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Aberrant DNA Methylation in Bladder Cancer among Saudi Arabia Population

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Abstract

Tumor biomarkers developed based on the aberrant deoxyribonucleic acid (DNA) methylation patterns in bladder cancer (BC) hold great promise due to their stability, specificity, and known associations with the disease. No study has investigated DNA methylation patterns in BC patients from Saudi population. We analyzed DNA methylation levels of 48 tumor suppressor genes loci in 24 bladder tissues (19 BC and 5 control samples) using Human Tumour Suppressor Genes EpiTect Methyl II Complete PCR Array (Qiagen, Hilden, Germany). We identified significant difference in DNA hypermethylation levels at *E2F1, ERBB2, HIC1, OPCML, SFN, SFRP1, SFRP2, SPARC,* and *TERT* gene loci between controls and cancerous samples. *SCGB3A1* was differentially methylated in nonmuscle invasive versus muscle invasive BC samples. Results suggest that these aberrant DNA methylation patterns in BC are disease and population specific and can be developed as distinct DNA methylation-based biomarkers for BC detection.

Keywords
► bladder cancer
► DNA hypermethylation
► biomarkers
► DNA methylation patterns
► epigenetic
► tumor
► MSP

Introduction

Bladder cancer (BC) is the ninth most commonly occurring cancer among men in the Kingdom of Saudi Arabia (KSA), accounting for 4.0% incidence rate[1]. Excessive tobacco consumption, exposure to increased levels of toxic chemicals, petroleum products, and infection with *Schistosoma haematobium* reflects the strong association with the continuous rise of BC in this region. Transitional cell carcinoma (TCC) represents the most common type (90%) of BC. Approximately, 80% of the new TCC of patients are nonmuscle invasive bladder cancer (NMIBC) and have good prognosis with more than 80% chance of 10 years survival rate; however, majority of the treated cases recur and progress to muscle invasive (MIBC). The most challenging aspect of BC is early diagnosis. Currently, periodic cystoscopy along with urine cytology is the essential prognostic and diagnostic tool for BC despite the fact that cystoscopy is invasive and has variable sensitivity.[2] Radiology imaging including utilization of computed tomography scan and/or magnetic resonance imaging scan are additional means of monitoring the progress and nonprogress of new TCC of the urinary bladder. Such limitations have increased interest in identifying nucleic acid-based biomarkers. CpG island hypermethylation has been reported as a strong indicator of bladder carcinogenesis.[3-6] Currently, aberrant modification of deoxyribonucleic acid (DNA) methylation patterns of tumor suppressor genes is of particular interest.[3-6] These aberrant DNA methylation patterns can be detected in blood, urine, or serum of the cancer patient as cfDNA.[2] Although these alterations are dynamic and respond to environmental influences[7] and have been studied worldwide,[8] to date no study has profiled similar alterations in Saudi population of BC patients. Therefore, in this study, we analyzed the DNA methylation patterns in BC, using a panel of 48 candidate genes loci.

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Materials and Methods

Sample Collection

This study was performed in the Urology Clinic, at tertiary care hospital in KSA after the approval from the Ethics Committee. Informed Consent was taken from all the participants of the study. Nineteen fresh samples of BC tissue from the patients were collected who underwent cystoscopy. As control, five corresponding normal appearing tissue samples from the same patients, adjacent to tumor, were also obtained. Patients were selected consecutively based on tissue availability with no age, gender, or ethnic restrictions. Exclusion criteria were patients with cystitis and tumor other than the BC.

Tumor tissue samples were obtained immediately after cystoscopy and were frozen as part of the routine protocol. All samples were stored at–80∘C. The presence and extent of tumor were evaluated by hematoxylin and eosin stains, to ensure at least 70% of tumor in the tumor samples. Tumor classification (World Health Organization [WHO]) and staging (tumor, nodes, metastasis, [TNM]) were routinely assessed for all tumor cases in formalin-fixed paraffin-embedded tissue. Detailed demographic and clinicopathologic parameters for all the samples are listed in ►**Table 1**.

Table 1 Summary of clinical pathological data of tumor and normal control samples

	Cancer cases (n = 19)	Control (n = 5)
Age		
Median	60	60
Range	30–85	42–72
Gender		
Male	14 (74%)	4 (80%)
Female	5 (26%)	1 (20%)
Extent		
Invasive (MIBC)	8 (42%)	
Noninvasive (NMIBC)	11 (58%)	
Pathological stage		
Stage CIS	1 (5.2%)	
Stage Ta	10 (52.6%)	
Stage T1	0	
Stage T2	7 (36.8%)	
Stage T3	0	
Stage T4	1 (5.2%)	
Grade		
High grade	12 (63%)	
Low grade	7 (37%)	
Relapse		
Primary	6 (31.5%)	
Recurrence	13 (68.5%)	

Abbreviations: MIBC, muscle invasive bladder cancer; NMIBC, nonmuscle invasive bladder cancer;

DNA Extraction

Genomic DNA was isolated using the Gentra Puregene Tissue kit (Qiagen). Briefly, DNA was digested overnight with proteinase K (1.5 µL) in the presence of 10% sodium dodecyl sulfate at 55°C, precipitated with isopropanol and 70% ethanol.

Selection of Genes

After a literature examination, 48 genes were selected for the evaluation of methylation abnormalities in BC. All genes analyzed in this study were previously reported as targets for epigenetic silencing in different human cancers[9-12] that could also contribute to the tumorigenesis process in BC.[3.]

EpiTect Methyl II qPCR array

The EpiTect Methyl II PCR Array (Qiagen) was used as a screening method to evaluate the promoter methylation status at 48 tumor suppressor genes in 24 samples. The assays were performed using the EpiTect Methyl II DNA Restriction Kit (Qiagen) according to the manufacturer's instructions. Briefly, 4 µg of genomic DNA was incubated overnight at 37∘C with a DNA methylation-sensitive restriction enzyme (Ms), which digests unmethylated DNA, with a DNA methylation-dependent restriction enzyme (Md) that digests methylated DNA, with both enzymes(Msd), and without enzyme added/"mock"(Mo) in four separate tubes. Following digestion, the remaining DNA in each "individual enzyme reaction was quantified by real-time polymerase chain reaction (PCR) using predesigned primers to the promoter region of the selected 48 genes. DNA amplification was carried on a 7,000 Sequence Detection System (Applied Biosystems, Foster City, California, United States), at 95∘C for 10 minutes followed by 40 cycles of 97∘C for 15 seconds and 72∘C for 1 minute. PCR product was marked with SYBR Green and Ct values were obtained. The analysis was performed using a SABiosciences Excel-Based Data Analysis Template, and the percentage of hypermethylated DNA was obtained by comparing the amount of DNA in each digest with that of a mock digest, representing the fraction of input DNA containing at least two methylated CpG sites in the targeted gene region. Statistically significant associations for promoter methylation for each gene were assessed using Student's t-test with a 5% significance level.

Results and Discussion

In our methylation-specific polymerase chain reaction analysis, significant difference in DNA hypermethylation frequencies was observed at nine genes (*E2F1, ERBB2, HIC1, OPCML, SFN, SFRP1, SFRP2, SPARC,* and *TERT*) in cancerous compared with normal tissue samples in regard to set of tumor existence (►**Table 2**). Only *SFN* gene showed significantly lower hypermethylation with p-value = 0.0011 in comparison to

Table 2 Genes showing statistically significant evidence of differential methylation among noninvasive, invasive cancer and controls

Genes	Cancer versus controls			Invasive versus noninvasive		
	Cancer (mean ± SD)	Controls (mean ± SD)	*p*-Value	Invasive (mean ± SD)	Noninvasive (mean ± SD)	*p*-Value
E2F1	17.71 ± 16.75	3.15 ± 3.5	0.002[a]	16.09 ± 14.48	17.5 ± 19.62	
EGFR	62.81 ± 3.85	62.28 ± 6.14		63.88 ± 4.48	61.3 ± 2.73	
ERBB2	59.6 ± 2.09	56.06 ± 1.25	0.0006[a]	60.29 ± 2.13	58.85 ± 2.02	
FAS	64.18 ± 3.16	56.67 ± 14.51		65.1 ± 3.06	63.24 ± 3.33	
FOS	60.59 ± 2.59	59.69 ± 2.21		59.87 ± 3.21	60.99 ± 1.77	
HIC1	79.84 ± 11.43	67.7 ± 2.87	0.0004[a]	77.27 ± 11.38	82.74 ± 12.11	
HOXA1	60.13 ± 3.36	59.33 ± 3.69		60.55 ± 3.33	59.29 ± 3.39	
HRAS	63.22 ± 8.55	61.98 ± 1.72		65.43 ± 3.9	60.46 ± 11.51	
IGF2R	64.08 ± 3.71	62.1 ± 1.46		63.58 ± 3.91	64.56 ± 3.9	
ING1	61.61 ± 7.01	65.06 ± 2.67		63.25 ± 2.84	59.78 ± 9.77	
JUN	58.41 ± 6.66	58.5 ± 1.73		59.43 ± 3.56	57.09 ± 9.11	
LOX	60.53 ± 3.5	63.07 ± 5.45		60.56 ± 2.73	61.13 ± 3.97	
MDM2	62.55 ± 3.08	56.26 ± 13.47		61.6 ± 2.46	63.5 ± 3.65	
MEN1	60.9 ± 3.74	60.54 ± 3.11		60.43 ± 3.94	60.82 ± 3.55	
MYC	62.7 ± 2.86	63.07 ± 1.79		62.07 ± 2.46	63.28 ± 3.38	
MYCN	62.34 ± 13.54	60.16 ± 5.73		64.26 ± 7.37	60.54 ± 18.71	
NF1	58.7 ± 6.82	59.28 ± 3.45		57.13 ± 9.3	59.37 ± 2.55	
NF2	55.12 ± 9.45	56.96 ± 1.67		57.48 ± 3.54	52 ± 12.86	
NFKB1	63.56 ± 3.89	62.4 ± 2.8		62.91 ± 3.42	63.83 ± 4.52	
NME1	60.34 ± 2.27	59.35 ± 1.03		60.36 ± 1.34	60.17 ± 3.09	
OPCML	74.62 ± 10.36	59.31 ± 14.04	0.0119[a]	77.35 ± 8.5	73.07 ± 12.01	
PRDM2	63.71 ± 3.44	62.79 ± 1.4		63.81 ± 3.45	63.64 ± 3.83	
PTCH1	60.56 ± 7.89	56.39 ± 12.84		62.88 ± 3.3	57.88 ± 10.667	
PTGS2	61.94 ± 3.49	62.58 ± 2.83		61.82 ± 2.68	62.04 ± 4.49	
PYCARD	29.25 ± 17.76	33.34 ± 23.44		30.78 ± 16.68	28.12 ± 20.63	
RB1	60.41 ± 3.18	57.72 ± 16.79		60.73 ± 3.38	59.38 ± 2.22	
RET	64.93 ± 8.45	60.28 ± 3.33		68.57 ± 11.36	61.45 ± 1.71	
SCGB3A1	73.4 ± 6.97	73.39 ± 3.72		77.06 ± 8.03	69.95 ± 4.01	0.0355
SFN	74.22 ± 8.97	93.09 ± 7.05	0.0011[a]	77.32 ± 9.47	71.31 ± 8.41	
SFRP1	71.51 ± 6.76	64.36 ± 3.43	0.0059[a]	74.34 ± 6.59	69.29 ± 6.42	
SFRP2	77.29 ± 12.41	64.83 ± 3.47	0.0009[a]	80.02 ± 11.48	75.52 ± 13.91	
SH3PXD2A	60.3 ± 3.04	60.73 ± 3.01		60.65 ± 1.72	59.78 ± 4.13	
SLC5A8	80.38 ± 11.57	72.73 ± 8.33		83.45 ± 10.67	78.66 ± 12.44	
SMAD4	61.43 ± 3.28	62.01 ± 1.44		60.95 ± 2.65	62.14 ± 3.97	
SMARCB1	56.57 ± 7.85	60.73 ± 2.6		54.81 ± 10.98	57.97 ± 3.34	
SPARC	51.24 ± 20.89	27.05 ± 19.04	0.0435	58.32 ± 17.79	48.44 ± 0.36	
TERT	77.68 ± 6.95	70.22 ± 4.31	0.0134[a]	79.45 ± 5.03	76.64 ± 8.57	

(*Continued*)

Table 2 (*Continued*)

Genes	Cancer versus controls			Invasive versus noninvasive		
	Cancer (mean ± SD)	Controls (mean ± SD)	*p*-Value	Invasive (mean ± SD)	Noninvasive (mean ± SD)	*p*-Value
TGFB1	60.03 ± 8.36	62.91 ± 1.83		61.26 ± 3.95	58.15 ± 11.49	
TGFBR2	64.83 ± 2.91	65.03 ± 3.21		65.48 ± 2.17	63.63 ± 3.03	
THBS1	61.9 ± 3.54	62.28 ± 2.66		62.75 ± 2.89	60.93 ± 4.23	
TP53	59.38 ± 6.82	60.97 ± 2.01		61.92 ± 3.53	56.15 ± 8.26	
TSC1	57.56 ± 7.78	59.38 ± 2.97		59.26 ± 4.21	55.17 ± 10.2	
TSC2	61.82 ± 2.9	58.28 ± 14.65		62.01 ± 2.66	61.26 ± 3.15	
VEGFA	62.06 ± 3.16	61.53 ± 1.93		62.16 ± 2.78	61.43 ± 3.38	
WT1	72.55 ± 9.46	68.17 ± 2.89		75.14 ± 10.15	70.12 ± 9.17	
WWOX	58.43 ± 7.86	60.29 ± 3.23		59.67 ± 2.57	56.69 ± 11.16	
XRCC1	61.53 ± 3.55	61.9 ± 3.9		61.36 ± 1.98	61.51 ± 4.9	
ZMYND10	63.05 ± 7.54	64.49 ± 2.1		64.33 ± 4.45	61.43 ± 10.1	

Abbreviation: SD, standard deviation.
Significant *p*-value = < 0.05.
[a]Highly significant.

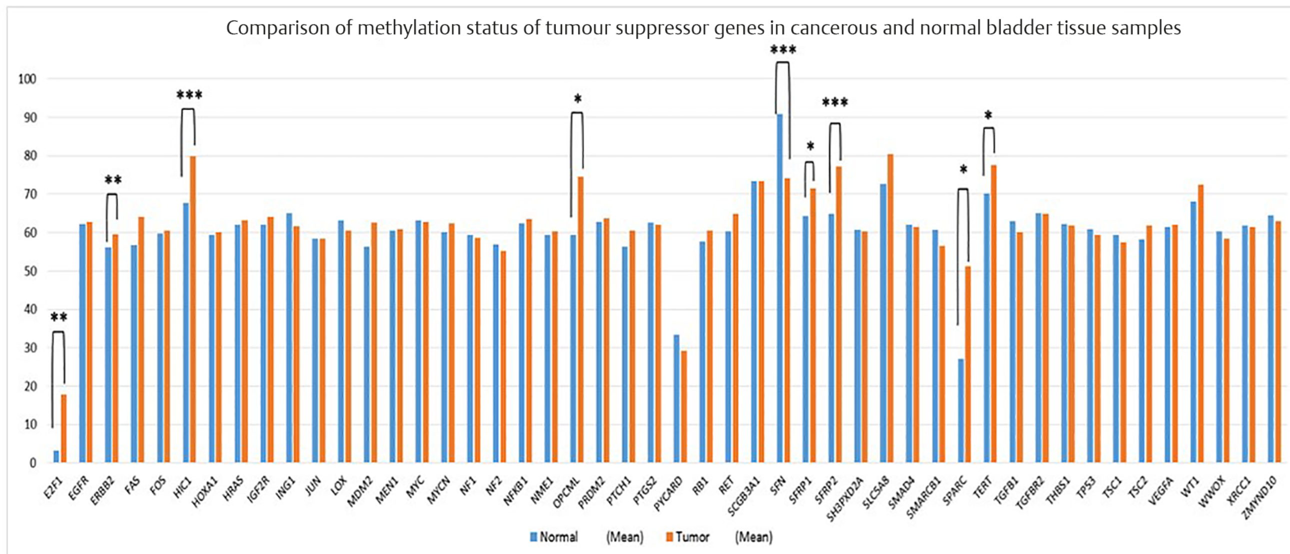

Fig. 1 The histogram showing deoxyribonucleic acid methylation profile between normal and cancerous bladder tissue among Saudi population. * and ** denotes *p*-value < 0.05 and 0.01, respectively (Student's *t*-test).

other eight genes that shows increase hypermethylation frequency in bladder cancerous tissue samples with most significant *HIC1*, *SFRP2*, and *ERBB2* genes (►**Fig. 1**). Silencing of most of these highly significant genes, due to methylation of their promoter regions, have been reported in multiple human cancer types. However, some of these genes have not been investigated, thus far, in BC. This suggests that the panel of candidate genes selected is appropriate to detect aberrant methylation profiles in BC patients and can be considered specific for Saudi Arabian population. It was also observed that *SCGB3A1* demonstrated the lower hypermethylation ($p = 0.0355$) in NMIBC versus MIBC samples. In contrast, no significant difference in DNA methylation levels at any gene promoter was found to be associated with tumor grade. Evidence suggests that these altered DNA methylation patterns are dynamic and respond to environmental influences. To our knowledge, this is the first study that has analyzed the methylation patterns in BC, using a panel of 48 candidate genes loci in 19 BC patients from Saudi Arabian population.

Limited studies in the past had observed the methylation patterns of *SFN, HIC1, OPCML* tumor suppressor genes in relation to BC.[13-15] One reason could be related to the methodologies used in the past or the dynamic patterns of DNA methylation due to diverse environmental exposure. *SFN (Stratifin)* is a protein kinase coding gene associated mainly with breast and pancreatic carcinoma. Although Negraes et al

have previously reported the hypermethylation of *SFN* gene in BC,[16] but due to its indistinct hypermethylation pattern in both cancerous and control group, it was not considered favorable for biomarker for BC detection. Similarly, silencing of *HIC1* gene due to methylation of their promoter regions has been reported in multiple human cancer types including BC[17] but no significant results were reported in BC to date. In contrast, our study has revealed significant DNA hypermethylation of both *SFN* and *HIC1* genes making them promising biomarkers for BC patients in Saudi Arabia. *OPCML* promoter hypermethylation in BC has previously been reported only by one study.[18] This study showed overall frequency of DNA methylation was 60% and methylation levels were significantly higher among BC patients when compared with normal mucosa ($p = 0.0001$). Our study was consistent with these findings and has shown that the significant hypermethylation of OPCML has a potential role in BC. It has been reported that *SCGB3A1* hypermethylation plays an important role in the carcinogenesis of several human malignancies including breast cancer.[19] Our study showed significant decrease of DNA methylation levels of *SCGB3A1* in patients with MIBC as compared with NMIBC. In this context, decrease in DNA methylation status of this putative tumor suppressor gene would require further observation so that it can be used specifically for the surveillance of invasive bladder tumors in future.

Conclusion

In conclusion, this is a pilot study and we have identified distinctive genes that show evidence of differential methylation between cancerous and normal tissue, noninvasive, and invasive cancer tissue specific for the Saudi Arabian population. Such DNA methylation-based biomarkers will later help us to develop biomarkers specifically for the Saudi Arabian population. However, larger studies on BC-associated changes in DNA methylation are required for better diagnosis and prediction of the disease.

Study Limitations

This is a pilot study to screen out potential DNA methylation markers that can used in future for detection and prognosis of BC on a large representing sample size of Saudi population. As we were screening 48 tumor suppressors, we needed large quantity of good quality DNA (4 µg) from our biopsy samples to conduct our experiments, which was not available from small size of the normal bladder biopsies. This is the reason we had only five control samples taken from the adjacent normal tissues of the BC patients. However, we compared the methylation differentiation for 48 genes in three different groups which was quite extensive and conducted for the very first time in this part of the region. Interestingly, a good number of genes showed significant results as well. Therefore, we can utilize this information from this pilot study as the screening tool for selecting some significant genes and run the similar analysis for bigger sample size with a smaller number of highly significant genes.

Note

All samples were collected from consenting patients/relatives according to institutional guidelines of participating hospital. Ethical approval was obtained from the Office of Research (ORA) of the Hospital and prior to the initiation of the study.

Conflict of Interest

None declared.

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