BMC Cancer

RESEARCH Open Access

A urine-based liquid biopsy for detection of upper tract urothelial carcinoma: a self-matched study

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Abstract

Background To establish the pathological diagnosis of UTUC before treatment is profitable. At present, the conventional pathological diagnostic methods have certain problems. Besides, the urine-based DNA methylation test have been already utilized to detect bladder cancer.

Objective To evaluate the sensitivity and specificity of DNA methylation plus 17 genes mutation test and compare the combined test with cytology.

Materials and methods We included 45 patients from April 2019 to May 2022, all of whom underwent radical nephroureterectomy (RNU), nephrectomy, diagnostic ureteroscopy or tissue biopsy. Before surgery, the urine samples were collected for DNA methylation plus 17 genes mutation test and cytology. The test performance was calculated, and comparative ROC curves were drawn.

Results The median age of the patients was 67 years. The Kappa value of the DNA methylation plus 17 genes mutation test and tissue pathology was 0.59 ($p<0.001$). The sensitivity/specificity/PPV/NPV of DNA methylation plus 17 genes mutation test was 86/80/94/62% compared with 29/100/100/29% for cytology. The AUC of DNA methylation plus 17 genes mutation test was 0.829 ($p<0.001$).The mutated gene proportion of UTUC patients was 51.43% for TERT and 25.71% for TP53.

Conclusion The test performance of DNA methylation plus 17 genes mutation test was satisfactory, which may replace cytology in the future. Further multicenter studies with larger samples are needed to confirm the clinical value of this promising method.

Novelty & impact statements We evaluated the diagnostic efficacy of a urine-based liquid biopsy for the detection of UTUC and compared the combined test with cytology. We found satisfactory results and concluded that the test could partly replace cytology. Further studies are needed.

Keywords Cytology, Diagnosis, DNA methylation, Upper tract urothelial carcinoma, Urine sample

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Introduction

Urothelial carcinoma (UC) is one of the most common malignant tumors worldwide, including bladder cancer (BCa) and upper tract urothelial carcinoma (UTUC). UTUC is a relatively rare type of UC, accounting for 5–10% of cases, with an incidence of approximately 2/100,000 in Western countries [1]. Hematuria is the most common symptom of urothelial carcinoma [2, 3]. Ultrasound is a common imaging modality used to detect bladder cancer, and the typical finding is intraluminal masses in the bladder. We can also perform computed tomography (CT) urography to detect BCa, with the characteristic of filling defects. While the diagnosis of BCa ultimately depends on the utilization of cystoscopy examination to search for the intraluminal mass and implement the tissue biopsy. The tumor could also be resected with the help of cystoscopy. In contrast to BCa, UTUC is more challenging to diagnose. Although the sensitivity and specificity of CT are satisfactory, there are still some patients who escape and miss the diagnosis. Moreover, CT cannot provide a pathological diagnosis, which is crucial to guide the treatment. At present, more and more medical centers choose kidney sparing surgery (KSS) for selected UTUC patients on account of the acceptable oncological outcomes and overall survival [4]. For some patients with locally advanced disease, neoadjuvant chemotherapy can significantly improve survival [5]. Consequently, it is important to obtain the pathological diagnosis before treatment. We could perform cytology examination, which has limited sensitivity. With the help of the diagnostic ureteroscopy (URS), we could directly visualize the abnormal lesion. In addition, we could acquire the tissue for biopsy. Nevertheless, URS may increase the risk of intraluminal recurrence [6] and may lead to underestimation of the final pathological stage [7].

Epigenetic modifications are heritable changes in the chromatin structure and gene expression that are not caused by alterations in the DNA sequence, of which DNA methylation has been most widely studied as a contributor to tumor progression and metastasis [8]. Urine, a type of body fluid, may contain cancer-specific DNA methylation markers that can be used for the detection of BCa [8]. Nowadays, several DNA methylation-based diagnostic kits are currently available for the detection of BCa. UTUC originates from the urothelium of the renal pelvis and ureter, which is histologically and molecularly similar to BCa [9]. However, there is a paucity of research on the use of DNA methylation markers for the detection of UTUC. To evaluate the sensitivity and specificity of DNA methylation test to detect UTUC and compare the diagnostic efficacy with exfoliative cytology, we conduct the self-matched study. Meanwhile, we include mutations of 17 genes in the urine test to acquire more information about UTUC.

Materials and methods

Patients and sample

The study was approved by the Ethics Committee of Henan Provincial People's Hospital (No.73 in 2019). We included patients admitted to Henan Provincial People's Hospital from April 2019 to May 2022 who met the following inclusion criteria: (a) hematuria (gross or microscopic); (b) renal pelvic or ureteral occupying lesion according to B-ultrasonography or CT scan. Both criteria were required. Urine samples were collected to perform the DNA methylation plus 17 genes mutation test and exfoliative cytology examination before radical nephroureterectomy (RNU), nephrectomy, diagnostic ureteroscopy or tissue biopsy. The exclusive criteria were: (a) urine samples without high quality or sufficient quantity; (b) patients who did not receive RNU, nephrectomy, diagnostic ureteroscopy or tissue biopsy; (c) concomitant bladder cancer. According to the sample evaluation, a minimum of 36 patients were required for the study. A total of 45 patients were eventually included.

DNA methylation plus gene mutation test

80 ml first-void urine was collected and processed within 12 h. The samples were centrifuged, the ctDNA of which was extracted and subjected to DNA methylation plus 17 genes mutation testing.

The CPG Island on ONECUT2 gene was detected by using EZ DNA Methylation-Lightning™ Kit (Zymo Research Corporation, Irvine, California, USA). Quantitative real-time PCR (qPCR) was performed on 20 nanograms (ng) of bisulfite-converted DNA. FAM and VIC signals were used to quantify the methylated and unmethylated components, respectively. The methylation score was calculated by subtracting the CT values of the two signals.

The Genetron-Health 17 genes panel was devised to cover most of the driver genes in UTUC. The regions were selected based on previous studies of frequently mutated genes in UC [10]. The specific genes included in the 17 gene panels are as follows: AKT1, ASXL2, CREBBP, ERBB2, ERBB3, ERCC2, FBXW7, FGFR3, HRAS, KDM6A, KRAS, PIK3CA, RHOA, SF3B1, TP53, TERT, and U2AF1. The sequencing libraries amplified using multiplex PCR methods were sequenced on the Ion Proton system (Thermo Fisher Scientific).

Exfoliative cytology examination

To improve the sensitivity of cytology, urine samples were collected for three consecutive days. After centrifugation treatment on the samples, the tumor cell was detected by microscope to diagnose UTUC. The samples

were classified into six categories according to The Paris System (TPS) [11]: nondiagnostic/unsatisfactory, negative for high-grade urothelial carcinoma (NHGUC), atypical urothelial cells, suspicious for high-grade urothelial carcinoma (SHGUC), high-grade urothelial carcinoma (HGUC), low-grade urothelial neoplasm (LGUN). Only specimens evaluated as SHGUC or HGUC could be reported as "positive", while others were reported as "negative" [12].

Collection of gross pathological specimens

Following the collection of urine samples for DNA methylation plus 17 genes mutation test, as well as exfoliative cytology examination, we conducted radical nephroureterectomy (RNU), nephrectomy, diagnostic ureteroscopy, or tissue biopsy to obtain gross specimens. After appropriate fixation and tissue processing, we established the pathological diagnosis, which served as the gold standard and reference.

Statistical analyses

Demographic and clinical data were collected from the electronic medical records (EMR) database. Numerical variables were described as median and interquartile range, and categorical variables were described as frequencies and percentages. The McNemar's test was performed to compare the differences in rates between the following tests: DNA methylation plus 17 genes mutation test, DNA methylation test, exfoliative cytology examination, and tissue pathology. The Kappa test was applied to assess the concordance between the urine tests mentioned above (DNA methylation plus 17 genes mutation test, DNA methylation test, exfoliative cytology examination) and tissue pathology. Sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), negative likelihood ratio (NLR), and positive likelihood ratio (PLR) with 95% confidence intervals (CIs) were calculated for the urine tests. Receiver Operating Characteristic (ROC) Curves were drawn to compare the diagnostic performance of the test (Delong's test). All p-values were two-tailed, and $p<0.05$ was considered statistically significant. All analyses were performed with SPSS26.0 software.

Table 1 Demographic and clinical characteristics of patients

Characteristics	Result($n=45$)
Gender, n(%)	
Female	16 (35.6%)
Male	29 (64.4%)
Age	
Median	67.0
P25, P75	57.5,72.5
Lateral, n(%)	
Right	24 (53.3%)
Left	21 (46.7%)
Location, n(%)	
Kidney/Renal Pelvis	28 (62.2%)
Ureter	17 (37.8%)
Preoperative Hydronephrosis, n (%)	22 (48.9%)
Tissue Pathology, n (%)	
Urothelial Carcinoma (UC)	35 (77.8%)
Renal Cell Carcinoma (RCC)	5 (11.1%)
Renal Leiomyosarcoma	1 (2.2%)
Renal Angiomyolipoma (RAML)	1 (2.2%)
Mucosal Inflammation	3 (6.7%)
Grade of Urothelial Carcinoma, n (%)	35 (100%)
High Grade (HG)	30 (85.7%)
Low Grade (LG)	2 (5.7%)
Carcinoma in Situ (CIS)	1 (2.9%)
Undefined	2 (5.7%)
Smoking history, n (%)	17(37.8%)
History of Bladder Cancer (BCa), n (%)	3 (6.7%)

Results

Clinical characteristics

A total of 45 patients were enrolled in the study, of whom 16 (35.6%) were female and 29 (64.4%) were male. The median age of the patients was 67 years (range:28–101 years). Preoperative hydronephrosis was observed on B-ultrasonography or CT scan in 22 (48.9%) patients. Of the patients, 35 (77.8%) had urothelial carcinoma (UC), 5 (11.1%) had renal cell carcinoma (RCC), 1 (2.2%) had renal leiomyosarcoma, 1 (2.2%) had renal angiomyolipoma (RAML), 3 (6.7%) had mucosal inflammation. Table 1 lists all the relevant clinical parameters.

Discrepancy and consistency in urine tests and tissue Pathology

Figure 1 lists the positive and negative results of these urine tests and tissue pathology. Figure 1 also shows the discrepancy in the rates between these tests, which were calculated by McNemar's test. We found that there was no statistical difference between the DNA methylation plus 17 genes mutation test and tissue pathology, while there were clear differences in the rates between the other tests. Supplemental table shows the consistency between these tests, which was calculated with Kappa test. The Kappa value for the DNA methylation plus 17 gene mutation test and tissue pathology was 0.59 ($p<0.001$), indicating moderate consistency. However, the Kappa value for cytology and tissue pathology was 0.15, ($p=0.055$), indicating poor consistency.

Test performance and ROC curve

The test performance of these urine tests is shown in Table 2. The DNA methylation plus 17 genes mutation test yielded a sensitivity/specificity/PPV/NPV of 86/80/94/62% vs. 29/100/100/29% for cytology. Table 2 also shows the PLR and NLR of the urine tests. The PLR/

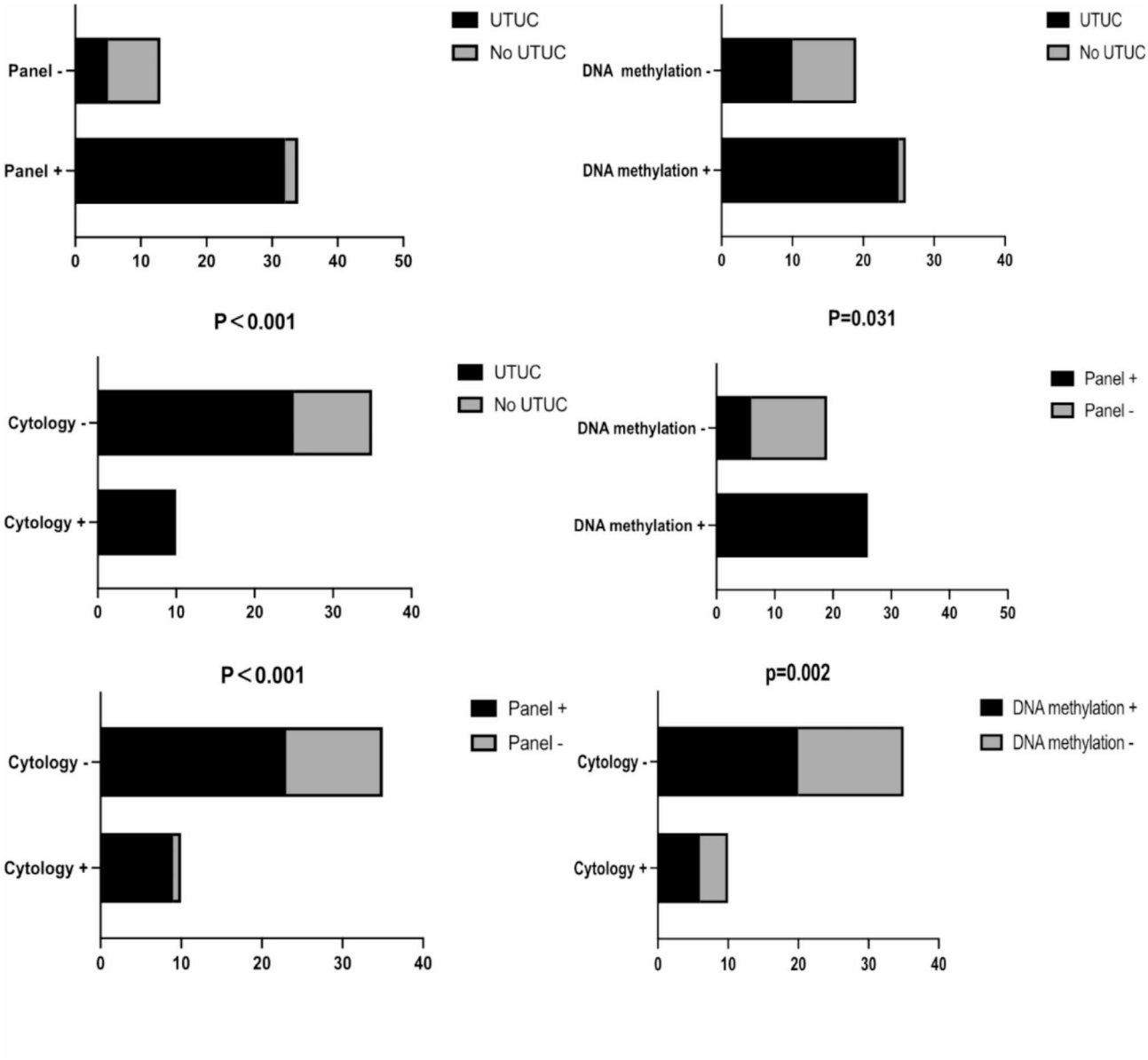

Fig. 1 *P* values denote the statistical significance of the difference between these tests, which was calculated using McNemar's test. Panel means DNA methylation plus 17 genes mutation test

Table 2 Test performance

Test Performance							
	Sensitivity(95%CI)	Specificity(95%CI)	PPV (95%CI)	NPV (95%CI)	PLR (95%CI)	NLR (95%CI)	AUC(95%CI)
DNA methylation	0.71(0.53–0.85)	0.90(0.54–0.99)	0.96(0.78-1.00)	0.47(0.25–0.70)	7.14(1.10–46.40)	0.32 (0.18–0.55)	0.807(0.683–0.931)
Panel※	0.86(0.69–0.95)	0.80(0.44–0.96)	0.94(0.78–0.99)	0.62(0.32–0.85)	4.29(1.23–14.91)	0.18(0.08–0.42)	0.829(0.685–0.972)
Cytology	0.29(0.15–0.47)	1(0.66-1)	1(0.66-1)	0.29(0.15–0.47)	Infinity	0.71 (0.58–0.88)	0.643(0.567–0.719)

Panel※ means DNA methylation plus 17 genes mutation test

PLR: positive likelihood ratio NLR: negative likelihood ratio

PPV: positive predictive value NPV: negative predictive value

NLR of the DNA methylation plus 17 genes mutation test was 4.29/0.18, while the PLR/NLR of the DNA methylation test was 7.14/0.32. Figure 2; Table 2 show the AUC of these urine tests, which are as follows: 0.829 (p<0.001) for DNA methylation plus 17 genes mutation test, 0.807 (p<0.001) for DNA methylation test, and 0.643 (p<0.001) for cytology. Figure 2; Table 3 show the area difference under the ROC curves. The difference between the DNA

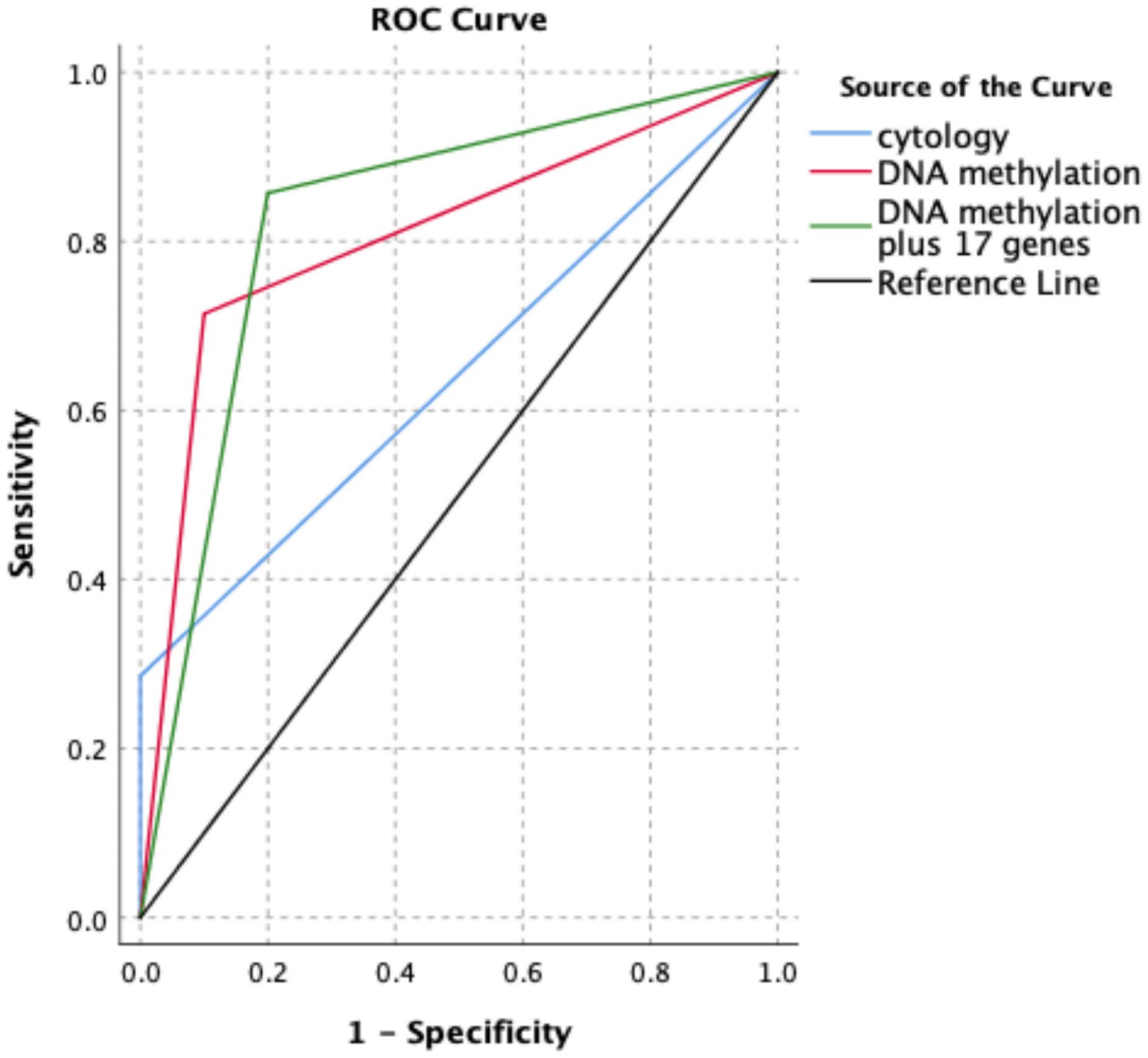

Fig. 2 The ROC curves of the urine tests

Table 3 Paired-sample area difference under the ROC curves

Test Result Pairs	Z	AUC Difference(95%CI)	*P*
DNA methylation - Panel※	-0.367-	-0.021(-0.136-0.093)	0.713
Panel※-Cytology	2.275	0.186(0.026–0.346)	0.023
DNA methylation -Cytology	2.124	0.164(0.013–0.316)	0.034

Panel※ means DNA methylation plus 17 genes mutation test

methylation plus 17 genes mutation test and cytology was 0.186 (p=0.023). Supplemental figure shows the overall model quality of these urine tests, which was drawn by the ROC module of SPSS26 software. A good model has a value above 0.5, while a value less than 0.5 indicates the model is no better than random prediction. The overall model quality of these urine tests was 0.69 for DNA methylation plus 17 genes mutation test, 0.68 for DNA methylation test, and 0.57 for cytology.

Gene mutation result

Figure 3 shows the gene mutation results of UTUC patients in the form of oncoprint. We found that although we performed 17 genes mutation test in urine samples, there were only 9 genes mutation detected in the study.

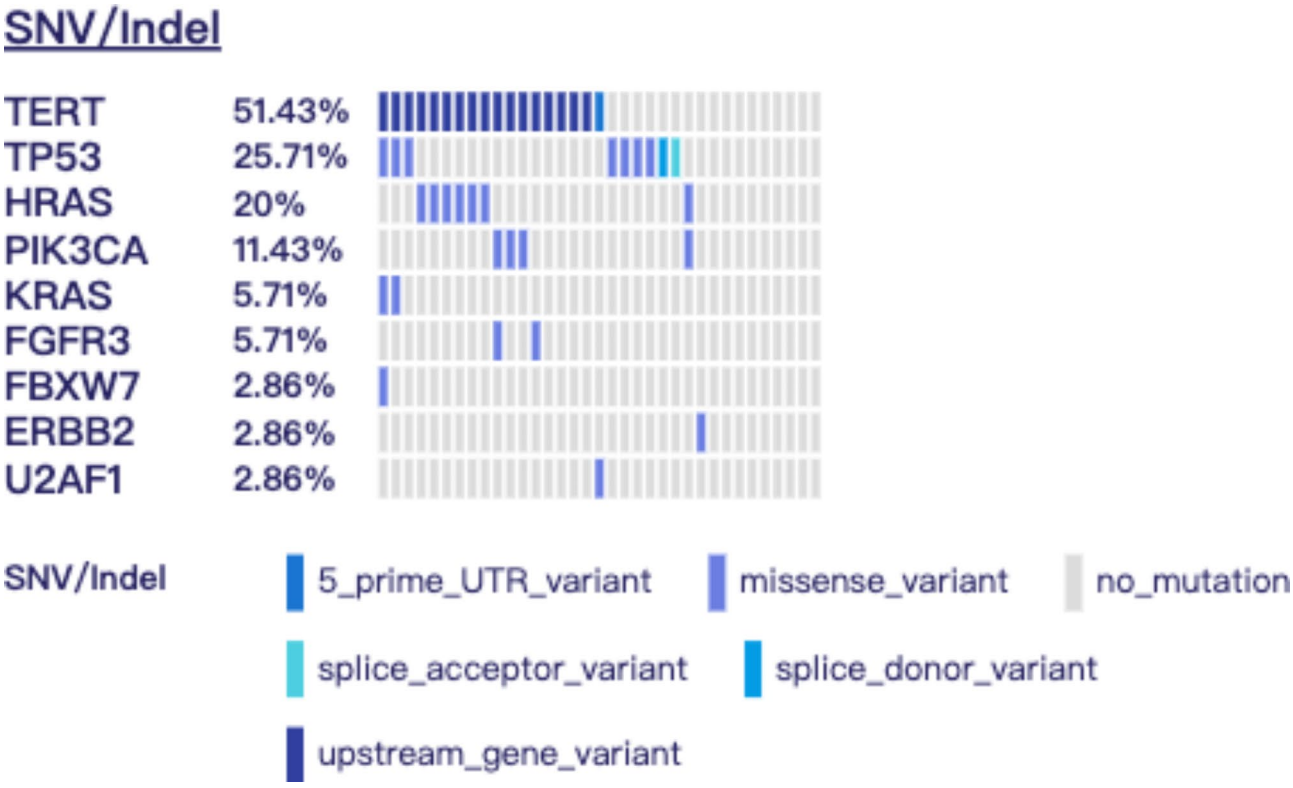

Fig. 3 Oncoprint of all variations in urine samples. Abbreviation: SNV, singe nucleotide variants; Indel, insertion-deletion; UTR, untranslated regions

We found the most frequent gene mutations were TERT (51.43%), TP53 (25.71%), HRAS (20%), PIK3CA (11.43%), KRAS (5.71%), which was generally consistent with Xu's report [10].

Discussion

Urothelial carcinoma of the upper urinary tract (UTUC) is a rare but aggressive cancer with a high mortality rate. Radical nephroureterectomy (RNU) is the standard of care for UTUC, but treatment is changing rapidly due to emerging new data. Hence, it is recommended to obtain pathological results before treatment. Although the tissue pathology could be obtained by diagnostic ureteroscopy, the surgical procedure could be challenging for some patients, and requires anesthetic support, which restricts the application of the operation. Additionally, as mentioned above, URS increases the risk of intravesical recurrence but does not affect overall survival. Nevertheless, Fredrik et al. [13] found invasive diagnostic modalities (IDM) was associated with an increased risk of urothelial cancer death (HR1.56, 95%CI 1.12–2.18), compared with no IDM after a median follow-up of 1.3 years. Therefore, non-invasive liquid biopsy is receiving increasing attention. There is a lack of self-matched studies of urine tests for the diagnosis of UTUC and comparison of these urine tests, which prompted us to conduct this study.

Exfoliative cytology is a diagnostic test that detects abnormal cells in urine samples, which is a valuable non-invasive tool. The void urine specimen is convenient to obtain, and cytology has satisfactory specificity but lower than desired sensitivity. The false negative rate of cytology for the diagnosis of UTUC ranges from 50–89% [14]. A meta-analysis [15] reported a sensitivity of 53.1% and a specificity of 90% for cytology. There are two reasons accounting for this phenomenon. The variable morphology of atypical cells increases the difficulty of diagnosing UTUC. The absence of specific diagnostic criteria contributes to inter-and intra-observer inconsistency. The identification of atypical cells is critical for the accurate diagnosis of UTUC. To address these challenges, TPS was introduced in 2016. TPS has improved the accuracy of cytology in diagnosing UTUC, but some misdiagnosis still occurs. X.Zheng et al. [12] found that when interpretating cytology cases with suspicious or positive for HGUC as positive, the performance of TPS in predicting high grade urothelial carcinoma on histology had values of 78.6% sensitivity, 86% specificity, 80.5% positive predictive value and 84.5% negative predictive value. The use of TPS in the evaluation of cytology specimens was specific and sensitive in identifying patients with HGUC by histology. We adopted the conclusion of the report, and in our report, the sensitivity/specificity of cytology was of 29/100%.

DNA methylation, an epigenetically important marker targeting human gene regulation, contributes to tumorigenesis and has been observed in a variety of cancers, including bladder cancer [16]. The hypermethylation of CpG islands is absent in normal tissues, which is highly cancer-type specific and could be applied to clinical practice. There are similar genomic and clinical characteristics between UTUC and UC, and many reports had corroborated the DNA methylation expression of UTUC. Studies showed that aberrant methylation was detected in 88.9% of UTUC patients [17]and could be found in various genes [10, 17, 18]. Francesco et al. [19] retrospectively compared the urine cytology with DNA methylation test in high grade UTUC. The sensitivity of DNA methylation test is much higher than that of cytology (97.4% vs. 59.0%). In our report, the sensitivity/specificity of DNA methylation test is 71%/90%, which is similar to another report [20]. Therefore, DNA methylation testing of urine samples could be used to diagnose UTUC, potentially replacing urine cytology and reducing the need for diagnostic ureteroscopy.

Circulating tumor DNA (ct DNA) shed into the body fluid has emerged as a promising biomarker for cancer detection. Some reports have focused on the utilization of next-generation sequencing (NGS) to detect DNA mutations in urine samples [21, 22]. Telomerase activity is detectable in many human cancers, while telomerase reverse transcriptase (TERT) gene is transcriptionally repressed in most normal human cells, which leads to telomerase silence [23]. Originally identified in melanomas, TERT promoter mutations have been shown to be common in certain other tumors, including UC [23, 24]. In view of this, there are potentially promising applications of TERT in UC, involving diagnosis, prognosis, and therapy. Hayashi Y detected TERT promoter mutations in 46.4% of UTUC patients and FGFR3 in 16.1% of patients [21]. In our study, we found that 51.43% of UTUC patients showed TERT mutation, and 25.71% of patients showed TP53 mutation, which contributed to the diagnosis of UTUC. As for prognostic evaluation, P Sivaramakrishna Rachakonda et al. [24] found that TERT promoter mutation could influence the survival and tumor recurrence of patients with bladder cancer. The patients with mutations showed poor survival (HR 2.19,95% CI 1.02–4.70) and higher disease recurrence (HR 1.85, 95% CI 1.11–3.08). Furthermore, the mutation may be used to guide treatment decisions. Immune checkpoint inhibitors (ICI) are increasingly used in patients with metastatic and locally advanced urothelial cancer. Ivan de Kouchkovsky [25]et al. found that the presence of a TERT promoter mutation was an independent predictor of improved OS (HR 0.32, $p=0.037$) in a cohort of advanced UC patients treated with an ICI.

We use the DNA methylation plus 17 genes mutation test to diagnose UTUC, which had higher accuracy than urine cytology alone. The Kappa value was 0.59, indicating that the combined test had a moderate consistency with tissue pathology. The sensitivity and specificity of the combined test is 0.86 and 0.80, respectively. The AUC difference between the combined test and cytology was 0.186 ($p=0.023$), indicating a statistically significant improvement in diagnostic accuracy. Although the AUC of the combined test (0.829) was higher than that of DNA methylation alone (0.807), the difference was not statistically significant ($p=0.713$). Nevertheless, the combined DNA methylation plus 17 genes mutation test offers valuable prognostic information and influences clinical decision-making in ways that DNA methylation alone cannot. Notably, a false positive result was observed in a patient who exhibited inflammation on pathological examination. This patient is under regular follow-up. In another study [26] evaluating the diagnostic accuracy and recurrence prediction of a urinary assay for mutation and methylation in patients with non-muscle invasive bladder cancer, it was found that the assay detected 48% of recurrences during follow-up that were missed by standard-of-care clinical methods. They believed that a false positive test was thought to have predictive value for future tumor recurrence. Further studies are needed to validate the predictive power of the test.

Our report has several limitations. First, the sample size was small, which may influence the outcomes. Second, all the patients were selected in one center, which may result in selection bias. However, we devised the paired sample study to control bias, and the control group (cytology) is widely used in clinic. What's more, we collected the urine samples before surgery, although we retrospectively conducted the study. There were some prospective reports [27] about DNA methylation test to diagnose UTUC, the control group in which patients underwent cytology test was lacking. It was reasonable to assume the reliability of the conclusion.

Conclusion

In conclusion, we compared the test performance of urine cytology and urine DNA methylation plus 17 genes mutation analysis. The test performance of the combined urine test is satisfactory, and we could perform the test to detect UTUC which may replace cytology in the future. And we discuss the potential value of the urine test to evaluate the prognosis and guide the treatment. Further multicenter studies with larger samples are needed to confirm the clinical value of the promising method.

Abbreviations

BCa	Bladder cancer
UC	Urothelial carcinoma
UTUC	Upper tract urothelial carcinoma

KSS	Kidney sparing surgery
RNU	Radical nephroureterectomy
UC	Urothelial carcinoma
RCC	Renal cell carcinoma
RAML	Renal Angiomyolipoma
CIS	Carcinoma in situ
HG	High grade
LG	Low grade
SNV	Singe nucleotide variants
Indel	Insertion-deletion

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-024-12913-3.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
Not applicable.

Author contributions
WW performed the data analysis and wrote the paper; PF and DW performed experiment and collected data; ZZ and JL performed data curation; LW performed the formal analysis; XD performed the validation; ZX and DD performed the supervision.All authors read and approved the final manuscript.

Funding
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Data availability
The data presented in the study are deposited in the NCBI Sequence Read Archive (SRA) repository, accession number PRJNA973017. Here is the reviewer link for BioProject PRJNA973017: https://dataview.ncbi.nlm.nih.gov/object/PRJNA973017?reviewer=77r924lfhap6dsqd6pvst29n5p.

Declarations

Ethics approval and consent to participate
Our study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Institutional Review Board of Henan Provincial People's Hospital. All patients signed an informed consent approved by the institutional Review Board.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 2 May 2023 / Accepted: 6 September 2024
Published online: 27 September 2024

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