Urine Cellular DNA Point Mutation and Methylation as Potential Biomarkers for the Detection of Urothelial Carcinoma

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Abstract

Background

Previously, our team identified a seven-gene mutation panel in urine sediment to discriminate UBC from benign urological diseases. In the present study, we aimed to validate the panel in an expanded and close to natural population cohort of hematuria. Also, we tried to optimize the panel by incorporating methylation biomarkers. We performed external validation to investigate the robustness and stability of the novel panel.

Methods

Patients with urothelial carcinomas and controls were prospectively recruited in clinical trial ChiCTR2000029980. The mutation panel was validated in the expanded cohort(n=333) from Hunan multicenter. Several UBC-specific methylation biomarkers were identified by comprehensive analyses of a series of TCGA, GEO and an independent cohorts, and examined in the expanded cohort. Random Forest algorithm was used to construct an optimal panel. External validation of the optimal panel was carried out in Beijing single center cohort(n=89). NGS technique was used to analyze the DNA point mutations and MS-PCR for methylation.

Results

The AUC, sensitivity and specificity of the mutation panel in expanded cohort were 0.81, 0.67 and 0.90, respectively. After screening, only cg16966315, cg17945976 and cg24720571 were left for further analysis. The optimal panel consisted of cg24720571 and 8 point mutations, including TERT.chr5_1295228 G_A (TERT 228), FGFR3.chr4_1803568 C_G (FGFR3 568), TERT.chr5_1295250 G_A (TERT 250), FGFR3.chr4_1806099 A_G (FGFR3 099), PIK3CA.chr3_178936091 G_A (PIK3CA 091), PIK3CA.chr3_178952085 A_G (PIK3CA 085), PIK3CA.chr3_178936082 G_A (PIK3CA 082), HRAS.chr11_533874 T_C (HRAS 874). The AUC, sensitivity and specificity of the optimal panel in training group were 0.89, 0.84 and 0.79, respectively, and in test group were 0.95, 0.91 and 0.95, respectively. In the external validation, the AUC, sensitivity and specificity were 0.98, 0.93 and 0.93, respectively.

Conclusions

The optimal panel was obviously superior to previous mutation panel and showed a highly specific and robust performance. The optimal panel may be used as a replaceable approach for early detection of UC.

Trial registration

This research was registered in Chinese Clinical Trial Registry(ChiCTR2000029980).

Background

Urothelial bladder carcinoma (UBC) is the most common malignancy of the urinary tract, with approximately an estimated 550,000 new cases and 200,000 deaths per year worldwide¹. The majority of newly diagnosed cases are non-muscle invasive bladder cancer (NMIBC). Nearly 70% of these patients will experience recurrence, and 10–30% progress to muscle-invasive bladder cancer (MIBC) inevitably^{2,3}.

Typical diagnosis and surveillance of UBC involves the use of cystoscopy, cytology, FISH and computed tomography(CT)^{4,5}. Cystoscopy is regarded as the gold standard for the detection of UBC, which exhibits relatively high clinical sensitivity but low patient acceptance owing to its invasive nature⁶. In contrast, urine cytology and FISH are noninvasive and specific, but lacks sensitivity, especially in low-grade tumors. CT is a good tool but still has the potential to cause radiation damage. These facts, together with the high cost and follow-up biopsy procedures, have led to many attempts to develop alternative noninvasive methods to detect UBC.

Given the special anatomical characteristic, noninvasive strategies to identify UBC mainly include urine-based genetic, epigenetic and protein assays. Currently, FDA has approved several tests for UBC diagnosis and surveillance, including NMP22 and UroVysion, with sensitivity ranging 30–100% and specificity of 55–98%^{7,8}. However, due to assay performance inconsistencies, technical expertise and high cost, integration of such assays into routine clinical practice has not yet occurred. In addition, none of these assays have been validated for detection of upper tract urothelial carcinoma (UTUC), accounting for 5% of urothelial carcinoma (UC).

Previously, we identified a seven-gene mutation panel in urine sediment to discriminate UBC from benign urological diseases in hematuria patients⁹. Seven genes include 33 different types of point mutation. The model was

' $X = -0.6685 + 416.2208 * TERT + 16.3065 * FGFR3 + 21.6375 * TP53 + 1030.8943 * HRAS + 269.6423 * KRAS + (-6.6597) * PIK3CA + 1365.2377 * ERBB2$ '. The value x was substituted into the sigmoid function $f(x) = 1 / (1 + e^{-x})$ to get a fit value, $f(x)$. A malignancy is considered when $f(x) > 0.4491049$, while the benign state is indicated otherwise. In the present study, we aim to validate the panel in an expanded and close to natural population cohort. Besides, we try to optimize the panel by incorporating methylation biomarkers. We also performed external validation to investigate the utility and stability of the novel panel.

Materials And Methods

Patients' characteristics and ethics statement

Participants were prospectively recruited (Chinese Clinical Trial Registry, ChiCTR2000029980) as approved by the Ethics Committee of Xiangya Hospital(XYH), The Second Xiangya Hospital(SXYH), Hunan Provincial People's Hospital(HPPH), Hunan Cancer Hospital(HCH) and Beijing Hospital(BJH) after written informed consents were obtained. Studies were conducted in accordance with the ethical principles in the Declaration of Helsinki.

Study design

In the prospective, multicenter expanded cohort, a total of 385 individuals with macroscopic or microscopic hematuria were initially enrolled from XYH(n=143), SXYH(n=87), HPPH(n=82) and HCH(n=73) (Hunan, China) between August 2019 and January 2020, of whom 333 were eligible for inclusion(Figure 1). Previous seven-gene panel was validated in this cohort to explore the possibility of discriminating UC from other urological diseases.

In the panel optimization stage, we identified several UBC-specific methylation biomarkers by comprehensive analyses of a series of TCGA, GEO and an independent cohorts from Hunan multicenter. Candidate methylation biomarkers were examined in 333 participants. We established important predictor features using Boruta feature selection algorithm based on the analysis of DNA mutations and methylation, and reconstructed a novel panel using Random Forest algorithm.

In the external validation stage, 99 participants with hematuria were recruited from BJH (Beijing, China) from May 2020 to August 2020, of whom 89 were eligible to evaluate the stability and reproducibility of the optimal panel.

Sample collection and DNA Isolation

For all participants, each urine sample (at least 30ml) was collected from the first miction in the morning. The urine samples were centrifuged at 1,600 g for 10 mins at 4 °C, the supernatant was discarded and the pellet was carefully collected into new vacant 2 mL tubes. Same procedure was performed again at 12,000 g for 10 mins at 25 °C. Then 200 µl of 1× PBS was added to each tube to resuspend the cells. DNA isolation was performed using Tissue Genomic DNA Extraction Kit (cat DP304, Tiangen Biotechnology, China) according to the manufacture's instruction. The modified DNA was stored at -80°C for further processing.

Library Preparation and Sequencing (Mutation)

50ng genomic DNA from each sample was fragmented and tailing by TIANSeq Fragment/Repair/Tailing Module (Tiangen, Cat: NG301), and then ligased to forward oligos with UMI. After two rounds purification with 1.2× AMPure XP beads (Beckman), the ligased-product was PCR-amplified using specific backward primers and universal primers. After another round of purification using 1× AMPure beads, the final library pool was quantified by ABI 7500 fast Real-Time PCR system (Applied Biosystems) and sequenced on a NextSeq 500 system (Illumina, USA) to obtain paired-end 150 bp reads.

All reads were quality trimmed and sequences of adapters were removed. The index sequences and UMI were appended to the read identifier for the next analysis. Sequence reads were mapped to the human genome (hg19) using the Burrows-Wheeler aligner (BWA-MEM). The reads with the same UID were cluster to get the final consensus sequence. Variant calling was performed using the Genome Analysis Toolkit (GATK v3.8) and emanated variants were annotated using ANNOVAR.

Methylation specific-PCR(MS-PCR)

Sodium bisulfite conversion and purification of 100ng genomic DNA were performed using EZ DNA MethylationLightning™ Kit (Zymo Research Corporation, Irvine, California, USA), according to the manufacturer's protocol. GAPDH was set as the internal reference. Ct values represented the relative methylation quantity of CpG markers and the internal reference gene (GAPDH), which was measured by FAM and VIC signals separately. The delta ct (Δ ct) values were calculated as methylation score.

Statistical analysis

The model performance was evaluated by the area under the curve (AUC) statistics. The sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) of the panel and cytology in detecting UC were obtained by comparison to pathology and presented as univariate values in bar graph. The percent of cases in each variant subgroup using different clinical characteristics were also presented as univariate values in bar graph. The Δ ct distribution were presented as boxplots with median and the interquartile range (IQR) marks. Random forest analysis was applied to highlight the most powerful mutation and methylation biomarker combination for distinguishing UC from controls. Chi-square test was used for categorical variables, t-test was used for continuous variables and Mann–Whitney U test for non-normally distributed variable data. All statistical analyses and data visualizations were carried out in R software (R version 3.4.3) and GraphPad Prism 8 (version 8.0.2). Adobe Illustrator (CC 2017) was used for image processing. All hypothesis tests were two-sided with a p value < 0.05 considered to be statistically significant.

Results

Baseline characteristics and flow chart

The baseline characteristics of cohort were shown in table 1. The exact flow chart is summarized in figure 1.

Validation of the seven-gene mutation panel

The seven-gene mutation panel achieved a sensitivity of 0.67 and a specificity of 0.90 in the expanded cohort, yielding an AUC of 0.81. For the subgroup analysis, the panel resulted in sensitivity of 0.71 and 0.56 for UBC and UTUC, and specificity of 0.90 and 0.91 for benign control and malignant control, respectively. For further analysis, the sensitivity of the panel were 0.73 and 0.64 for NMIBC and MIBC, respectively(Figure 2).

Optimization of panel by incorporating methylation biomarkers

Discovery of DNA methylation biomarkers

We analyzed DNA methylation data of 21 pairs of UBC and adjacent tissue, 412 UBC tissues and 656 normal blood samples, 412 UBC tissues and 533 Kidney renal clear cell carcinoma (KIRC) tissues, and 412 UBC tissues and 498 Prostate carcinoma (PRAC) tissues from TCGA or GEO database (Supplementary Figure 1 A~D). Through differential methylation analysis and a series of statistical filters to reduce the number of markers, we finally identified 9 most powerful markers, including cg13974773, cg16966315, cg17945976, cg21472506, cg23229261, cg24720571, cg25510609, cg25947619, cg27404023 (Table S1).

Verification of putative methylation biomarkers

We then recruited an independent set of 71 UC patients (38 UBC and 33 UTUC) and 70 controls (31 benign controls and 39 malignant controls) to verify the methylation status of 9 biomarkers using MS-PCR (Table 2&Figure 3&Supplementart Figure 2&3). Based on the comprehensive analysis of AUC, cutoff value, sensitivity and specificity, cg16966315, cg17945976 and cg24720571 were selected for optimization of the panel. 4.50, 9.19 and 9.73 were set as the cut-off value for cg16966315, cg17945976 and cg24720571, respectively. The 3 biomarkers were then examined in Hunan multicenter cohort using MS-PCR. Any Δ ct equal to or below the determined cut-off value is considered as being positive(+), while negative(-) is indicated otherwise.

Construction of the novel panel

The frequency was set at > 0.5% as abnormal cut-off value, and 33 point mutations status were converted to '+' or '-'. Boruta feature selection algorithm was used to rank the 33 point mutation biomarkers and 3 methylation biomarkers by their importance, and highlight the most powerful biomarker combinations for distinguishing UC from controls. Cg24720571, TERT 228(G_A), FGFR3 568(C_T), TERT 250(G_A), FGFR3 099(A_G), PIK3CA 091(G_A), PIK3CA 085(A_G), PIK3CA 082 (G_A) and HRAS 874(T_C) were determined(Figure 4). The 9 biomarkers were used to construct a novel panel using random forest algorithm. Then 333 participants were randomly divided to training and test sets with ratio 7:3. The novel panel achieved an excellent performance which exhibited a high AUC of 0.95 in test group. For the total cohort, the model gave a diagnosis with sensitivity of 0.86 and specificity of 0.84 (Figure 5 A~E).

External validation of the optimal panel

To further test the robustness of the novel panel, an additional set of 89 participants was obtained from BJH to carry out external validation. The optimal model achieved excellent discrimination, with an overall AUC of 0.98 in the external cohort). Resulting in a sensitivity of 0.93, and a specificity of 0.93(Figure 5 J&H).

Integrated analysis of two cohorts

Combining data from the two cohorts, a total of 422 participants consisting of 236 UC participants and 186 controls, the novel panel showed an overall sensitivity of 0.88 and specificity of 0.86 (Figure 6 A&B).

In subgroup analysis, the sensitivity of the optimal panel reached 0.91 for UBC and 0.74 for UTUC, with a significant difference. In addition, the specificity of the panel was 0.89 for benign controls and 0.81 for malignant controls. To better understand how each biomarker contributes to the panel, we calculated the percent of cases in each variant subgroup. A significantly lower frequency of TERT 250(G_A) and cg24720571 in NMIBC versus MIBC&UTUC was observed(Figure 6 C&D).

From further analysis of the sensitivity and specificity using various clinical variables, no obvious difference was observed in gender and smoking status. Also, no significant difference of gene mutation frequency or methylation degree was found with gender and smoking history except TERT 228(Figure S4).

Application of the novel panel for early detection of UC

We then assessed the model for differentiating different grade and stage tumors. The model achieved a high sensitivity of 0.50(1/2), 0.90 and 0.89 in the PUNLMP, low grade and less than T2 tumors(Figure 7 A&D). For detailed analysis, the model showed an overall sensitivity of 0.75, 0.76 and 0.91 in carcinoma in situ(CIS), Ta and T1 tumors, with no significant difference(Figure 7 G). Besides, FGFR3 568(C_G) and PIK3CA 082 (G_A) occurred with higher frequency in low grade tumors, while TERT 250(G_A) and cg24720571 exhibited with lower frequency in low grade tumors, both with significant differences. FGFR3 568(C_G) occurred with higher frequency in less than T2 tumors, while cg24720571 exhibited with lower frequency in less than T2 tumors, both with significant differences(Figure 7 B&C&E&F).

Novel panel and cytology comparison

In the present study, urine cytology was available for only 210 UC patients and 119 controls (Table S2). Compared with cytology, novel panel showed significantly improved sensitivity (0.88 vs. 0.39, $P < 0.001$) and comparable specificity (0.86 vs. 0.91, $P = 0.05$). In tumor subgroup analysis, novel panel achieved a great improvement in sensitivity over cytology in NMIBC(0.91 vs. 0.41, $P < 0.001$), MIBC(0.90 vs. 0.44, $P < 0.001$) and UTUC(0.74 vs. 0.23, $P < 0.001$) detection, respectively. Further analysis showed that novel panel outperformed cytology in low (0.90 vs. 0.21, $P < 0.001$) and high grade(0.89 vs. 0.48, $P < 0.001$) tumors, and $< T2$ (0.89 vs. 0.39, $P < 0.001$) and $\geq T2$ (0.91 vs. 0.42, $P < 0.001$) tumors(Figure 8).

Discussion

Compared with previous mutation panel, the focus was shifted from genes to point biomarkers. And the number of point mutation reduced from 33 to 8, which significantly improved the detection efficiency. Also, the novel panel was more precise and specific. In addition, the cohort is expanded and closed to natural hematuria population. This may suggest that the novel panel could be applied for hematuria population screening in the future.

Genetic mutations are often the subject of investigation and play basic roles in the malignant transformation of urothelial cells¹⁰. However, not all UC harbor mutations in the most commonly altered

oncogenes. Mutation panel only produced sensitivities of 0.70 and 0.67 in previous validation group and present expanded cohort, respectively. The abnormal DNA methylation status is also an important mark in the development of UC, and could be the first detectable neoplastic changes associated with tumorigenesis^{5,11}. The novel panel, consisting of 8 point mutations and 1 methylation biomarker, showed a significant improvement in sensitivity. Epigenetic and genetic biomarkers therefore can complement and reinforce each other, resulting a more stable diagnostic performance^{12–14}.

Cytology is highly specific, and in expert hands nearly always indicates the presence of urothelial malignancy when positive. It is noninvasive, inexpensive, simple, and valuable for high-grade and flat lesions^{15,16}. However, cytology is not particularly sensitive, especially for low grade and early stage tumors. In the present study, cytology only achieved a sensitivity of 0.21 and 0.39 in low grade and < T2 tumors, respectively. The novel panel significantly outperformed cytology in nearly all aspects and exhibited comparable specificity. Besides, novel panel correctly identified 53 cases of low-grade UC while none were detected by cytology. This highlighted that the novel panel might replace the cytology for early detection of UC.

UTUC is an uncommon disease, accounting for only 5%~10% of UC¹⁷. Currently, most UC biomarkers focus on UBC, and UTUC associated biomarkers are relatively rare. Non-invasive and sensitive methods to screen at-risk individuals for UTUC are clearly desirable. Xu et al. constructed a diagnostic panel for UTUC detection, resulting a sensitivity of 0.94 and a specificity of 0.93¹². Zeng et al. developed a panel based on the analysis of copy number variant, producing a sensitivity of 1.0 and 0.64 in the training and validation cohort, respectively¹⁸. In our study, the sensitivity of the optimal panel reached 0.74 for UTUC, demonstrating the potential for UTUC screening purposes. However, the value of novel panel in detecting UTUC still needs to be validated given the small number of patients evaluated.

Neuritin 1 (NRN1), also named cpg15-1, is a GPI-anchored protein mainly involved in neuronal plasticity¹⁹. Neuritin 1 is associated with mental illness, such as schizophrenia, bipolar disorder and depression^{20,21}. Recently, aberrant methylation of *nm1* gene promoter region is associated with tumor development, such as gastric cancer and melanoma^{22,23}. In our study, the CpG site cg24720571 located on the promoter region of *nm1* gene, was first discovered as a useful biomarker to detect UC in urine. However, the biological function and methylated mechanism of NRN1 remain largely unknown, and further clarification is needed.

Conclusions

In summary, we developed an optimized model consisting of 1 methylation and 8 point mutation biomarkers for UC detection, which showed a highly specific and robust performance. It may be used as a replaceable approach for early detection of UC, resulting in less extensive examinations in patients at low risk.

Abbreviations

UBC: urothelial bladder carcinoma, NMIBC: non-muscle invasive bladder cancer, MIBC: muscle invasive bladder cancer, CT: computed tomography, UTUC: upper tract urothelial carcinoma, UC: urothelial carcinoma, XYH: Xiangya Hospital, SXYH: second Xiangya Hospital, HPPH: Hunan Provincial People's Hospital, HCH: Hunan Cancer Hospital, BJH: Beijing Hospital, AUC: area under the curve, PPV: positive predictive value, NPV: negative predictive value, IQR: interquartile range, KIRC: Kidney renal clear cell carcinoma, PRAC: Prostate carcinoma, CIS: carcinoma in situ,

Declarations

Ethics approval and consent to participate: Participants were prospectively recruited (Chinese Clinical Trial Registry, ChiCTR2000029980) as approved by the Ethics Committee of Xiangya Hospital, The Second Xiangya Hospital, Hunan Provincial People's Hospital, Hunan Cancer Hospital and Beijing Hospital. Studies were conducted in accordance with the ethical principles in the Declaration of Helsinki.

Consent for publication: Written informed consent for publication of all participants in this study has been obtained.

Availability of data and materials: The datasets used and/or analysed are available from the corresponding author on reasonable request.

Competing interests: None.

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Authors' contributions: JZ and LW contribute to conception and design. RX, QL, ZX, YZ, CZ and LL contribute to acquisition of data. JZ, JL and PL contribute to analysis of data. JZ contributes to drafting the article. JZ, RX, QL, WT, MC, ZW, GX and LW contribute to revising it critically for important intellectual content. All authors read and approved the final manuscript.

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References

1. Bray F, Ferlay J, Soerjomataram I, Siegel R, Torre L, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians 2018,68:394-424.
2. Avogbe PH, Manel A, Vian E, et al. Urinary TERT promoter mutations as non-invasive biomarkers for the comprehensive detection of urothelial cancer. EBioMedicine 2019,44:431-8.

3. Springer S, Chen C, Rodriguez Pena M, et al. Non-invasive detection of urothelial cancer through the analysis of driver gene mutations and aneuploidy. *eLife* 2018,7.
4. Cheng THT, Jiang P, Teoh JYC, et al. Noninvasive Detection of Bladder Cancer by Shallow-Depth Genome-Wide Bisulfite Sequencing of Urinary Cell-Free DNA for Methylation and Copy Number Profiling. *Clin Chem* 2019,65:927-36.
5. Chen X, Zhang J, Ruan W, et al. Urine DNA methylation assay enables early detection and recurrence monitoring for bladder cancer. *J Clin Invest* 2020.
6. Ge G, Peng D, Guan B, et al. Urothelial Carcinoma Detection Based on Copy Number Profiles of Urinary Cell-Free DNA by Shallow Whole-Genome Sequencing. *Clin Chem* 2020,66:188-98.
7. Breen V, Kasabov N, Kamat A, et al. A holistic comparative analysis of diagnostic tests for urothelial carcinoma: a study of Cxbladder Detect, UroVysion® FISH, NMP22® and cytology based on imputation of multiple datasets. *BMC medical research methodology* 2015,15:45.
8. Mowatt G, Zhu S, Kilonzo M, et al. Systematic review of the clinical effectiveness and cost-effectiveness of photodynamic diagnosis and urine biomarkers (FISH, ImmunoCyt, NMP22) and cytology for the detection and follow-up of bladder cancer. *Health technology assessment (Winchester, England)* 2010,14:1-331, iii-iv.
9. Ou Z, Li K, Yang T, et al. Detection of bladder cancer using urinary cell-free DNA and cellular DNA. *Clin Transl Med* 2020,9:4.
10. van Kessel KE, Van Neste L, Lurkin I, Zwarthoff EC, Van Criekinge W. Evaluation of an Epigenetic Profile for the Detection of Bladder Cancer in Patients with Hematuria. *The Journal of urology* 2016,195:601-7.
11. Chen X, Xie R, Gu P, et al. Long Noncoding RNA LBCS Inhibits Self-Renewal and Chemoresistance of Bladder Cancer Stem Cells through Epigenetic Silencing of SOX2. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2019,25:1389-403.
12. Xu Y, Ma X, Ai X, et al. A Urine-Based Liquid Biopsy Method for Detection of Upper Tract Urinary Carcinoma. *Frontiers in oncology* 2020,10:597486.
13. van Kessel KE, Beukers W, Lurkin I, et al. Validation of a DNA Methylation-Mutation Urine Assay to Select Patients with Hematuria for Cystoscopy. *The Journal of urology* 2017,197:590-5.
14. Wu Y, Jiang G, Zhang N, et al. HOXA9, PCDH17, POU4F2, and ONECUT2 as a Urinary Biomarker Combination for the Detection of Bladder Cancer in Chinese Patients with Hematuria. *European urology focus* 2020,6:284-91.
15. Dimashkieh H, Wolff D, Smith T, Houser P, Nietert P, Yang J. Evaluation of urovysion and cytology for bladder cancer detection: a study of 1835 paired urine samples with clinical and histologic correlation. *Cancer cytopathology* 2013,121:591-7.
16. Black P, Brown G, Dinney C. Molecular markers of urothelial cancer and their use in the monitoring of superficial urothelial cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2006,24:5528-35.

17. Sfakianos J, Gul Z, Shariat S, et al. Genetic Differences Between Bladder and Upper Urinary Tract Carcinoma: Implications for Therapy. *European urology oncology* 2020.

18. Zeng S, Ying Y, Xing N, et al. Noninvasive Detection of Urothelial Carcinoma by Cost-effective Low-coverage Whole-genome Sequencing from Urine-Exfoliated Cell DNA. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2020.

19. Zito A, Cartelli D, Cappelletti G, et al. Neuritin 1 promotes neuronal migration. *Brain structure & function* 2014,219:105-18.

20. Fatjó-Vilas M, Prats C, Pomarol-Clotet E, et al. Involvement of NRN1 gene in schizophrenia-spectrum and bipolar disorders and its impact on age at onset and cognitive functioning. *The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry* 2016,17:129-39.

21. Prats C, Arias B, Ortet G, et al. Neurotrophins role in depressive symptoms and executive function performance: Association analysis of NRN1 gene and its interaction with BDNF gene in a non-clinical sample. *Journal of affective disorders* 2017,211:92-8.

22. Bosserhoff A, Schneider N, Ellmann L, Heinzerling L, Kuphal S. The neurotrophin Neuritin1 (cpg15) is involved in melanoma migration, attachment independent growth, and vascular mimicry. *Oncotarget* 2017,8:11117-31.

23. Wisnieski F, Santos L, Calcagno D, et al. The impact of DNA demethylation on the upregulation of the NRN1 and TNFAIP3 genes associated with advanced gastric cancer. *Journal of molecular medicine (Berlin, Germany)* 2020,98:707-17.

Tables

Table 1. Clinical and pathological data of the cohort

	Expanded Validation Cohort		External Validation Cohort		Total		<i>P</i>
	UC	Control	UC	Control	UC	Control	
Sample Size	191	142	45	44	236	186	0.28
#Age, Years, Median (IQR)	63 (54-70)	58 (43-69)	64(56.5-70)	57 (47-62)	63 (54-70)	58(43-67)	0.945 ^a /0.537 ^b
Gender							
Male	149	92	34	35	183	127	0.071
Female	42	50	11	9	53	59	0.470
Smoking History							
Smoker	113	59	25	4	138	63	0.030*
Non-smokers	78	83	20	40	98	123	0.049*
Stage							
<pT2	123	/	31	/	154	/	0.035*
≥pT2	66	/	11	/	77	/	
Tx	2	/	3	/	5	/	
Grade							
PUNLMP	2	/	0	/	2	/	0.019*
Low grade	67	/	11	/	78	/	
High grade	121	/	31	/	152	/	
Gx	1	/	3	/	4	/	
Subtype							
UBC	157	/	44		201		0.005*
UTUC	34	/	1	/	35	/	
Tumor Organ							
Pelvis	12	/	1	/	13	/	0.063*
Ureter	12	/	0	/	12	/	
Bladder	157	/	44	/	201	/	
Multiple locations	10	/	0	/	10	/	

Tumor Type							
Primary	180	/	31	/	211	/	<0.0001*
Recurrent	11	/	14	/	25	/	
Control Group	/	142	/	44	/	186	0.002*
Benign control	/	84	/	37	/	121	0.245
Urolithiasis	/	47	/	28	/	75	
Infection	/	21	/	4	/	25	
BPH	/	3	/	1	/	4	
Renal cyst	/	5	/	2	/	7	
Renal hamartoma	/	7	/	1	/	8	
Ureteral polyp	/	1	/	1	/	2	
Maligant control		58		7		65	0.260
Renal cell carcinoma	/	35	/	4	/	39	
Prostate cancer	/	15	/	1	/	16	
OPTBC	/	4	/	2	/	6	
OPTRC	/	4	/	0	/	4	

PUNLMP: papillary urothelial neoplasm of low malignant potential; UBC: urothelial bladder carcinoma; UTUC: upper tract urothelial carcinoma; BPH: benign prostate hyperplasia; OPTBC: other pathological types of bladder cancer; OBTRC: other pathological types of renal cancer; a: UC group comparison; b: control group comparison; #: Non-normally distributed variable data was analyzed with the Mann–Whitney U test; Categorical variable data was analyzed with the Chi-square test; *: statistically significant difference;

Table 2. Diagnostic performance of the candidate biomarkers

Biomarker	UC	Control	AUC	95% CI	Cutoff	Sensitivity	Specificity
cg13974773	UBC	Control	0.98	0.95~1.00	3.57	0.94	0.92
cg13974773 ^a	UTUC	Control	0.93	0.88~0.98	5.31	0.86	0.91
cg13974773 ^b	UTUC	Control	/	/	3.64	1.00	0.21
cg16966315	UBC	Control	0.94	0.90~0.98	4.50	0.97	0.74
cg16966315 ^a	UTUC	Control	0.94	0.89~0.98	4.13	0.99	0.70
cg16966315 ^b	UTUC	Control	/	/	4.58	0.96	0.70
cg23229261	UBC	Control	0.73	0.63~0.82	10.37	0.76	0.61
cg23229261 ^a	UTUC	Control	0.76	0.66~0.85	9.63	0.86	0.55
cg23229261 ^b	UTUC	Control	/	/	10.47	0.74	0.55
cg21472506	UBC	Control	0.69	0.58~0.79	10.72	0.59	0.71
cg21472506 ^a	UTUC	Control	0.75	0.66~0.85	9.06	0.66	0.70
cg21472506 ^b	UTUC	Control	/	/	10.74	0.57	0.79
cg17945976	UBC	Control	0.95	0.91~0.99	9.19	0.93	0.87
cg17945976 ^a	UTUC	Control	0.91	0.85~0.97	9.15	0.87	0.85
cg17945976 ^b	UTUC	Control	/	/	9.29	0.84	0.85
cg24720571	UBC	Control	0.94	0.88~0.99	9.73	0.94	0.89
cg24720571 ^a	UTUC	Control	0.92	0.87~0.97	10.15	0.87	0.88
cg24720571 ^b	UTUC	Control	/	/	9.80	0.90	0.84
cg25510609	UBC	Control	0.79	0.70~0.88	9.12	0.71	0.82
cg25510609 ^a	UTUC	Control	0.74	0.64~0.84	10.03	0.60	0.82
cg25510609 ^b	UTUC	Control	/	/	9.18	0.71	0..61
cg25947619	UBC	Control	0.78	0.68~0.87	7.67	0.83	0.66
cg25947619 ^a	UTUC	Control	0.68	0.57~0.78	10.31	0.49	0.85
cg25947619 ^b	UTUC	Control	/	/	7.69	0.83	0.30
cg27404023	UBC	Control	0.66	0.55~0.76	7.37	0.63	0.68

Biomarker	UC	Control	AUC	95% CI	Cutoff	Sensitivity	Specificity
cg27404023 ^a	UTUC	Control	0.60	0.48~0.73	5.41	0.20	0.94
cg27404023 ^b	UTUC	Control	/	/	7.74	0.61	0.52

UBC: urothelial bladder carcinoma; UTUC: upper tract urothelial carcinoma; a: the optimal cutoff value for UTUC vs control; b: the cutoff value in UTUC group was adjusted to the closest to UBC group;

Figures

Figure 1

Flow chart of the study design

Figure 2

ROC curve and the AUC of the mutation panel in the expanded cohort (A). The sensitivity and specificity of the mutation panel in overall cohort and subgroup cohort (B~D).

Figure 3

The ΔC_t distribution of candidate methylation biomarkers in the independent cohort (n=141). Statistical analysis was assessed using independent sample t-test. The data were presented as median with the interquartile range. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; NS represents No significance

Figure 4

The 9 important prediction features screened by Boruta feature selection algorithm. TERT 228: TERT.chr5_1295228 G_A; FGFR3 568: FGFR3.chr4_1803568 C_G; TERT 250: TERT.chr5_1295250 G_A; FGFR3 099: FGFR3.chr4_1806099 A_G; PIK3CA 091: PIK3CA.chr3_178936091 G_A; PIK3CA 085: PIK3CA.chr3_178952085 A_G; PIK3CA 082: PIK3CA.chr3_178936082 G_A; HRAS 874: HRAS.chr11_533874 T_C;

Figure 5

ROC curves and the associated AUCs of the optimal panel using urine DNA mutation and methylation analysis in the training (A), test (B) and validation(F) cohorts. The sensitivity and specificity of the optimal panel in training (C), test (D), and validation cohort (J).

Figure 6

The sensitivity and specificity of the optimal panel in Hunan-Beijing mixed cohort and subgroup cohort (A~B). Comparison of mutations and methylation across different disease subgroups profiled in the study (C~D).

Figure 7

The sensitivity of the optimal panel in different stage and grade tumors (A&D&G). Comparison of mutations and methylation across different stage and grade tumors (B&C&E&F).

Figure 8

Comparison of optimal panel and cytology across different subgroups.

Supplementary Files

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- [TableS1.docx](#)
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