CD24 regulates cancer stem cell (CSC)-like traits and a panel of CSC-related molecules serves as a non-invasive urinary biomarker for the detection of bladder cancer

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BACKGROUND: CD24 is a cornerstone of tumour progression in urothelial carcinoma of the bladder (UCB). However, its contribution to cancer stem cell (CSC)-like traits and the clinical utility of CD24 as a urinary biomarker for cancer detection have not been determined.

METHODS: The functional relevance of CD24 was evaluated using in vitro and in vivo approaches. The clinical utility of CSC-related molecules was assessed in urine samples by quantitative RT-PCR.

RESULTS: The knockdown of CD24 attenuated cancer stemness properties. The high-CD24-expressing cells, isolated from patient-derived UCB xenograft tumours, exhibited their enhanced stemness properties. CD24 was overexpressed not only in primary tumours but also in urine from UCB subjects. By assessment of 15 candidate CSC-related molecules in urine samples of a training cohort, a panel of three molecules (CD24, CD49f, and NANOG) was selected. The combination of these three molecules yielded a sensitivity and specificity of 81.7% and 74.3%, respectively, in an independent cohort. A combined set of 84 cases and 207 controls provided a sensitivity and specificity of 82% and 76%, respectively.

CONCLUSION: CD24 has a crucial role in maintaining the urothelial cancer stem-like traits and a panel of CSC-related molecules has potential as a urinary biomarker for non-invasive UCB detection.

INTRODUCTION

Urothelial carcinoma of the bladder (UCB) is the most common malignancy of the urinary tract, with an estimated 79,030 new cases and 16,870 deaths from the disease per year in the United States. The 5-year relative survival rate is > 90% when detected as a non-muscle invasive bladder cancer (NMIBC), while it drops to < 50% for muscle invasive disease. Diagnosis at early stage of the initial and recurrent disease is crucial for favourable outcomes. However, the estimated recurrence rate of NMIBC is 60–70%, and 10–30% of these patients will progress to muscle invasive bladder cancer (MIBC) despite curative intensive therapy. This high recurrence rate requires patients to undergo frequent and lifelong monitoring. Although non-invasive, highly-specific urine cytology assay is commonly used for the surveillance of UCB patients, its sensitivity is relatively low, specifically for low-grade tumours. Clinically robust, sensitive, and specific urinary biomarkers are needed to supplement urine cytology test.

Tumours are hierarchically organized by a rare population of cancer stem cells (CSCs) that contributes to cancer initiation, progression, and treatment failure. A better understanding of the molecular mechanisms underlying urothelial CSC regulation and the identification of key molecules associated with CSC generation and maintenance are pivotal for the determination of biology-based accurate biomarkers for early cancer detection, monitoring following transurethral resection of the bladder tumor (TURBT), and molecular-targeting therapy. We recently demonstrated that CD24 is a crucial CSC marker that is overexpressed in urothelial CSCs. It was also reported previously that CD24 acts as a hub of tumorigenesis and metastatic progression, and associated with a poor outcome in UCB. Moreover, CD24 deficiency reduced urothelial tumorigenesis and metastasis in a mouse model, and treatment with an anti-CD24 monoclonal antibody resulted in a decreased metastatic tumour burden. Thus, CD24 has been implicated in tumour initiation and progression as an oncogene, and it is a potential therapeutic target for UCB. However, the oncogenic role of CD24 in UCB is incompletely understood. Although CD24 has been characterized as a major determinant of stemness in other cancer types, including liver and colorectal carcinoma, it is still unclear whether CD24 functionally contributes to urothelial CSC-like traits. Furthermore, although meta-analysis indicated that CD24 is an important marker of malignancy, including for UCB, its clinical utility as a biomarker for cancer detection has not been tested yet.
We recently demonstrated that chronic arsenic exposure endows urothelial cells with malignant stemness properties, including increased expression of several CSC-related molecules such as SOX2, CD24, and NANOG.19 Furthermore, we observed incremental expression of SOX2 in urine samples from carcinogen (arsenic)-exposed non-cancer subjects and UCB subjects compared with urine samples from non-exposed control subjects. Given these findings and the central role of CSCs at the top of the cellular hierarchy in tumour initiation, we hypothesized that urothelial CSC-related molecules may serve as urinary biomarkers for discriminating between subjects with and without UCB. In this study, we performed a quantitative expression assessment of 15 CSC-related molecules in urine samples from 24 non-cancer control and 24 UCB subjects to construct a candidate panel of urinary biomarker for UCB detection. After determining the analytical and clinical sensitivity of a panel of three genes (CD24, CD49f, and NANOG) in a set of primary tumours with the matched urine, we evaluated the clinical utility of this panel of three CSC-related molecules in an independent cohort of urine samples from 60 UCB and 183 control subjects. Furthermore, we evaluated the functional contribution of CD24 to urothelial cancer stem-like traits using in vitro and in vivo approaches.

**Materials and Methods**

**Cell lines and tissue samples**

UCB cell line 5637 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). BFTC 905 and BFTC 909 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). A total of 5637 cells were grown in RPMI 1640 medium and the other cells were grown in Dulbecco’s modified Eagle’s medium (DMEM). Re-authentication of cells was performed using PowerPlex 16 HS for short tandem repeats analysis at the Johns Hopkins University School of Medicine (JHUSOM), Institute of Genetic Medicine core facility, and all cell lines have been confirmed as authentic. Urine samples from a total 84 UCB subject (24 for training s and 60 for validation cohorts) and 207 from population-matched subjects (24 for training and 183 for validation cohort) were analysed. These urine samples were obtained from a urinary tract specimen bank maintained within the JHUSOM, Department of Pathology. Control subjects of both the cohorts had no history of genitourinary malignancy. Diagnosis of all UCB specimens was confirmed by a board-certified cytopathologist. Detailed clinicopathological information of UCB cases and controls are provided in Table 1. Thirty human primary UCB and the corresponding adjacent histologically non-cancer urothelial tissue samples were obtained from JHU-SOM, Department of Pathology. Informed consent was obtained from the patients before sample collection. Approval to conduct research on human subjects was obtained from the JHU institutional review boards. This study qualified for exemption under the U. S. Department of Health and Human Services policy for protection of human subjects [45 CFR 46.101(b)].

RNA extraction and quantitative reverse-transcriptase PCR

Total RNA from cell lines and formaldehyde-fixed paraffin-embedded human tissues was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, USA) and the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Austin, USA), respectively. Urine samples were centrifuged for 5 min at 1500 r.p.m. and the supernatant was used for RNA extraction as described previously.19 Total RNA extraction from urine was performed using the MirVana miRNA Isolation Kit (Ambion). Quantitative reverse-transcriptase PCR (qRT-PCR) was performed using the Fast SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, USA) on a 7900HT Fast Real-Time PCR System (Life Technologies, Carlsbad, USA) in triplicate. Primer sequences and the thermal cycling conditions were shown in Supplementary Table S1. SDS software (Applied Biosystems) was used to determine cycle threshold (CT) values. Expression levels were quantified relative to β-actin using the 2−ΔΔCT method.

Candidate gene selection to evaluate as a urinary biomarker

To construct a panel of urinary biomarker for cancer detection, 15 potential CSC-related molecules were selected based on our previous findings associated with malignant stemness properties in UCB.11,17 A receiver operating characteristic (ROC) analysis was used for evaluating the UCB detection accuracy using urine. ROC analysis method circumvents fluctuations caused by the arbitrarily chosen cut-off value of expression level to differentiate cases and controls as a selection criteria. The optimal cut-off value for distinguishing between UCB and control urine samples was determined using the ROC analysis for each gene. The performance of ROC analysis for each gene was evaluated by the area under the curve that is a combined measure of sensitivity and specificity. In addition, the positive and negative likelihood ratio, which are not affected by the prevalence of the disease, were measured to assess the strength of UCB detection accuracy for each gene.

**Table 1 The clinicopathological features of urine cohorts in this study**

| Samples       | Training cohort | Validation cohort |
|---------------|-----------------|-------------------|
|               | Tumour (n = 24) | Control (n = 24)  |
|               | Tumour (n = 60) | Control (n = 183) |
| Age (years)   | 71.29 ± 2.13    | 62.79 ± 2.56      |
|               | 62.87 ± 1.25    | 63.21 ± 1.19      |
| Median (years)| 71              | 64                |
| Range (years) | 54–90           | 26–81             |
|               | 46–83           | 21–92             |
| Race          | White           | Black             |
|               | Female          | Male              |
|               | 7               | 17                |
|               | 6               | 18                |
|               | 3               | 0                 |
|               | 3               | 0                 |
|               | 3               | 0                 |
| Gender        | Female          | Black             |
|               | Male            | Others            |
|               | Unknown         | 0                 |
|               | 7               | 1                 |
|               | 15              | 0                 |
|               | 6               | 0                 |
| Histological grade | High       | Low               |
|               | 17              | 6                 |
|               | 1               | 1                 |
|               | 38              | 15                |
|               | 7               | 0                 |
| Invasion of the muscularis propria | NMIBC | MIBC |
|               | 14              | 0                 |
|               | 47              | 9                 |
|               | 4               | 0                 |
| Cytology      | Negative        | Positive          |
|               | 16              | 8                 |
|               | 17              | 40                |
|               | 3               | 3                 |

**MIBC** muscle invasive bladder cancer, **NA** not applicable, **NMIBC** non-muscle invasive bladder cancer

*Negative cytology includes atypical urothelial cells and suspicious urothelial cancer cells
The Cancer Genome Atlas analysis
The gene expression data of 19 primary UCB samples and the matched tumour adjacent histologically normal samples in the The Cancer Genome Atlas (TCGA) cohort was downloaded from the MethHIC database to determine the expression level of our gene of interest in this external dataset.

Western blotting analysis
Whole-cell lysates were extracted using the RIPA buffer (Thermo Scientific) supplemented with 10 μL/mL of the Halt Protease Inhibitor Cocktail Kit (Life Technologies) and 30 μL/mL of the Halt Phosphatase Inhibitor Cocktail Kit (Life Technologies). CD133 (A3G6K) and ATP-binding cassette subfamily G member 2 (ABC2) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Yes-associated protein 1 (YAP1) (ab52771) and CD24 (AF5247-SP) were obtained from Abcam (Cambridge, USA) and R&D Systems (Minneapolis USA), respectively. β-Actin (A2228) was obtained from Sigma-Aldrich (St. Louis, USA). Secondary horseradish peroxidase (HRP)-conjugated antibodies were obtained from Cell Signaling Technology. Chemiluminescent detection of HRP-labelled antibodies was performed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Piscataway, USA). Expression levels of all candidates were quantified by myImageAnalysis™ Software (Thermo Scientific) and normalized to β-actin.

Gene silencing
CD24 short hairpin (shRNA) Lentiviral Particles (Cat # sc-29978-V) was used for the knockdown of the gene expression (CD24-sh; Santa Cruz Biotechnology, Dallas, USA). shRNA Lentiviral Particles (Cat # sc-108080) was used as a control (CD24-Ctrl; Santa Cruz Biotechnology). Cells were seeded in 24-well plates (5 × 10⁴ cells per well) for transduction. After 24 h, lentiviral particles were added to the cells in the presence of 8 μg/mL polybrene (EMD manufacturer) for transduction. After 24 h, lentiviral particles were added to the cells in the presence of 8 μg/mL polybrene (EMD Millipore) and incubated at 37 °C for 4 h. The medium was then replaced with fresh medium. Stable cells harbouring CD24 shRNA were established by antibiotic selection and expression level of CD24 was confirmed by RT-PCR and western blotting in the respective clone.

Sphere-formation assay and self-renewal assay
Sphere formation was performed by culturing cells (2 × 10³/well) in DMEM/Ham’s F12 50/50 Mix (Mediatech) supplemented with B-27 (Life Technologies), 20 ng/mL of fibroblast growth factors-basic (Peprotech, New Jersey, USA), and 20 ng/mL epidermal growth factor (Peprotech). Cell culture was performed in ultra-low attachment six-well plates (Coming, Lowell, USA) for 14 days. The medium was replaced every other day. Sphere formation was monitored and tumour volume was calculated from caliper measurements of two orthogonal diameters (larger (x) and smaller (y) diameters) using the following formula: volume = πx²y/2. The mice were killed when tumour reached 2 cm in diameter or 70 days later.

Preserved patient-derived tumour xenograft (PDX) tissues (CTG1388 and CTG1061) were obtained from Champion Oncology (Maryland, USA). For magnetic-activated cell sorting for CD24, PDX tumours were minced and digested with collagenase type IV (Sigma-Aldrich), hyaluronidase (Sigma-Aldrich), and DNase type IV (Sigma-Aldrich) into Hank’s buffered salt solution, followed by depletion of red blood cells using ACK lysing buffer (Quality Biological, Gaithersburg, USA). Tumour cells were isolated using Tumour Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer’s protocol. Then, tumour cells (1 × 10⁸) were labelled with PE-conjugated anti-human CD24 antibody (Miltenyi Biotec, Auburn, USA) and subsequently labelled with Anti-PE MultiSort MicroBeads (Miltenyi Biotec). After washing, separation for CD24-negative and -positive fraction was performed using MACS Columns and MidiMACS Separator (Miltenyi Biotec) twice. This process led to the separation of low-C2D and high-C2D, enriched cell population. To confirm the separation, flow cytometric analysis was carried out using PE-conjugated anti-human CD24 antibody (Miltenyi Biotec). PE-conjugated anti-IgG1k Isotype (Miltenyi Biotec) was used as controls for CD24 staining.

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**RESULTS**

Knockdown of CD24 attenuates urothelial cancer stemness properties

To our knowledge, there is no report whether CD24 functionally contributes to urothelial cancer stem-like traits. As spheroid cells contain enriched stem cell populations, we first assessed the expression levels of CD24 in spheroid cells compared with the matched parental cells in BFTC 905, BFTC 909, and 5637 cell lines. The spheroid cells showed higher expression levels of CD24 than the matched parental cells (Fig. 1a). To determine the functional role of CD24 in urothelial CSCs, a lentiviral-based stable knockdown clones of CD24 (CD24-sh) were established in BFTC 905, BFTC 909, and 5637 cell lines (Fig. 1b). The effect of CD24 knockdown on sphere-forming and self-renewal abilities was assessed by sphere-formation assays. CD24-sh cells generated fewer and smaller spheres compared with the control (CD24-Ctrl) cells through their first and second passages (Fig. 1c). To assess whether spheroid CD24-sh cells sustain an aggressive phenotype comparable to the spheroid CD24-Ctrl cells, we performed invasion assays and found that spheroid CD24-sh cells demonstrated decreased invasion (Fig. 1d).

As CSCs are resistant to conventional chemotherapies that efficiently eliminate bulk tumour cells, the viability of spheroid CD24-sh cells was assessed by treating with cisplatin (CDPP), an important chemotherapeutic agent for the treatment of UCB. As expected, spheroid CD24-sh cells were more sensitive to CDPP treatment than spheroid CD24-Ctrl cells (Fig. 2a). Furthermore, we determined that CD24 knockdown significantly attenuated the anti-apoptotic ability against CDPP treatment in spheroid cells (Fig. 2b).

To determine the effect of CD24 knockdown on candidate CSC-related molecules, we tested mRNA expression levels of 15 potential CSC-related molecules in CD24-sh and CD24-Ctrl cells by qRT-PCR. CD24 knockdown led to the downregulation of numerous CSC-related molecules, among which CD133, YAP1, and the drug efflux transporter ABCG2 were found to be consistently altered due to loss of CD24 in the three UCB cell lines (Supplementary Fig. S1). We confirmed similar findings at the protein level (Fig. 2c).

Knockdown of CD24 attenuates in vivo tumorigenicity

As an important feature of CSCs is efficient in vivo tumorigenesis at a limiting-dilution xenograft, we performed the tumour formation assays with serial dilutions of spheroid CD24-sh cells and CD24-Ctrl cells. Serially diluted spheroid CD24-sh or CD24-Ctrl cells (1 × 104, 1 × 103, or 1 × 102 cells per flank) were injected subcutaneously into both flanks of NSG mice (five mice per...
CD24 regulates cancer stem cell (CSC)-like traits and a panel of cancer stem-like markers in UCB cell lines.

To test whether cancer stem-like traits are associated with CD24 expression, we isolated high- and low-CD24-expressing cells from PDX models using the magnetic-activated cell sorting approach. One model was established from a primary site (CTG1388) and a second model was established from a metastatic site (CTG1061). We confirmed the expression status of CD24 by flow cytometric analysis (Fig. 3a). The high-CD24-expressing cells exhibited greater sphere-forming and chemoresistant abilities than the low-CD24-expressing cells (Fig. 3b, c). In addition, the link of CD24 with CD133, YAP1, and ABCG2 was further validated by our observation of high expression of these molecules in PDX-derived high-CD24-expressing cells, as compared with low-CD24-expressing cells (Fig. 3d). Tumour-initiating capacity is shown as the numbers of tumours/the number of injections after 70 days from subcutaneous injection of serially diluted spheroid CD24-sh cells (1 × 10^4, 1 × 10^3, or 1 × 10^2 cells per flank) into both flanks of NSG mice (five mice per group). Each error bar indicates mean ± SEM. *P < 0.05; **P < 0.01 (Wilcoxon–Mann–Whitney test).

CD24 has potential as a urinary biomarker for UCB detection. To test the cancer specificity of CD24 expression in primary UCB, we analysed mRNA expression levels of 30 primary UCB and compared with low-CD24-expressing cells (Fig. 3d). Furthermore, the high-CD24-expressing cells grew faster and generated larger tumours than the low-CD24-expressing cells after subcutaneous injection of 1 × 10^4 cells per flank into NSG mice (Fig. 3e). Collectively, our findings suggest a crucial role of CD24 in urothelial cancer stem-like traits.

Fig. 2 The chemoresistant and tumorigenic abilities attenuated by CD24 knockdown in BFTC 905, BFTC 909, and 5637 cell lines. a Cell viability after 5 μM cisplatin (DDP) treatment for 72 h in the spheroid CD24-sh cells, as measured by MTT assay. Cell viability was expressed as the ratio of absorbance values of the spheroid CD24-sh cells related to the spheroid CD24-Ctrl cells considered as 1.0. Data are from three independent experiments. b An apoptosis assay of spheroid CD24-sh cells treated with 5 μM CDPP for 72 h. Upper, representative images of early apoptosis (bottom right quadrant) and late apoptosis (top right quadrant); lower, percentage of apoptotic cells. c Western blotting analysis of ABCG2, YAP1, and CD133 in stable CD24-sh and CD24-Ctrl cells. d Limiting-dilution xenograft assays in stable spheroid CD24-sh BFTC 909 (upper) and BFTC 905 (lower) cells. Tumour growth was measured after subcutaneous injection of serially diluted spheroid cells (1 × 10^4, 1 × 10^3, or 1 × 10^2 cells per flank) into both flanks of NSG mice (five mice per group). SP, spheroid. e Tumour initiation frequency after the xenotransplantation of spheroid CD24-sh BFTC 909 (upper) and BFTC 905 (lower) cells. Tumour-initiating capacity is shown as the numbers of tumours/the number of injections after 70 days from subcutaneous injection of serially diluted spheroid CD24-sh cells (1 × 10^4, 1 × 10^3, or 1 × 10^2 cells per flank) into both flanks of NSG mice (five mice per group). Each error bar indicates mean ± SEM. *P < 0.05; **P < 0.01 (Wilcoxon–Mann–Whitney test).
corresponding adjacent normal tissues (Fig. 4a). Furthermore, analysis of the TCGA UCB cohort generated similar findings in tumour and the matched adjacent normal tissues (Fig. 4b).

The clinical utility of CD24 as a biomarker for cancer detection has not been determined. Given the cancer-specific elevation of CD24 expression in primary tumours, we next assessed the potential for non-invasive cancer detection using a total of 48 urine samples (24 UCB and 24 control subjects) as a training cohort (Table 1). The expression level of CD24 in urine was significantly higher in UCB subjects than in controls (Fig. 4c). The optimal cut-off value for distinguishing between urine samples from UCB and control subjects was calculated using a ROC analysis. By using the optimal cut-off value of CD24 expression, the sensitivity and specificity of CD24 for cancer detection were 45.8% and 95.8%, respectively (Supplementary Table S2). The high specificity indicates that CD24 may be a potential urinary biomarker for UC detection. We hypothesized that the low sensitivity was due to heterogeneity among UCB, and that the addition of other markers could improve sensitivity.

We previously reported SOX2 as a potential urine-based biomarker for non-invasive early detection of UCB, and this molecule is an established regulator of CSCs and have a considerable role in tumour initiation. To further identify CSC-associated urine-based biomarkers, we tested 15 CSC-related molecules by candidate gene approach in urine from 24 UCB and 24 controls subjects (total 48 urine samples as a training cohort). Among these 15 molecules, NANOQ, CD49f, LGR5, DNp63, SOX2, and CD24 showed significantly higher expression levels in urine from UCB subjects compared with control samples (Supplementary Table S2). By determining the optimal cut-off using ROC curves for each molecule, the individual sensitivity and specificity of these six molecules (NANOQ, CD49f, LGR5, DNp63, SOX2, and CD24) for cancer detection ranged from 29.2% to 62.5% and 83.3 to 100%, respectively (Supplementary Table S2). We further assessed the expression pattern spectrum of these six molecules. When the positive expression of at least one of the six molecules was considered, the sensitivity was 95.8%, whereas the specificity decreased to 50.0% (Supplementary Table S3). When combination of CD24, CD49f, and NANOQ was considered, a high UCB detection accuracy was achieved, with a sensitivity of 83.3% and specificity of 87.5% (Fig. 4d and Table 2).

To determine the analytical sensitivity, we analysed expression of later 3 molecules (CD24, CD49f, and NANOQ) in 17 primary UCB tissues with matched urine samples. The expression levels of these three molecules (CD24, CD49f, and NANOQ) in primary tumour tissues were significantly higher in subjects with positive expression in urine samples than in those with negative urine expression (Supplementary Fig. S2). The concordance rate between primary tumours and the matched urine samples (analytical sensitivity) was 77.8% (7/9) for CD24, 70.0% (7/10) for
Analytical sensitivity is defined as 'The fraction of cases in which overexpression of a marker was found in urine RNA for case patients who had confirmed overexpression of the same marker in the primary tumour RNA'. Cells in colour represent the positive expression, defined by optimal cut-off value determined by ROC curve, for each molecule in primary tumour (P) and the matched urine (U) samples. Each data indicates mean ± SEM. The paired t-test (a and b) were performed.

Validation of a panel of three CSC-related genes (CD24, CD49f, and NANOG) in an independent cohort of urine sample for early detection of UCB

To confirm the detection accuracy of combination of three CSC-related molecules (CD24, CD49f, and NANOG), we tested an independent validation cohort consisting of 60 UCB and 183 control subjects (Table 1). Again, higher expression levels of these three molecules were observed in the urine samples from UCB subjects than controls (Supplementary Fig. S3). Using the same cut-off as of training cohort, the individual sensitivity of CD24, CD49f, and NANOG was overexpressed in primary tumours and urines of all the samples analysed. Based on reasonable analytical and clinical sensitivity in the training cohort, a combination panel of these three CSC-related molecules may have potential to detect UCB with high sensitivity and specificity using clinical samples.

CD24 and 64.3% (9/14) for NANOG (Fig. 4e). The clinical sensitivity (detection of cancer by urine test of these three genes) of CD24, CD49f, and NANOG were 47.1% (8/17), 52.93% (9/17), and 52.93% (9/17), respectively (Fig. 4e). At least one of the three genes was overexpressed in primary tumours and urines of all the samples analysed. Based on reasonable analytical and clinical sensitivity in the training cohort, a combination panel of these three CSC-related molecules may have potential to detect UCB with high sensitivity and specificity using clinical samples.

Fig. 4 The cancer detection accuracy of a combination panel of three CSC-related molecules (CD24, CD49f, and NANOG) in urine samples. a Box plots of the relative expression levels of CD24 mRNA in 30 primary tumour and the matched adjacent normal tissues. Scatter plots show the distribution of individual expression value of CD24 determined by qRT-PCR. The expression levels of tumour and the matched adjacent tissues were connected with a line. b Box plots of the expression levels of CD24 mRNA in 19 UCB samples and the matched adjacent, histologically normal samples in the TCGA cohort. The expression values (RSEM log2) of tumours and the matched adjacent tissues were connected with a line. c Box plots of the expression levels of CD24 mRNA in urine samples from 24 UCB and 24 control subjects. d Sensitivity and specificity of the combination panel for cancer detection in urine samples of the training cohort and independent validation cohort. The schematic representation shows true positives, false negatives, true negatives, and false positive detected by the combination panel of three molecules (CD24, CD49f, and NANOG). e Analytical sensitivity (AS) of CD24, CD49f, and NANOG in 17 primary UCB and the matched urine samples. Analytical sensitivity is defined as 'The fraction of cases in which overexpression of a marker was found in urine RNA for case patients who had confirmed overexpression of the same marker in the primary tumour RNA'. Cells in colour represent the positive expression, defined by optimal cut-off value determined by ROC curve, for each molecule in primary tumour (P) and the matched urine (U) samples. Each data indicates mean ± SEM. The paired t-test (a and b) were performed.
CD24 is a lynchpin of tumorigenesis and metastatic progression in UCB. However, the relevance of CD24 in urothelial cancer stem-like traits remains unclear. In this study, for the first time we characterized CD24 as a major determinant of urothelial stemness, supporting previous findings that CD24-expressing cells exhibit an aggressive phenotype. Although we genetically inhibited CD24 using a lentiviral-based approach that may not be suitable for clinical use, Overdevest et al. demonstrated that treatment with an anti-CD24 monoclonal antibody led to reduced tumour growth and metastasis, resulting in prolonged survival in UCB xenograft model. Collectively, our pre-clinical data suggests that CD24 could be a promising therapeutic target to efficiently eliminate urothelial CSCs.

The exact molecular mechanisms for CSC generation and maintenance via CD24 are incompletely understood. We observed the downregulation of several CSC-related molecules such as CD133, ABCG2, and YAP1 in CD24-sh cells and expression level of these molecules were higher in high-CD24-expressing cells, indicating a potential crosstalk between CD24 and these CSC-related molecules. CD133, a pentaspan transmembrane glycoprotein, has been used as a surface marker for isolation of urothelial CSCs. ABCG2 is a drug transporter and this molecule actively effluxes varieties of chemotherapeutic agents that may provide CSCs with a selective survival advantage against chemotherapy. YAP1 is a downstream transcription effector of the Hippo pathway and we recently demonstrated that urothelial cancer stem-like traits are driven by the YAP1-SOX2 signalling axis that is an upstream regulator of CD24 expression. CD24 interacts with Src to promote its kinase activity and activated Src has been implicated in regulating YAP1. Thus, the regulatory circuitry between YAP1 and CD24 may accelerate urothelial CSC maintenance and progression. Further research is needed to elucidate these complex crosstalk mechanisms.

Urine cytology analysis is a non-invasive approach for cancer detection with a high specificity (> 95%), but it is limited by its low sensitivity (35–55%), especially for low-grade (< 20%) and low-stage (< 40%) disease. Sensitivity is generally considered more important than specificity for screening and surveillance, as the missing of early disease increases the risk of progression to advanced disease and a poor clinical outcome. Although several urine-based diagnostic assays have been approved by the U.S.

### Table 2 The bladder cancer detection accuracy of a panel of three genes in urine samples

| Characteristics                              | CD24          | NANOG        | CD49f        | CD24/NANOG/CD49f |
|----------------------------------------------|---------------|--------------|--------------|------------------|
|                                              | Sensitivity   | Specificity  | Sensitivity  | Specificity      |
| **Training cohort**                          | 45.8% (11/24) | 95.8% (23/24)| 45.8% (11/24)| 100% (24/24)     |
| Tumour invasion                              | 54.2% (13/24) | 91.7% (22/24)|             | 88.3% (20/24)    |
| NMIBC                                        | 21.4% (3/14)  | 57.1% (8/14) | 64.3% (9/14) | 78.6% (11/14)    |
| Grade of urothelial cancer                   | 16.7% (1/6)   | 50.0% (3/6)  | 66.7% (4/6)  | 66.7% (4/6)      |
| Low                                          | 58.8% (10/17) | 47.1% (8/17) | 52.9% (9/17) | 94.1% (16/17)    |
| High                                         |              |              |              |                  |
| CytoLOGY                                     | 43.8% (7/16)  | 50.0% (8/16) | 56.3% (9/16) | 81.3% (13/16)    |
| Negativea                                    | 37.5% (3/8)   | 50.0% (4/8)  | 87.5% (7/8)  |                  |
| Positive                                     |              |              |              |                  |
| **Independent cohort**                       | 35.0% (21/60) | 91.3% (167/183)| 51.7% (31/60) | 88.5% (162/183) |
| Tumour invasion                              | 35.0% (21/60) | 91.3% (167/183)| 51.7% (31/60) | 88.5% (162/183) |
| NMIBC                                        | 44.7% (21/47) |              | 40.4% (19/47) | 80.9% (38/47)    |
| Grade of urothelial cancer                   | 40.0% (6/15)  | 33.3% (5/15) | 33.3% (5/15) | 80.0% (12/15)    |
| Low                                          | 39.5% (15/38) | 50.0% (19/38)| 39.5% (15/38)| 78.9% (30/38)    |
| High                                         |              |              |              |                  |
| CytoLOGY                                     | 41.2% (7/17)  | 52.9% (9/17) | 23.5% (4/17) | 82.4% (14/17)    |
| Negativea                                    | 47.5% (19/40) | 40.0% (16/40)| 80.0% (32/40)|                  |
| Positive                                     |              |              |              |                  |
| **Combined cohort**                          | 38.1% (32/84) | 91.9% (190/207)| 50.0% (42/84) | 89.9% (186/207) |
| Tumour invasion                              | 40.5% (34/84) | 84.5% (175/207)|              |                  |
| NMIBC                                        | 39.3% (24/61) | 47.5% (29/61)| 45.9% (28/61)| 80.3% (49/61)    |
| Grade of urothelial cancer                   | 33.3% (7/21)  | 38.1% (8/21) | 42.9% (9/21) | 76.2% (16/21)    |
| Low                                          | 45.6% (25/55) | 49.1% (27/55)| 43.6% (24/55)| 83.6% (46/55)    |
| High                                         |              |              |              |                  |
| CytoLOGY                                     | 42.4% (14/33) | 51.5% (17/33)| 39.4% (13/33)| 81.8% (27/33)    |
| Negativea                                    | 37.5% (18/48) | 45.8% (22/48)| 41.7% (20/48)| 81.3% (39/48)    |
| Positive                                     |              |              |              |                  |

**MIBC** muscle invasive bladder cancer; **NMIBC** non-muscle invasive bladder cancer;

*Negative cytology from our cohorts includes atypical urothelial cells and suspicious urothelial cancer cells
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Food and Drug Administration, these assays do not overcome the low sensitivity for low-grade disease. Therefore, improvement of the detection sensitivity for low-stage and low-grade disease is one of the central goals of urine-based tests. In this study, the combination panel (CD24, CD49f, and NANOG) yielded high sensitivity for cancer detection not only for NMIBC (80.9%), but also for low-grade UCB (80.0%). Most importantly, 82.4% of UCB specimens negative by cytology were positive by CSC marker test in urine. Therefore, this CSC-related panel with high sensitivity could provide a valuable adjunct to urine cytology for UCB detection, if confirmed by a larger prospective study.

CD49f mediates the stem cell niche via interactions with the extracellular matrix and communication between tumour cells and the tumour microenvironment. In UCB, CD49f is downregulated during differentiation and has been utilized to enrich CSC population. NANOG is a key pluripotent transcription factor and predominantly expressed in urothelial CSCs. Thus, all the three molecules (CD24, CD49f, and NANOG) act as biologically relevant CSC factors in UCB. As we did not observe concordant changes in CD49f and NANOG expression due to alteration of CD24 expression in CD24-sh cells and CD24-expressing cells, the combination panel may be able to detect different CSC populations and each of these molecules could be an independent marker for this heterogeneous disease. In fact, 39 of 60 UCB urine samples in the validation cohort showed negative expression of CD24, and 9 (23.1%) and 21 (53.8%) out of 39 urine samples without CD24 expression showed positive for CD49f and NANOG, respectively. This observation also supports the multiclonal origin of this heterogeneous disease. Although its needs further validation, some inconsistency of findings between primary tumours and urine may be due to the site of the primary tumours that were analysed. It could happen that we analysed the tumour site that did not shed tumour cells in the urine and the source of tumour cell may be other sites of the same bladder.

Several studies reported promising panels of urinary mRNAs determined by qRT-PCR for UCB detection, including the commercially available Cxbladder assay. Compared with these assays, our overall sensitivity of our assay was similar, but specificity was relatively low, partially due to the use of different reference genes and selection approaches. Cxbladder adapted CXCR2 as a reference gene to reduce the false positive rate. Other assays considered genes that are expressed stably and with little variability in exfoliated urinary cells. Thus, the use of suitable reference genes may improve the specificity of our assay—say. In addition, the novel transcriptome profiling approach may yield more sensitive and specific CSC-related biomarkers in urine.

Study limitations include the relatively small sample size and possible bias due to retrospective analysis and empirical selection of CSC-related molecules for the urinary biomarker. In addition, our cohort may not be representative of the general population at risk for UCB because of the lack of relevant clinical data, including smoking history, occupational exposure history, and patient outcome. Infections or any other inflammatory disease in the urinary tract may influence expression of these biomarkers. Therefore, although promising, our findings cannot be considered conclusive, and extensive validation is needed in a larger independent cohort including various urologic conditions to assess the clinical utility of combination panel of three CSC-related molecules.

In summary, we demonstrated that CD24 drives cancer stem-like traits and serves as a promising non-invasive urinary biomarker for UCB detection. In addition, we also identified a panel of CSC-related molecules that has potential as a urinary biomarker for UCB detection with high sensitivity and specificity. These findings may facilitate the development of improved therapeutic strategies and non-invasive detection of UCB.

Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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AUTHORS’ CONTRIBUTIONS

A.O. and M.H. designed the research and wrote the paper. C.V., M.K., N.H., D.M., and T.B. collected human samples and clinical data, and participated in the writing of the paper. All authors read and approved the final manuscript.

ADDITIONAL INFORMATION

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