Clinical Validation of a Urine Test (Uromonitor-V2®) for the Surveillance of Non-Muscle Invasive Bladder Cancer Patients

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ABSTRACT

Background: Current follow-up for non-muscle invasive bladder cancer (NMIBC) consists of regular cystoscopies combined with urine cytology. Due to its costly and burdensome nature new follow-up methods are being developed. The Uromonitor-V2® is a urine assay based on the detection of hotspot alterations in three different genes (*TERT*, *FGFR3* and *KRAS*) for detecting disease recurrence.

Objective: to investigate the test properties of the Uromonitor-V2® for detecting NMIBC recurrence.

Design, Setting, and Participants: This was a prospective, blinded, single-visit, case-enriched cohort study. From February 2018 to September 2019 patients were enrolled. All subjects underwent a standard-of-care (SOC) cystoscopy, either as part of their follow-up for NMIBC or for a non-malignant urological pathology. A final number of 97 subjects were enrolled in the study. Twenty patients were non-malignant, 29 patients had a history of NMIBC with current disease recurrence and 49 patients had a history of NMIBC without a recurrence at time of enrollment.

Outcome Measurements and Statistical Analysis: The Uromonitor-V2® test characteristics for detecting disease recurrence were analyzed and compared with cytology results.

Results and Limitations: A total of 105 patients were enrolled of whom 97 were eligible for the study. In NMIBC the Uromonitor-V2® showed a sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of respectively 93,1%, 85.4%, 79.4% and 95.3%. Urine cytology was available for 52 patients and showed a sensitivity, specificity, PPV and NPV of respectively 26.3%, 90.9%, 62.5% and 68.2%.

Conclusions: The Uromonitor-V2® shows promising properties for the follow-up of patients with NMIBC. With its high NPV of 95.3% the test might be considered as an alternative for cystoscopy and cytology.

Patient summary: The Uromonitor-V2® has shown disease detecting properties that make it a considerable alternative for the current follow-up methods in patients with superficial bladder cancer.

1. Introduction

Bladder cancer (BCa) is the 10th most common type of cancer worldwide with 549,393 newly diagnosed cases in 2018. [1] The disease can be divided into two subtypes: non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). There is a distinct difference in the behavioral patterns of the two subtypes. NMIBC is infamous for its high number of disease recurrences with the 5-year recurrence rate ranging from 40% to 78%. [2-6] MIBC is known as the more aggressive counterpart with high disease mortality rates in spite of radical treatment. At initial diagnosis approximately 75% of the patients are diagnosed with NMIBC. [7] The majority of these patients will develop disease recurrence following diagnosis. Less frequent, although more ominous is the development of muscle invasive disease by patients previously diagnosed with a non-muscle invasive tumor. Disease progression in NMIBC occurs in up to 20% of the patients, with patients with CIS and/or T1HG disease being most at risk. [2-6]. Due to the high rates of disease recurrence and the lingering risk of developing progressive disease frequent follow-up is needed. The current follow-up schedule consists of regular surveillance cystoscopies possibly combined with urine cytology that should be maintained throughout many years following initial diagnosis, but can even span throughout life. [8] These extensive and lengthy schemes in NMIBC patients lead to high costs, making BCa the most expensive cancer when looking at patients' life time expenses. [9] Other drawbacks from the current follow-up strategies are the invasive nature of the cystoscopies and the low sensitivity of urine cytology, especially in low grade NMIBC. [10] There is an undisputable need for cheaper, better and less invasive methods for the follow-up of patients with a history of NMIBC. One way to potentially improve the current regime might be through the introduction of urinary biomarkers, either as an addition to current standard practice or even as a (partial) replacement of the current methods. Over the last decades, a multitude of biomarker based urine tests have been developed, but none of these have truly been implemented into the clinical practice to date. [11] Recently, a new urine test (the Uromonitor-V2®) was developed (U-monitor, Porto, Portugal). The test analyses a subset of hotspot alterations in three different genes (*TERT*, *FGFR3* and *KRAS*) using Real-Time qPCR. In a previous multicentre study on 122 NMIBC patients in follow-up, the test showed promising results with a sensitivity, specificity

and NPV of respectively 100%, 83.8% and 100% [12]. With our current study we aimed to validate these results in an independent cohort of NMIBC patients in follow-up.

2. Patients/Material and methods

2.1. Patients

This was a prospective, blinded, single-visit, case-enriched cohort study. All patients in this study were in follow-up at the Radboud University Medical Center in Nijmegen, the Netherlands, either because of a history of NMIBC or because of a benign, non-BCa related urological pathology. All were enrolled prior to undergoing a cystoscopy at the hospital's outpatient clinic. Three groups of patients were subsequently identified: 1) patients with a history of NMIBC with a recurrence at time of cystoscopy; 2) patients with a history of NMIBC without a recurrence at cystoscopy; and 3) patients without a history of BCa, undergoing cystoscopy for benign urological causes (bladder stones, BPH, bladder pain syndrome, etc.). The last group was used as a control to explore the effect of non-malignant urological pathologies on the Uromonitor-V2® test results. Patients were eligible for inclusion if they were ≥ 18 years of age, able to give written consent and provide a minimum of 10 ml of urine prior to undergoing SOC cystoscopy. NMIBC patients in follow-up could be enrolled if they had an event (initial or recurrent NMIBC) within five years prior to enrollment. Exclusion criteria were inadequate material for testing; a failed Uromonitor-V2® test; previous diagnosis with muscle invasive bladder, or in case of the benign control group any prior history of bladder cancer. Clinical information of all patients on e.g. age, sex, and smoking status was collected. Informed consent was obtained from all patients. All procedures described in this study were in accordance with national and institutional ethical standards and the Declaration of Helsinki.

2.2. Urine collection, sampling handling and testing

All urine collections were carried out during the standard clinical surveillance program of the participating patients, in which patients were asked to provide additional urine in parallel to their regular follow-up program. Additional cytology was collected for those patients with a history of NMIBC. Following urine collection, all patients underwent a SOC cystoscopy by an urologist or

an urologist in training who inspected the bladder for any abnormalities. After collection the urine was filtered using a pre-treated 0.80µm nitrocellulose syringe filter (Whatman® Filter - Z612545, Merck, Germany) containing a housemade conservative storage buffer [12].

Per patient ≥ 10ml of urine was collected. A minimum of two filters per patient was required to perform adequate in-duplo testing, with a minimum amount of 5ml being used per filter. After the filtration process the filters were shipped to the laboratory (Uromonitor Porto, Portugal) for further testing. The Uromonitor-V2® has been developed and optimized for the detection of pre-specified hotspot alterations (TERTp c.1-124C>T, TERTp c.1-146C>T, FGFR3 p.R248C, FGFR3 p.S249C, KRAS p.G12X, KRAS p.Q61X and KRAS p.A146X) through improved Locked Nucleic Acid (LNA) based real-time allelic discrimination assays and competitive allele-specific real-time detection PCRs. High molecular weight DNA was extracted from the filters using Norgen® Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen Biotek Corp, Canada), as described [12]. TERTp, FGFR3 and KRAS testing was performed on 25-50 ng of the extracted DNA. The extracted DNA was amplified and detected on a qPCR real-time machine (which machine?) using the proprietary chemistry for amplification and detection as provided in the Uromonitor-V2® test kit. Amplification signals were analyzed as recommended by the manufacturer (Uromonitor, Porto, Portugal). If at least one of the screened alterations provided a positive result then the test result was deemed positive.

2.3. Statistical analyses

Statistical analysis was carried out using 21.0 SPSS Statistical Package (SPSS, Inc.,220 2003). Descriptive statistics were performed and differences between groups were tested by the Student's t-test, Mann-Whitney test or One-Way ANOVA, according to variables and groups.

3. Results

3.1. Patients

From the 105 patients who were initially enrolled eight subjects were deemed ineligible for inclusion, leading to 97 eligible patients being included in the study. Of the eight ineligible patients five did not fit the inclusion criteria since they had been previously diagnosed with a muscle-invasive tumor, one sample had a failed Uromonitor-V2® test, while for the two remaining samples there was insufficient material for testing. Of the 97 eligible patients, there were 77 who had a history of NMIBC. Of these, 29 were found to be positive for recurrence during enrollment cystoscopy. Recurrence was defined as either pathologically proven disease following transurethral resection of the bladder tumor (TURBT) and/or based on the clinical decision of the urologist based on cystoscopy and/or cytology results. An overview of the clinicopathological information of the 97 included patients can be found in table 1. The three subject groups had no significant differences between them, except for the time since last treatment between the NMIBC patients with and without current recurrence. The time since last treatment was significantly longer in patients with recurrence (10.81 months (SD ± 18.84) than in those without recurrence (5.20 months (SD ± 6.31) ($p = 0.004$).

Table 1 - Clinicopathological information on all enrolled study subjects (n=97)

Characteristic	***Non bladder cancer (n=20)***	***NMIBC recurrence (n=29)***	***NMIBC non-recurrence (n=48)***
Age			
Median (min - max)	71 (22-82)	68 (50-85)	72,5 (49-93)
Gender			
Female	8 (40%)	9 (31%)	12 (25%)
Male	12 (60%)	20 (69%)	36 (75%)
Smoking			
No	1 (5%)	4 (13,8%)	6 (12,5%)
Yes, former	2 (10%)	19 (65,5%)	35 (72,9%)
Yes, current	0 (0%)	5 (17,2%)	7 (14,6%)
Unknown	17 (85%)	1 (3,4%)	0 (0%)
Most recent intravesical treatment			
Chemotherapy	N.A.	13 (44,8%)	21 (43,8%)
BCG	N.A.	5 (17,2%)	11 (22,9%)
Synergo	N.A.	8 (27,6%)	13 (27,1%)
Other	N.A.	1 (3,4%)	1 (2,1%)
None	N.A.	2 (6,9%)	2 (4,2%)
Time since last treatment (in months)			
Mean (min - max)	N.A.	10,81 (1-56)	5,20 (0-28)
Stage initial tumor			
PUNLMP	N.A.	2 (6,9%)	0 (0%)
pTa	N.A.	17 (58,6%)	27 (56,3%)
pT1	N.A.	3 (10,3%)	8 (16,7%)
CIS	N.A.	7 (24,1%)	13 (27,1%)
Grade initial tumor			
Low grade	N.A.	13 (44,8%)	19 (39,6%)
High grade	N.A.	16 (55,2%)	29 (60,4%)
Stage last recurrence			
pTa	N.A.	6 (20,7%)	N.A.
pT1	N.A.	2 (6,9%)	N.A.
CIS	N.A.	5 (17,2%)	N.A.
Not available	N.A.	15 (51,7%)	N.A.
Grade last recurrence			
Low grade	N.A.	2 (6,9%)	N.A.
High grade	N.A.	12 (41,4%)	N.A.
Not available	N.A.	15 (51,7%)	N.A.
Cytology of last recurrence			
TPS2	N.A.	13 (44,8%)	28 (58,3%)
TPS3	N.A.	1 (3,4%)	2 (4,2%)
TPS4	N.A.	3 (10,3%)	2 (4,2%)
TPS5	N.A.	2 (6,9%)	1 (2,1%)
Not available	N.A.	10 (34,5%)	15 (31,3%)

3.2. Test results

Out of the 97 patients who were successfully tested using the Uromonitor-V2® a total of 36 (37.1%) patients showed a positive result, while the other 61 (62.9%) were tested negative. Of those 97 patients, 29 subjects were positive for disease recurrence at time of enrollment. The Uromonitor-V2® was able to detect 27 of the recurrent samples, while leaving two samples undetected. Histological material from TURBT or biopsy of the tumor was available in 14 of the recurrence positive patients. Of the pathologically proven recurrences six were diagnosed with a Ta tumor, two with a T1 tumor, five patients presented with CIS, while one patient showed progression into a muscle invasive tumor. All 14 of the histologically proven patients showed a positive result for the Uromonitor-V2® test.

In total 36 samples were tested Uromonitor-V2® positive, of which 27 had an actual positive recurrence. For the other positive samples seven were of patients with a history of NMIBC without a current detectable recurrence by cytoscopy, while two were of patients without a history of bladder cancer. In the two benign patients with a positive Uromonitor-V2® result one patient underwent a cystoscopy for LUTS due to BPH, while the other patient presented with urge incontinence.

The Uromonitor-V2® showed an overall sensitivity of 93.1% (27/29), specificity of 86.8% (59/68), a PPV of 75.0% (27/36) and a NPV of 96.7% (59/61). In table 2 an overview can be found comparing the recurrence positive patients versus non-recurrence patients (control patients + non-recurrent NMIBC patients). When only including patients with a history NMIBC in the analysis, and excluding the 'healthy' control patients, the test characteristics remain comparable with a sensitivity of 93.1% (27/29), a specificity of 85.4% (41/48), a PPV of 79.4% (27/34) and a NPV of 95.3% (41/43). See table 3 for the test characteristics per patient group.

Table 2 - Test performances in enrolled patients (n=97), comparing recurrence negative (control patients + non-recurrent NMIBC patients) and recurrence positive patients (recurrent NMIBC patients).

Test result	Reccurence positive	Recurrence negative	Total
Positive	27	9	36
Negative	2	59	61
Total	29	68	97

Table 3- Test performances in enrolled patients (n=97), per subgroup.

Test result	NMIBC recurrence	NMIBC non-recurrence	Non bladder cancer	Total
Positive	27	7	2	36
Negative	2	41	18	61
Total	29	48	20	97

3.3. Cytology

Cytology results from time of enrollment cystoscopy were available for 52 samples. Nineteen of these were from patients with recurrent disease, of which five had a positive cytology; one cytology result was equivocal; while the other results were negative. All patients who had a positive cytology also tested positive for the Uromonitor-V2®. In these small numbers urine cytology showed a sensitivity, specificity, PPV and NPV of respectively 26.3%, 90.9%, 62.5% and 68.2%. An overview of the Uromonitor-V2® test characteristics in comparison with cytology can be seen in table 5.

Table 5 - Comparing Uromonitor-V2® test characteristics with urine cytology

Patients	Parameter	n/N	Result, % (95% CI)
Uromonitor-V2® *NMIBC patients*	Sensitivity	27/29	93,1 (75,8-98,8)
	Specificity	41/48	85,4 (75,8-93,4)
	PPV	27/34	79,4 (57,5-87,3)
	NPV	41/43	95,3 (87,6-99,4)
Uromonitor-V2® *All patients*	Sensitivity	27/29	93,1 (75,8-98,8)
	Specificity	59/68	86,8 (71,6-93,5)
	PPV	27/36	75,0 (61,6-90,7)
	NPV	59/61	96,7 (82,9-99,2)
Cytology	Sensitivity	5/19	26,3 (10,1-51,4)
	Specificity	30/33	90,9 (74,5-97,6)
	PPV	5/8	62,5 (25,9-89,8)
	NPV	59/65	68,2 (52,3-80,9)

Discussion

The high number of disease recurrences in NMIBC patients may partially be explained by shortcomings of the current follow-up methods. While white light cystoscopy (WLC) is an adequate method for detecting papillary lesions, it lacks adequacy in the detection of flat lesions (e.g. carcinoma in situ), dysplasia, multifocal tumors and microscopic lesions. [2] Methods to improve tumor visualization, like blue light cystoscopy (BLC) and narrow-band imaging (NBI) have therefore been developed. In BLC a fluorescent 'dye' is used which accumulates in neoplastic cells, while in NBI the red spectrum of white light is used to enhance the visual differentiation between hypovascularized BCa tissue and normal bladder tissue. These methods seemed promising in reducing the amount of disease recurrence, however current evidence is weak and the methods are not widely used. [8, 12-14]
The main advantage of urine cytology lies in the non-invasive nature of this method. However, also urine cytology is flawed since it is known to have low sensitivity, especially in patients with low-grade disease. Consequently, it is only of value as an additive to cystoscopy in patients with high-grade disease. [8, 10]

Not only are the current follow-up methods deficient, they also lead to high expenses, with BCa being accountable for 3% of all cancer costs within the European Union. [15] These high expenses and considerable flaws have led to the development of new and non-invasive methods of follow-up in NMIBC. Using Uromonitor-V2®, a non-invasive, urine-based test to monitor NMIBC patients recurrence, we found a sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of respectively 93,1%, 85.4%, 79.4% and 95.3%. Urine cytology was available for 52 patients and showed a sensitivity, specificity, PPV and NPV of respectively 26.3%, 90.9%, 62.5% and 68.2%.

Over the last decades multiple biomarker based urine assays have been developed to act as an additive to the current follow-up or even as a (partial) replacement of the current follow-up methods. Tests like the NMP22, BTA stat or UroVysion have been FDA-approved, but none of these tests are widely used in the clinic today. [16]. A major shortcoming of these assays is their relatively low NPVs. A urine assay that aims to (partially) replace cystoscopy in the follow-up of

NMIBC is required to have a very high NPV, as to assure the urologist no tumors are being overlooked. None of the FDA-approved tests are able to provide a NPV that is clinically relevant. [16]

Some of the latest additions (the CxBladder Monitor; the Xpert Bladder Monitor; and the Bladder EpiCheck) have shown more promising results, In the overall BCa population the Xpert Bladder Monitor proved to have a NPV of 93%, while the CxBladder and the Bladder Epicheck displayed a NPV of 95%, Despite overall performance is lower in low-grade tumors, for example Epicheck demosntrated overall sensitivity and specificity of 68.2% and 88.0%, both the Xpert Bladder Monitor and the Bladder Epicheck have shown higher NPVs in high-grade tumors, with NPVs of respectively 98% and 99.3%. [17-19] .

With an overall NPV of 95.3%, and a NPV of 100% for high grade tumors, the Uromonitor-V2®,has shown a comparable high performance in screening the cohort of NMIBC patients with clear advantages in the implement ability across different centers or laboratories. First it is a real-time PCR-based method, a methodology that is well implemented in most laboratories, and not requiring a specialized technician to execute the test; second, it uses affordable equipment and has a reduce cost; and last, it is a fast test that has the capacity to output a result in 6 hours. The alternatives tests are next generation sequencing (NGS)-based methods that required a sample and library preparation, failing in a short-time response, the costs would also increase with run and equipment requirements and NGS equipment and specialized staff is not widely available.

A definitive, evidence-based answer to the question of which molecular test has the best performance will only come from a head-to-head comparison study performed in the same urine samples of the same patients. Such study is still missing and without such scientific evidence no definitive conclusion can be drawn. Histological evidence of recurrence was available in 14 of the 29 NMIBC recurrent patients. The decision whether a patient with disease recurrence was referred for tumor removal was based on the visual appearance of the tumor and prior tumor history, but noteworthy 100% of the resected bladder tumors were detected by the Uromonitor-V2®. Only two tumors that were detected by cystoscopy and/or cytology were missed by the Uromonitor-V2®. Both were of patients previously diagnosed with pTa, low-grade tumors and the tumors were relatively small in diameter (max. 5mm and 12mm). However, missing these

small, low-grade tumors could be considered a defendable act since they do not determine the course of the disease. Without histological confirmation, there is also the possibility that these may also consist of cystoscopy or cytology false positives.

Due to the relatively low number of pathologically confirmed recurrences in this study no sub-analysis on high-grade recurrences was conducted. Therefore, it would be interesting to test the Uromonitor-V2® in a larger cohort of high-grade NMIBC patients in future research. A limitation in our study is the absence of follow-up data, especially in NMIBC patients who presented with a positive Uromonitor-V2® test, but who were considered recurrence negative according to current follow-up methods. These false-positive patients are of interest, since they might harbor microscopical recurrences that could not be detected by the current combination of cystoscopy and cytology.

4. Conclusions

With a sensitivity of 93.1%, specificity of 85.4% and a NPV of 95.3% this study showed that the Uromonitor-V2® has promising test characteristics for detecting disease recurrence in patients under follow-up for NMIBC. The presence of non-malignant urological pathologies does not seem to interfere with the Uromonitor-V2® test results, while only being tested in a limited number of samples. It has shown its potential as an alternative to the current follow-up methods. Additional research in a larger cohort of high-grade NMIBC patients should be conducted to determine whether the Uromonitor-V2® could serve as a (partial) replacement for cystoscopy and cytology.

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