Research Article

Liquid Biopsy Analysis of FGFR3, TERT Promoter and STAG2 Hotspot Mutations for Disease Surveillance in Bladder Cancer

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ABSTRACT

Patients with non-muscle invasive bladder cancer (NMIBC) are followed by frequent cystoscopies. Innovative approaches partly replacing cystoscopy (uncomfortable, expensive, low sensitive procedure) are demanded. The current study aims to establish a fast, reliable, non-invasive, and inexpensive procedure for NMIBC patient surveillance. Liquid biopsy is a reliable source of biomarkers for cancer patient monitoring. Urine is the most suitable biological liquid to search for bladder cancer biomarkers. Cell-free DNA in urine represents tumor-related mutations for several cancers, including the bladder. We investigated mutations in FGFR3, TERT promoter, and STAG2 as markers for diagnostics and follow-up in NMIBC. Digital PCR was used to detect mutations in urine-derived cell-free DNA. The sensitivity and specificity of the markers in relation to clinical outcomes served as criteria of the assay efficiency. The sensitivity with a single marker (TERT) reached 87%, with a specificity of 77%. Combining two biomarkers (TERT+FGFR3) increased the specificity of the assay to 100% with a sensitivity of 72%. Different mutational status of STAG2 can indicate NMIBC presence or recurrence. Therefore, applying the suggested combination of biomarkers with simple detection procedures to larger patient cohorts will allow developing procedures for BC detection and surveillance with optimal sensitivity and specificity. Based on the results of this proof-in-concept study, we conclude that this simple, fast and inexpensive assay can add diagnostic and prognostic value to cystoscopy/cytology analysis of NMIBC patients.

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Introduction

The combination of urine cytology and cystoscopy is the current gold standard to monitor bladder cancer (BC) and to detect the recurrence. The main advantage of urine cytology is that it is non-invasive, cheap, and easy to perform. The high specificity (>90%) of urine cytology makes it a very attractive option, but selectivity is relatively low (<30%), especially for low-grade tumors. The sensitivity of cystoscopy is approximately 80%; so some tumors escape detection. Cytoscopy is expensive and uncomfortable for patients and can be damaging. In addition, it might be subjective and operator dependent [1]. Therefore, other, preferentially non-invasive techniques are desirable for the detection and surveillance of bladder tumors. These techniques must provide access to reliable biomarkers for BC monitoring. Biological fluids are the best source of such biomarkers [2]. For cancer research, plasma and other biological fluids were successfully used for the analysis of such biomarkers [3]. For BC monitoring, urine is the most convenient and logical source of biomarkers [4]. Proteins, fragments of mRNA and genomic DNA are the most valuable of such biomarkers. Fragments of genomic DNA derived from malignant cells (ctDNA) are very promising biomarkers, and the current study is focused on the use of these molecules for BC monitoring. Accurate detection of ctDNA (circulating

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tumor DNA) in plasma is challenging because ctDNA concentrations can be very low. This could greatly impair reliable and valid measurements of tumor dynamics [5]. Several groups performed the analysis of plasma and urine as a source of fragments of genomic DNA derived from BC cells [4, 6-8]. For BC, the urine is the most suitable and reliable source of ctDNA.

Urinary cfDNA consists of slightly larger fragments than plasma cfDNA with a broad size distribution peaking between 200 and 300 bp and a median concentration of 4 ng/µL in BC patients [9, 10]. Urine DNA presents in two forms: genomic DNA in exfoliated cells (the cell pellet) and cfDNA in the supernatant/filtrate. Analysis of cfDNA was proved as a reliable and effective technique for tumor surveillance. Analysis of cfDNA in the urine of BC patients was shown to be more efficient than the analysis of DNA from exfoliated cells. It was shown that urinary cfDNA of BC patients had a higher tumor genomic burden and greater detection potential as a genomic biomarker (90%) than urinary pellet DNA (61%) [10]. However, data about the diagnostic implications of cfDNA in urine for the detection of BC is still sparse [11]. Therefore, for the current study, we decided to use for cfDNA NMIBC from a patient’s urine.

Heterogeneity of the disease determines the use of multiple biomarkers for the analysis [8, 12]. Therefore, for the current study, we selected three alterations (biomarkers) in 3 genes highly mutated in BC. Accurate selection and validation of potential biomarkers can help to develop a clinically valuable assay. Two of the most frequently mutated genes in bladder cancer with point mutation hotspots are FGFR3 and TERT. Both have been assessed as biomarkers for detecting bladder cancer in urinary DNA in separate studies and were combined for NGS-based detection of mutations. The TERT promoter is mutated in approximately 65% of bladder tumours regardless of stage and grade and represents the best single biomarker for bladder cancer with a recent report of 62% sensitivity at 90% specificity for detecting primary bladder tumours [13]. TERT promoter mutations have previously been described at high frequencies across stages in BC, but their prognostic value in urine is unclear [13, 14]. This motivated us to include the TERT promoter mutation (C228T) in the current study.

About 70% of low-grade NMIBC tumors have an activating point mutation in the fibroblast growth factor receptor 3 (FGFR3) gene [15]. Patients with the FGFR3 mutation have a good prognosis and could serve as a significant predictor of intravesical recurrence [16]. Based on these data, we decided to include the FGFR3 mutation (S249C) in this study as a possible biomarker for BC surveillance. STAG2 is one of the most commonly mutated genes in NMIBC, and the most frequent mutations are truncation [17]. Although STAG2 mutation and inactivation is a common event in BC and there are data showing in a pilot cohort that STAG2-mutant tumors recurred less frequently than STAG2 wild-type, it is difficult yet to make a final conclusion regarding the role of STAG2 in BC [17, 18]. Therefore, we decided to include this marker (as negative) in our panel and further investigate its potential role as a diagnostic and prognostic marker.

There are multiple approaches to detect rare mutations in DNA samples. Digital PCR (dPCR) is an innovative PCR technology based on the dividing of the sample into multiple separate reactions. This allows for the determination of exact numbers of mutated copies relative to the wild type allele with high specificity [8]. However, the necessity to develop one dPCR assay for each biomarker (mutation) is a limiting factor. Several groups recently published their data regarding the use of dPCR for the detection of mutations in urinary cell-free DNA (cfDNA) [8, 19, 20]. The exploring of this technology established a correlation between the molecular and clinical status of the tumor. In one of these studies, for example, it was shown that increased levels of FGFR3 and PIK3CA mutated DNA in urine are indicative of later progression and metastasis in BC [20].

The major objective of the current proof-of-principle study is to develop and to evaluate a simple, quantitative, reliable and affordable assay for monitoring NMIBC status. We want to determine if the addition of a urine test can help in the early recognition of potential recurrences or progression in patients during follow-up after resection of NMIBC.

Materials and Methods

I Patients

In total 109 urine samples were collected during consecutive office visits (usually cystoscopy or cytology was performed) from 34 patients at the Department of Urology at Stony Brook University Hospital (Stony Brook, NY, USA) followed for urothelial bladder carcinoma irrespective of the histological stage (2-3 visits per patient). Urine samples were collected with intervals of 1-5 months. Informed consent was obtained from patients to use their specimens for research purposes, as required. This study complies with the latest version of the Declaration of Helsinki and general guidelines for good clinical practice. Urine samples from 8 subjects without diagnosed BC were included as controls (1 visit). Additional demographic and clinical data were collected from hospital charts of selected patients (Table 1). Recurrence was defined as a histologically proven tumor. Since primary bladder carcinoma in situ (CIS) is a rare lesion, patients with primary CIS (5 patients) were excluded as we did not have sufficient numbers to be able to draw valid conclusions. With the exception of 8 patients, all patients were disease-free on the date of urine collection.

II cfDNA Isolation from Urine

For effective analysis of a biospecimen, it is essential that it contains a sufficient amount of high-quality tDNA. Among 2 types of urine tDNA (DNA from exfoliated cells and cell-free DNA) cf DNA better suited for the chosen analytical technique (digital PCR), possibly because of the better quality after isolation and good availability in most urine samples (unpublished observation). Therefore, in the current study, we exploited dPCR analysis of 3 hot mutations in urine-derived cfDNA.

A 15-50 ml volume of fresh urine was transferred to the laboratory. Urine was centrifuged at 800 g for 10 min and cell pellet (urine sediment) was discarded. DNA extraction was carried out in 2-12 h after collection with two methods: 1. Urine DNA isolation kit (Norgen Biotek, Canada) was used as described by the manufacturer’s manual. 2. A second method was based on the published procedure with some modifications [21]. Briefly, silica particles (silica gel 60N, 250 mg) and poly-Lys (100 mg, powder form) were combined in a tube along with 10 mL of 100
mM Tris–HCl buffer (pH 7.0) and mixed for 30 min using a vortex mixer. The particles were washed three times with 10 ml of 100 mM Tris–HCl. 20 μl of silica particles were incubated with 25–50 ml of urine supernatant. Beads were washed 3 times with water and DNA was eluted with 30 μl of elution buffer [21]. Eluted DNA was quantified with QuantiFluor dsDNA kit (Promega) or by Qubit fluorometric quantitation (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's recommendations. Only samples with DNA concentration higher than 1 ng/μl were used for dPCR.

### III cfDNA Mutation Screening

Digital PCR assays were designed with Thermo Scientific software to have short amplicons and dual-labeled fluorescent probes with complementarity to the mutated DNA sequence. FGFR3 probe (S249C) was available from the premade list for TagMan qPCR and was validated by us for dPCR, TERT promoter probe (C228T) (the most prevalent mutation for BC) was specially designed for dPCR and was validated for use with dPCR by the manufacturer [22]. STAG2 TagMan probe was synthesized specifically for this study by Thermo Scientific and was also a subject for dPCR validation.

Digital PCR reaction mixtures contained 7.5 μL Digital PCR Master Mix, 0.75 μL 20× TagMan Assay (primer/probe mix), 6.75 μL diluted DNA (25 ng) and then loaded into the QuantStudio™ 3D Digital PCR Chip, with 20000 compartments. Experimental conditions for dPCR were set based on the recommendations of the developmental team. Total DNA copies and percentile of mutated copies were quantified using QuantStudio 3D digital PCR machine and related software (Thermo Fisher Scientific). To ensure experiment quality, samples with total counts of less than 10,000 were excluded from the analysis.

The linearity and sensitivity of the dPCR assays were evaluated by spiking increasing amounts of mutated synthetic DNA with related mutation (0.1%, 1%, 10%) into a background of 30000 wild-type DNA copies (90 ng human genomic DNA). Based on the data we determined the lowest detection rate (LOD) of mutations for all 3 genes in urine cfDNA <1%.

### IV Data Analysis

Thresholds for the FGFR3, TERT, and STAG2 (negative) assay were set as 3%. This decision was made based on the estimated sensitivity and specificity of the related assay. Sensitivity and Specificity were calculated based on dPCR data.

### Results

#### I Patient Characteristics and Liquid Biopsy Analysis

In total, 29 patients (Table 1) that underwent trans-urothelial resection of bladder tumor (TURBT) and diagnosed with NMIBC were involved in the study. In addition, 9 non-BC subjects were screened for hotspot mutations for FGFR3 (S249C) and TERT promoter (C228T) in urine cfDNA. cfDNA from the urine of 15 patients and 9 control subjects were tested for STAG2 (R216Stop) mutations. Table 1 illustrates demographic and clinical patient information.

| Table 1: Clinical characteristics of patients. |
|-----------------|-----------------|-----------------|
| Gender | BC n 21 (%) | T+ n 8 (%) | no BC n 9 (%) |
|-----------------|-----------------|-----------------|
| M | 12(57) | 7(87) | 7(77) |
| F | 9(43) | 1(13) | 2(23) |
| Smoking | | | |
| No | 11(52) | 1(13) | 9(100) |
| Yes | 10(48) | 7(87) | 0 |
| Stage | | | |
| Ta | 13(61) | 4(50) | 0 |
| T1 | 8(39) | 4(50) | 0 |
| T2 | 0 | 0 | 0 |
| PUNLMP | 0 | 0 | 0 |
| CIS | 0 | 0 | 0 |
| Grade | | | |
| LG | 7(33) | 4(50) | 0 |
| HG | 14(66) | 4(50) | 0 |
| Multiplicity | | | |
| Solitary | 4(19) | 6(75) | 0 |
| Multiple | 2(6) | 2(25) | 0 |
| Unclear | 15(75) | 0 | 0 |
| BC cases with previously diagnosed, removed, but not recently confirmed tumors; T+ cases with tumor confirmed within 6 months of DNA analysis; no BC= control subjects. |

Urine samples (109 samples) were collected from all NMIBC patients during office visits. In 8 instances, tumor presence was confirmed within 6 months of urine collection. Urine samples (9) were collected once from non-BC patients (control group). The longest follow-up time (after the first urine collection) was 1.5 years. Cell-free DNA samples were isolated from urine samples and after measurement of concentration were used for digital PCR assays with related probes. DNA samples with concentration of at least 1 ng/μl was sufficient for 3 digital PCR reactions and 52 urine samples already satisfied this criterion (Figure 1).

For the analysis of mutations in this study, we utilized QuantStudio™ 3D Digital PCR.

To analyze sensitivity and specificity of the particular assay we first showed linearity and sensitivity of this approach for each selected probe by spiking related mutated DNA in wild type DNA as described in the Material and Methods. All 3 probes satisfied the demand for linearity in

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**Figure 1:** Flow-chart for patients and related urine samples.
these dilution tests (data not shown). The limit of detection was identified for each probe as the dilution that is statistically different from the negative control (WT DNA only). The threshold for mutation presence was set as described in the Materials and methods.

II Correlation Between Tumor Presence at the Time of Analysis and Positive Mutation Load in Urine Samples

The tumor was detected by cystoscopy and/or cytology in 8 patients at no longer than 6 months before DNA analysis. We observed FGFR3 mutations in 5 patients in this group (62.5% sensitivity). Five urine samples collected at different time points from 21 patients (23%) without recently confirmed tumors were positive for FGFR3 mutations (Table 2) (80% specificity).

Only one sample from the control group was positive for FGFR3 mutations (Table 2) (Figure 2A). When several sample collections were performed, mutation status for tumor–positive patients were determined at the most recent office visit.

Table 2: Mutational status of DNA from urine samples.

<table>
<thead>
<tr>
<th>dPCR reaction</th>
<th>BC n 21 (%)</th>
<th>T+ n 8 (%)</th>
<th>No BC n 9 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>16 (76)</td>
<td>3 (38)</td>
<td>8 (88)</td>
</tr>
<tr>
<td>Mutated</td>
<td>5 (24)</td>
<td>5 (62)</td>
<td>1 (12)</td>
</tr>
<tr>
<td>Not determined</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TERT promoter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>11 (65)</td>
<td>1 (13)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>Mutated</td>
<td>6 (35)</td>
<td>7 (87)</td>
<td>0</td>
</tr>
<tr>
<td>Not determined</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>STAG2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>1 (15)</td>
<td>5 (63)</td>
<td>8 (88)</td>
</tr>
<tr>
<td>Mutated</td>
<td>6 (85)</td>
<td>3 (37)</td>
<td>1 (12)</td>
</tr>
<tr>
<td>Not determined</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FGFR3m and TERTm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>4 (23)</td>
<td>4 (50)</td>
<td>0</td>
</tr>
<tr>
<td>Mutated</td>
<td>5 (71)</td>
<td>7 (87)</td>
<td>0</td>
</tr>
<tr>
<td>Not determined</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>All 3 mutated</td>
<td>22 dPCRs</td>
<td>4 (28)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>No mutations</td>
<td>22 dPCRs</td>
<td>8 (72)</td>
<td>9 (100)</td>
</tr>
</tbody>
</table>

BC-patients with diagnosed BC; T+ -patients with tumor confirmed within 6 months of recent DNA analysis; no BC-control subjects.

TERT promoter was mutated in 7 of 8 cases with detected tumors (87% sensitivity). TERT mutations were identified in 6 patients without tumor detectable in 6 months before the most recent dPCR test. TERT mutations were not detected in the control group (9 subjects). Therefore, specificity for this test was 77% (Figure 2A). The relatively high presence of TERT mutation-related false positive (specificity 77%) can be explained by the possible appearance of secondary tumors that cannot be detected by cystoscopy and/or cytology at the moment of the DNA analysis. Although all these samples derived from the patients that were currently considered as disease-free, these patients were previously diagnosed with NMIBC.

It was previously shown that a STAG2 mutation (truncated) is a mark of low expression of STAG2 and related to better prognosis (less recurrence rate), as compared to wild type STAG2 [17, 23]. In the current study, STAG2 mutations were analyzed in 15 patients and in 9 healthy volunteers. STAG2 mutations were detected in 3 of 8 cases where tumor was present at the time of DNA analysis or 6 months before (37%). (Figure 2B).

Figure 2: Marker performance for tumor presence in patients with primary NMIBC A) FGFR3 and TERT marker performance separately and in combination for tumor detection; B) STAG2 marker performance for tumor detection (mutated form) or for recurrence (wild type).
STAG2 mutations were detected in cfDNA samples derived from 6 patients without a recently detected tumor (of 7 analyzed) (85%). One STAG2 mutation was detected in control group (9 subjects never diagnosed with BC). Therefore, specificity of this assay for detection potential tumor was 91 %. STAG2 mutation was detected in 3 of 8 cases with recently confirmed tumors (Table 2, Figure 2B). It was shown recently that STAG2 mutation could serve as NMIBC marker, but not as a marker for recurrence [24]. However, since the role of STAG2 in BC development is not completely clear yet, it is premature to make a decision about the relation of STAG2 mutation to the clinical status of the tumor based on the limited data obtained in this study.

Combining several biomarkers often improve diagnostic and/or prognostic power [20, 25-27]. Therefore, we analyzed the performance of assays combinations. TERT promoter assay revealed the best sensitivity (87%) among 3 probes, and specificity was also high (77%). (Table 2, Figure 2A). A combination of 2 assays (TETR+FGFR3) allowed to record improved specificity (100%) but sensitivity at these conditions was lower than for single probes (72%) (Figure 2). However, increased specificity allows considering multiple assays as a promising approach.

III Predictive Value of Hot Mutations for NMIBC during Follow-Up

Prediction of recurrence or tumor progression is the major task for the evaluation of NMIBC after TURBT surgery. In the current study 8 cases with tumor detected at the time of DNA analysis (<6 months) could be considered as recurrent. Since in this study all cases were positive for the tumor presence in 6 months period these cases might be considered as both tumor positive and recurrent. However, small number of those cases and not completely defined role of STAG2 in this situation make the potential role of 3 tested biomarkers in recurrence prediction preliminary.

Discussion

Results of this small cohort proof-of-concept study demonstrate diagnostic and monitoring the potential of the FGFR3, TERT promoter and STAG2 mutations detected by dPCR in cfDNA extracted from urine supernatant of patients with NMIBC.

Even one marker assay showed that this analysis could increase the sensitivity of tumor detection or recurrence/progression as compared to cytology or cystoscopy. To improve the predictive value of all three tested biomarkers an increased number of samples and observation time for individual patients is necessary. FGFR3 and TERT promoter mutations were used previously as urine biomarkers for monitoring BC separately and in combination [11, 15, 28-30]. An addition of STAG2 as a predictor for recurrence will substantially increase the analytical potential of the assay. This was observed even in the small cohort in this proof of concept study. The specificity of the single assays and their combinations might be further improved by applying them to a larger patient population.

Furthermore, the specificity of the urine DNA assays might be higher if one considers that at the time of the analysis some of the false-positive (negative by cystoscopy and/or cytology but positive by mutation detection) cases can present as a future recurrence. It was already shown that a false positive urine assay was frequently followed by a later recurrence [25]. The presence of false-positive samples for these mutations can be explained by the possible presence of secondary tumors at the time of analysis. This hypothesis might be confirmed or rejected after further surveillance of these patients. In addition, case stratification can further increase assay efficiency and data interpretation [25].

Although the observation time is short (18 months) we already can conclude, based on our analysis, that the developed assays are suited for earlier stages of NMIBC patient surveillance and can possibly be valuable for the prediction of progression to invasive cancer forms or to recurrence. The major difference of the current assay from previously suggested procedures is the simplicity and affordability. Analysis of mutations in cfDNA can be performed in several ways. We selected dPCR for this study because this approach is inexpensive, quantitative, special equipment may be purchased for any clinical laboratory, and requires a very low demand for skills and additional training for personnel. The average price of the 3 markers assay will be approximately $60 including labor expenses. Any clinical laboratory that is equipped for molecular analysis and dPCR will be able to produce and analyze results within 3-4 h of urine collection.

We present herein the proof of concept that this fast and simple assay is effective for detecting bladder tumors that confirmed by other tests (cystoscopy, cytology). Mutation in cfDNA very tightly matched somatic mutation in the original tumor. High concordance rates of mutation allele frequencies in the FFPE tumor tissue with urinary cfDNA was shown [11]. Another group showed that 80.7% of somatic mutations detected in tumors were found in cfDNA [31]. In this proof-of-concept study, we showed that dPCR-based mutation assays using urine samples represent a cheap and fast monitoring method, although these data need to be validated with more samples and for longer observation periods. Further studies with more patients and longer observation time will help to select the most efficient combinations of biomarkers.

Author Contributions

Conceptualization, VR, DG, WW, and EF; methodology, VR, DG, EF; validation, TW, OP, WW and LX; formal analysis EF, VR, resources DG; data curation, EF, DG, LX and TW; writing-original draft preparation, VR; writing-review and editing, EF, VR, TW; supervision, VR.

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None.
Conflicts of Interest

None.

Abbreviations

cfDNA: cell-free DNA
ctDNA: cell-free tumor DNA
dPCR: digital polymerase chain reaction
FGFR: fibroblast growth factor receptor
NMIBC: non-muscle-invasive bladder cancer
TERT: telomerase reverse transcriptase
TURBT: transurethral resection of bladder tumor
UBC: urothelial bladder cancer
STAG2: Cohesin subunit SA-2

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