A 3-plex methylation assay combined with the FGFR3 mutation assay sensitively detects recurrent bladder cancer in voided urine

Running title: Urine biomarkers for recurrent bladder cancer detection

Raju Kandimalla¹, Roy Masius¹, Willemien Beukers¹, Chris H. Bangma², Torben F. Orntoft³, Lars Dyrskjot³, Nikki van Leeuwen⁴, Hester Lingsma⁴, Angela A. G. van Tilborg¹, Ellen C. Zwarthoff¹

¹Department of Pathology, Erasmus MC, Rotterdam, The Netherlands
²Department of Urology, Erasmus MC, Rotterdam, The Netherlands
³Department of Molecular Medicine, Aarhus University Hospital, Aarhus, Denmark
⁴Department of Public Health, Erasmus MC, Rotterdam, The Netherlands

Corresponding author

Prof. dr. E.C. Zwarthoff

Department of Pathology

Erasmus MC, P.O. Box 2040

3000 CA Rotterdam, the Netherlands

Phone: +3110 7043929, Fax: +31107044762

E-mail: e.zwarthoff@erasmusmc.nl

Conflict of interest

Potential conflicts do not exist with authors
Translational relevance

70% of patients presenting with non-muscle invasive bladder cancer (NMIBC) will develop recurrences necessitating long-term monitoring by cystoscopy. Molecular tests using voided urine have been developed to replace cystoscopy. What is still lacking are tests that address the patient population in question, i.e. patients under surveillance after a primary G1/G2 NMIBC. The authors developed a 3-plex assay for the diagnosis of recurrent BC in voided urine that achieved a sensitivity of 68 and 74% in the test and validation sets at 90% specificity. Combination with the FGFR3 mutation assay increased sensitivity to 79%. The sensitivity of this assay combination is higher than cytology and similar to the sensitivity of the current gold standard white light cystoscopy. We suggest that a subsequent study should investigate a modified surveillance protocol consisting of the urine test followed by cystoscopy, only when the urine test is positive, for patients in the low/intermediate risk BC group.
Abstract

Purpose

DNA methylation is associated with bladder cancer and these modifications could serve as useful biomarkers. FGFR3 mutations are present in 60-70% of NMIBC. Low-grade bladder cancer recurs in over 50% of patients. The aim of this study is to determine the sensitivity and specificity of a urine assay for the diagnosis of recurrences in patients with a previous primary NMIBC G1/G2 by using cystoscopy as the reference standard.

Experimental Design

We selected eight CGIs methylated in BC from our earlier genome-wide study. Sensitivity of the CGIs for recurrences detection was investigated on a test set of 101 preTUR urines. Specificity was determined on 70 urines from healthy males >50 years. A 3-plex assay for the best combination was developed and validated on an independent set of 95 preTUR, recurrence free and non-malignant urines (n=130).

Results

The 3-plex assay identified recurrent BC in voided urine with a sensitivity of 74% in the validation set. In combination with the FGFR3 mutation assay a sensitivity of 79% was reached (specificity of 77%). Sensitivity of FGFR3 and cytology was 52% and 57% respectively.

Conclusion

The combination of methylation and FGFR3 assays efficiently detects recurrent BC without the need for stratification of patients regarding methylation/mutation status of the primary tumor. We conclude that the sensitivity of this combination is in the same range as cystoscopy and paves the way for a subsequent study that investigates a modified
surveillance protocol consisting of the urine test followed by cystoscopy only when the urine test is positive.

**Key words:** Bladder cancer, DNA methylation, Biomarkers, Sensitivity and specificity, Recurrence, Surveillance.
Introduction

Bladder cancer is the fifth most common cancer in the western world with an estimated 386,300 new cases and 150,200 deaths in the year 2008 worldwide (1). Tumors of the urinary bladder present either as non-muscle or as muscle-invasive carcinomas. Five-year survival for patients with muscle-invasive bladder cancer (> stage pT1) is 50%; for patients with non-muscle-invasive bladder cancer (stages pTa, pT 1 and CIS) it is 90% (2, 3). These different forms of bladder cancer are also associated with different genetic changes. Somatic mutations in the \textit{FGFR3} gene accompanied with losses of chromosome 9 are more frequent in NMIBC, while TP53 mutations are associated with MIBC (4-6). Unfortunately, 70% of patients with NMIBC will have one or more recurrence after transurethral resection (TUR) and 10-20% of patients will eventually have progression to MIBC (7-9). Patients with NMIBC are currently stratified into low/intermediate and high risks groups regarding progression and recurrence based on clinicopathological parameters (10, 11). Currently cystoscopy is the gold standard for surveillance; however, it is an invasive and uncomfortable procedure (12). Moreover with cystoscopy, there is 20 to 30% chance to miss a tumor (13). The stringent follow-up of patients by cystoscopy every 3-12 months after TUR makes BC one of the most expensive cancers to treat (14).

Cytological examination of voided urine can identify tumor cells with a high sensitivity if a high-grade tumor is present (15). However, for low stage and grade tumors the sensitivity is low. This low sensitivity induced the development of urine-based assays in the past decade. Although some assays have been approved by the FDA, they have so far not been taken up in routine clinical practice (16-20). In most studies the sensitivity of the markers was assessed on a convenience set of urines that comprises urines from primary and recurrent tumors and urines from patients with high stage and grade tumors. Hence when tested on urines from patients under surveillance the sensitivity of these markers is disappointing (17, 21). Because
of these problems our group has specifically focused on markers that are better in detecting recurrent BC in patients who presented with a primary NMIBC (22-24). Up to 80% of the pTa tumors have a mutation in the fibroblast growth factor receptor 3 oncogene (25, 26). We showed that a multiplex assay for the most common mutations was able to detect about 75% of tumors smaller than 1.5 cm and 100% if the tumors were larger than 3 cm (27). A great advantage of the FGFR3 assay is that since these mutations are extremely rare in normal cells, an assay to detect FGFR3 mutations has a specificity of 100%. A disadvantage is that the patients need to be stratified up front for the presence of an FGFR3 mutation and therefore other assays are required for patients with wild-type tumors.

It is evident from many studies that methylated CpG islands (CGIs) may present useful biomarkers. In order to find the most promising CGIs for BC prognosis and diagnosis we have previously performed a genome-wide study (28). The purpose of this study is to determine the diagnostic accuracy of a 3-plex methylation assay combined with FGFR3 mutation assay in the diagnosis of recurrence of bladder cancer in patients with previous low-grade bladder cancer.
Materials and methods

Study population

We used samples from the Departments of Pathology and Urology, Erasmus MC and Aarhus University Hospital, Denmark. Patient characteristics are presented in Table 1. Written informed consent was obtained from all patients. The test and validation set pre-TUR urines were collected before transurethral resection of the corresponding recurrent tumor. Inclusion criteria were presence of a previous primary NMIBC G1/G2 tumour and histologically confirmed recurrence at TUR. Exclusion criteria were the presence of a G3 primary tumor.

Test samples and validation samples

Forty-eight FFPE BC DNAs from a mixture of primary and recurrent tumors, 101 pre-TUR urines from patients with a previous primary NMIBC G1/G2, and 39 pre-TUR urines from patients with primary tumors (pTaG1 and pTaG2) were included as test cases, and 70 urines from healthy males (> 50 y) as controls.

An additional set of 103 pre-TUR urines from patients with a previous primary NMIBC G1/G2 was obtained from Aarhus University Hospital, Denmark to perform external validation to test the robustness of our final model. Eight samples were discarded because they were from patients with G3 tumors. As controls, we included 40 urines that were collected during surveillance in a period in which no recurrences occurred within 6 months. Out of these 40 patients, 24 were under and 16 were over 65 years. There were 27 males and 13 females. The median follow-up was 24 months. We have validated our urine assay further on the following number of non-bladder cancer related urological afflictions: high leucocyte count (n=25, Multistix® 8 SG), cystitis patients (n=18), lower urinary tract symptoms (n=30), prostate cancer patients (n=14) and renal cancer patients (n=3). All samples were collected prior to cystoscopy at the Department of Urology, Erasmus MC. Cystitis was...
defined visually during cystoscopy. There is no overlap in the samples used. Five control samples failed due to low DNA concentration. In this case-control validation study, data were collected retrospectively. The study adhered to the STARD guidelines and the guidelines presented by Pepe for the reporting of studies of diagnostic accuracy (29, 30).

Bisulfite conversion, methylation and FGFR3 assays

Urine samples (25-100 ml) were collected one day before TUR and stored at 4 °C. Within 6 hrs after voiding, samples were centrifuged at 4000 rpm for 10 minutes, followed by washing the pellet twice with cold PBS. DNA was isolated using DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany). Bisulfite conversion and quantitative assessment of methylation was done as described previously (28). In short: DNA was converted with sodium bisulfite (EZ DNA methylation gold kit, Zymo Research Corp, Orange, CA, USA). PCR of selected CGIs was performed using 20 ng of converted DNA, 20 pmols of primers and 10 µL of KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, USA) in a total volume of 20 µl. The PCR products were treated with 2 units of Exonuclease I (ExoI) and 3 units of Shrimp Alkaline Phosphatase (SAP) (USB, Cleveland, Ohio USA). This was followed by a single-nucleotide probe extension assay using a SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA) and probes designed to anneal to either the forward or reverse strand adjacent to the investigating CpG site. These probes were fitted with T-tails of different length at their 5’ ends to allow separation of the extension products by size. The single nucleotide primer extension reactions were performed in a total volume of 10 µl containing 2 µl SAP/ExoI treated PCR product, 2.5 µl SNaPshot Multiplex Ready Reaction mix, 1 x Big Dye sequencing buffer, and 1 µl probe mix. Thermal cycler conditions were: 25 cycles of 10 seconds at 95°C, 5 seconds at 50°C, and 30 seconds at 60°C. The products were treated with 1 unit SAP at 37°C for 60 min, and at 75°C for 15 min, and were analyzed on an automatic sequencer (ABI PRISM 3130 XL Genetic Analyzer, Applied Biosystems) with the
fluorescent label on the incorporated ddNTP indicating incorporation of a C or T or an A or G depending on the strand investigated. We further developed a multiplex BS-SNaPshot assay aimed at detecting the methylation status of the three most interesting CGIs (OTX1, ONECUT2 and OSR1) in a single PCR and a single nucleotide primer extension reaction. FGFR3 mutation analysis was performed as described previously (31). Concentrations of all primers and probes are given in Supplementary Table S1.

**Statistical analysis and selection of the optimal combination of markers for the detection of recurrent BC in voided urine**

For the BS-SNaPshot data, we used GeneMarker Software version 1.7 (SoftGenetics, State College, PA, USA). The methylation percentage for a CpG in a sample was calculated as the ratio of the height of the C/G peak divided by the height of the C/G plus T/A peak*100. To predict the best combination, we performed logistic regression on all possible combinations of the eight markers using test set of 101 preTUR urines with recurrence and 70 control urines. Logistic regression will assign a beta (Coefficient) value for each marker based on the contribution in predicting an outcome. From these values we calculated the probability value for that particular combination using a formula 1/1+e^{-z}, where z = constant + % methylation A * \beta value A + % methylation B * \beta value B etc, with A and B being the markers. We chose a cut-off value allowing 10% false positives in the control group (specificity=90%). From this analysis we picked the best 3-marker combination that showed the highest sensitivity in the test set. We calculated the sensitivity and specificity by plotting the receiver operating characteristic curve (ROC) and calculating the area under the curve (AUC) using SPSS statistical software V17.0.
Independent validation of the 3-marker combination

We then validated the three marker combination in an independent set of 95 preTUR urines from patients with a previous NMIBC G1/G2 and 130 controls. In addition, we analysed sensitivity of each marker regarding detection of different stage and grade recurrences. To this 3-plex methylation assay we added \textit{FGFR3} and Cytology outcome as 0 or 1 and determined the sensitivity of recurrence detection with different combinations, i.e. Methylation+FGFR3, Methylation+Cytology and Methylation+FGFR3+Cytology.
Results

Selection of methylated CGIs in BC

Study design is described in Supplementary Figure 1. Patient characteristics for all cohorts are given in Table 1. Previously we identified 110 CGIs methylated in BC but not in control urine (28) (PCT(NL2010)/050213). For this study the 21 CGIs with the largest methylation difference were selected. First we tested the performance of 42 CpGs from these 21 CGIs in BS-SNaPshot assays on DNA obtained from BC cell lines (results not shown). We selected 8 candidate CpGs that performed best regarding PCR efficiency and probe signal. We then validated the methylation status of these eight CpGs in an independent set of 48 bladder tumors and 70 normal urines. Figure 1 shows the methylation % of the 8 markers.

Selection of the optimal combination of markers for the detection of recurrent BC in voided urine

The eight markers were then analyzed on a test set of 101 voided urines collected from different patients before resection of a recurrent tumor. We calculated the power of each methylation marker by calculating the AUC using the 101 recurrent urines against the 70 control samples. Specificity was set at 90%. The best single marker in the test set was OTX1 with a sensitivity of 65%. Performance of the 8 markers on the test set is shown in Table 2 and Figure 1. The p-values in Table 2 indicate that the markers are significantly different between urines from patient and healthy individuals. In addition we analyzed sensitivity of each marker regarding detection of different stage and grade recurrences. Sensitivity increased with stage and grade as shown in Supplementary Table 2.

A 3-plex sensitive and reproducible methylation assay

Next we performed logistic regression with all possible combinations and picked the combination of three CGIs (in the OTX1, ONECUT2 and OSR1 genes) with the highest
sensitivity. The probability value was calculated using the following formula: 
\[ z = -1.618 + \% \text{methylation} \, OTX1 \times 0.168 + \% \text{methylation} \, ONECUT2 \times 0.050 + \% \text{methylation} \, OSR1 \times 0.016. \]

A probability cut-off value of 0.580 was chosen, allowing 10% false positives in the normal urines, which is shown in Supplementary Figure 2. This resulted in a sensitivity of 68% in the test set. Sensitivity, AUC, PPV, NPV of this combination are presented in Table 2. The 3 markers were subsequently combined in a 3-plex BS-SNaPshot assay as depicted in Supplementary Figure 3. The 3-plex methylation assay showed sensitivities of 64, 77 and 86% for Ta, T1 and T2 recurrences, respectively and 57, 65 and 81% for detecting G1, G2 and G3 tumors (Supplementary Table 2). Reproducibility of the assay was investigated independently by two investigators on 16 urine samples. Percentages of methylation were highly correlated (Supplementary Figure 4). In order to get an impression of methylation in the case of multiple metachronous tumors, we selected patients with multiple tumors (30 tumors from 11 patients) from the original validation array (28). Methylation of the 3 genes was highly consistent within a patient (Supplementary Table 3).

**Validation of the best combination of markers in an independent set of urines**

Subsequently, the 3-plex assay was tested on an independent validation set of 95 preTUR urines from patients with a previous NMIBC G1/G2. A design for this case-control validation study is given in Figure 2. The best single marker in the test set *OTX1* also showed the highest sensitivity (72%) in the validation set. The 3-plex assay achieved a sensitivity of 74% with an AUC of 0.86 (Table 3, Supplementary Figure 5). We achieved a sensitivity of 80% for the detection of primary NMIBC G1/G2 tumors. We further observed 22% false positives in 40 urines investigated from BC patients who did not have a recurrence 6 months following urine collection (Figure 1 and Supplementary Figure 2). We have tested our urine assay on the following number of non-bladder cancer related urological afflictions: High leucocyte count (n=25), cystitis patients (n=18), lower urinary tract symptoms (n=30), prostate cancer.
patients (n=14) and renal cancer patients (n=3). We have observed 2/25 as positive in high leucocyte samples, 7/18 as positive in cystitis patients, 7/30 as positive in lower urinary tract symptoms patients and 4/14 as positive in prostate cancer patients. In renal cancer patients 1 out of 3 patients was positive, but this patient was diagnosed with an upper urinary tract urothelial tumor.

Next we combined the 3-gene methylation panel with FGFR3 status of the preTUR urine. This lead to an increase of 5% sensitivity in the validation set achieving 79% sensitivity with an AUC of 0.89 for the detection of recurrent BC in voided urine (Table 3). The p-values in Table 3 indicate that the markers/assays are significantly different between urines from patient and healthy individuals. The AUC curve for the combination of the methylation and the FGFR3 assay is shown in Supplementary Figure 5. The data of cytology, FGFR3 and methylation was available for 72 urines. For this set of urines we calculated the sensitivity, PPV, NPV and AUC of cytology alone, methylation+cytology and methylation+cytology+FGFR3. Results are shown in Table 3. Spearman correlation showed a significant correlation among three methylation markers. The 3-plex methylation assay also showed a significant correlation with Cytology and FGFR3. This is shown in supplementary Table 4.

The 3-plex methylation assay is more sensitive than the FGFR3 and cytology

Next, we compared the 3-plex methylation assay with the FGFR3 assay and cytology in the validation set. Methylation, FGFR3 mutation status and cytology information was available for 72 urine samples. The methylation assay showed a sensitivity of 74%, while it was 57% for cytology and 52% for FGFR3, respectively. When we split this group according to grade, the sensitivity increased from low to high grade as shown in Table 4.

The sensitivities of the combinations Methylation+FGFR3, Methylation+Cytology and Methylation+FGFR3+Cytology were 79, 77 and 82%, respectively. These results show that
the methylation assay is more sensitive than cytology and the FGFR3 assay (when patients were not stratified based on the FGFR3 or methylation status of their primary tumor).
Discussion

A major problem in the management of patients presenting with NMIBC is that 70% will develop one or more recurrences and that recurrences can keep on developing for up to 25 years (9). Surveillance of these patients by cystoscopy is warranted (32). However, cystoscopy is an invasive diagnostic procedure that is not well tolerated by many patients. Cytology has a high sensitivity for high-grade lesions, but lacks sensitivity for low-grade tumors (17). To provide an alternative for cystoscopy and cytology, the development of molecular non-invasive tests using voided urine has been a major undertaking in the last decade. However, what is still lacking are tests that address the patient population in question, i.e. patients under surveillance for potential recurrences after a primary G1/G2 NMIBC. One-third of these patients do not develop recurrences at all and low/intermediate risk patients may develop only few recurrences over a long period of time. Hence, surveillance by too frequent cystoscopies can be considered as overtreatment of many of these patients. An ideal test for surveillance of BC that can replace cystoscopy should be urine based, sensitive, cost-effective, easy to perform with limited material, and with no intraobserver variability.

The low sensitivity of cytology induced the development of urine-based assays in the past decade (20, 33). In summary, these assays are based on immunological assays to detect tumor cells, differentially expressed genes, tumor-associated proteins and tumor-specific DNA alterations (17). Three of these tests have been approved by the FDA (Federal Drug Administration, USA), namely NMP22, UroVysion, and ImmunoCyt (15). Although most tests have better sensitivity than urinary cytology, their specificity is lower and their sensitivity for low grade recurrent tumors is also insufficient. Hence, none of them have been accepted as a standard diagnostic procedure in routine urology to date. The FGFR3 mutations test is an excellent diagnostic test for recurrent cancer in patients presenting with NMIBC if
their primary tumor harbors a mutation. Evidently, other tests are required for those patients with \textit{FGFR3} wild-type tumors.

To develop an accurate urine based assay next to the \textit{FGFR3} assay, we developed a 3-plex methylation assay for the diagnosis of recurrent BC. To our knowledge this is the first study, where the methylation markers were assessed during follow-up of patients with a primary Ta/T1G1/G2 tumor. Our three gene methylation panel consisting of \textit{OTX1}, \textit{ONECUT2} and \textit{OSR1} had a sensitivity of 68 and 74% in the test and validation set respectively with a specificity of 90% for the detection of recurrent bladder tumors in voided urine. Previously we and Serizawa et al., (34) showed an inverse correlation between \textit{FGFR3} mutation and methylation, therefore a combination of these assays could increase sensitivity for the detection of recurrent BC. We therefore combined the 3-plex methylation assay with the \textit{FGFR3} mutation assay. The combination of both assays increased sensitivity to 79% in the validation set.

It is our experience that many urine samples contain low amounts of cells and a yield of 50 nanogram DNA from 50 ml of urine is no exception. The \textit{FGFR3} and 3-plex assays require 5 and 30 nanogram DNA each. Hence, there will be sufficient DNA in most urine samples to perform these assays. Both assays are easy to perform in a standard molecular diagnostic laboratory. Moreover, we have shown that they are highly reproducible between different operators. Combined material costs of the two assays, including DNA isolation, amount to about 30€. Personnel costs depend on the number of assayed samples, being cheaper when many samples are analyzed simultaneously.

The fact that not all recurrences are detected with the urine assays is most probably due to the absence or low concentration of tumor cells in the urine sample. This could probably be improved by analyzing more than one urine sample as we showed previously for the \textit{FGFR3} mutation assay (27). Another possibility would be to increase the analytical sensitivity of the
assays. For the $FGFR3$ assay we obtained an analytical sensitivity of about 5% (the mutation is detected when more than 5% of the cells harbor the mutation (31)). For the similar 3-plex methylation assay this would be the same. Higher analytical sensitivity can possibly be obtained by using next-generation sequencing (NGS). However, at the moment the cost of methylation+$FGFR3$ as performed in this work is still much cheaper than NGS. We further observed that multiple tumors from a patient have highly concordant methylation of the 3 markers, which underlines the usefulness of the markers for surveillance. The sensitivity of our assay combination is similar to the sensitivity of the current gold standard white light cystoscopy with sensitivity in the range of 68 to 83% when compared to the more sensitive blue light cystoscopy (35). Given the comparable sensitivities of urine testing and cystoscopy, we suggest that, a subsequent study should be performed investigating a modified surveillance protocol consisting of the urine test followed by cystoscopy, only when the urine test is positive, for patients in the low/intermediate risk BC group.

A number of recent studies have reported high sensitivities with DNA methylation biomarkers for the detection of BC in voided urine (36-43). Three of these studies used methylation specific PCR (MSP) and reported sensitivities in the range of 85 to 94% at a specificity in the range of 93 to 100%. Renard et al showed a high sensitivity for the combination of the $TWIST1$ and $NID2$ genes for the diagnosis of primary BC. Reinert and colleagues used a methylation sensitive high resolution melting analysis and reported 84% sensitivity at 96% specificity. The study by Zuiverloon (44) et al., reported a four gene methylation panel to detect recurrent bladder tumors with a sensitivity of 72% at a specificity of 55%. However, all these studies, with the exception of Zuiverloon et al, used urines from patients with primary and recurrent tumors, including high-grade and MIBC. Hence the detection rate of recurrent tumors developing after a primary NMIBC G1/G2 using these markers is probably much lower. The CGIs found by others to be methylated in BC were
largely also found to be methylated in our genome-wide study, but to a lesser extent than the ones we chose for our diagnostic assay.

The *OTX1* (orthodenticle homeobox 1) gene is methylated in lung cancer (45) and its expression is regulated by p53 in breast cancer (46). The *OSR1* (odd-skipped related 1) gene is methylated in lung and breast cancer (45, 47). The *ONECUT2* (one cut homeobox 2) gene participates in the network of transcription factors regulating liver differentiation and metabolism (48). This gene is methylated in lymphomas and lung cancer (49, 50). The methylation of these genes in BC was not reported before.

In conclusion here we report a genome-wide methylation investigation in BC, followed by a selection and validation strategy to develop a 3-plex methylation assay specific for the detection of recurrent BC. The combination of the 3-plex methylation assay and the *FGFR3* assay efficiently detects recurrent BC without the need for up-front stratification of patients. Given the comparable sensitivities of urine testing and cystoscopy, we suggest that, a subsequent study should be performed investigating a modified surveillance protocol consisting of the urine test followed by cystoscopy, only when the urine test is positive, for patients in the low/intermediate risk BC group. Further validation of the presented markers in a prospective longitudinal study is underway.
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Author contributions

R. Kandimalla conceived, carried out experiments, analyzed data and wrote the manuscript. R. Masius did data analysis, carried out experiments and helped in manuscript preparation. N. van Leeuwen and H. Lingsma supervised the statistical analysis and helped with the presentation of patient and control cohorts. R. Kandimalla, R. Masius, W. Beukers, C.H. Bangma, T. Orntoft, L. Dyrskjot, A. van Tilborg did data and sample collection and involved in manuscript preparation. C.H. Bangma, T. Orntoft, L. Dyrskjot also helped in providing patient samples. E. Zwarthoff did the study design, supervised the study, interpreted the data, reviewed the MS, obtained funding and is the corresponding author.
References


Figure legends

Figure 1. Scatter plots of methylation percentage of each marker in the samples investigated. 
A) Urines from healthy individuals (n=70) B) Urines from patients without recurrence (n=40) 
C) Tumor tissue (n=48) D) Test set urines (n=101).

Figure 2. Design of the validation case-control study.
Table 1. Patient characteristics

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<th>Validation set</th>
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Table 2. Sensitivity, PPV, NPV and AUC of the individual markers in the test set (n=101). Specificity is set at 90%. The combination of OTX1, ONECUT2 and OSR1 was found to be the most sensitive combination of markers.

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<td>HOXA9</td>
<td>62 (62/101)</td>
<td>90</td>
<td>63</td>
<td>.829</td>
<td>.770</td>
<td>.888</td>
<td>.000</td>
</tr>
<tr>
<td>OSR1</td>
<td>44 (44/101)</td>
<td>86</td>
<td>53</td>
<td>.705</td>
<td>.630</td>
<td>.781</td>
<td>.000</td>
</tr>
<tr>
<td>OTX1_ONECUT2_OS1</td>
<td>68 (68/101)</td>
<td>91</td>
<td>67</td>
<td>.801</td>
<td>.734</td>
<td>.867</td>
<td>.000</td>
</tr>
</tbody>
</table>
Table 3. Validation of 3-plex methylation assay in an independent set of 95 preTUR urines (validation set, n=95) alone and in combination with the FGFR3 assay and cytology. Specificity of 3-plex assay, 3-plex assay plus FGFR3 assay, 3-plex assay plus cytology and 3-plex assay together with cytology and FGFR3 is set at 90% (test set), while it is 100% for the FGFR3 assay and cytology.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity</th>
<th>PPV</th>
<th>NPV</th>
<th>AUC</th>
<th>95% CI-</th>
<th>95% CI-</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>Methylation (OTX1+ONECUT2+OSR1)</td>
<td>74</td>
<td>91</td>
<td>72</td>
<td>.864</td>
<td>.808</td>
<td>.919</td>
<td>.000</td>
</tr>
<tr>
<td>FGFR3</td>
<td>52</td>
<td>100</td>
<td>58</td>
<td>.762</td>
<td>.692</td>
<td>.832</td>
<td>.000</td>
</tr>
<tr>
<td>Methylation + FGFR3</td>
<td>79</td>
<td>92</td>
<td>76</td>
<td>.886</td>
<td>.835</td>
<td>.938</td>
<td>.000</td>
</tr>
<tr>
<td>Cytology</td>
<td>57</td>
<td>100</td>
<td>70</td>
<td>.785</td>
<td>.707</td>
<td>.863</td>
<td>.000</td>
</tr>
<tr>
<td>Methylation + Cytology</td>
<td>77</td>
<td>89</td>
<td>79</td>
<td>.890</td>
<td>.833</td>
<td>.947</td>
<td>.000</td>
</tr>
<tr>
<td>Methylation + Cytology + FGFR3</td>
<td>82</td>
<td>89</td>
<td>83</td>
<td>.904</td>
<td>.850</td>
<td>.959</td>
<td>.000</td>
</tr>
</tbody>
</table>

Table 4. Comparison of the sensitivities of the methylation assay, FGFR3 and cytology and sensitivities of the various combinations (validation set, n=95)

<table>
<thead>
<tr>
<th>Assay</th>
<th>G1 (n=33)</th>
<th>G2 (n=52)</th>
<th>G3 (n=9)</th>
<th>Overall (n=94)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylation</td>
<td>67 (22/33)</td>
<td>77 (40/52)</td>
<td>78 (7/9)</td>
<td>74 (70/94)</td>
</tr>
<tr>
<td>FGFR3</td>
<td>60 (20/33)</td>
<td>48 (25/52)</td>
<td>56 (5/9)</td>
<td>52 (49/94)</td>
</tr>
<tr>
<td>Cytology</td>
<td>36 (9/25)</td>
<td>67 (26/39)</td>
<td>75 (6/8)</td>
<td>57 (41/72)</td>
</tr>
<tr>
<td>Methylation + FGFR3</td>
<td>73 (24/33)</td>
<td>81 (42/52)</td>
<td>78 (7/9)</td>
<td>79 (74/94)</td>
</tr>
<tr>
<td>Methylation + Cytology</td>
<td>68 (17/25)</td>
<td>82 (32/39)</td>
<td>87 (7/8)</td>
<td>77 (55/72)</td>
</tr>
<tr>
<td>Methylation + FGFR3 + Cytology</td>
<td>72 (18/25)</td>
<td>87 (34/39)</td>
<td>87 (7/8)</td>
<td>82 (59/72)</td>
</tr>
</tbody>
</table>
Clinical suspicion: n=238

Reference standard: cystoscopy

Gate criteria: previous primary tumor, Ta/T1 G1/G2, and histologically proven tumor at TUR

Target condition present: n=103

Target condition absent: n=135

Excluded: n=8 (primary tumor G3)

Excluded: n=5 (DNA concentration too low)

Index test: 3-plex and FGFR3

- n=95
  - Sensitivity 79% (75 of 95 positive)

- n=130
  - Specificity 77% (100 of 130 negative)
A 3-plex methylation assay combined with the FGFR3 mutation assay sensitively detects recurrent bladder cancer in voided urine

Raju Kandimalla, Roy Masius, Willemien Beukers, et al.

*Clin Cancer Res* Published OnlineFirst July 10, 2013.

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