OncoTargets and Therapy

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ORIGINAL RESEARCH

Promoter methylation analysis of *CDH1* and *p14ARF* genes in patients with urothelial bladder cancer

This article was published in the following Dove Press journal:
OncoTargets and Therapy

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Background/aim: Urothelial bladder cancer arises from the accumulation of multiple epigenetic and genetic changes. We aimed to investigate the specificity and sensitivity of gene-specific promoter methylation of *CDH1* and *p14ARF* genes in the early diagnosis of bladder cancer and compare those with other diagnostic tests in our population.

Patients and methods: In the current study, 65 patients with urothelial bladder cancer and 35 controls without any history of cancer were recruited. Methylation profiles of *CDH1* and *p14ARF* genes from tumor and urine samples were determined by methylation-specific polymerase chain reaction method.

Results: Methylation of *CDH1* and *p14ARF* genes in tumor samples was 95.4% and 78.5%, respectively. The methylation frequencies were found to be 68.8% for *CDH1* gene and 72.9% for *p14ARF* gene in urine samples. Sensitivities of *CDH1*, *p14ARF* and urine cytology were found to be 67.4%, 72.1% and 34.9%, respectively, while their specificities were 93.9%, 63.6% and 93.9%, respectively.

Conclusion: Aberrant promoter methylation of *CDH1* and *p14ARF* genes can be used to detect urothelial bladder cancer. In low-grade tumors, when compared with urine cytology, combined methylation analysis of *CDH1* and *p14ARF* genes may not increase the sensitivity to identify malignant cells in urine samples.

Keywords: biomarker, *CDH1*, urine cytology, urothelial bladder cancer, *p14ARF*, urine

Introduction

Urothelial bladder cancer (UBC) is the most prevalent type of urinary tract malignancy, particularly in men.[1,2] Numerous genetic and epigenetic factors play a significant role in progression, recurrence and metastasis of UBC.[3] Certain genetic factors such as activation of proto-oncogenes (*EGFR*, *FGFR*, *HER/neu* [*c-erb-B2*], *c-myc*, *MDM2* and others), inactivation of tumor suppressor genes (*p53* mutation, *Rb* homozygous deletion and others) and alteration of cell cycle regulators (p21, p27, Ki-67, cyclin D1, cyclin E and others) and cell adhesion molecules (MMP-2, E-cadherin, etc) have been observed to be related to bladder cancer.[4–7] Aberrant promoter methylation of tumor suppressor genes might change normal cellular growth properties by causing decrease in gene expression. Methylation occurs in the early stages of carcinogenesis and can be determined in body fluids, indicating that a noninvasive and early cancer detection method can be developed. Bladder cancer studies of hypermethylation in urine DNA samples are in progress from day to day and refer to the potential of epigenetics in cancer diagnosis.[8]

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http://dx.doi.org/10.2147/OTT.S158259

Cadherin 1 (CDH1, E-cadherin, epithelial cadherin) is a Ca^{2+}-dependent transmembrane glycoprotein that mediates cell–cell adhesion and is highly expressed in normal epithelial tissues.[9,10] Cadherins (CDHs) make cell–cell connection with extracellular, transmembrane and intracellular domains.[11] Loss of CDH1 expression is one of the characteristics of epithelial–mesenchymal transition (EMT) frequently observed in carcinogenesis. Somatic mutations, transcriptional repressors (*SNAI1* and *SNAI2*, *TWIST*, *ZEB1*/*ZEB2*), loss of heterozygosity and promoter hypermethylation of *CDH1* are involved in transcriptional repression and reduced *CDH1* expression.[12]

The INK4a/ARF locus is localized on chromosome 9p21 and encodes tumor suppressor proteins such as p16INK4a and p14ARF, which are the negative regulators of the cell cycle via 16INK4a-Rb and p14ARF-p53 pathways.[13–16] p14ARF proteins prevent abnormal cell growth and division in response to oncogene (*Ras*, *c-myc*, *E1A* and *E2F1*) activation. Overexpression of E2F1, radiations, genotoxic drugs and DNA damage induce the expression of *p14ARF* and prevent the aberrant cell cycle progression.[17,18] Activation of p14ARF inhibits E3 ubiquitin–protein ligase, mouse double minute 2 (MDM2) protein, and prevents degradation of p53 by ligase activity of MDM2. Activated p53 arrests abnormal cell cycle at G1 and G2 phases.[17,19,20]

The current study aimed to investigate and validate the methylation patterns of *CDH1* and *p14ARF* genes in urothelial bladder carcinoma tissues and voided urine samples as epigenetic diagnostic biomarkers and compared those with other diagnostic methods including urine cytology. In addition, we investigated whether aberrant methylation of *CDH1* and *p14ARF* genes can replace urine cytology in bladder cancer.

Patients and methods

Study subjects

The study protocol was approved by the institutional review board of the Ondokuz Mayis University (OMU), and all participants signed an informed consent form stating their full consent and their own free will to participate in the study after receiving detailed information about the study. A total of 65 histologically confirmed UBC patients diagnosed at the urology clinic of OMU and 35 healthy control individuals were enrolled in this study. All patients were investigated by standard urological evaluation including urine cytology and flexible cystoscopy. Tumors were examined and classified according to the World Health Organization/International Society of Urological Pathology (WHO/ISUP) (2010) and were staged according to TNM (2010). Paired preoperative voided urine samples were collected from 48 patients. The exclusion criteria for controls included urinary infections, a history of cancer and benign conditions. A total of 35 healthy voided urine samples were selected from gender- and age-matched (±5) volunteers. The healthy control individuals with suspicious results for urine cytology were examined in the OMU Urology Clinic, and inflammatory, benign or malignant conditions were not observed.

DNA isolation

The tumor samples were immediately frozen and stored at –80°C until the DNA isolation procedure was performed. Fresh urine samples were used for DNA extraction. DNA isolation from tumor and urine samples was performed with ZR Genomic DNA™ Tissue MiniPrep Kits (Zymo Research, Irvine, CA, USA) and ZR Urine DNA Isolation Kit™ (Zymo Research), respectively, according to the manufacturer's instructions. DNA samples were stored at –20°C until bisulfite modification.

Bisulfite modification and methylation-specific polymerase chain reaction (MS-PCR)

Bisulfite modification of extracted DNA samples was carried out by EZ DNA Methylation™ Kit (Zymo Research). Approximately 200 ng DNA/20 µL and 500 ng DNA/20 µL were used for bisulphite conversion of urine and tumor samples, respectively.

MS-PCR was run for the promoter regions of both *CDH1* and *p14ARF* genes using methylated and unmethylated primer pairs. The reaction was carried out in a final volume of 50 µL containing 2 µL of bisulfite-treated DNA, 0.25 mM of each deoxynucleotide triphosphate (dNTP) (Zymo Research), 0.5 µM of each of the primers and 2 U of *Taq* polymerase (Zymo Research). After initial denaturation at 95°C for 10 minutes, the subsequent steps of denaturation at 95°C for 30 seconds, annealing at 57°C (*CDH1*, for both methylated [M] and unmethylated [U]) and 60°C (*p14ARF*, for both M and U) for 30 seconds, and extension at 72°C for 60 seconds were repeated for 40 cycles.[21,22] Half of the PCR products were electrophoresed on a 2.5% agarose gel. Methylated and unmethylated products of *CDH1* were 116 bp and 97 bp, respectively, while methylated and unmethylated products of *p14ARF* were 122 bp and 132 bp, respectively. The gel images of methylation status of the *CDH1* and *p14ARF* promoters in tumor and urine samples are shown in Figure 1. Universal methylated human DNA standard (in vitro-methylated DNA [IVD]; Zymo Research) was used as a positive control for methylation. Fifty base pair DNA ladder (New England BioLabs, Ipswich, MA, USA) was used as a marker.

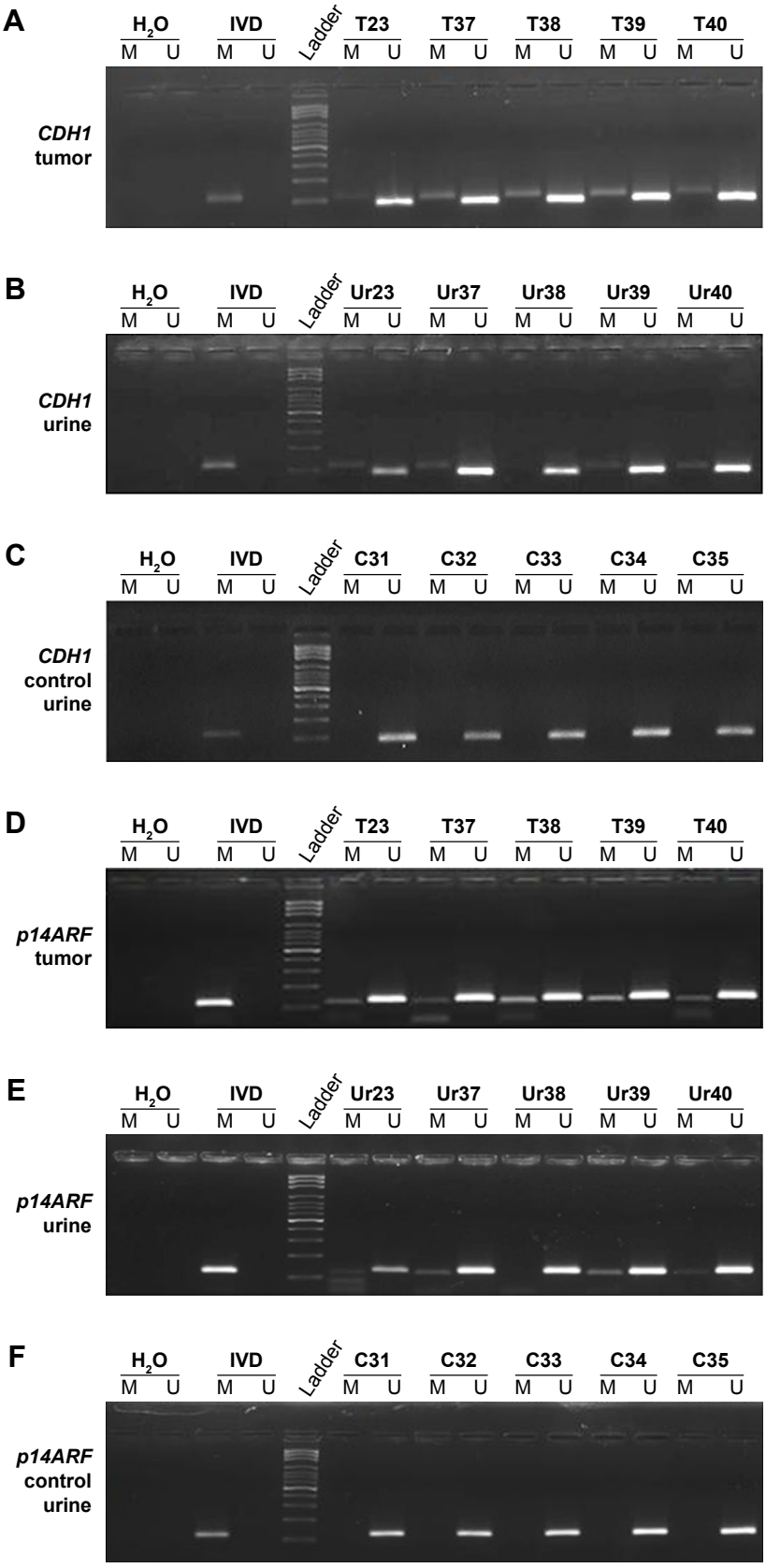

Figure 1 Promoter methylation analysis of *CDH1* and *p14ARF* genes in tumor tissues and paired urine samples of bladder cancer patients and controls.
Notes: Methylation analysis of CDH1 promoter, (**A**) in tumor samples; (**B**) in urine samples; (**C**) in urine samples of controls. Methylation analysis of p14ARF promoter, (**D**) in tumor samples; (**E**) in urine samples; (**F**) in urine samples of controls.
Abbreviations: M, methylation; U, unmethylation; C, urine samples of controls; H_2O, water (negative control); IVD, in vitro-methylated DNA (positive control); T, tumor tissue samples; Ur, urine samples.

The gene methylation status was indicated as methylated when amplification products were detected in the reactions performed with primers M or both M and U.

Statistical analyses

For statistical analyses, MedCalc Statistical Software, version 17.2 (MedCalc Software bvba, Ostend, Belgium) was used. After testing for normal data distribution using the Kolmogorov–Smirnov test, the nonparametric Spearman's rank correlation and Mann–Whitney *U*-test were applied as needed. κ test was performed to compare the methylation status of *CDH1* and *p14ARF* genes in tumor and paired urine samples. *P*-value of <0.05 was considered significant.

Results

In the current study, we analyzed the methylation status of *CDH1* and *p14ARF* promoters by MS-PCR in 65 bladder tumor samples, 48 paired urine samples and 35 urine samples of healthy individuals. The clinical and pathological data of patients and controls are summarized in Table 1.

Frequency of methylation in tumor

Methylation analyses of *CDH1* and *p14ARF* genes were performed on 65 tumor samples from bladder cancer patients.

Table 1 Clinicopathological characteristics of UBC patients and controls

Characteristics	Patients (n = 65; %)	Controls (n = 35; %)
Gender		
Male	55 (84.6)	32 (91.4)
Female	10 (15.4)	3 (8.6)
Age		
Range	41–89	38–83
Mean ± SD	65.6 ± 12.6	61.2 ± 9.8
Pathological grade		
Low	19 (29.2)	
High	42 (64.6)	
Unknown	4 (6.2)	
Pathological stage		
Ta	12 (18.5)	
T1	28 (43.0)	
T2	17 (26.2)	
T3	3 (4.6)	
Unknown	5 (7.7)	
Recurrence		
Primary	35 (53.9)	
Recurrence	19 (29.2)	
Unknown	11 (16.9)	
Smoking status		
Yes	16 (24.6)	1 (2.9)
No	18 (27.7)	21 (60.0)
Quit	11 (16.9)	9 (25.7)
Unknown	20 (30.8)	4 (11.4)

Abbreviation: UBC, urothelial bladder cancer.

Table 2 Promoter methylation frequencies of *CDH1* and *p14ARF* genes in the patient and control groups

Samples	Methylated *CDH1* (%)	Methylated *p14ARF* (%)
Patient group		
Tumor tissue (n = 65)	62 (95.4)	51 (78.5)
Urine sample (n = 48)	33 (68.8)	35 (72.9)
Control group		
Urine sample (n = 35)	2 (5.7)	13 (37.1)

Overall, 95.4% (62/65) and 78.5% (51/65) of tumor samples had methylation in *CDH1* and *p14ARF* promoter, respectively (Table 2).

Frequency of methylation in urine samples

Methylation pattern of *CDH1* and *p14ARF* genes in paired preoperative voided urine samples was analyzed in 48 patients and 35 controls. The methylation frequencies of urine samples of patients were 68.8% (33/48) for *CDH1* and 72.9% (35/48) for *p14ARF* gene. Moreover, methylation frequencies for *CDH1* and *p14ARF* genes were 5.7% (2/35) and 37.1% (13/35; Table 2), respectively, in urine samples of controls without cancer history. The paired urine samples of the patient and control groups have shown a significant difference ($P < 0.001$) regarding the promoter methylation profile of *CDH1* and *p14ARF* genes. κ test was performed to compare the promoter methylation status of *CDH1* and *p14ARF* in 48 tumor and paired urine samples. The results of κ test demonstrated the methylation status of two genes in both samples showed reliability (Table 3).

The methylation pattern of paired urine samples was identical to tumor samples in almost in all cases; however, the methylation pattern of tumor samples was not identical in a few urine samples. The agarose gel images of the methylation analysis of *CDH1* and *p14ARF* genes are shown in Figure 1.

We evaluated the positive predictive value (PPV) and negative predictive value (NPV) for *CDH1* and *p14ARF* genes. The PPV and NPV for *CDH1* methylation analysis

Table 3 The agreement between methylation status of tumor samples according to paired urine samples

Samples	*CDH1* (%)	*p14ARF* (%)
Tumor (n = 48)	45 (93.8)	40 (83.3)
Urine (n = 48)	33 (68.8)	35 (72.9)
k	0.256	0.700
P-value	0.008	0.000

Notes: >0 = poor agreement; 0.0–0.20 = slight agreement; 0.21–0.40 = fair agreement; 0.41–0.60 = moderate agreement; 0.61–0.80 = substantial agreement; 0.81–1.00 = almost perfect agreement.

Table 4 Predictive values of methylation status of *CDH1* and *p14ARF* genes and urine cytology

	Patients (tissue)	Controls (urine)	Total
***CDH1* MS-PCR[a]**			
+	True positive (n = 62)	False positive (n = 2)	64
−	False negative (n = 3)	True negative (n = 33)	36
***p14ARF* MS-PCR[b]**			
+	True positive (n = 51)	False positive (n = 13)	64
−	False negative (n = 14)	True negative (n = 22)	36
Urine cytology[c]			
+	True positive (n = 15)	False positive (n = 0)	15
−	False negative (n = 28)	True negative (n = 33)	61

Notes: [a]PPV: true positive/true positive + false positive = (62/62 + 2) × 100 = 96.9%; NPV: true negative/true negative + false negative = (33/33 + 3) × 100 = 91.7%; ACC: (62 + 33/100) × 100 = 95%. [b]PPV: true positive/true positive + false positive = (51/51 + 13) × 100 = 79.7%; NPV: true negative/true negative + false negative = (22/22 + 14) × 100 = 61.1%; ACC: (51 + 22/100) × 100 = 73%. [c]PPV: true positive/true positive + false positive = (15/15 + 0) × 100 = 100%; NPV: true negative/true negative + false negative = (33/33 + 28) × 100 = 54.1%; ACC: (15 + 33/76) × 100 = 63.2%.
Abbreviations: ACC, accuracy; MS-PCR, methylation-specific PCR; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value.

were 96.9% and 91.7%, respectively, with 95% accuracy. These values were lower for *p14ARF* gene, 79.7% and 61.1%, respectively, with 73% accuracy (Table 4).

Matching of MS-PCR and urine cytology results

A urine cytology test was performed in urine samples of 48 patients and 35 healthy individuals. However, five of the 48 patients voided urine samples and two of the 35 urine samples of the controls could not be diagnosed cytologically. Urine cytology and MS-PCR results were compared in 43 patient samples with tests being positive in 34.9% (15/43) and negative in 23.3% (10/43) of urine samples. The remaining patients' urine samples were undiagnosed. Urine cytology test results of low-grade bladder tumors demonstrated 41.7% (5/12) suspicious results, and there was no positive result for 12 low-grade patient samples. In low-grade tumor specimens, the sensitivity of the test was 66.7% (8/12) for *CDH1* gene and 75% (9/12) for *p14ARF* gene with the methylation of one of the two genes being 83.3% (10/12) of the samples. In addition, the results of the urine cytology test of the control group were suspicious and negative in 6.1% (2/33) and 93.9% (31/33) of the samples, respectively.

Sensitivity and specificity of urine cytology and MS-PCR results for *CDH1* and *p14ARF* genes are summarized in Table 5. The test was significant for *CDH1* gene with a sensitivity of 67.4% and a specificity of 93.9% ($P < 0.0001$). In addition, sensitivity and specificity for *p14ARF* were calculated to be 72.1% and 63.6%, respectively. On the other hand,

Table 5 Sensitivity and specificity of MS-PCR and urine cytology for *CDH1* and *p14ARF* genes

Tests	Sensitivity (%)[a]	Specificity (%)[b]
CDH1	67.4 (29/43)	93.9 (31/33)
p14ARF	72.1 (31/43)	63.6 (21/33)
Methylation of one of the two genes	86.0 (37/43)	97.0 (32/33)
Urine cytology	34.9 (15/43)	93.9 (31/33)

Notes: [a]Positive/total. [b]Negative/total.
Abbreviations: MS-PCR, methylation-specific PCR; PCR, polymerase chain reaction.

the sensitivity and specificity in the control group for *CDH1* were 34.9% (15/33) and 93.9% (31/33), respectively.

The PPV and NPV for the urine cytology test were 100% and 54.1%, respectively (Table 4). Our results have demonstrated a significant relation between the smoking status in patients and controls with regard to the methylation of *CDH1* ($P = 0.01$) and *p14ARF* ($P = 0.0015$). In addition, we have found a significant positive association between age and promoter methylation of *CDH1* and *p14ARF* genes ($P < 0.05$).

We have not observed any correlation among tumor grade, stage, recurrence, gender factor, intravesical therapy and routine second transurethral resection (Re-TURB) with methylation of *CDH1* and *p14ARF* genes (Table 6).

Discussion

UBCs are the mixture of heterogeneous cell populations; therefore, tumors with similar pathological characteristics may behave differently.[2] Numerous diagnostic methods are used to diagnose and follow up the recurrence and progression

Table 6 Correlations among the methylation status of *CDH1* and *p14ARF* genes and tumor stage, recurrence, intravesical therapy and Re-TURB

Parameters	n	*CDH1*	*P*-value	*p14ARF*	*P*-value
Bladder tumor					
≤pT1	40	38 (95.0%)	>0.05	29 (72.5%)	>0.05
≥pT2	20	20 (100%)		17 (85.0%)	
Recurrence	19	19 (100%)	>0.05	14 (73.7%)	>0.05
Primary	35	32 (91.4%)	>0.05	29 (82.9%)	>0.05
Urine					
≤pT1	32	22 (68.8%)	>0.05	22 (68.8%)	>0.05
≥pT2	13	8 (61.5%)		10 (76.9%)	
Recurrence	16	12 (75%)	>0.05	12 (75.0%)	>0.05
Intravesical therapy					
Positive	22	21 (95.5%)	0.56	18 (81.8%)	1.00
Negative	22	20 (90.9%)		18 (81.8%)	
Re-TURB					
Positive	4	3 (75.0%)	0.26	4 (100%)	0.36
Negative	38	36 (94.7%)		31 (81.6%)	

Abbreviation: Re-TURB, routine second transurethral resection.

of the UBC.[5] Flexible cystoscopy is the current standard for the early detection of UBC; however, this method is expensive and invasive under the local anesthesia in bladder cancer patients for histopathological diagnosis.[23,24] Urine cytology is a useful noninvasive diagnostic test in the detection of high-grade tumors in urine samples with high specificity, whereas the sensitivity is low in low-grade bladder tumors.[5,25] These limitations of the currently available diagnostic techniques have increased the focus on identifying other clinically useful, reliable and noninvasive early diagnostic markers with high specificity for the diagnosis of bladder cancer.

We have found a significant positive association between urothelial bladder carcinoma and methylation of *CDH1* and *p14ARF* genes. The methylation level in our study has been found higher than in previous studies using the same methods.[9,22,26] However, the sample size of our study was larger than those of previous studies. Lin et al[22] studied the methylation status of four genes (*CDH1*, *p14*, *CDKN2A* and *RASSF1A*) in 57 bladder tumor specimens and associated preoperative urine samples with the urine samples of 20 healthy control individuals using MS-PCR. Hypermethylation of *CDH1*, *p14* and *RASSF1A* genes has been suggested as a valuable diagnostic biomarker to urine cytology for low-grade bladder cancer.

Lin et al[22] calculated the overall sensitivity of *E-cadherin* and *p14ARF* genes to be 35% and 33%, respectively, in detecting bladder carcinoma. In contrast, these researchers found 7.7% positive results for low-grade tumors by urine cytology. In this study, the sensitivity of *CDH1* and *p14ARF* genes separately and combined was higher than the sensitivity of urine cytology in urine samples. In addition, the specificity of urine cytology was the same as the specificity of *CDH1* methylation, but higher than the specificity of *p14ARF* methylation in urine samples and slightly lower than the combined specificity based on two genes. Therefore, our results indicate that combined methylation analysis of these genes may be a useful marker compared with the urine cytology to increase the sensitivity for detecting bladder cancer.

Although the specificity of MS-PCR analysis of urine samples of patients was significantly higher than the results of urine cytology, methylation could not be detected in paired urine samples of six patients despite positive methylation in their tumor samples. This situation can be explained with the low number of malignant cells in urine samples from tumor tissues or a low level of methylated alleles in cancer cells within the urine samples, which could therefore not be enough to be detected by MS-PCR.[27] Indeed, methylation results of tumor and urine samples demonstrated a fair and substantial agreement for *CDH1* and *p14ARF*, respectively. Hoque et al[28] investigated multiple tumor suppressor genes (*APC*, *ARF*, *CDH1*, *GSTP1*, *MGMT*, *CDKN2A*, *RARβ2*, *RASSF1A* and *TIMP3*) as potential biomarkers for the diagnosis of bladder cancer. Similarly, they observed promoter methylation in tumor samples but not in all paired urine samples.

Numerous studies demonstrated a correlation between promoter methylation of certain genes with tumor grade, stage, recurrence, intravesical therapy and Re-TURB.[8,29,30] The methylation of 10 tumor suppressor genes in bladder cancer patients (N = 98) was examined. Methylation of *CDH1* was found to be significantly associated with poor survival ($P = 0.003$) and an independent predictor of survival in a multivariate analysis ($P = 0.02$).[31] In a recent study, Xiong et al[32] conducted a large trial involving 687 patients with bladder cancer using MS-PCR. The promoter methylation level of *CDH1* was found to be significantly associated with a higher tumor stage compared with lower tumor stage. However, there was no association between the aberrant methylation of the *CDH1* and *p14ARF* genes and tumor stage, grade and recurrence in the current study. Interestingly, several studies showed similar findings in the different types of malignancies.[33–35]

In our study, the control group consisted of age- and gender-matched individuals, and a significant relationship was observed among the increased methylation of *CDH1* and *p14ARF* genes, aging and malignity. Similarly, Yates et al[36] reported an increase in the frequency and extent of methylation with age and malignity in bladder cancer patients. Nevertheless, two studies demonstrated an association between promoter methylation of *CDH1* gene and aging independent of cancer.[26,37] These findings might indicate that aging may be a risk factor for bladder cancer. On the other hand, Chan et al[38] analyzed the methylation of *RARβ*, *DAPK*, *E-cadherin*, *p16*, *p15*, *GSTP1* and *MGMT* genes in bladder transitional cell carcinoma and found no association between age and methylation status of these. Two studies observed no methylation in tumor suppressor gene panels including *E-cadherin*, *p16*, *p14ARF*, *APC* and *RASSF1A* in urine DNAs from normal healthy individuals.[22,36] We have found a significant relation between smoking status of patients and controls regarding the methylation of *CDH1* and *p14ARF*. Indeed, numerous studies indicate that smoking is a risk factor for bladder cancer in both men and women.[39,40]

Finally, it is important to note that our study is limited by the nonquantitative nature of the detection method of the methylation status of *CDH1* and *p14ARF* genes as well as the sample size. These results must be verified in large cohorts using different approaches.

Conclusion

Our preliminary results have demonstrated that the combined methylation analysis of *CDH1* and *p14ARF* genes as biomarkers may be a sensitive method to detect malignant cells in urine samples. However, larger cohorts and well-defined subgroups of bladder cancer patients are required to reveal the potential role of *CDH1* and *p14ARF* genes as novel biomarkers.

Acknowledgment

This study was supported by OMU Research Foundation (project no PYO.TIP.1904.14.017).

Disclosure

The authors report no conflicts of interest in this work.

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