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Cancer Epidemiology, Biomarkers & Prevention

Aberrant Methylation of *RARβ2* and *APC* Genes in Voided Urine as Molecular Markers for Early Detection of Bilharzial and Nonbilharzial Bladder Cancer

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Research Article

Aberrant Methylation of *RARβ₂* and *APC* Genes in Voided Urine as Molecular Markers for Early Detection of Bilharzial and Nonbilharzial Bladder Cancer

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Abstract

Background: Bladder cancer cells illustrate major disruptions in their DNA methylation patterns as compared with normal ones. Authors aimed to identify epigenetic molecular markers in urine for early detection of bladder cancer.

Materials and Methods: We retrospectively analyzed the methylation status of *RARβ₂* and *APC* genes in urine samples from 210 bladder cancer patients, 61 patients with benign urological diseases, and 49 healthy volunteers by using methylation-specific PCR.

Results: Methylated *RARβ₂* and *APC* were significantly higher in bladder cancer patients (62.8%, 59.5%) than benign (16.4%, 5%) but not detected in healthy volunteers (0%) at ($P < 0.0001$). Both methylated genes showed no significant difference among clinicopathologic factors; however, they were detected in all grades and stages. Among the 128 patients with bilharzial bladder cancer, 94 (73.4%) showed methylated *RARβ₂* and 86 (67.2%) showed methylated *APC*. Homoplasmic methylation pattern of both genes were only detected in bilharzial bladder cancer cases. Both sensitivities and specificities of the methylated genes for bladder cancer detection were superior to urine cytology and when altogether combined, the sensitivities improved to (91.8%), (93.5%), (91.9%), and (80.9%) in detection of: bladder cancer, non-muscle invasive bladder cancer, low-grade tumors, and bilharzial associated bladder cancer, respectively.

Conclusion: Thus, methylated *RARβ₂* and *APC* genes might be valuable urinary molecular markers for early detection of bilharzial and nonbilharzial bladder cancer. *Cancer Epidemiol Biomarkers Prev; 20(8); 1657–64.*

Introduction

Bladder cancer is a global problem, the highest frequency of bladder cancer worldwide is observed in Egypt, this high incidence is believed to be due to endemic infestation by *Schistosoma haematobium* (1, 2).

The gold standard for bladder cancer detection remains to be cystoscopy. Not only this technique is invasive, but also 10% to 40% of malignancies may be undetected by this procedure (3). Alternatively, urine cytology is known for its good specificity but poor sensitivity for early stages and low grades. An array of urine markers were evaluated to improve the diagnostic ability of urine cytology and, perhaps, to reduce the need for frequent cystoscopies during follow-up, especially in those with low-risk disease (4–11).

Promoter hypermethylation of CpG islands is strongly associated with tumor development and survival in bladder carcinogenesis (1). Several reports indicate that body fluids, including urine can be used for noninvasive detection of bladder cancer by hypermethylation of DNA (12, 13).

The aim of this study was to evaluate the usefulness of 2 methylated genes: *RARβ₂* and *APC* as urine markers to detect bladder cancer in urine sediments, especially those with bilharzial infestation in comparison to urine cytology.

Materials and Methods

Clinical samples

This study was approved by the Medical Ethical Committee of the Ain Shams University, Faculty of Medicine,

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and informed written consent to participate in the study was obtained from each participant ($n = 320$) before being enrolled. Patients were admitted to Ain Shams Hospital suffering from urological symptoms from January 2007 to December 2009. After cystoscopy, based on histopathologic examination, 210 patients were diagnosed as bladder cancer (mean age, 60 years ± 11; range, 28–61 years) and 61were diagnosed as benign urological lesions (mean age, 55 years ± 16; range, 22–65 years). A group of 49 healthy volunteers (mean age, 50 years ± 9.4; range, 25–55 years) were recruited from the hospital laboratory staff as controls. Of the malignant bladder cancer patients, 112 were diagnosed by histopathology as transitional cell carcinoma (TCC), 53 cases as squamous cell carcinoma (SCC), and 45 as adenocarcinoma. Tumor staging and grading were determined according to TNM and World Health Organization classification (14, 15).

Collection of samples

Sera (5 mL) and voided urine (30–60 mL) samples were obtained from all individuals before they received any treatment and before they underwent surgery. Each urine sample was centrifuged and then the urinary sediment was washed with PBS. A portion of the pellet was used for cytological and microscopic examinations (16) and the other portion was treated with protease inhibitor cocktail (8) and stored at −80°C for further processing to extract DNA to be used for detection of methylated *APC* and *RARβ*$_2$.

Detection of schistosomiasis antibodies in serum

The sera were used for detection of schistosomiasis antibodies by using the Cellognost Schistosomiasis H Kit (Dade Behring Marburg GmbH; ref. 17).

Detection of methylated *RARβ*$_2$ and *APC* genes

This was accomplished blindly in all samples malignant, benign, and control in 4 phases. In the first phase, DNA was extracted from urine pellets. In the second phase, DNA was treated by bisulfite. In the third phase, we amplified the gene by PCR. Finally, the fourth phase included detection of the PCR products.

DNA isolation from urine pellets. DNA extraction was done by using AllPrep DNA/RNA Micro kit (QIAGEN Ltd). DNA integrity was checked by 2% agarose electrophoresis and 2 samples with degraded DNA were discarded.

DNA modification. The bisulfite modification was done by using the EZ DNA Methylation Kit (Zymo Research) as follows: 130 μL CT conversion reagent was added to 20 μL of purified DNA sample in a PCR tube. Then PCR tubes were placed in Hybaid Thermal Cycler (Thermo Electron) and temperature conditions were as follows: 98°C for 10 minutes; 64°C for 2.5 hours; and storage at 4°C overnight. Then the modified DNA was loaded into Zymo-Spin IC Column after addition of 600 μL of M-binding buffer. After centrifugation at full speed for 30 seconds, the flow-through was discarded, and 100 μL of the M-wash buffer were added to the column and centrifuged at full speed for 30 seconds. Then 200 μL of M-desulfonation buffer were added to the column and incubated at room temperature for 20 minutes, followed by centrifugation at full speed for 30 seconds. Two hundred microliters of M-wash buffer were added twice to the column and after each time, centrifugation at high speed for 30 minutes was done. At the end of the procedure, DNA was eluted after the addition of 10 μL elution buffer and stored at −20°C for methylation-specific PCR (MSP).

MSP of RARβ*$_2$ *and APC genes. *MSP of RARβ*$_2$. Amplification of the *RARβ*$_2$ gene region of the modified DNA samples was first done by an outer PCR reaction by using external primers for *RARβ*$_2$ gene (18). The amplified products of this PCR were used as DNA templates in the nested PCR. The reaction was conducted by using puReTaq Ready-To-Go PCR beads (GE Healthcare), in brief: a 635-bp region, encomposing 27 *RARβ*$_2$ CpG sites in the *RARβ*$_2$ regulatory region was amplified by using first primer set [*RARβ*$_2$ sense 5\-TTA AGT TTT GTG AGA ATT TTG-3\ and set *RARβ*$_2$ antisense 5\-CCT ATA ATT AAT CCA AAT AAT-3\ (provided by Biolegio BV, Netherlands)] and 2 μL modified DNA with a down-step protocol in the Thermal Cycler (PCR Express, Hybrid) as follows: activation at 95°C for 5 minutes; then 35 cycles of denaturation at 94°C for 30 seconds; annealing at 50°C for 30 seconds; and extension at 72°C for 1 minute followed by final extension at 72°C for 5 minutes and hold at 4°C. The expected modified amplified DNA product was 404 to 425 bp. As control, 2 human MLH1 primers were used provided by Zymo Research [5′-GGA GTG AAG GAG GTT ACG GGT AAG T-3′ (sense) and 5′-AAA AAC GAT AAA ACC CTA TAC CTA ATC TAT C-3′ (antisense)], the expected amplified product was 182 bp. Then the PCR products obtained from the first PCR were reamplified by using 2 sets of nested primers provided by Biolegio BV. The first set of primer [5′-TCG AGA ACG CGA GCG ATT CG-3′ (sense) and 5′-GAC CAA TCC AAC CGA AAC GA-3′ (antisense)] was specially designed to amplify initially methylated DNA at critical CpGs on *RARβ*$_2$ gene, whereas the second primer [5′-TTG AGA ATG TGA GTG ATT TGA-3′ (sense) and 5′-AAC CAA TCC AAC CAA AAC AA-3′ (antisense)] was used to amplify only initially unmethylated DNA at critical CpGs on *RARβ*$_2$ gene. The PCR conditions for nested *RARβ*$_2$ were as follows: activation at 95°C for 5 minutes; then 35 cycles of denaturation at 94°C for 30 seconds; annealing at 60°C for 30 seconds; and extension at 72°C for 1 minute followed by final extension at 72°C for 10 minutes and hold at 4°C.

MSP of APC. The primer sequences designed for the promoters 1A spanned 7 CpGs and were provided by Biolegio BV. Primer sequences of unmethylated *APC* promoter 1A were 5′-GTG TTT TAT TGT GGA GTG TGG GTT-3′ (sense) and 5′-CCA ATC AAC AAA CTC

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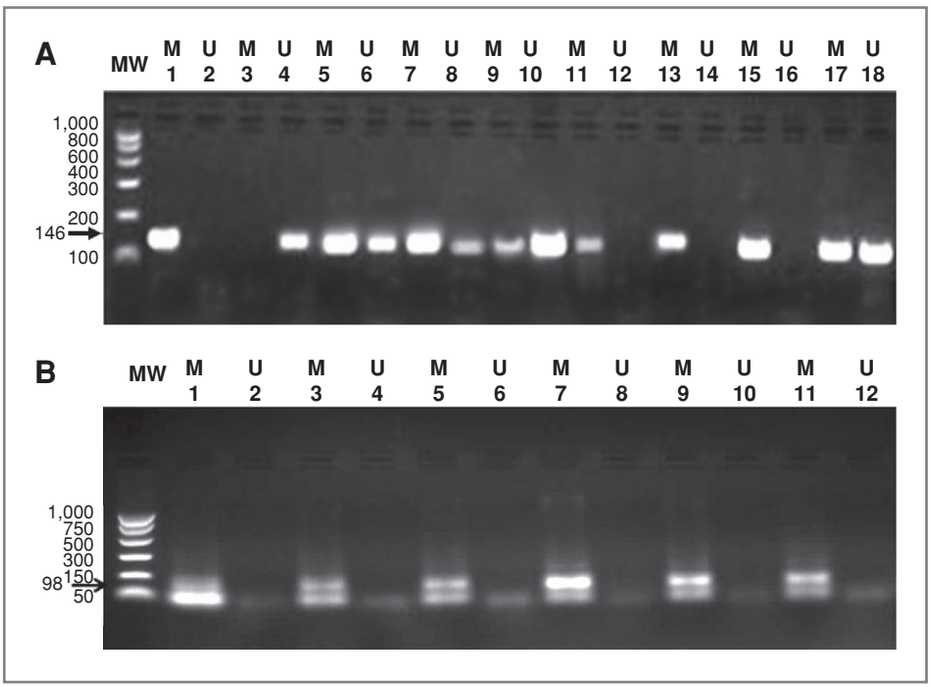

Figure 1. MS-PCR product analysis of urinary $RAR\beta_2$ and *APC* genes by agarose gel electrophoresis and ethidium bromide staining. A, $RAR\beta_2$ positive bands for both unmethylated and methylated bands are shown at 146 bp. Lane (MW) molecular weight ladder standard marker (100–1,000 bp). Lanes 3–4 normal urine samples, lanes 9–10 benign urine samples, and lanes 1, 2, 5–8, 11–18 malignant urine samples. Each sample was represented by 2 successive lanes, one for unmethylated (U) band and the second for methylated (M) band. B, *APC* positive bands for methylated bands are shown at 98 bp. Lanes 1–2 benign urine samples, lanes 3–12 malignant urine samples. Each sample was represented by 2 successive lanes, one for unmethylated (U) band and the second for methylated (M) band.

CCA ACA A-3′ (antisense), which amplify a 108-bp product; and for the methylated *APC*, 5′-TAT TGC GGA GTG CGG GTC-3′ (sense) and 5′-TCG ACG AAC TCC CGA CGA-3′ (antisense), which amplify a 98-bp product. The 5′ position of the sense unmethylated and methylated primers corresponds to bp 696 and 702 of GenBank sequence no. U02509, respectively (19). PCR was done by using HotStarTaq Master Mix Kit (provided by QIAGEN) and done for 39 cycles consisting of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, and extension at 72°C for 1 minute and hold at 4°C.

Detection of PCR products. Agarose gel electrophoresis (2%) was used to separate DNA fragments on the basis of size and visualized by ethidium bromide staining as previously described (20). The PCR products were compared with molecular weight marker (provided by Promega, GE Healthcare Bio-science, UK limited) by using Gel-pro (version 3.1; Media cybernetics) software.

Methylated and unmethylated $RAR\beta_2$ appear as bands at 146 bp, respectively (Fig. 1A), whereas methylated and unmethylated *APC* appears as bands at 98 bp and 108 bp, respectively (Fig. 1B).

Statistical analysis

Univariate analyses were done by using a χ^2 square test. Significant markers by univariate analysis were further tested by multivariate analysis using general linear model. Efficacies of investigated markers were calculated according to standard statistical methods (21). All analyses were done using Statistical Package for the Social Sciences software (SPSS Inc.).

Results

A total of 320 participants were included in this study. Two hundred and ten were diagnosed with bladder cancer; 128 of them were bilharzial bladder cancer and the remaining ($n = 82$) were nonbilharzial bladder cancer. Among the 61 patients diagnosed with benign urological diseases, 28 showed benign bilharzial lesions (18 with bilharzial dysplasia, 10 with bilharzial cystitis), and the rest ($n = 33$) were nonbilharzial lesions (17 with renal stones, 9 with bladder polyp, and 7 with pyelonephritis). All healthy volunteers ($n = 49$) were nonbilharzial. Clinicopathologic and demographic characteristics of bladder cancer group are shown in Table 1.

Distribution of $RAR\beta_2$ and *APC* methylation patterns among the studied groups

Methylated $RAR\beta_2$ was detected in 62.8% malignant group and 16.4% benign group, and it was not detected in normal individuals ($\chi^2 = 64$, $P = 0.009$). Similarly, methylated *APC* promoters were detected in 59.5% (125/210) and in 5% (3/61) of the malignant and benign cases, respectively, although it was not detected in normal individuals 0% ($\chi^2 = 47.79$, $P < 0.0001$; Table 2). For early bladder cancer detection, receiver operating characteristic curve (ROC) was plotted for the discrimination between malignant and nonmalignant groups by using $RAR\beta_2$ and *APC* (Fig. 2). Multivariate MANOVA revealed statistical significant effect of investigated methylated $RAR\beta_2$ and *APC* genes, and cytology on diagnosis of bladder cancer (F = 55.916, Wilks'

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Table 1. Clinicopathologic and demographic characteristics of bladder cancer group ($n = 210$) among investigated patterns of *RARβ2*, *APC* methylation, and cytology

Clinicopathologic factors ($n = 210$)	Methylation of urinary *RARβ2* promoter		Methylation of urinary *APC* promoter		Positive urine cytology ($n = 91$)
	Unmethylated ($n = 193$)	Methylated ($n = 130$)	Unmethylated ($n = 196$)	Methylated ($n = 125$)	
Age					
≤52 years ($n = 62$)	57 (92%)	30 (48.4%)	52 (83.3%)	41 (66.7%)	31 (50%)
>52 years ($n = 148$)	136 (91.8%)	100 (67.6%)	144 (97.3%)	84 (56.8%)	60 (40.5%)
Gender					
Male ($n = 145$)	136 (93.8%)	90 (62.1%)	136 (93.5%)	86 (59.6%)	67 (46.2%)
Female ($n = 65$)	57 (87.7%)	40 (61.5%)	60 (92.3%)	39 (60%)	24 (36.9%)
Bilharziasis					
−ve ($n = 82$)	79 (96.3%)	49 (59.8%)	82 (100%)	55 (67.7%)	23 (28%)
+ve ($n = 128$)	114 (89%)	81 (63.3%)	114 (89%)	70 (54.7%)	68 (53.1%)
					$\chi^2 = 4.4$, $P = 0.036$
Smoking					
−ve ($n = 90$)	88 (97.5%)	65 (72.2%)	85 (94.4%)	40 (44.4%)	25 (27.8%)
+ve ($n = 120$)	105 (87.5%)	65 (54.2%)	111 (92.5%)	85 (70.8%)	66 (55%)
					$\chi^2 = 6.65$, $P = 0.01$
Pathologic type					
SCC ($n = 53$)	50 (94.3%)	33 (62.3%)	53 (100%)	39 (73.6%)	14 (26.4%)
TCC ($n = 112$)	102 (91.1%)	75 (66.9%)	104 (93.6%)	63 (56.3%)	52 (46.4%)
Adenocarcinoma ($n = 45$)	41 (91.1%)	22 (48.9%)	39 (86.7%)	23 (51.1%)	25 (55.6%)
Histologic grades					
1 ($n = 43$)	39 (90.7%)	36 (83.7%)	38 (88.4%)	24 (55.8%)	14 (32.6%)
2 ($n = 110$)	101 (91.8%)	64 (58.2%)	104 (94.5%)	65 (59.1%)	51 (46.4%)
3 ($n = 57$)	53 (92.9%)	30 (52.6%)	54 (94.7%)	36 (63.2%)	26 (45.6%)
Clinical stages					
I ($n = 77$)	74 (96.1%)	54 (70%)	70 (90.9%)	45 (58.4%)	41 (53.2%)
II ($n = 67$)	62 (92.5%)	35 (52.2%)	63 (94%)	43 (64.2%)	29 (43.3%)
III ($n = 30$)	23 (76.6%)	16 (53.3%)	30 (100%)	22 (73.3%)	7 (23.3%)
IV ($n = 36$)	34 (94.4%)	25 (69.4%)	33 (91.7%)	15 (41.7%)	14 (38.9%)

Lambda = 0.456 at $P < 0.0001$), the test between subject effect revealed significant efficacy of *RARβ2* and *APC* genes (F = 31.9, 73.3, and 53.5, respectively, at $P < 0.0001$) and superiority to cytology as diagnostic markers (F = 53.5 at $P = 0.002$).

Considering bilharzial status among the enrolled patients collectively, methylation of *RARβ2* and *APC* promoters were significantly higher in patients (benign vs. malignant) with bilharzial infection as compared with those with no bilharzial infection. Bilhariziasis was associating 50% (5/10) and 100% (3/3) of methylated *RARβ2* and *APC* among the benign cases, and 71.2% (94/132) and 68.8% (86/125) of methylated *RARβ2* and *APC* among the bladder cancer cases, respectively. Homoplasmic methylated (M/M) *RARβ2* and *APC* genes were only detected in bilharzial bladder cancer. Heteroplasmic methylated (M/U) *RARβ2* and *APC* genes were detected in 17.9% (5/28) and in 10.7% (3/28) in bilharzial benign cases (bilharzial dysplasia and bilharzial cystitis), whereas among the bilharzial bladder cancer patients, they were detected in 64.8% (83/128) for both, respectively, as shown in Table 2.

Correlation between *APC* and *RARβ2* promoter methylation and clinicopathologic factors in bladder cancer

Methylated *APC* and *RARβ2* promoters revealed no significant correlation with any of the clinicopathologic factors.

No statistical difference was detected between bilharzial bladder cancer and clinicopathologic factors apart from cytology. Methylated *RARβ2* and *APC* patterns were highly expressed in bilharzial bladder cancer as compared with nonbilharzial bladder cancer, although statistical difference was not reached.

Concordance between *RARβ2*, *APC* methylation patterns, and urine cytology

Authors have investigated the concordance between *APC*, *RARβ2* methylation patterns, and urine cytology

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Table 2. *RARβ2* and *APC* methylation among different investigated groups

Investigated markers	Control ($n = 49$)	Benign ($n = 61$)			Malignant ($n = 210$)		
		Total	Bilharzial status of benign group		Total	Bilharzial status of malignant group	
			N-Bilh. ($n = 33$)	Bilh. ($n = 28$)		N-Bilh. ($n = 82$)	Bilh. ($n = 128$)
Methylated urinary *RARβ2* ($n = 142$)	0 (0%)	10 (16.4%)	5 (15.2%)	5 (17.9%)[a]	132 (62.8%)	38 (46.3%)	94 (73.4%)
Homoplasmic methylation ($n = 11$)	—	—	—	—	11 (5.2%)	—	11 (8.6%)
Heteroplasmic methylation ($n = 131$)	—	10 (16.4%)	5 (15.2%)	5 (17.9%)	121 (57.6%)	38 (46.3%)	83 (64.8%)
Methylated urinary *APC* promoter ($n = 128$)[b]	0 (0%)	3 (5%)	0 (0%)	3 (10.7%)[b]	125 (59.5%)	39 (47.6%)	86 (67.2%)
Homoplasmic methylation ($n = 3$)	—	—	—	—	3 (1.4%)	—	3 (2.3%)
Heteroplasmic methylation ($n = 125$)	—	3 (5%)	—	3 (10.7%)	122 (58.1%)	39 (47.6%)	83 (64.8%)
Positive urine cytology ($n = 91$)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	91 (43.3%)	20 (24.4%)	71 (55.5%)

NOTE: Nonbilharzial (49; control, 33 benign, and 82 malignant cases), bilharzial [28 benign (bilharzial dysplasia and bilharzial cystitis) and 128 malignant cases].

Statistical significance was detected between the 3 studied groups (control, benign, and malignant) regarding *RARβ2, APC,* and cytology (χ^2 = 64, 47.2, and 62.8, at $P < 0.0001$, respectively).

Significant between bilharzial benign group (bilharzial dysplasia and bilharzial cystitis) versus bilharzial malignant group: at [a]$\chi^2 = 19.96$, $P \leq 0.0001$, and at [b]$\chi^2 = 11.31$, $P = 0.003$. No statistical difference between bilharzial (bilharzial dysplasia, and bilharzial cystitis) and nonbilharzial benign groups and no statistical difference between bilharzial and nonbilharzial malignant groups.

among the study population ($n = 320$), as shown in Table 3.

Overall sensitivity, specificity, PPV, NPV, and accuracy of methylated *RARβ2* and *APC* and urine cytology

All these statistical analyses were carried out to evaluate the efficiency of these urine markers to discriminate between malignant and nonmalignant groups (normal individuals and benign group), as shown in Table 4. The sensitivities of urinary methylated *RARβ2* and *APC* were superior to urine cytology for detection of bladder cancer, nonmuscle invasive bladder cancer (early stages), low grades, bilharzial cases, and SCC. As infection (i.e., bilhariziasis) may induce promoter methylation of certain genes; authors have investigated the diagnostic efficacy of *RARβ2* and *APC* among those with nonbilharzial infection. Similarly, their sentitivities were superior to urine cytology (sensitivity: 76.4%, 71.4%, and 50% for *RARβ2* and *APC* and cytology, respectively). Combination between urinary methylated *RARβ2* and *APC* increased their sensitivity with slight decrease in their

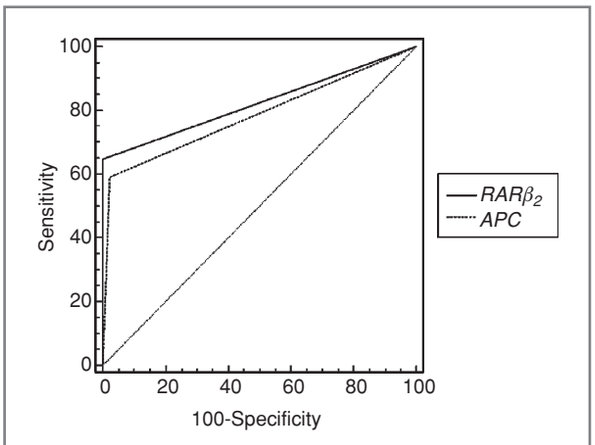

Figure 2. ROC curve analysis for *RARβ2* and *APC* genes to discriminate between malignant and nonmalignant groups. For *RARβ2* the sensitivity = 63.6% and specificity = 90%. Area under the curve (AUC; SE) = 0.736 (0.034), 95% confidence limits range = 0.671–0.795, $P < 0.0001$) and for *APC*: the sensitivity = 59.3% and specificity = 98%. AUC (SE) = 0.824 (0.033), 95% confidence limits range = 0.715–0.847, $P < 0.0001$).

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Table 3. Concordance between methylated *APC* and *RARβ2* patterns among all investigated groups ($n = 320$)

Urine markers	Methylated urinary *RARβ2* promoter		Urine cytology	
	Unmethylated ($n = 178$)	Methylated ($n = 142$)	Negative ($n = 229$)	Positive ($n = 91$)
Methylated urinary *APC* promoter				
Unmethylated ($n = 192$)	119	73	157	35
Methylated ($n = 128$)	59	69	72	56
	$\chi^2 = 5.51$, $P = 0.019$		$\chi^2 = 14.5$, $P < 0.0001$	
Urine cytology				
Negative ($n = 229$)	154	75	—	—
Positive ($n = 91$)	23	67	—	—
	$\chi^2 = 19.01$, $P < 0.0001$			

specificity except for bilharzial association, where the sensitivity increased with absolute specificity.

Discussion

Previous studies suggested that DNA methylation in urine could be useful diagnostic tests but were limited by bladder cancer type (e.g., no bilharzial bladder cancer), and inappropriate controls (e.g., no benign urological group). In this study, authors have investigated 2 DNA methylation biomarkers *RARβ2* and *APC* (13), using MSP PCR (12), which can distinguish cytosine from methylated cytosine residues and can detect as few as 1 to 10 tumor cells among 10^4 normal cells in clinical samples, including the urine sediments (22).

Table 4. Combined sensitivity and specificity for investigated parameters in detection of bladder cancer, especially superficial, low-grade, bilharzial bladder cancer, and SCC

Investigated parameters	Bladder Cancer		Non-muscle invasion (superficial bladder cancer)		Low grade Bladder cancer		Bilharzial Bladder cancer		SCC	
	Sens. %	Spec., %	Sens., %	Spec., %	Sens., %	Spec., %	Sens., %	Spec., %	Sens., %	Spec., %
Urine cytology	43.3	100	48.9	100	45.3	100	36.8	100	26.3	100
Urinary *RARβ2* promoter hypermethylation	62.8	87.9	60.9	87.9	64	87.9	50.9	87.9	62.3	87.9
Urinary *APC* promoter hypermethylation	59.5	98	62.6	98	58.8	98	50.6	98	73.6	98
Urine cytology + Urinary *RARβ2* promoter hypermethylation	79.1	89.4	80.4	89.4	81.4	89.4	66	89.4	78.9	89.4
Urine cytology + Urinary *APC* promoter hypermethylation	75.5	98	80.4	98	74.4	98	66.3	98	73.7	98
Urinary *RARβ2* promoter hypermethylation + Urinary *APC* promoter hypermethylation	87.3	97.6	88	97.6	87.2	97.6	77.5	97.6	89.5	97.6
Urine cytology + Urinary *RARβ2* promoter hypermethylation + Urinary *APC* promoter hypermethylation	91.8	97.6	93.5	97.6	91.9	97.6	80.9	97.6	89.5	97.6

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Our results revealed significant urinary *APC* and *RARβ*$_2$ promoter hypermethylation in malignant group as compared with benign and healthy normal groups confirming previous reports (13, 23). The DNA hypermethylation of both genes was neither stage nor grade dependent, indicating that their usefulness is not limited to high-grade or high-stage tumors. DNA methylation of *APC* and *RARβ*$_2$ improves the sensitivity of urine cytology to 93.5%, 91.9%, and 80.9% in detecting superficial bladder cancer (nonmuscle invasive bladder cancer), low-grade tumors, and bilharzial associated bladder cancer, respectively.

Among all groups of this study, there was a positive correlation between smoking and bilharziasis ($P < 0.01$), which are well-known risk factors of bladder cancer (24). Meanwhile, there was no difference between bilharzial bladder cancer and nonbilharzial bladder cancer as regard to clinicopathologic criteria (25).

Studies proved that there is epigenetic disparity between nations. A unique profile of both environmental and genetic factors in every nation may contribute to such a geographically distinct methylation pattern. For this reason, it was important to investigate gene methylation in bilharzial bladder cancer.

To the best of our knowledge, this study is the first to investigate urinary *RARβ*$_2$ and *APC* methylation patterns in bilharzial bladder patients in comparison with nonbilharzial cases. There was a significant difference between bilharzial benign cases (bilharzial dysplasia and bilharzial cystitis), and bilharzial malignant cases regarding methylated *RARβ*$_2$ and *APC*; Table 2. Interestingly, when considering bilhariziasis among benign patients (bilharzial dysplasia and bilharzial cystitis), half of them showed methylated *RARβ*$_2$, whereas all of the benign patients with methylated *APC* suffered from bilhariziais. Moreover, homoplasmic methylation pattern for both *RARβ*$_2$ and *APC* was only found in bilharzial bladder cancer, indicating that bilharzial infestation might play a role in the aberrant hypermethylation underlying carcinogenesis. Thus, these findings can add to the diagnostic value of methylated *RARβ*$_2$ and *APC* in early detection of bladder cancer.

Concordance between methylated *RARβ*$_2$ and *APC* as well as urine cytology was investigated (Table 3). A significant difference between them indicates that these 3 urine markers were independent and their combinations will improve their sensitivities in detecting bladder cancer.

When a urine molecular marker is used for diagnostic work-up of bladder cancer, it should have a high sensitivity (26). Accordingly, urinary methylated *RARβ*$_2$ and *APC* are significantly superior to urine cytology (Table 4). Methylated urinary *RARβ*$_2$ and *APC* were detected in 37 cases (31.1%) and 35 cases (29.4%), respectively, with negative urine cytology ($n = 119$), signifying their values in the early diagnosis of bladder cancer. Thus, combination of *RARβ*$_2$ or *APC* methylation with urine cytology improved the sensitivity of urine cytology. Moreover, the sensitivity of urine cytology was the highest when combined with methylated *RARβ*$_2$ and *APC* altogether in bilharzial and nonbilharzial cases (Table 4).

Thus, the detection of methylated *RARβ*$_2$ and *APC* by MSP in urine sediment DNA is a powerful noninvasive approach for the detection of bilharzial and nonbilharzial bladder cancer over urine cytology. However, further multicentric studies will be required to define the impact of these molecular markers on early detection and disease monitoring before clinical application, also, longitudinal follow-up of bilharzial cases is needed to understand the association of methylation of candidate genes in cancer development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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