A Novel and Sensitive DNA Methylation Marker for the Urine-Based Liquid Biopsies to Detect Bladder Cancer

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Research Article

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Abstract

Background

Better prognostic outcome is closely correlated with early detection of bladder cancer. Current noninvasive urinalysis relies on simultaneously testing multiple methylation markers to achieve relatively high accuracy. Therefore, we have developed an easy-to-use, convenient, and accurate single-target urine-based DNA methylation test for the malignancy.

Methods

By analyzing TCGA data, 366 candidate markers with 446 primer pairs and probe sets were synthesized and systematically screened in cancer cell lines, paired tissue specimens, and urine sediments from bladder cancer patients and normal controls. The identified marker was further validated in large case-control cohorts. Wilcoxon rank sum tests and χ^2 tests were performed to compare methylation levels between case-control groups and correlate methylation levels with demographic and clinical characteristics. In addition, MSP, qMSP and RT-PCR, western blot analysis, and immunohistochemistry were performed to measure levels of DNA methylation, mRNA expression, and protein level in cancer cell lines and tissues.

Results

A top-performing *DMRTA2* marker identified was tested in both training and validation sets, showing similar sensitivity and specificity for bladder cancer detection. Overall sensitivity in the aggregate set was 82.9%(179/216). The specificity, from a control group consisting of patients with lithangiuria, prostatoplasia, and prostatitis, is 92.5%(468/506). Notably, the methylation assay had the highest sensitivities for tumors at stages of T1(90.4%) and T2(95.0%) compared with Ta(63.0%), T3(81.8%), T4(81.8%), and unknown (85.2%) stages. Furthermore, the test showed admirable detection rate of 80.0% (24/30) for recurring cancers. While methylation was observed in 39/54(72.2%) urine samples from patients with carcinomas of renal pelvis and ureter, it was detected at extremely low rate of 6.0%(8/133) in kidney and prostate cancers. Compared with SV-HUC-1, the normal bladder epithelial cell line, *DMRTA2* was hypermethylated in 8/9 bladder cancer cell lines, consistent with the results of MSP and qMSP, but not correlated with mRNA and protein expression levels in these cell lines. Similarly, *DMRTA2* immunostaining was moderate in some tissues but weak in others. Further studies are needed to address functional implications of *DMRTA2* hypermethylation.

Conclusions

Our data demonstrated that a single-target DNA methylation signature, m*DMRTA2*, could be highly effective to detect both primary and recurring BC via urine samples.

Introduction

Bladder cancer (BC) is the 4th most frequently occurring malignancies and the 9th most common cause of death worldwide in men [1]. In China, about 53000 patients were newly diagnosed BC with incidence rate ranked 6th in men before prostate cancer and continuously increasing [2]. The invasive and metastatic form of the cancer is the main cause of death or unfavorable prognosis for BC patients. The 5-year survival rate for patients with localized tumors can reach as high as 92%, but only 45% for those with tumors spreading to nearby regions [3]. Around 70% of the patients with superficial BC can be completely rid of tumor cells after surgical resection. However, the overall 5-year survival rate did not significantly improve in the past thirty years even though numerous therapeutic approaches have been developed for its clinical treatment [4]. Therefore, it is imperative to detect BC at early stage for the therapeutic interventions to be more effective and survival rate to be further increased.

Certain urinary tests for detection of BC have been approved by the US Food and Drug Administration (FDA) for clinical practice. BladderChek (Matritech Inc., Newton, MA), an immunoassay of a nuclear matrix protein NMP22 in urine, is more sensitive than cytology in detecting low-grade and early stage BC [5]. Cytological examination of urine using fluorescence in situ hybridization (FISH) technique, UroVysion (Vysis Inc., Downers Grove, IL), can identify BC at Ta, G1, and T1 with very high specificity but poor sensitivity [5, 6]. Another adjunct test to cytology, Immunocyt™, is a immunocytological assay particularly suited for monitoring BC recurrence [7]. The common drawback of the aforementioned urinalyses is their significantly lower specificity and prone to interference from benign conditions of the urinary tract. Other routine diagnostic methods including computed tomography (CT) and ultrasound are not particularly adept at detecting early stage BC [8]. Cystoscopy combined with tissue biopsy—the gold standard for diagnosing BC—can miss 10–40% of the cancer cases due to multiple factors [9, 10]. Meanwhile, the method is invasive, causing physical discomfort and psychological trauma for the patients. Furthermore, BC recurrence after surgery is frequent, and postoperative patients are recommended to undergo lifetime surveillance by cystoscopy, putting continuous physical stress and financial burden on individuals and their families [11]. Therefore, development of a noninvasive, sensitive, and specific diagnostic alternative is needed for early detection and postoperative surveillance of BC.

In recent years, detection of aberrantly methylated DNA in urine has emerged as a promising and noninvasive approach to aid BC diagnosis. Hypermethylation in specific genomic regions has been well characterized for bladder tumors versus normal epithelia [12–13]. A plethora of individual genes and gene panels have been tested on urine samples for their diagnostic potential for BC mostly *via* methylation-specific PCR method [14–15]. Several methylation markers or their combinations, including *ZNF154*, *POU4F2*, *EOMES*, *HOXA9*, *TWIST1*, *etc.*, demonstrated highest sensitivities in early diagnosis or recurrence surveillance of BC [14]. However, based on most up-to-date reports, current noninvasive urinalysis for detecting BC have only been able to achieve relatively high accuracy

by simultaneously testing multiple markers [16–21]. A commercial test, Bladder EpiCheck (Nucleix, Rehovot, Israel), demonstrated higher diagnostic accuracy than most other urinary tests for BC detection in multiple clinical trials, but it employed a total of 15 DNA methylation biomarkers to achieve robust performance [17]. By analyzing TCGA data and improving analytic technology currently available, we have developed an easy-to-use, convenient, and accurate detection approach for BC relying on a single methylation marker. In current investigation, we systematically evaluated the performance indexes of the exclusive marker in terms of its sensitivity, specificity, AUC values in a large number of urine samples from BC patients, healthy donors, and control individuals with benign conditions of urinary tract. In addition, we also assessed its detection capacity for other types of urothelial carcinomas and the effect of interference diseases such as prostate and kidney cancers on its performance in detecting BC. Finally, we examined its levels of mRNA and protein expression in correlation with its methylation.

Materials And Methods

BC cell lines

Seven BC cell lines, BIU-87, SW780, T24, 5637, SCaBER, TCCSUP, and J82, were used to test primer sets for candidate genes by methylight method. Two additional BC cell lines, UM-UC-3 and RT4, were also included for subsequent measurement of DNA methylation levels and quantitative RT-PCR experiments. SV-HUC-1, a bladder epithelial cell line, was used as normal control.

Sample collection

The study was approved by the Institutional Review Board of the Second Affiliated Hospital of Nanchang University ([2018]No(027)) and performed to Helsinki Declaration. Additionally, written informed consents were obtained for all participants. All samples including paraffin-embedded blocks, fresh frozen tissues, and urine specimens were collected from May, 2018 to February, 2021. Frozen tissues were stored at -80°C until use. All cancer tissue specimens were reviewed by an experienced pathologist, and all cancers were classified according to the 7th edition of American Joint Committee on Cancer (AJCC).

Microdissection and DNA extraction

BC tissue sections were examined by an experienced pathologist who circled out histologically distinct lesions with more than 70% tumor cells to direct careful microdissection. Different types of DNA were extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

Bisulfite treatment

DNA was treated with bisulfite using EZ DNA Methylation Kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. For cell line and tissue DNA samples, approximately 500 ng genomic DNA was added into the bisulfite treatment reaction and eluted out in 20 µL M-Elution Buffer. For urine DNA samples, 0-400 ng extracted DNA was bisulfite-treated and eluted out in 100 µL TE buffer.

Methylation-specific PCR (MSP)

MSP was performed to determine the methylation status of *DMRTA2* in BC cell lines. Methylated and unmethylated *DMRTA2* (m*DMRTA2* and um*DMRTA2*) primers were designed in its CpG islands. Briefly, 1 µL bisulfite-treated DNA was amplified in a total volume of 25 µL containing 2×iQ Universal SYBR@ Green Supermix (Bio-Rad, Hercules, CA) and 100 nmol/L of each primer. Amplification included hot-start at 95°C for 5 minutes, denaturing at 95°C for 30s, annealing at 60°C for 30s, extension at 72°C for 30s for 35 cycles, and a final 5 minutes extension step at 72°C [22]. Bisulfite treated human genomic DNA (Merck Milipore, Darmstadt, Germany) and CpGenome Universal Methylated DNA (Sigma-Aldrich, St. Louis, MO) were used as unmethylation and methylation controls, respectively. Water was used as no template control. All MSP products were verified by 2% agarose gel electrophoresis.

Real-time quantitative methylation-specific PCR (qMSP)

An improved methylight assay was performed for bisulfite-treated DNA[23]. The sequences of primers and TaqMan probes designed for m*DMRTA2* as well as *ACTB* were included in Supplementary Table S1. *ACTB* was included as a reference gene to assess the quality of isolated DNA. The qMSPs for urine samples and BC cancer cell lines were conducted as previously described [22]. Briefly, the total volume of each reaction was 30 µL, amplified via 95°C, 5min followed by 45 cycles of 95°C, 15s, 58°C, 30s, and 72°C, 30s and a final step at 40°C for 30s on Roche LightCycler 480 II (Roche, Basel, Switzerland) [22]. The probes used for qMSP of *CHAD*, *MEIS1*, *CMTM2*, *DRD4*, *PENK*, and *DMRTA2* are CGGTTGCGGTTAGGGTTATCGTAT, CGAGAGGGGTCGGGCGAGTTAG, CGTTGCGTTCGCGGAGTTTAGG, CGTGA GTTTGGCGGTCGTCGATTT, CGAACCAACTACGAACTCTAAACGCC, and CTATTACCGCCGCCCGCTCG, respectively.

Interpretation and data analysis of real-time qMSP of *DMRTA2*

Abs Quant/2nd Derivative Max method in Roche LightCycler 480 II (Roche, Basel, Switzerland) was used to calculate cycling threshold (CT value) by assigning a prespecified cut-off value for each amplification curve as previously reported [24]. Every batch of PCR reactions were performed with three controls, an *ACTB* internal control, m*DMRTA2* as a positive control, and um*DMRTA2* as a negative control. If a sample showed no amplification of m*DMRTA2*, no CT value would be assigned for the sample. All valid samples should satisfy the requirement of CT value of *ACTB* ≤ 35. If a sample has CT value of *ACTB* > 35, the result would be considered invalid. Target gene capture, bisulfite treatment, and PCR amplification would be rerun using a second aliquot from the sample. The CT threshold of 37 was selected to dichotomize the result of qMSP for m*DMRTA2* mainly to maximize sensitivity and minimize false positive rate. Therefore, urine samples with CT values ≤ 37 for m*DMRTA2* were called "positive" and were most likely associated with BCs. In contrast, urine samples with CT value > 37 or no CT value assigned were reported negative and were unlikely associated with bladder neoplasia. All negative samples without CT values assigned from Roche LightCycler 480 II would be arbitrarily given a value of 43 each in order to compare m*DMRTA2* levels between BCs and normal controls.

5-aza-2'-deoxycytidine treatment

To assess the impact of methylation on the expression of *DMRTA2* gene, demethylation agent 5-aza-2'-deoxycytidine (5-Aza-dC, Sigma, St. Louis, MO) was used to treat all nine bladder cancer cell lines and one normal cell lines as reported previously [25]. Treat the cells with 10 μ M 5-aza for 6 consecutive days, change the medium every day. The mRNA expression of *DMRTA2* in cell lines was quantified with RT-PCR. *GAPDH* was used as an internal reference gene to normalize cDNA input. The RT-PCR primers of *DMRTA2* and *GAPDH* are listed in Supplementary Table S4.

IHC and western blot

IHC was used to detect *DMRTA2* expression in tissues. Tissue sections of normal bladder and bladder tumor were used. The procedure was conducted as previously reported [26]. The commercially available antibodies *DMRTA2* (PA5-60237) in 1:20 dilution were used to stain sections. The intensity of the specific immunohistochemical staining reactions were evaluated using a semi-quantitative method (IRS-score), as previously described [27]. H&E staining was carried out using a Hematoxylin and Eosin Staining Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Western blot analysis was also conducted to detect *DMRTA2* protein expression in cell lines. Total protein was extracted, electrophoresed, and transferred to polyvinylidene fluoride membranes. Membranes were incubated with *DMRTA2* and *GAPDH* primary antibodies (Invitrogen, Carlsbad, CA) and then with appropriate HRP-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). Fluorescent signals were detected with ChemiDoc™ Imaging System (Bio-Rad, Hercules, CA).

RNA extraction and RT-PCR

Total RNA was isolated using TRIzol™ Reagent (Invitrogen, Carlsbad, CA) from various cell lines. First-strand cDNA was synthesized using the ReverAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). Real Time-PCR (RT-PCR) was performed with Applied Biosystems ABI 7500. *GAPDH* was used as an internal control. Primer sequences are shown in Supplementary Table S2.

Statistical analysis

Wilcoxon rank sum tests were performed to compare methylation levels between different types of sample groups. Paired t test was used in paired samples. c2 test was applied to evaluate the correlation of methylation levels with demographic and clinical characteristics, such as age, sex, tumor-node-metastasis (TNM) stage, tumor location, tumor size, and dysplasia. ROC curve was constructed to compare *DMRTA2* methylation levels between sample types. The associated AUC value was calculated for each ROC curve. All experiments examining levels of methylation, mRNA and protein expression in BC cell lines were independently performed at least three times. Data is shown as mean $\pm$ SD, with the significance between the means calculated using Two-tailed Student's t-test. A *p* value less than 0.05 was considered statistically different (* *p* < 0.05, ** *p* < 0.01). Statistical analyses were conducted with GraphPad Prism Version 5.0 (Graph Pad Software Inc. San Diego, CA).

Results

Screening for best-performing methylation biomarkers in urine specimens

In the current investigation, we initially searched literature covering a wide variety of cancers in addition to TCGA and GEO databases to select 344 candidate markers and designed 424 primer pairs for MSP of a plethora of methylation sites (Supplementary Table S1). The panel was subsequently whittled down to 176 genes and 204 primer pairs based on results of MSP assays in five selected BC cell lines, further analysis of Illumina's HM450K methylation data from 21 pairs of BC and matched normal tissue specimens, and methylation data of 20 BC cell lines available in TCGA and GEO databases, respectively (Supplementary Table S1). All 204 selected primer pairs were further tested via MSP assays in 4 BC cell lines including 5637, T24, SW780, and UM-UC-3 and 1 additional immortalized epithelial cell line SV-HUC-1. A total of 69 genes with detectable methylation in at least 3 out of 4 BC lines were further selected for quantitative assessment of their methylation level in the aforementioned cell lines *via* SYBR Green qMSP (Supplementary Table S2). Using the same quantifying method, the group was further narrowed down to a panel of 23 genes whose methylation was further quantified in 10 pairs of BC and normal tissue specimens. The top 6 genes including *CHAD*, *MEIS1*, *DMRTA2*, *PENK*, *CMTM2*, *DRD4* with highest sensitivity and specificity were selected for subsequent examination in 40 urine samples from 20 BC patients and 20 controls by TaqMan probe-based qMSP (Supplementary Table S2). The top 4 candidate genes were tested again in a total of 127 urine samples including 44 BC patients and 83 normal controls. Finally, two best markers, *DMRTA2* and *PENK*, were subjected to the final round of screening in 237 urine samples including 100 BC patients and 137 controls to further evaluate their performance (Figure 1, Supplementary Table S3).

DMRTA2 and *PENK* showed very similar assay performance in detecting BC from 237 urine samples. With specificity from a control group consisting of patients with lithangiuria, prostatoplasia, and prostatitis fixed at 95%, the two markers had sensitivities of 84.37% and 78.12%, respectively (Table 1). The AUC value for *DMRTA2* was 0.958, higher than the 0.937 for *PENK* (Table 1, Figure 2). Furthermore, the sensitivity remained unchanged when the two best markers were combined, and AUC value was 0.955, slightly lower than *DMRTA2* alone (Table 1). Hence, the subsequent assessment of the uDNA test's performance in independent training and validation sets was exclusively performed for *DMRTA2* as the most accurate marker.

Table 1
Performance characteristics of mDMRTA2 and mPENK in urine samples

Gene	Specificity (%)	Sensitivity (%)	95% CI	Ct value	AUC (95% CI)
mDMRTA2	95	84.37	68.75 to 95.83	≤35.6	0.958 (0.922 to 0.979)
mPENK	95	78.12	60.47 to 89.58	≤35.9	0.937 (0.893 to 0.963)
mDMRTA2 + mPENK	95	84.37	68.75 to 94.79	>0.564	0.955 (0.905 to 0.976)

Performance characteristics of DMRTA2 in a training set

The best methylation biomarker, mDMRTA2, was further tested in a group of 477 urine samples, consisting of 137 BCs, 202 normal controls, 31 renal carcinomas, 36 carcinomas of renal pelvis and ureter, 28 benign tumors of the bladder, 22 recurring cancers, 13 prostate cancers, and 30 postoperative patients (Supplementary Table S4). Overall, the single-target uDNA methylation test had a sensitivity of 85.4% (95% CI: 0.781–0.906), a specificity of 93.1% (95% CI: 0.884–0.960), and an AUC value of 0.937 for BC detection (Table 2, Figure 3A). Notably, the methylation assay had the highest sensitivities for tumors at stages of T1 (94.1%) and T2 (96.4%) compared with T3 (77.8%), T4 (71.4%), and unknown stage (88.9%) (Table 3). While methylation was observed in 25/36 urine samples from patients with carcinomas of renal pelvis and ureter, a good sensitivity of 69.4%, it was detected at extremely low rate of 2.3% (1/44) in those with interfering cancers of kidney and prostate (Table 2). The test was also sensitive in detecting recurring cancers in the bladder at 77.3% (17/22) and less sensitive in detecting benign bladder tumors at 10.7% (3/28) (Table 2). Overall, the assay seems to be a feasible methylation-specific testing for early-stage BCs and recurring cancers.

Table 2
The sensitivity of DMRTA2 by different types of disease in urine samples

Training set				Validation set			Aggregate set		
Type of disease	Samples	DMRTA2-positive	Sensitivity/Specificity* (95%CI)	Samples	DMRTA2-positive	Sensitivity/Specificity* (95%CI)	Samples	DMRTA2-positive	Sensitivity/Sp (95%CI)
Normal	202	14	93.1%* (88.4–96.0%)	304	24	92.1%* (88.3–94.8%)	506	38	92.5%* (89.7–
Bladder cancer	137	117	85.4% (78.1–90.6%)	79	62	78.5% (67.5–86.6%)	216	179	82.9% (77.0–
Carcinomas of renal pelvis	23	18	78.3% (55.8–91.7%)	12	11	91.7% (59.8–99.6%)	35	29	82.9% (65.7–
Carcinomas of ureter	13	7	53.8% (26.1–79.6%)	6	3	50% (13.9–86.1%)	19	10	52.6% (29.5–
Renal carcinomas	31	0	0	40	2	5% (0.8–18.2%)	71	2	2.8% (0.5–10
Prostate cancers	13	1	7.7% (0.4–37.9%)	49	5	10.2% (3.8–23.0%)	62	6	9.7% (4–20.5
Benign tumors of bladder	28	3	10.7% (2.8–29.4%)	22	7	31.8% (14.7–54.9%)	50	10	20% (10.5–34
Recurring cancers	22	17	77.3% (54.2–91.3%)	8	7	87.5% (46.7–99.3%)	30	24	80% (60.8–91

*Normal controls were not healthy donors but subjects with benign diseases of the urinary tract.

Table 3
The sensitivity of DMRTA2 to detect BC at different TNM stages in training and validation sets.

Training set				Validation set		
Stage	Bladder cancer (n = 137)	DMRTA2- positive	Sensitivity (95%CI)	Bladder cancer (n = 79)	DMRTA2- positive	Sensitivity (95%CI)
Total	137	117	85.4% (78.1–90.6%)	79	62	78.5% (67.5–86.6%)
Ta	33	22	66.7% (48.1–81.4%)	21	12	57.1% (34.4–77.4%)
T1	51	48	94.1% (82.8–98.5%)	22	18	81.8% (59.0–94.0%)
T2	28	27	96.4% (79.8–99.8%)	12	11	91.7% (59.8–99.6%)
T3	9	7	77.8% (40.2–96.1%)	2	2	100% (19.8–100%)
T4	7	5	71.4% (30.3–94.9%)	4	4	100% (39.5–100%)
NA	9	8	88.9% (50.7–99.4%)	18	15	83.3% (57.7–95.6%)

Further validation of DMRTA2 as the exclusive methylation marker

After evaluation of the methylation levels of *DMRTA2* in our training set for BC detection, we further validated its performance in an additional and independent set of 520 urine samples from which 79 were from BC patients, 22 benign growths of the bladder, 304 from control individuals, 107 other types of malignancies, as well as 8 postoperative patients (Supplementary Table S5). The *mDMRTA2* test was able to identify 62 out of 79 BC cases with a sensitivity of 78.5% (95% CI: 67.5–86.6), which is similar to that of the training set (Table 2). For 34 cases whose stage T1 or T2 tumors were confined to bladder walls, the sensitivity was drastically improved to 29 out of 34, at 85.3% (95% CI: 68.1–94.5), significantly higher than that of stage Ta at 57.1% and consistent with the data from the training set (Table 3). The sensitivity for recurring BC stood at 87.5% (7/8), which was similar to that for all BC cases, and the specificity of the *mDMRTA2* test was 92.1% (95% CI: 88.3–94.7) for 304 normal controls (Table 2). The AUC value for BC detection was 0.910, representing an excellent diagnostic accuracy with cystoscopy combined with tissue biopsies (Figure 3B).

When the two independent sample sets (training and validation) were combined, the performance indexes for BC detection in a total of 216 urine samples from BC patients and 506 urine samples from controls were 82.9% (95% CI: 77.0–87.5) for sensitivity and 92.5% (95% CI: 89.7–94.6) for specificity, resulting in an AUC value of 0.926 (Table 2, Figure 3C). In further stratified analysis according to TNM stage, the methylation test retains highest sensitivity for stage T1 and T2 tumors, which is similar to the trend observed in both training and validation sets (Table 3, Table 4, and Supplementary Table S6). The test performs better in older men (≥ 60 y) and for high grade neoplasia than low grade ones ($p < 0.001$), but no significant association was observed between level of *mDMRTA2* and gender ($p > 0.05$) (Table 4, Supplementary Table S6). Notably, the uDNA test of *mDMRTA2* was also sensitive in detecting carcinomas of renal pelvis (29/35, 82.9%), and to a lesser extent, ureter (10/19, 52.6%), but performed poorly in detecting prostate cancer (6/62, 9.7%), clear cell carcinoma of kidney (2/71, 2.8%), and benign tumors of bladder (10/50, 20.0%) (Table 2).

Table 4
The sensitivity and specificity of *DMRTA2* by different clinical characteristics in aggregate set.

Clinical characteristics	<i>DMRTA2</i> -positive	Bladder cancer	Sensitivity(95%CI)	<i>DMRTA2</i> -negative	Non-cancer disease	Specificity (95%CI)
Total	179	216	82.9% (77.0%-87.5%)	468	506	92.5% (89.7–94.6%)
Age						
≤60	29	44	65.9% (50.0–79.1%)	218	233	93.6% (89.4–96.2%)
60~69	63	74	85.1% (74.5–92.0%)	156	169	92.3% (86.9–95.7%)
70~79	62	68	91.2% (81.1–96.3%)	76	85	89.4% (80.4–94.7%)
≥80	25	30	83.3% (64.5–93.7%)	18	19	94.7% (71.9–99.7%)
Sex						
Male	152	179	84.9% (78.6–89.7%)	281	306	91.8% (88.0–94.5%)
Female	30	37	81.1% (64.3–91.4%)	187	200	93.5% (88.9–96.3%)
Grade						
Low	60	84	71.4% (60.4–80.5%)	0	0	NA
High	106	112	94.6% (88.2–97.8%)	0	0	NA
NA	16	20	80% (55.7–93.4%)	0	0	NA
Stage						
Ta	34	54	63% (48.7–75.4%)	0	0	NA
T1	66	73	90.4% (80.7–95.7%)	0	0	NA
T2	38	40	95% (81.8–99.1%)	0	0	NA
T3	9	11	81.8% (47.8–96.8%)	0	0	NA
T4	9	11	81.8% (47.8–96.8%)	0	0	NA
NA	23	27	85.2% (65.4–95.1%)	0	0	NA

DNA methylation status and gene expression of DMRTA2 in BC cell lines and tissues

Additionally, we detected level of *mDMRTA2* and its mRNA expression in 9 BC cell lines and 1 normal bladder epithelial cell line by MSP, qMSP and RT-qPCR methods. Compared with SV-HUC-1, the normal bladder epithelial cell line, *DMRTA2* gene was hypermethylated in 8 out of 9 BC cell lines, which is consistent with the results of MSP and qMSP (Figure 4A–B). Notably, BIU-87 was an outlier, which may be attributable to potential cross-contamination of this line [28]. The hypermethylation could be reversed by 5'-aza treatment, drastically reducing methylation level and stimulated mRNA expression in certain cell lines including SV-HUC-1, T24, and RT4 (Supplementary Table S7 and Figure 4C). The mRNA levels of *DMRTA2* were low in some bladder cancer cell lines, such as T24, J82, UM-UC-3 and RT4, however, were drastically higher in 5637, SCaBER, TCCSUP and SW780 (Figure 4D). The protein levels in all 10 of the above-mentioned cell lines were not significantly increased after demethylation even in the aforementioned 4 cell lines with significant mRNA up-regulation, implying

a more complex pattern of gene expression at mRNA and protein synthesis levels for *DMRTA2* (Figure 4E–H). Further IHC staining of a total of 11 pairs of BC and adjacent normal tissue specimens as well as 8 standalone BC carcinoma sections showed mainly weak staining of *DMRTA2* in cancerous cells compared with no recognizable staining in normal tissues (Figure 4I–L, Supplementary Table S8), which is consistent with the results from western analysis of BC cell lines (Figure 4F). The unclear mechanism *via* which the hypermethylated region of *DMRTA2* gene regulates its own expression needs to be further addressed.

Discussion

Numerous genes are aberrantly methylated in primary BC as revealed by Illumina HM450K methylation data in TCGA database. *De novo* screening for methylated genes based on deposited data rather than testing tumor suppressors in signaling pathways related to growth, differentiation, and epithelia-to-mesenchymal transition (EMT), etc maybe a more reliable and convincing strategy to identify optimal diagnostic and prognostic biomarkers. Multigene panels with superior sensitivity and specificity for the detection of BC have been identified by such a systematic approach [29–30]. In our study, a large number of candidate genes that have differential methylation levels between cancer and normal tissues have been selected and sequentially screened in BC cell lines, tissue specimens, and urine sediments. After multiple rounds of screening, we focused on a single marker, *mDMRTA2*, for further modeling of the uDNA test in retrospective and independent training and validation cohorts. The single-target testing exhibited an overall sensitivity of 82.9% and 92.5% specificity in a final aggregate cohort, again demonstrating better performance characteristics than most of the other combinations previously reported. The functional role of *DMRTA2* in the development and progression of BC remains unclear. The targeted methylation site is located in the 5'-untranslated region of *DMRTA2* mRNA. Demethylation of the hypermethylated region did not result in increased mRNA expression in every cancer cell line we tested and protein level was relatively unperturbed in most of the cells as a result of 5'-aza treatment. Therefore, we speculate that *mDMRTA2* maybe a collateral by-product as the malignancy progresses instead of playing a causative role in cancer development.

The goal of our study is to find a single methylation biomarker for urine detection of BC with comparable performance characteristics to BC diagnostic tests currently available on the market. However, none of the biomarkers with published performance in up-to-date literature meets this requirement. Hence, we searched TCGA database for candidate methylation sites that could distinguish between BC tumor and adjacent normal tissues and subsequently tested them in clinical tissue and urine sample specimens in hope to find such a novel biomarker. The *de novo* approach did generate two top-performers in *DMRTA2* and *PENK*, who showed similar sensitivity and specificity in detecting BC. Since *PENK* had been analyzed in multigene panels as biomarkers for BC detection in some previous studies [31–32], and we did not find increased sensitivity when two genes were combined in a test of a group of 237 urine samples, we evaluated the performance of *mDMRTA2* as the sole marker in a large hospital-based cohort. Overall, the single-target uDNA methylation test achieved 82.9% of sensitivity and 92.5% specificity. The single biomarker is particularly useful in detecting early BC such as T1 and T2 stage tumors, with increased sensitivity up to 92.0%, a much desirable feature for any *in vitro* diagnostic test. The *mDMRTA2* test also had an admirable detection rate for recurring BC at 80.0%, comparable to some of the tests currently available on the market. Put together, the simple, non-invasive, and convenient single-marker test offered a much more affordable and attractive option than multigene panel test such as EpiChek with comparable performance for aiding early diagnosis and monitoring recurrence of the malignant disease.

Most of the reported methylation markers tested in relatively large cohorts had poor specificity [17, 33–36]. Benign diseases of the bladder and upper urinary tract often caused elevated methylation in certain markers and hence a large number of false positive calls. In addition, the biomarker is required to distinguish between BC and other types of cancers in the urinary tract such as those of the prostate and kidney that may also excrete tumor cells into the urine. To reduce false positives and increase specificity, we included a large proportion of urine samples from patients with lithangiuria, prostatoplasia, and prostatitis, benign diseases routinely seen in outpatient visits. Furthermore, benign tumors from the bladder, certain urothelial carcinomas including those from ureter and pelvis, as well as cancers of the prostate and kidney were also included in the testing. Under such conditions, the *mDMRTA2* test achieved 92.5% specificity at a cut-off Ct value of 37 among a total of 506 controls, higher than most of those reported for studies of multigene panels in large-scale cohorts. It could also detect other urothelial cancers including those of ureter and renal pelvis, implying that the test would be sensitive to carcinomas originated in the epithelia lining the urinary tract and in close contact with liquid urine. However, its sensitivity was much reduced for benign tumors of the bladder, kidney and prostate cancers with 165 out of 183 cases tested negative, a 90.2% specificity, another desirable characteristic of the uDNA methylation test to aid the diagnosis of BC.

Multi-tiered cross-validation is a powerful and widely used approach to demonstrate effectiveness of a diagnostic test. In the current study, we were able to divide the urine specimens into a pair of training and validation sets as a result of the large scale of the cohort. The cross-validation between two independent sets allowed us to critically evaluate the robust sensitivity and specificity of the *mDMRTA2* test. Adequately powered, the training set, validation set, and the aggregate set demonstrated similar overall sensitivity and specificity for the detection of primary BC. More importantly, in both training and validation sets, we observed a significantly higher sensitivity in detecting T1 and T2 stage BC. Since early-stage tumors are surgically removable and curable, this suggests that the *mDMRTA2* test would be potentially suitable for mass screening purpose to effectively reduce mortality rate of BC. In addition, another similar observation across all three sets was the high detection rate for recurring BC. If the methylation threshold was increased to CT = 38 for recurrence detection, the sensitivity in training, validation, and aggregate sets would be 89.8%, 84.8%, and 88.0%, respectively, and the specificity would be slightly reduced to 89.1%, 83.2%, and 85.6% in corresponding sets, still satisfactorily high for the test. In conclusion, our extensive data suggests that *mDMRTA2* maybe a promising biomarker suitable for detecting both primary and recurring bladder tumors.

In conclusion, the non-invasive, simple, and user-friendly uDNA test of *mDMRTA2* had robust sensitivity, superior specificity, and admirable accuracy. However, certain limitations are associated with the test. First, the *mDMRTA2* test was not as sensitive in detecting Ta stage tumor as it was in detecting T1–T4 stage tumors. Additional markers may be required to increase its sensitivity in detecting non-invasive tumor confined to the epithelia of the bladder. Secondly, even though the *mDMRTA2* test had a decent detection rate for recurring cancers, the number of cases tested was still limited compared to a couple of previous studies [17]. More urine specimens need to be collected to have a large-scale cohort to give a more accurate and meaningful estimate of the test in its ability to detect recurring BC. Thirdly, the test kit was designed based on the genetic make-up of Chinese population. Alignment of the sequences of different ethnic

backgrounds, including Asians, Caucasians, African Americans, European Descent, and Hispanics, from the amplified region indicated that no single nucleotide polymorphism (SNP) greater than 0.1% exist, suggesting that the qMSP should generally work for the DNA isolated from urine sediments once it had been methylated irrespective of ethnic backgrounds. It would be interesting to see how the uDNA test perform in other ethnic groups for early detection of BC. Fourth, the proportion of cases of various types of urothelial malignancies does not necessarily reflect their prevalence in the Chinese population because we wanted to evaluate all the cases that had been collected and were available to us. When the number in cancers of bladder, ureter, and renal pelvis was weighted against their actual incidence (carcinomas of ureter and renal pelvis account for 10% of the total), we estimated that the sensitivity for urothelial carcinomas would improve from 82.1–86.9% [37]. Fifth, the current investigation is a feasibility study performed at a standard laboratory bench but not in a real clinical setting. Hence, the clinical utility of the uDNA test is needed to be validated in a multicenter clinical trial before *mDMRTA2* can be used as a reliable and marketable biomarker for both diagnosis and recurrence surveillance of BC.

Declarations

Ethics approval and consent to participate

This study was performed in accordance to the principles of the Helsinki Declaration and approved by the Institutional Review Board of the Second Affiliated Hospital of Nanchang University. All participants have acknowledged and signed and informed consent.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

R Zhao, L Huang, Y Gong, Y Zhu, Q Wang, F Li, L Liu, L He, Z Tang, C Liao, Y Qi, X Wang are employees of Creative Biosciences (Guangzhou) Co., Ltd. H Zou is the shareholder of Creative Biosciences (Guangzhou) Co., Ltd. Other authors declared no competing interests.

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Author contributions

T Zeng and H Zou performed conceptual design, obtained funding, and supervised the study. L Deng, H Chao, H Deng, Z Yu, R Zhao, L Huang, Y Gong, Y Zhu, Q Wang, F Li, L Liu, Y Qi acquired data and performed analyses. X Wang drafted and revised the manuscript. Z Tang, C Liao, X Wang, T Zeng, and H Zou performed critical review of the manuscript. H Chao, L Deng, H Deng, R Zhao, L Huang, L He, and Y Qi provided administrative, technical, and material support.

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Figures

Figure 1

Diagram of work-flow to screen for top-two urine biomarkers, m*DMRTA2* and m*PENK*, for BC detection.

Figure 2

ROC curves and associated AUCs of the diagnostic prediction model using DNA methylation analysis of *DMRTA2* and *PENK*.

Figure 3

ROC curves and associated AUCs of the diagnostic prediction model using DNA methylation analysis of *DMRTA2* in the training(A), validation(B), and aggregate (C) sets.

Figure 4

(A) *DMRTA2* methylation in BC cell lines and normal cell lines detected by MSP. MSP products in lanes U and M indicate the presence of unmethylated and methylated *DMRTA2*, respectively. (B) Reexpression of *DMRTA2* in BC cell lines and normal cell lines by qMSP. Data shown as mean±s.d. of three independent experiments (n = 3). (C) Reexpression of *DMRTA2* in BC cell lines and normal cell lines by demethylation. Data shown as mean±s.d. of three independent experiments (n = 3). (D) Expression of *DMRTA2* in BC cell lines and normal cell lines by qPCR. Data shown as mean±s.d. of three independent experiments (n = 3). (E) and (F), Expression of *DMRTA2* was detected in BC cell lines with western blot analysis. Data shown as mean±s.d. of three independent experiments (n = 3). (G) and (H), Expression of *DMRTA2* was detected in BC cell lines by demethylation with western blot analysis. Data shown as mean±s.d. of three independent experiments (n = 3). (I) and (J), Expression of *DMRTA2* was examined in adjacent normal and BC tissues by IHC. (K) and (L), Morphology of tumor and adjacent normal cells shown by H&E staining. Scale bar is 100 μm.

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