of *MLH1* and *DAPK1* in cases of concomitant TCC and SCBC. Similar rates of methylation were found in pure and concomitant histopathologies, with the exception of *MGMT*, which was much less frequently methylated in pure TCC. These findings suggest that SCBC and TCC have common origins, establish DNA methylation of some tumor suppressors as frequent occurrences in both histopathologies, and suggest that *MGMT* methylation may be an SCBC-specific epimutation.
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DNA methylation-induced silencing of gene expression is now recognized as an important contributor in all stages of carcinogenesis.[1] Methylation of CpG-dense regions, or CpG islands (CpGIs), associated with the first exons of many genes, serves to recruit transcriptional silencing machinery, including methyl-CpG-binding proteins, histone deacetylases and methyltransferases, and ATP-dependent chromatin-remodeling enzymes.[2,3] Silencing of key tumor and metastasis suppressors,[4,5] drug-metabolizing enzymes,[6] and DNA-repair proteins[7] is an event that contributes to carcinogenesis, acquisition of invasiveness and metastatic potential, angiogenesis, and therapy refractoriness. DNA methylation is a target for both therapeutic and biomarker purposes.[8] Importantly, the study of DNA methylation in clinical samples may provide useful and novel insights into the pathobiology of disease processes such as cancer.

Transitional-cell carcinoma (TCC) arises from urothelium, which lines the bladder, as well as the renal pelvis, ureter, and portions of the urethra. Although infrequent in occurrence, small-cell bladder cancer (SCBC) may evolve or co-evolve from pre-existing TCC.[9] We hypothesized that DNA methylation of specific genes may underlie the pathogenesis of SCBC. In this study, we quantitatively analyzed the methylation status of *RASSF1*,

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MLH1, *DAPK1*, and *MGMT*, in cases of SCBC, which coexisted with TCC. These genes are tumor suppressors that are commonly methylated in malignancies of many tissues in humans. In addition, *MLH1* was chosen because of its known role in mismatch repair pathway,[7,10–12] which is known to govern sensitivity to platinum based therapies, which are part of the typical chemotherapy regimen for TCC. We were able to separately assess the CpGI methylation status in synchronous SCBC and TCC in 13 patients.

Materials and methods

Sample Selection and Preparation

Tissues from 13 patients with transitional-cell (urothelial) carcinoma (TCC) concurrent with small-cell carcinoma of the urinary bladder were included in our study. Archival materials were retrieved from the surgical pathology files of the participating institutions. Clinical and pathological information was available for all the patients. Patients ranged in age from 63 to 83 years, with a mean age of 73 years. Eleven patients were pathological stage pT2 and two were pathological stage pT3. Tumors were diagnosed by light microscopy, with each case fulfilling the criteria established for urothelial carcinoma and small-cell carcinoma, according to the World Health Organization classification system.[13] For pathological staging, the 2002 tumor, lymph node, and metastasis (TNM) classification system was used.[14] Tissues were microdissected and DNA was extracted as previously described.[9,15] This research was approved by the Indiana University Institutional Review Board.

In addition, 14 patients with pure TCC were analyzed in comparison. All these patients had advanced-stage (pT2 or above) and high-grade bladder cancer.

Bisulfite Conversion of Genomic DNA

Before conversion, a quantitative PCR reaction was performed using 2 μl of genomic DNA as template, to ensure that >400 pg of DNA was present in the extracted sample. This was accomplished using the eukaryotic elongation factor 1α gene (*EEF1A1*) in a Taqman®-based assay (primer sequences in Table 1, CG dinucleotides in boldface). DNA was treated with bisulfite as described previously.[16] Converted DNA was purified using Zymo Spin IC Columns (Zymo Research, Orange, CA, USA) as described by the manufacturer. DNA was eluted with 20 μl of sterile water.

Quantitative Methylation-Specific PCR

Quantitative methylation-specific PCR (qMSP) is a TaqMan-based assay based on conventional MSP.[17]

Table 1 Primer and probe sequences used to quantify DNA methylation at corresponding CpGIs

Gene	*GenBank number*	*Amplicon*	*Forward primer*	*Reverse primer*	*Probe*
DAPK1	X76104	137–204	T**CG**T**CG**T**CG**TTT**CG**GTTAGTT	TCCCTC**CG**AAA**CG**CTAT**CG**	fam-**CG**ACCATAAA**CG**CCAA**CG**C**CG**-tamra
MLH1	U26559	254–341	AGGAAGAG**CG**GATAG**CG**ATTT	TCTT**CG**TCCCTCCTAAAAA**CG**	fam-C**CG**C**CG**CTACCTAAAAAAAATATA**CG**CTTA**CG**C**G**-tamra
MGMT	X61657	1067–1149	G**CG**TTT**CG**A**CG**T**CG**TAGGT	CACTCTTC**CG**AAAA**CG**AAA**CG**	fam-**CG**CAAA**CG**ATA**CG**CAC**CG**C**G**A-tamra
RASFF1A	AC002481	18 107–18 171	ATTGAGTTG**CG**GGAGTTGGT	ACA**CG**CTCCAAC**CG**AATA**CG**	fam-CCCTTCCCAA**CG**C**G**CCCA-tamra
EEF1A1	NC000006	2778–2836	CTGAACCATCCAGGCCAAAT	GCCGTGTGGCAATCCAAT	fam-AGCGCCGGCTATGCCCCTG-tamra

Two microliters of bisulfite-converted DNA were used as template in 20-μl qMSP reactions. Reactions were optimized for each reactant in separate control reactions before the study, and contained 1 × PCR buffer, 8.75 mM $MgCl_2$, 0.3 mg/ml BSA, 125 nM forward and reverse primers, 100 nM probe, 62.5 μM each dNTP, and 0.5 U of Platinum *Taq* (Invitrogen, Carlsbad, CA, USA). Primers were synthesized by MWG (High Point, NC, USA). Probes were modified by 5′ FAM and 3′ TAMRA. Primer and probe sequences, GenBank accession numbers, and amplicon locations are listed in Table 1 and are previously described.[18] PCR was performed for 50 cycles using the Light Cycler machine (Roche, Indianapolis, IN, USA). Each set of PCR reactions included positive and negative controls. Negative controls used were blanks (water) or non-bisulfite-treated DNA. Second-derivative maximum points were used to quantitate samples. All samples were referenced to an empty fluorescence channel to reduce background signal. To quantitate sample values, linear regressions were constructed using serial dilutions of a known amount of DNA (before bisulfite treatment), which was methylated *in vitro* using enzyme *Sss*I (New England Biolabs, Ipswitch, MA, USA). A minimum threshold of detection for methylation of each gene was empirically determined using control DNA. This threshold was reproducibly 20–100 pg of fully methylated normal human male genomic DNA (Promega, Madison, WI, USA) for each primer. Reactions with an amount of template less than the reliable minimal detectable threshold amplified products erratically on different days. Therefore, sample scores were deemed positive or negative for gene methylation based on this threshold. This prevented the need for an internal quantitative control.

Statistical Analysis

Methylation of *RASSF1*, *MGMT*, and *MLH1* in TCC, SCBC, and normal tissues was compared using McNemar's exact test for matched pairs. The association between methylation in TCC and SCBC tissues was also analyzed across *RASSF1* and *MGMT* by investigating methylation of both *RASSF1* and *MGMT*, and methylation of either *RASSF1* or *MGMT*, using McNemar's exact test. The association between pathological stage and gene methylation was examined by Fisher's exact test. All *P*-values were two-sided and a *P*-value <0.05 was considered statistically significant.

Results

Tissues were microdissected for DNA isolation (Figure 1). Amplifications using the *RASSF1* qMSP primers were performed on standardized samples and all tissues used in the study (Figure 2a and b). A linear regression for *RASSF1* primers was constructed (Figure 2c). MGMT, *DAPK*, and *MLH1* primers produced similar slopes and intercepts after linear regressions were constructed for each primer (data not shown).

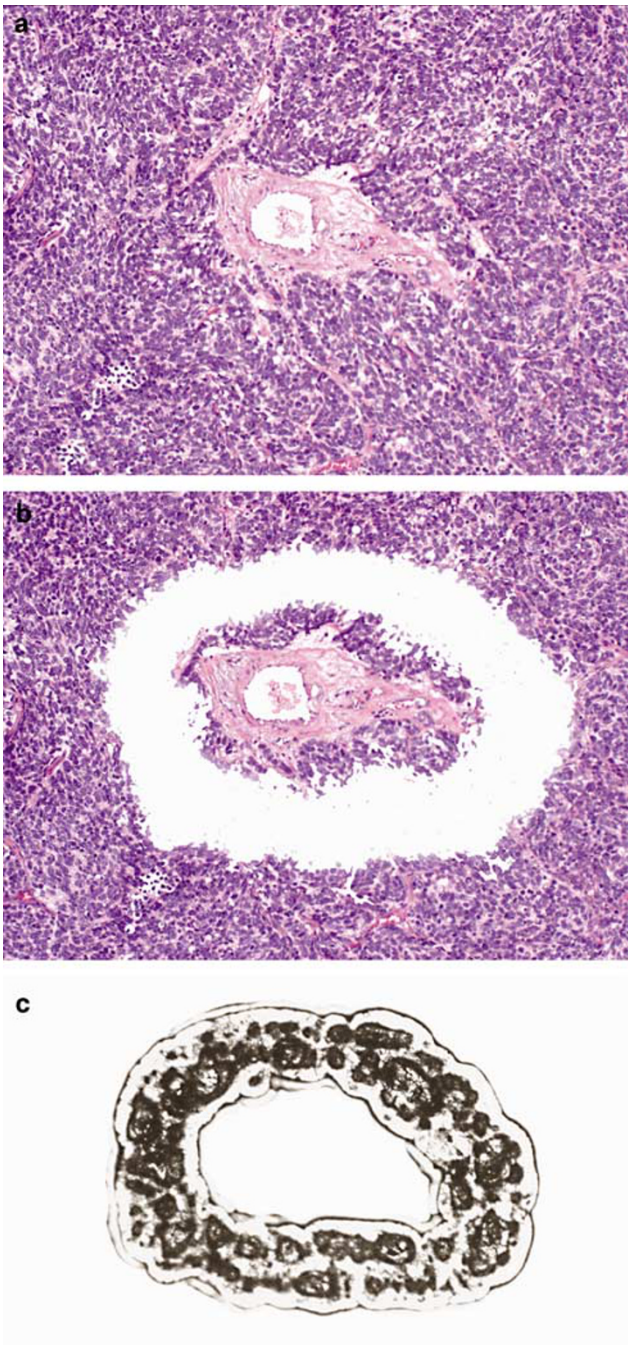

Figure 1 Laser-capture microdissection of small-cell carcinoma of the urinary bladder. Hematoxylin and eosin-stained sections showed small-cell carcinoma before microdissection (**a**) and after microdissection (**b**). (**c**) Laser-captured tumor cells.

All 13 TCC tissues (associated with coexisting small-cell carcinoma) selected for this study showed methylation of at least one gene (Table 2). Twelve of 13 SCBC samples showed methylation of at least one gene, and 12 of 13 TCC samples showed methylation

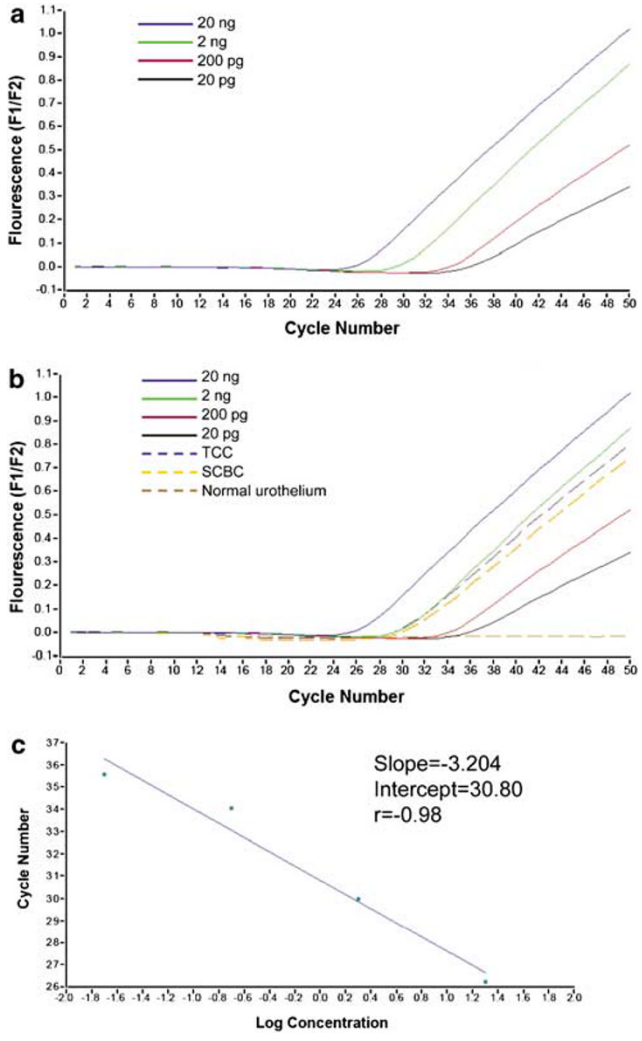

Figure 2 (**a**) Known amounts of *in vitro* methylated, bisulfite-treated DNA were amplified using *RASSF1* qMSP primers. (**b**) Fluorescence/amplification curves for selected tissues used in this study are shown. (**c**) A linear regression was constructed from the second-derivative maximum points for each standard. Other primer/probe sets used in the study produced similar slopes and *y*-intercepts.

of at least one gene. Three TCC tissues showed methylation of both *RASSF1* and *MGMT*, and six SCBC tissues showed methylation of both *RASSF1* and *MGMT*. Only one tissue (an SCBC) showed methylation of three or more genes.

RASSF1 methylation was observed in 46% (6 of 13) of TCC tissues and 77% (10 of 13) of SCBC tissues, respectively. Out of 13 matched pairs of TCC and SCBC samples, concordant methylation of *RASSF1* was found in six matched pairs (46%), and concordant lack of *RASSF1* methylation was found in three matched pairs (23%). Four matched pairs had discordant *RASSF1* methylation (31%). There was no significant difference between *RASSF1* methylation frequencies in matched TCC and SCBC samples ($P = 0.125$).

Table 2 Methylation status of *RASSF1*, *MGMT*, and *MLH1* in patients with coexisting TCC and SCBC

Sample	*RASSF1a*	*MGMT*	*MLH1*	*DAPK1*
1 N	M	M		
1 TCC	M	M		
1 SCBC	M	M	M	
2 N				
2 TCC	M			
2 SCBC	M	M		
3 N				
3 TCC		M		
3 SCBC		M		
4 N	M			
4 TCC		M		
4 SCBC				
5 N	M			
5 TCC	M	M		
5 SCBC	M			
6 N		M		
6 TCC	M			
6 SCBC	M	M		
7 N	M	M		
7 TCC		M		
7 SCBC	M	M		
8 N	M	M		
8 TCC		M		
8 SCBC	M	M		
9 N		M		
9 TCC		M		
9 SCBC	M			
10 N		M		
10 TCC				
10 SCBC	M			
11 N	M	M		
11 TCC		M		
11 SCBC		M		
12 N		M		
12 TCC	M	M		
12 SCBC	M			
13 N	M	M		
13 TCC	M			
13 SCBC	M	M		
Adjacent normal	7	9		
Both TCC and SCBC	6	5	0	0
TCC only	0	4	0	0
SCBC only	4	3	1	0

M, methylated; N, adjacent normal tissue; SCBC, small-cell carcinoma; TCC, transitional-cell carcinoma.

MGMT methylation was observed in 69% (9 of 13) of TCC tissues and 62% (8 of 13) of SCBC tissues, respectively. Out of 13 matched pairs of TCC and SCBC samples, concordant methylation of *MGMT* was found in six matched pairs (46%), and concordant lack of *MGMT* methylation was found

in one matched pair (8%). Seven matched pairs (54%) had discordant *MGMT* methylation. *MGMT* methylation frequency was very similar in TCC and SCBC tissues ($P=1.000$).

Methylation of both *RASSF1* and *MGMT* was found in 46% (6 of 13) of SCBC samples and 23% (3 of 13) of TCC samples, respectively. Methylation of both genes was more frequently harbored in SCBC only than in TCC only (6 *vs* 3), but this was not statistically significant ($P=0.453$).

Using Fisher's exact test, TNM stages, gender, history of tobacco use, and age were evaluated to determine if any association with DNA methylation was present. Methylation status of *RASSF1*, *MGMT*, and of both genes was considered. No significant association between TNM stage and any methylation status was found in either TCC or SCBC.

MLH1 was only methylated in one tissue, an SCBC, which also harbored methylation of *MGMT* and *RASSF1*, whereas *DAPK1* was not methylated in any tumor tissues.

To determine if field defects were involved in the carcinogenesis of SCBC, we also measured methylation levels in adjacent normal appearing tissue, which was microdissected from the original samples. *RASSF1* methylation was detectable in 54% (7 of 13) of normal tissues. *RASSF1* was concordantly methylated in normal tissue and at least one histopathology in 5 of these 7 patients. The concordance was not statistically significant ($P=0.453$). *MGMT* methylation was detectable in 69% (9 of 13) of normal tissues. *MGMT* was concordantly methylated in normal tissue and at least one histopathology in 8 of these 9 patients. Again, concordance was not statistically significant ($P=0.375$).

To determine if TCC that gives rise to SCBC is epigenetically different from TCC that does not give rise to SCBC (pure TCC), we performed the same analyses on a separate set of 14 TCC tissues from patients without SCBC. We found that *RASSF1*, *MGMT*, *MLH1*, and *DAPK1* were methylated in 11, 1, 0, and 0 tumors within this set, respectively (Table 3). The frequency of *MGMT* methylation in classical TCC *vs* SCBC-associated TCC was statistically significantly different ($P=0.002$, Fisher's Exact Test). Other relationships were not significant.

Table 3 Methylation status of *RASSF1*, *MGMT*, and *MLH1* in microdissected bladder cancer specimens containing only TCC

Sample	*RASSF1*	*MGMT*	*MLH1*	*DAPK1*
14 TCC	M			
15 TCC		M		
16 TCC	M			
17 TCC	M			
18 TCC	M			
19 TCC				
20 TCC	M			
21 TCC	M			
22 TCC	M			
23 TCC	M			
24 TCC				
25 TCC	M			
26 TCC	M			
27 TCC				
Sum	10	1	0	0

M, methylated; TCC, transitional-cell carcinoma.

Discussion

SCBC has a poor prognosis despite aggressive surgical and medical management. In a large series of patients with SCBC that we recently reported, outcomes were poor and survival did not appear to be influenced by whether or not the patient underwent cystectomy.[19] Therefore, the diagnosis of SCBC has grave implications. It is critical to understand the molecular changes occurring in this disease in order to design better detection assays, prognostic algorithms, and therapy for patients. Epigenetic changes are a particularly attractive avenue to pursue because (i) they are reversible, (ii) occur in many if not all types of cancer, (iii) many molecular alterations can be targeted at once with chromatin-altering drugs, and (iv) reversing epigenetic changes is one of the few ways, if not the only way, to reactivate tumor suppressors. Additionally, several noteworthy studies have used detection of DNA methylation in urine sediment as a screening tool for several types of genitourinary cancer.[20–22] Therefore, identifying epigenetic changes in SCBC may provide fruitful targets for earlier detection and novel therapies. However, few studies have described DNA methylation in SCBC.[23,24] We describe the methylation status of four genes, which are commonly silenced by epigenetic mechanisms in multiple cancers.

RASSF1 is a gene with multiple transcripts, one of which encodes RASSF1A, which is transcribed from an alternate promoter.[25] This gene was first identified by virtue of its location on chromosome 3p,[25,26] loss of which is one of the earliest and/or most common events in lung and other cancers.[27] *RASSF1* resides in the minimal region of homozygous deletion in human cancers of many histologies. It functions as a bona fide tumor-suppressor gene when artificially overexpressed in cancer cells, which lack its expression.[25] The mechanism of loss of function RASSF1A in cancer is almost exclusively related to deletion of 3p or DNA methylation-induced silencing, as sequencing studies have shown infrequent point mutations.[25,26] At this point, more is known about the mechanism of loss of RASSF1A function than about its actual function, although it is known to interact with and stabilize microtubules.[28–31] Its loss in model systems is known to cause hypersensitivity to microtubule-targeting agents[32] and therefore, its loss of function by methylation or deletion may herald chemosensitivity to microtubule-targeted drugs. In support of

this theory, novel chemotherapy regimens, which include microtubule-targeting agents for TCC, are currently being explored and have shown promising results.[33,34] In addition, one case of a sustained response of SCBC to a paclitaxel-based regimen has been reported.[35] In our study, 86% of patients had methylation in at least one component of their bladder tumors.

Similarly, *MGMT* is methylated at the gene level in a wide range of cancers including bladder cancer.[22,36,37] The function of MGMT is well known: it participates in DNA repair of alkylated O^6-methylguanine,[38,39] a lesion that occurs as a result of alkylation by anti-neoplastic agents. Consequently, therapeutic measures, which result in alkylation of guanine, may be effective for tumors that lack the repair mechanisms imparted by the participation of MGMT activity. Indeed, *MGMT* methylation is known to be a strong predictor of response to multiple alkylating agents in glioma.[6,40] Therefore, agents which produce O^6-methylguanine may be a viable chemotherapeutic approach for patients with SCBC tumors in which *MGMT* expression has been silenced.

We found that *MLH1* was infrequently methylated in the bladder tumors that we studied. MLH1 has been shown to be required for the response of tumors to cisplatin exposure in model systems.[7,10–12] Platinum-based chemotherapy has been the mainstay of treatment for more than 20 years.[41] TCC is often sensitive to this chemotherapy regimen, theoretically in part because its promoter is not hypermethylated and MLH1 expression is maintained. In support of this hypothesis, the lone patient with detectable MLH1 methylation survived only 3 months after being diagnosed with T2N1M1 SCBC. This patient received carboplatin and etoposide. In comparison, the eight other patients for whom survival information was available, lived an average of 15.8 months, with six of them surviving 6 months or more.

SCBC resembles small-cell lung cancer (SCLC) morphologically and clinically in terms of its propensity to metastasize, its chemoresistance, and its poor prognosis.[42] Like SCBC, SCLCs frequently coexist with non-small-cell lung cancer. Smoking is the most important risk factor for the development of SCLC, as $<1\%$ of SCLCs develop in non-smokers. Similarly, 65% of patients with SCBC had a history of smoking in the largest reported case series.[19] Smoking may cause similar genetic lesions in bladder and pulmonary epithelium. *MGMT* and *RASFF1* methylation are frequent occurrences in SCLC.[25,43–45] It is notable that we observed a similar high frequency of methylation of these genes in our reported cohort of patients with SCBC.

Other studies of methylation of specific gene loci in TCC have been reported.[23,24] The rate of methylation of *RASSF1* that we detect in pure TCC and TCC with SCBC are similar to those in previous reports.[37,46–49] However, the frequency of *MGMT* methylation in TCC with SCBC that we observed was significantly higher than the 2–5% rates reported in two prior studies comprising 196 combined specimens of pure TCC.[36,37] We believe this reflects an innate propensity for TCCs harboring *MGMT* methylation to develop clones of SCBC. This belief is based on the fact that we found *MGMT* methylation in only 1 of 14 cases containing pure TCC, but in 9 of 13 TCC samples where the histologies were admixed and genetically related.[9] Our findings may distinguish *MGMT* methylation as an SCBC-specific epimutation. In this setting, *MGMT* methylation has a sensitivity of 69% and a specificity of 92% in the detection of SCBC. In contrast, *DAPK1* methylation is found at higher levels (4–25%) in pure TCCs[36,37,49] compared with the tumors examined in our study, in which it was not detectably methylated in either histology. Alterations of *MLH1*, both in primary sequence and DNA methylation, have been thoroughly explored in one study and apparently do not play a part in bladder carcinogenesis.[50] Similarly, we found that methylation of *MLH1* was infrequent in TCC with or without SCBC.

Urothelial carcinogenesis has been postulated to proceed through epithelium with field cancerization.[51–54] To address this possibility for SCBC, we analyzed the methylation of these genes in adjacent normal appearing tissue in patients with coexisting TCC and SCBC. Methylation of *MGMT* and *RASSF1* was detected in a majority of normal tissues. Additionally, most tumors associated with normal tissues harboring DNA methylation were also methylated at these loci. These findings suggested that methylation of a locus in normal tissue predicted methylation in the tumor sample. Our finding of epimutations in normal appearing mucosa and concordant tumor samples supports the idea that the entire epithelium may be predisposed to premalignant change.[55] Tumor contamination of normal samples is possible but unlikely since these samples were carefully microdissected. Although not statistically significant, our findings do suggest a role for field cancerization through epigenetic changes.

DNA methylation studies may ultimately serve a pivotal role in the diagnosis and management of cancer. DNA methylation status is an ideal target for development of biomarkers, diagnosis, treatment, and surveillance. This study provides additional information concerning DNA methylation in small-cell carcinoma of the urinary bladder, and increases our understanding of the pathobiology of this disease.

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