

A 3-Plex Methylation Assay Combined with the *FGFR3* Mutation Assay Sensitive Detects Recurrent Bladder Cancer in Voided Urine

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Abstract

Purpose: DNA methylation is associated with bladder cancer and these modifications could serve as useful biomarkers. *FGFR3* mutations are present in 60% to 70% of non-muscle invasive bladder cancer (NMIBC). Low-grade bladder cancer recurs in more than 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 CpG islands (CGI) methylated in bladder cancer 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 aged more than 50 years. A 3-plex assay for the best combination was developed and validated on an independent set of 95 preTUR, recurrence free, and nonmalignant urines ($n = 130$).

Results: The 3-plex assay identified recurrent bladder cancer 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 bladder cancer 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. *Clin Cancer Res*; 19(17); 4760–9. ©2013 AACR.

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 (NMIBC; stages pTa, pT1 and CIS) it is 90% (2, 3). These different forms of bladder cancer are also associated with different genetic changes. Somatic mutations in the *FGFR3* gene accompanied with losses of chromosome 9 are more

frequent in NMIBC, whereas TP53 mutations are associated with muscle-invasive bladder cancer (MIBC) (4–6). Unfortunately, 70% of patients with NMIBC will have one or more recurrence after transurethral resection (TUR) and 10% to 20% of patients will eventually have progression to MIBC (7–9). Patients with NMIBC are currently stratified into low/intermediate and high-risk groups regarding progression and recurrence based on clinicopathologic 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 to 12 months after TUR makes bladder cancer one of the most expensive cancers to treat (14).

Cytologic 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 U.S. Food and Drug Administration (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

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Translational Relevance

Seventy percent 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 bladder cancer 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 bladder cancer group.

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 was disappointing (17, 21). Because of these problems, our group has specifically focused on markers that are better in detecting recurrent bladder cancer 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 (*FGFR3*) 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 as 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. To find the most promising CGIs for bladder cancer prognosis and diagnosis, we previously conducted a genome-wide study (28). The purpose of this study was 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 (Rotterdam, the Netherlands) 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 TUR of the corresponding recurrent tumor. Inclusion criteria were presence of a previous primary NMIBC G1/G2 tumor and histologically confirmed recurrence at TUR. Exclusion criteria were the presence of a G3 primary tumor.

Test samples and validation samples

Forty-eight formalin-fixed paraffin embedded (FFPE) bladder cancer 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 years) 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 to carry out 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 urine samples 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), patients with cystitis ($n = 18$), patients with lower urinary tract symptoms ($n = 30$), patients with prostate cancer ($n = 14$), and patients with renal cancer ($n = 3$). All samples were collected before 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 Standards for Reporting of Diagnostic accuracy (STARD) guidelines and the guidelines presented by Pepe and colleagues 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 hours after voiding, samples were centrifuged at 4,000 rpm for 10 minutes, followed by washing the pellet twice with cold PBS. DNA was isolated using DNeasy Tissue kit (Qiagen GmbH). 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). PCR of selected CGIs was conducted using 20 ng of converted DNA, 20 pmols of primers, and 10 µL of KAPA2G Robust HotStart ReadyMix (Kapa Biosystems) 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).

Table 1. Patient characteristics

Characteristics	Test set			Validation set
	Pre-TUR urines (recurrence; <i>n</i> = 101)	Pre-TUR urines (primary tumor; <i>n</i> = 39)	FFPE DNA (<i>n</i> = 48)	Pre-TUR urines (recurrence; <i>n</i> = 95)
Gender				
Male	78	29	39	52
Female	23	10	9	43
Age				
<65	38	17	15	36
>65	63	22	33	59
Stage				
Ta	73	39	39	93
T1	13	0	8	0
> = T2	14	0	1	1
NA	1	0	0	1
Grade				
G1	23	16	15	33
G2	46	23	25	52
G3	27	0	2	9
NA	5	0	0	1
Multiplicity				
Solitary	36	3	NA	69
Multiple	48	26	NA	26
NA	17	10	NA	0
Tumor size				
<3 cm	27	25	NA	90
>3 cm	5	4	NA	4
NA	69	10	NA	1
<i>FGFR3</i>				
Mutant	10	15	39	49
Wild-type	91	24	9	46

This was followed by a single-nucleotide probe extension assay using a SNaPshot Multiplex kit (Applied Biosystems) 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 conducted in a total volume of 10 μ L containing 2 μ L SAP/ExoI-treated PCR product, 2.5 μ L SNaPshot Multiplex Ready Reaction mix, 1 \times Big Dye sequencing buffer, and 1 μ L 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 minutes, and at 75°C for 15 minutes, 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 3 most interesting CGIs (*OTX1*, *ONECUT2*, and *OSR1*) in a single PCR and a single nucleotide primer extension reaction. *FGFR3* muta-

tion analysis was conducted 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 bladder cancer in voided urine

For the BS-SNaPshot data, we used GeneMarker Software version 1.7 (SoftGenetics, State College). 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 \times 100. To predict the best combination, we conducted logistic regression on all possible combinations of the eight markers using test set of 101 pre-TUR urines with recurrence and 70 control urine samples. 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 $z = \text{constant} + \% \text{ methylation A} \times \beta \text{ value A} + \% \text{ methylation B} \times \beta \text{ value B}$ 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 three-marker combination that showed the highest sensitivity in the test set. We calculated the sensitivity and specificity by plotting the receiver operating characteristic curve and calculating the area under the curve (AUC) using SPSS statistical software V17.0.

Independent validation of the three-marker combination

We then validated the three-marker combination in an independent set of 95 pre-TUR urines from patients with a previous NMIBC G1/G2 and 130 controls. In addition, we analyzed sensitivity of each marker regarding detection of different stage and grade recurrences. To this 3-plex methylation assay, we added *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 Fig. S1. Patient characteristics for all cohorts are given in Table 1. Previously, we identified 110 CGIs methylated in bladder cancer 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-SNaP-shot assays on DNA obtained from bladder cancer 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 8 CpGs in an independent set of 48 bladder tumors and 70 normal urines. Figure 1 shows the percentage of methylation of the 8 markers.

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

The 8 markers were then analyzed on a test set of 101 voided urine samples 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 Fig. 1. The *P* values in Table 2 indicate that the markers are significantly different between urine samples 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 S2.

A 3-plex sensitive and reproducible methylation assay

Next, we conducted logistic regression with all possible combinations and picked the combination of 3 CGIs (in the *OTX1*, *ONECUT2*, and *OSR1* genes) with the highest sen-

sitivity. 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 Fig. S2. This resulted in a sensitivity of 68% in the test set. Sensitivity, AUC, positive predictive value (PPV), negative predictive value (NPV), of this combination are presented in Table 2. The 3 markers were subsequently combined in a 3-plex BS-SNaP-shot assay as depicted in Supplementary Fig. S3. 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 S2). Reproducibility of the assay was investigated independently by two investigators on 16 urine samples. Percentages of methylation were highly correlated (Supplementary Fig. S4). 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 S3).

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 pre-TUR urines from patients with a previous NMIBC G1/G2. A design for this case-control validation study is given in Fig. 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 Fig. S5). 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 patients with bladder cancer who did not have a recurrence 6 months following urine collection (Fig. 1 and Supplementary Fig. S2). We have tested our urine assay on the following number of non-bladder cancer related urological afflictions: High leucocyte count ($n = 25$), patients with cystitis ($n = 18$), patients with lower urinary tract symptoms ($n = 30$), patients with prostate cancer ($n = 14$), and patients with renal cancer ($n = 3$). We have observed 2 of 25 as positive in high leucocyte samples, 7 of 18 as positive in patients with cystitis, 7 of 30 as positive in patients with lower urinary tract symptoms, and 4 of 14 as positive in patients with prostate cancer. In patients with renal cancer, 1 of 3 patients was positive, but this patient was diagnosed with an upper urinary tract urothelial tumor.

Next, we combined the three-gene methylation panel with *FGFR3* status of the pre-TUR urine. This led to an increase of 5% sensitivity in the validation set achieving 79% sensitivity with an AUC of 0.89 for the detection of recurrent bladder cancer 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

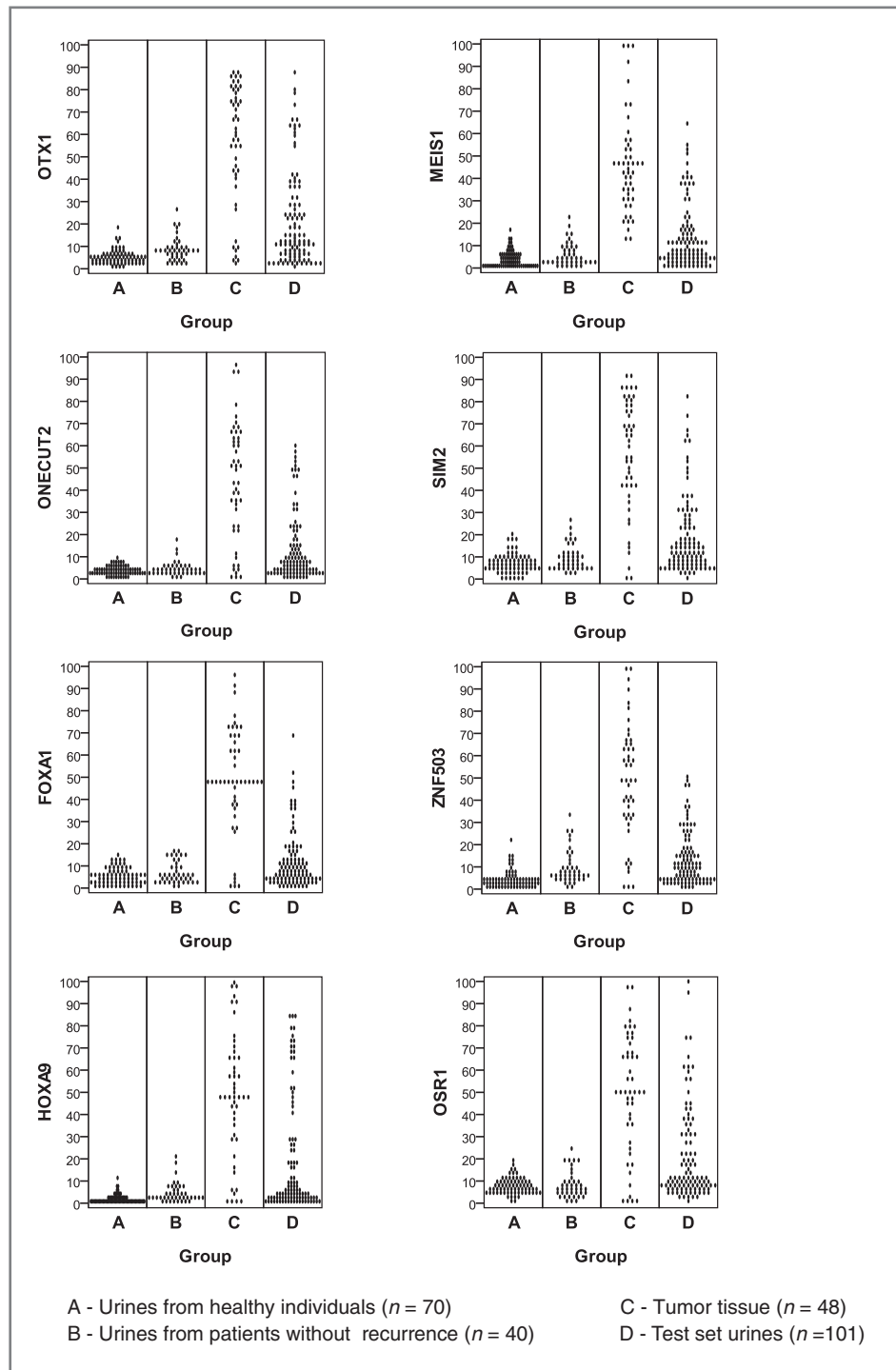


Figure 1. Scatter plots of methylation percentage of each marker in the samples investigated. A, urines from healthy individuals ($n = 70$). B, urines from patients without recurrence ($n = 40$). C, tumor tissue ($n = 48$). D, test set urines ($n = 101$).

methylation and the *FGFR3* assay is shown in Supplementary Fig. S5. 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 S4.

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

Table 2. Sensitivity, PPV, NPV and AUC of the individual markers in the test set (n = 101)

Gene	Sensitivity	PPV	NPV	AUC	95% CI	P
<i>OTX1</i>	65 (65/101)	90	65	0.805	0.740–0.871	0.000
<i>MEIS1</i>	46 (46/101)	87	54	0.749	0.678–0.821	0.000
<i>ONECUT2</i>	52 (52/101)	88	57	0.737	0.664–0.810	0.000
<i>SIM2</i>	49 (49/101)	88	56	0.753	0.683–0.824	0.000
<i>FOXA1</i>	38 (38/101)	84	50	0.659	0.579–0.739	0.000
<i>ZNF503</i>	52 (52/101)	88	57	0.784	0.717–0.852	0.000
<i>HOXA9</i>	62 (62/101)	90	63	0.829	0.770–0.888	0.000
<i>OSR1</i>	44 (44/101)	86	53	0.705	0.630–0.781	0.000
<i>OTX1_ONECUT2_OSRI</i>	68 (68/101)	91	67	0.801	0.734–0.867	0.000

NOTE: Specificity is set at 90%. The combination of *OTX1*, *ONECUT2* and *OSR1* was found to be the most sensitive combination of markers.

available for 72 urine samples. The methylation assay showed a sensitivity of 74%, whereas 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 on the basis of 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 noninvasive 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 bladder cancer that can replace cystoscopy should be urine based, sensitive, cost-effective, easy to perform with limited material, and with no intra-observer 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 immunologic 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, 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 *FGFR3* wild-type tumors.

To develop an accurate urine-based assay next to the *FGFR3* assay, we developed a 3-plex methylation assay for the diagnosis of recurrent bladder cancer. 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 *OTX1*, *ONECUT2*, and *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 and colleagues, (34) showed an inverse correlation between *FGFR3* mutation and methylation, therefore a combination of these assays could increase sensitivity for the detection of recurrent bladder cancer. We therefore combined the 3-plex methylation assay with the *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 ng DNA from 50 mL of urine is no exception. The *FGFR3* and 3-plex assays require 5 and 30 ng DNA each. Hence, there will be sufficient DNA in most urine samples to carry out these assays. Both assays are easy to conduct 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

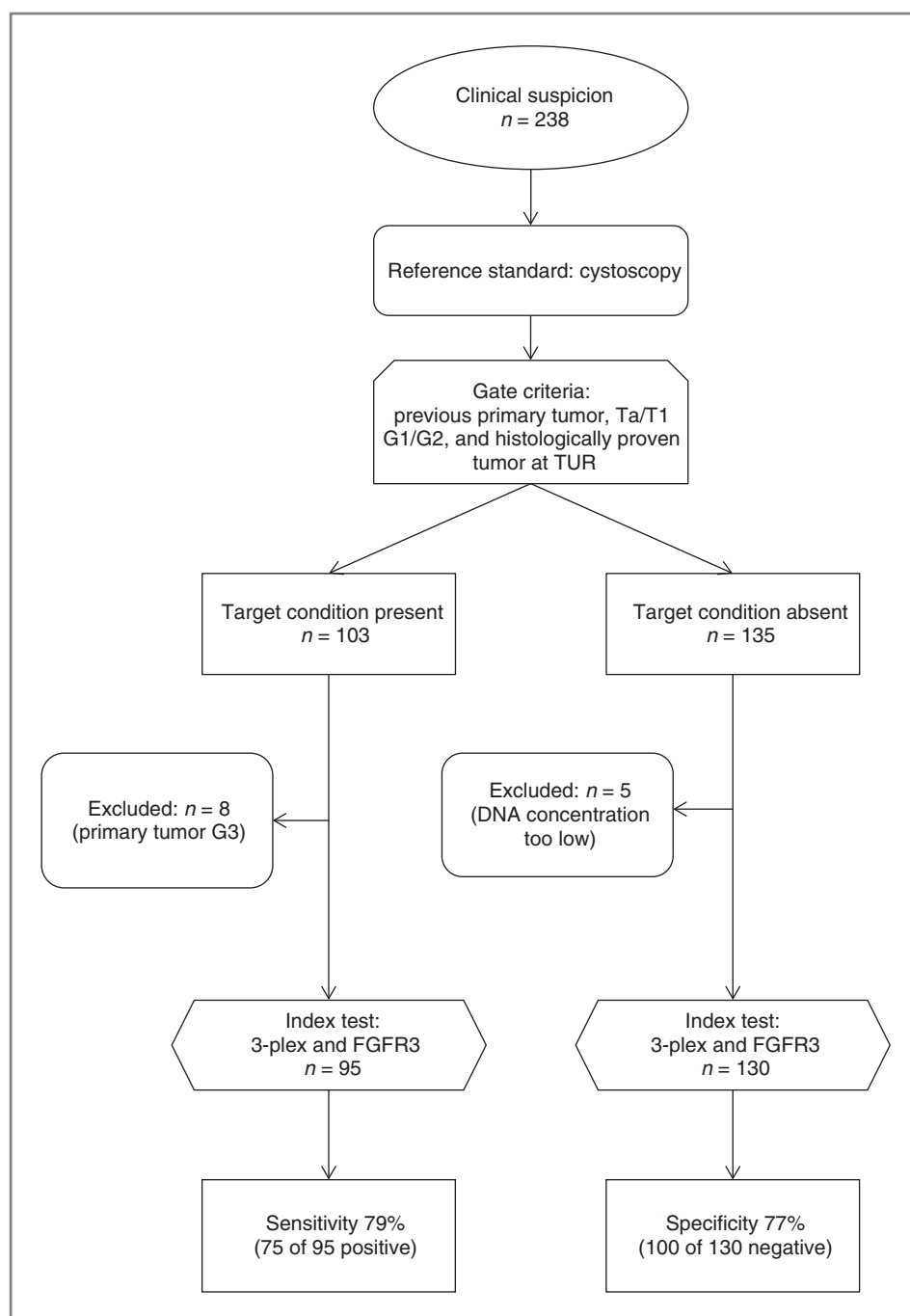


Figure 2. Design of the validation case-control study.

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 *FGFR3* mutation assay (27). Another possibility would be to increase the analytical sensitivity of the assays. For the *FGFR3* assay, we obtained an analytic 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 analytic 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

Table 3. Validation of 3-plex methylation assay in an independent set of 95 pre-TUR urines (validation set, $n = 95$) alone and in combination with the *FGFR3* assay and cytology

Assay	Sensitivity	PPV	NPV	AUC	95% CI	P
Methylation (<i>OTX1</i> + <i>ONECUT2</i> + <i>OSR1</i>)	74	91	72	0.864	0.808–0.919	0.000
<i>FGFR3</i>	52	100	58	0.762	0.692–0.832	0.000
Methylation + <i>FGFR3</i>	79	92	76	0.886	0.835–0.938	0.000
Cytology	57	100	70	0.785	0.707–0.863	0.000
Methylation + cytology	77	89	79	0.890	0.833–0.947	0.000
Methylation + cytology + <i>FGFR3</i>	82	89	83	0.904	0.850–0.959	0.000

NOTE: 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.

similar to the sensitivity of the current gold standard white light cystoscopy with sensitivity in the range of 68% to 83% when compared with the more sensitive blue light cystoscopy (35). Given the comparable sensitivities of urine testing and cystoscopy, we suggest that a subsequent study should be carried out 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 bladder cancer group.

A number of recent studies have reported high sensitivities with DNA methylation biomarkers for the detection of bladder cancer 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 and colleagues showed a high sensitivity for the combination of the *TWIST1* and *NID2* genes for the diagnosis of primary bladder cancer (42). Reinert and colleagues used a methylation-sensitive high-resolution melting analysis and reported 84% sensitivity at 96% specificity. The study by Zuiverloon and colleagues (44), 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 and colleagues, 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 bladder cancer 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 bladder cancer was not reported before.

In conclusion, here we report a genome-wide methylation investigation in bladder cancer, followed by a selection and validation strategy to develop a 3-plex methylation assay specific for the detection of recurrent bladder cancer. The combination of the 3-plex methylation assay and the *FGFR3* assay efficiently detects recurrent bladder cancer 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 carried out 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 bladder cancer group. Further validation of the presented markers in a prospective longitudinal study is underway.

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

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

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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