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An accurate diagnostic approach for urothelial carcinomas based on novel dual methylated DNA markers in small-volume urine

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Diagnostic methods for urothelial carcinomas (UCs) are often invasive or have suboptimal accuracy. Methylation of exfoliated cell DNA or cell-free DNA in urine has shown great promise in the diagnosis of UCs. However, most current studies have focused on bladder cancer (BCa), and only a few high-plex methylated DNA panels based on large-volume urine have been reported to exhibit both high sensitivity and specificity.[1,2] The purpose of this study was to identify universal biomarkers for BCa and upper tract urothelial carcinoma (UTUC) using a small volume of urine. We developed a dual-target diagnostic panel comprising the novel marker *AL021918.2* and the well-known BCa biomarker Vimentin (*VIM*). This panel can accurately detect UCs using only 1.8 mL of full-voided urine.

This study was approved by the Ethics Committee of Peking University First Hospital (No. 2021 scientific research 481), and written informed consent was obtained from each participant. The clinical trial was registered on the Chinese Clinical Trial Registry website (No. ChiCTR2200066864).

First, we identified differentially methylated regions (DMRs) between BCa tissues and their normal adjacent tissues (NATs) using a sliding window method as previously described.[3] If two or more DMRs overlapped, we obtained a longer region by taking their union. Potential DMRs were selected based on receiver operating characteristic (ROC) analysis performed using the methylation level (mean β value of probes within each window). To select the top 25 DMRs, two criteria were used. First, the $\Delta\beta$ between the cancer tissue and NAT must be greater than 0.3. Second, the DMRs must meet either criterion 1 (with specificity >95% and top 20 sensitivity) or criterion 2 (with the top five sensitivity and >90% specificity).

Second, the β values of the 25 DMRs were analyzed in 440 BCa, 21 BCa-NAT, 324 kidney renal clear cell carcinoma (KIRC), 275 kidney renal papillary cell carcinoma (KIRP) samples from The Cancer Genome Atlas (TCGA) database, and 656 healthy blood samples from the Gene Expression Omnibus (GEO) database. After considering the background noise in healthy blood/NATs and the feasibility of primer design, eight DMRs were selected for further testing in 30 healthy blood samples and 20 normal urine samples using polymerase chain reaction (PCR) and Sanger sequencing. DMRs with methylated sites in amplicons of more than 10% of the samples were excluded, and the remaining DMRs were analyzed in tissues consisting of 47 bladder, 14 ureter, and 14 renal pelvis transitional cell carcinomas, as well as 28 NATs paired with the 28 UTUCs (clinical information can be found in Supplementary Table 1, http://links.lww.com/CM9/B647), using methylation-specific PCR (MSP). Two well-known markers of BCa (*VIM* and *Twist* family bHLH transcription factor 1 [*TWIST1*]) were also analyzed for comparison. Non-general DMRs of BCa and UTUC were excluded.

Next, candidate biomarkers identified at the tissue level were validated in 264 carcinoma (199 bladder, 39 renal pelvis, and 26 ureter) and 213 negative (other urological carcinomas, including KIRC, KIRP and prostate adeno-

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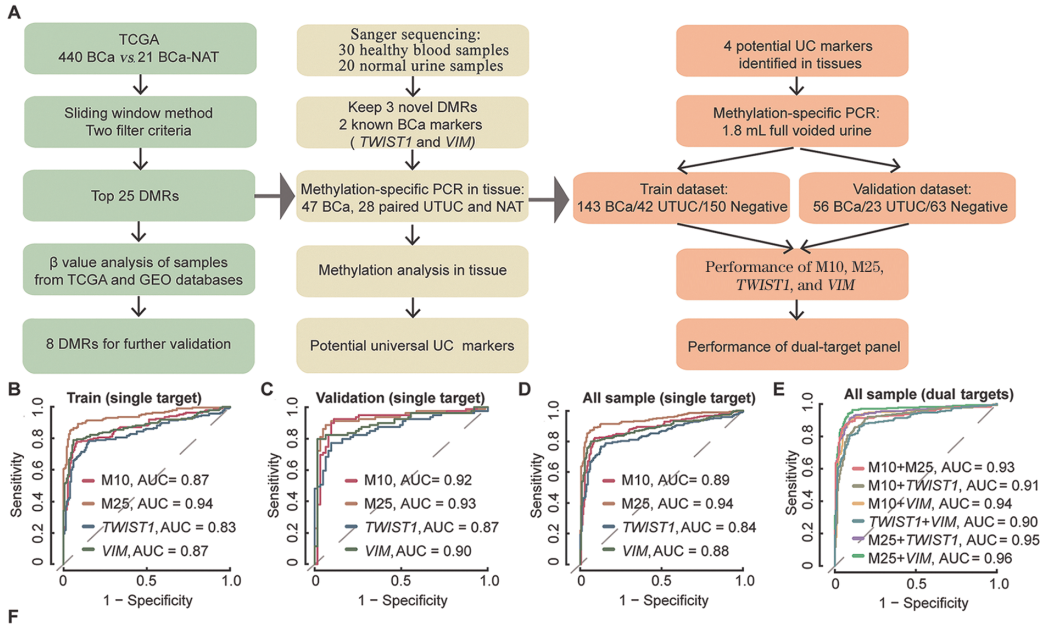
carcinoma (PRAD)/benign diseases) urine samples using MSP. All cancer samples with different grades (papillary urothelial neoplasms of low malignant potential [PUNLMP], low-grade [LG], and high-grade [HG]) and Tumor-Node-Metastasis (TNM) stages were transitional cell carcinomas confirmed by surgical pathology (clinical information can be found in Supplementary Table 2, http://links.lww.com/CM9/B647).

ROC analysis was performed to evaluate the distinguishing ability of the UC and negative tissue/urine samples using methylation levels indicated by $2^{-\Delta\Delta Ct}$. The carcinoma and negative urine samples were separated randomly into a training set, and validation set in a ratio of 7:3. The accuracy of dual-marker detection was assessed by constructing a logistic regression model.

The study design is shown in Figure 1A. In the TCGA/GEO databases, we used the mean β value of 0.2/0.1 as the threshold, and defined mean $\beta \geq 0.2$ as hyper-methylated, mean $\beta < 0.2$ as hypo-methylated, and mean $\beta < 0.1$ as extremely hypo-methylated. The baseline methylations of the 25 DMRs in healthy blood and BCa-NAT samples exhibited distinct patterns [Supplementary Table 3, http://links.lww.com/CM9/B647]. Five DMRs (M10 [Chr10: 101140373-101140735], M17 [*GRASP*], M18 [*GDF7*], M24 [*ALX1*], and M25 [*AL021918.2*]) were hypo-methylated in the two types of normal samples and were extremely hypo-methylated in at least one of their types. M25 was the only one that was extremely hypo-methylated in both healthy blood and BCa-NAT. M10 and M25 were also found to be hypo-methylated in KIRC and KIRP.

M10, M17, M18, M24, and M25 were selected for PCR and Sanger sequencing in 50 normal samples, accompanied by four hyper-methylated DMRs in one type of normal samples in TCGA/GEO (M1, M11, M20, and M21) as controls. Unfortunately, the appropriate primers for M24 could not be obtained. The sequencing results of the remaining eight DMRs showed that M10, M17, and M25 did not have any methylated CpG sites in 90% or more of the samples [Supplementary Figure 1, http://links.lww.com/CM9/B647], which was consistent with their low methylation levels in the TCGA/GEO cohort. Therefore, the three novel DMRs were retained for tissue validation, along with two BCa markers, *VIM* and *TWIST1*.

Among the DMRs studied, only M17 showed no significant difference in methylation levels between cancer tissue and NAT samples [Supplementary Figures 2A–F, http://links.lww.com/CM9/B647]. M25 had the highest area under curve (AUC) value (0.79) in classifying UCs and



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Accuracy	Dataset	Single target				Dual targets					
		M10	M25	*TWIST1*	*VIM*	M10+M25	M10+*TWIST1*	M10+*VIM*	*TWIST1*+*VIM*	M25+*TWIST1*	M25+*VIM*
Sensitivity	Train	77.84%	84.86%	78.38%	78.92%	/	/	/	/	/	/
	Validation	92.41%	88.61%	77.22%	79.75%	/	/	/	/	/	/
	All samples	82.20%	87.12%	76.52%	79.92%	87.12%	85.61%	92.42%	80.30%	91.29%	93.94%
Specificity	Train	92.00%	96.00%	84.67%	94.00%	/	/	/	/	/	/
	Validation	88.89%	93.65%	90.48%	98.41%	/	/	/	/	/	/
	All samples	91.08%	93.90%	87.32%	93.43%	93.90%	87.79%	88.26%	91.08%	90.61%	92.02%

Figure 1: The study design of developing an accurate diagnostic approach and its clinical performance in urine samples. (A) The in-house developed workflow outlines the study design. (B–D) ROC curves of M10, M25, *TWIST1*, and *VIM* are shown for the train dataset (B), validation dataset (C), and all sample dataset (D). (E) ROC curves of the six dual-target panels are shown for the all sample dataset. (F) Sensitivities and specificities of single/dual marker(s) for different datasets. /: Not available. AUC: Area under curve; BCa: Bladder cancer; DMRs: Differentially methylated regions; GEO: Gene Expression Omnibus; HG: High-grade; LG: Low-grade; M10: Chr10:101140373-101140735 (hg38); M25: *AL021918.2*; MSP: Methylation-specific PCR; NAT: Normal adjacent tissue; PCR: Polymerase chain reaction; PUNLMP: Papillary urothelial neoplasms of low malignant potential; ROC: Receiver operating characteristic; TCGA: The Cancer Genome Atlas; TNM: Tumor-Node-Metastasis; *TWIST1*: Twist family bHLH transcription factor 1; UC: Urothelial carcinoma; UTUC: Upper tract urothelial carcinoma; *VIM*: Vimentin.

NATs, followed by *VIM* (0.75), *TWIST1* (0.73), and M10 (0.72) [Supplementary Figure 2G, http://links. lww.com/CM9/B647]. The suboptimal AUC values of *VIM* and *TWIST1* were consistent with their inferior sensitivities in the TCGA cohort [Supplementary Figure 2H, http://links.lww.com/CM9/B647]. Therefore, M25 may be a more effective diagnostic marker for UCs, particularly for UTUC. M25 had a sensitivity of 92.86% for UTUC tissue, which is significantly higher than the second value of 71.43% [Supplementary Table 4, http://links.lww.com/CM9/B647].

M10, M25, *VIM*, and *TWIST1* were tested on urine samples. Of these, M25 had the highest accuracy in the training, validation, and all sample datasets [Figures 1B–F]. In the all sample dataset, the overall AUC value, sensitivity, and specificity of M25 were 0.94, 87.12%, and 93.90%, respectively. The dual-target panels substantially improved performance, and the AUC values for all six dual-target combinations were ≥0.90, and M25 plus *VIM* was the optimal combination, with an overall AUC of 0.96, a sensitivity of 93.94%, and a specificity of 92.02%.

The sensitivities of M25 and *VIM* varied depending on the histological grade [Supplementary Figure 3, http://links.lww.com/CM9/B647], TNM stage, and tumor size/focality. For LG-BCa, Ta stage UCs, and single small-sized UCs (<3 cm), the sensitivities of M25 were comparable to those of *VIM* (75.56% *vs*. 77.78%, 83.33% *vs*. 83.33%, and 74.60% *vs*. 68.25%, respectively). However, M25 appeared to be a more suitable detection target for UTUC, particularly for low-grade-upper tract urothelial carcinoma (LG-UTUC). M25 detected all five LG-UTUCs with a sensitivity of 100%, while *VIM* missed two of them. The dual markers had combined sensitivities of 50.00%, 91.11%, 100%, 94.44%, and 87.30% for PUNLMP, low-grade-bladder cancer (LG-BCa), LG-UTUC, Ta stage, and single small-sized carcinomas (<3 cm), respectively, indicating their utility in early-stage detection. Additionally, except for PRAD, the combined specificities were >90% in all subgroups of negative samples.

In this study, we developed a diagnostic panel for UCs with an accuracy of over 90% using only two methylated DNA markers. One of these markers, M25, was completely novel. Unlike other multiplex strategies, we used the sliding-window method to search for DMRs on a genome-wide scale. We mainly focused on DMRs with the highest specificity in the first filtering criterion to prevent the loss of specificity when different markers were combined into a diagnostic panel. However, we also emphasized high sensitivity in criterion 2, and M25 met this standard. The high sensitivity of M25 in the TCGA cohort provided a cornerstone for its utility in diagnosing UCs using as little as 1.8 mL of full-voided urine. M25 had the best performance at both tissue and urine levels, with its sensitivity for the hardest-to-detect Ta BCa and LG-UTUC being as high as 83.33% and 100% in urine, respectively. Notably, an assay based on small-volume full-voided urine can avoid the usage of large centrifugal equipment, and make the sample easily stored.

Our diagnostic panel has an overall specificity of 92.02%, which is comparable to that of cytology (95%).[4] Its sensitivity to LG and early-stage carcinomas was, surprisingly, higher than 90%. Most importantly, the panel exhibited outstanding performance in diagnosing UTUCs, which are easily missed by traditional cytology and fluorescence *in situ* hybridization. Currently, there are fewer UTUCs cases owing to their low prevalence, and a multi-center clinical trial is underway to further prove the panel's clinical performance.

The aforementioned advantages will assist medical workers in various clinical scenarios. For instance, the diagnostic tool can be used for risk stratification in the hematuria population, reducing the workload of endoscopy. In addition, an accurate non-invasive method like this can help confirm or rule out the presence of UTUC without ureteroscopy, when used in combination with imaging modalities. Furthermore, a positive detection of this molecular urine test, followed by more sensitive narrow-band imaging or photodynamic diagnosis, could decrease false-negative rates when compared with white light cystoscopy. Therefore, this simple and efficient diagnostic test has great clinical application prospects.

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Conflicts of interest

Di Cai, Tingting Li and Lanlan Dong were employees of wuhan Ammunition Life-tech Company, Ltd.

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