Non-invasive detection of bladder cancer through the analysis of driver gene mutations and aneuploidy

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Abstract: Current non-invasive approaches for bladder cancer (BC) detection are suboptimal. We report the development of non-invasive molecular test for BC using DNA recovered from cells shed into urine. This “UroSEEK” test incorporates assays for mutations in 11 genes and copy number changes on 39 chromosome arms. We first evaluated 570 urine samples from patients at risk for BC (microscopic hematuria or dysuria). UroSEEK was positive in 83% of patients that developed BC, but in only 7% of patients who did not develop BC. Combined with cytology, 95% of patients that developed BC were positive. We then evaluated 322 urine samples from patients soon after their BCs had been surgically resected. UroSEEK detected abnormalities in 66% of the urine samples from these patients, sometimes up to 4 years prior to clinical evidence of residual neoplasia, while cytology was positive in only 25% of such urine samples. The advantages of UroSEEK over cytology were particularly evident in low-grade tumors, wherein cytology detected none while UroSEEK detected 67% of 49 cases. These results establish the foundation for a new, non-invasive approach to the detection of BC in patients at risk for initial or recurrent disease.

Introduction
Bladder cancer (BC) is the most common malignancy of the urinary tract. According to the American Cancer Society, 79,030 new cases of bladder cancer and 18,540 deaths are estimated to occur in the United States alone in 2017 [1]. Predominantly of urothelial histology, invasive BC arises from non-invasive papillary or flat precursors. Many BC patients suffer with multiple relapses prior to progression, providing ample lead-time for early detection and treatment prior to metastasis [2]. Urine cytology and cystoscopy with transurethral biopsy (TURB) are currently the gold standards for diagnosis and follow-up in bladder cancer. While urine cytology has value for the detection of high-grade neoplasms, it is unable to detect the vast majority of low-grade tumors [3-5]. This fact, together with the high cost and invasive nature of repeated cystoscopy and TURB procedures, have led to many attempts to develop novel noninvasive strategies. These include urine or serum based genetic and protein assays for screening and surveillance [6-21]. Currently available U.S. Food and Drug Administration (FDA) approved assays include ImmunoCyt test (Scimedx Corp), nuclear matrix protein 22 (NMP22) immunoassay test (Matritech), and multitarget FISH (UroVysion) [6-12]. Sensitivities between 62% and 69% and specificities between 79% and 89% have been reported for some of these tests. However, due to assay performance inconsistencies, cost or required technical expertise, integration of such assays into routine clinical practice has not yet occurred.

Much is now known about the genetic pathogenesis of BC. High rates of activating mutations in the upstream promoter of the TERT gene are found in the majority of BC as well as in other cancer types [22-24]. TERT promoter mutations predominantly affect two hot spots, g.1295228 C>T and g.1295250 C>T. They lead to the generation of CCGGAA/T or GGAA/T motifs altering binding site for ETS transcription factors and subsequently increased TERT promoter activity [22, 25]. TERT promoter mutations occur in up to 80% of invasive urothelial carcinomas of the bladder and upper urinary tract as well as in several of its histologic variants [14, 23, 26-28]. Moreover, TERT
promoter mutations occur in 60-80% of BC precursors, including Papillary Urothelial Neoplasms of Low Malignant Potential [29], non-invasive Low Grade Papillary Urothelial Carcinoma, non-invasive High Grade Papillary Urothelial Carcinoma and “flat” Carcinoma in Situ (CIS), as well as in urinary cells from a subset of these patients [14]. TERT promoter mutations have thus been established as the most common genetic alteration in BC [14, 30]. Other important oncogene-activating mutations include those in FGFR3, RAS and PIK3CA, which have been shown to occur in a high fraction of non-muscle invasive bladder cancers [31, 32]. In muscle-invasive bladder cancers, mutations in TP53, CDKN2A, MLL2 and ERBB2 are also frequently found [33-40].

The current study assesses the performance of a massively parallel sequencing-based assay, termed UroSEEK, for the detection of BC through the analysis of urinary cells. UroSEEK has three components: detection of intragenic mutations in regions of ten genes (FGFR3, TP53, CDKN2A, ERBB2, HRAS, KRAS, PIK3CA, MET, VHL and MLL2) that are frequently mutated in BC [33-40]; detection of mutations in the TERT promoter [22-24]; and detection of aneuploidy [41, 42]. UroSEEK was applied to two independent cohorts of patients. The first (called the Early Detection cohort) involved patients with microscopic hematuria or dysuria, which are both risk factors for BC. Only a relatively small fraction (4 to 5%) of micro-hematuria patients are at risk for developing urothelial malignancy [43, 44], so the decision about which of these patients should undergo cystoscopy is often difficult. The second cohort (called the Surveillance cohort) involved patients who had already been diagnosed with BC. These patients are at high risk for recurrence [43]. Because urine cytology is relatively insensitive for the detection of recurrence, cystoscopies are performed as often as every three months in such patients in the U.S. In fact, the cost of managing these patients is in aggregate higher than the cost of managing any other type of cancer, and amounts to 3 billion dollars annually [45]. A non-invasive test that could predict which of these patients were most likely to develop recurrent BC could thereby be both medically and economically important.

Results

A schematic of the approach used in this study is provided in Figure 1A, and a flow diagram indicating the number of patients evaluated in this study and the major results is provided in Figure 1B.
Figure 1A Schematic of the approach used to evaluate urinary cells in this study.
Figure 1B  Flow diagram indicating the number of patients in the two cohorts evaluated in this study and summarizing the salient findings. Cytology was performed on only a subset of the patients (see main text).
Early Detection Cohort

Cohort characteristics. A total 570 patients were included in the Early Detection cohort, each with one urine sample analyzed. 90% of the patients had hematuria, 3% had lower urinary tract symptoms (LUTS), and 9% had other indications suggesting they were at risk for BC. The median age of the participants was 58 years (range 5 to 89) (Supplementary Table 1). As expected from prior studies of patients at risk for BC, 70% of the patients were male [1, 43]. 175 (31%) of patients developed BC after a median follow-up period of 18 months (range 0 to 40 months). For each patient who developed BC, we selected two other patients who presented with similar symptoms but did not develop BC during the follow-up period. By design, then, the fraction of cases in this cohort developing BC was higher than the fraction (5%) of patients with similar presentations that would have developed BC in standard clinical practice. The characteristics of the tumors developing in the 570 patients are summarized in Supplementary Table 1 and detailed in Supplementary File 1.

Genetic analysis. We performed three separate tests for genetic abnormalities that might be found in urinary cells derived from BC (Figure 2). First, we evaluated mutations in selected regions of ten genes that have been shown to be frequently altered in urothelial tumors (Supplementary File 2). For this purpose, we designed a specific set of primers that allowed us to detect mutations in as few as 0.03% of urinary cells. The capacity to detect such low mutant fractions was a result of the incorporation of molecular barcodes in each of the primers, thereby substantially reducing the artifacts associated with massively parallel sequencing [41]. Second, we evaluated TERT promoter mutations. A singleplex PCR was used for this analysis because the unusually high GC-content of the TERT promoter precluded its inclusion in the multiplex PCR design. Third, we evaluated the extent of aneuploidy using a technique in which a single PCR is used to co-amplify ~38,000 members of a subfamily of long interspersed nucleotide element-1 (L1 retrotransposons, also called LINEs). L1 retrotransposons, like other human repeats, have spread throughout the genome via retrotransposition and are found on all 39 non-acrocentric autosomal arms [41].
**Figure 2.** Fraction of mutations found in the ten-gene panel in 231 urinary cell samples assessed in the (A) early detection cohort and 132 urinary cell samples assessed in the (B) surveillance cohort.
The multiplex assay detected mutations in 68% of the 175 urinary cell samples from the individuals that developed BC during the course of this study (95% CI 61% to 75%) (Supplementary Table 1 and Supplementary File 2). A total of 246 mutations were detected in 8 of the ten target genes (Figure 2A and Supplementary File 2). The mean mutant allele frequency in the urinary cells with detectable mutations was 18% and ranged from 0.17% to 99%. The most commonly altered genes were TP53 (45% of the total mutations) and FGFR3 (20% of the total mutations; Fig. 2A). The distribution of mutant genes was roughly consistent with expectations based on previous exome-wide sequencing studies of BCs [46]. At the thresholds used, 1.7% of the 395 patients in the Early Detection Cohort who did not develop BC during the course of the study had a detectable mutation in any of the ten genes. At the same thresholds, none of the 188 urinary cell samples from healthy individuals had any mutation in any of the ten genes assayed (100% specificity, 95% CI 98% to 100%).

Mutations in the TERT promoter were detected in 57% of the 175 urinary cell samples from the patients that developed cancer during the study interval (95% CI 49% to 64%; Supplementary Table 1 and Supplementary File 3). The mean TERT mutant allele frequency in the urinary cells was 14% and ranged from 0.18% to 78%. Mutations were detected in 3 positions: 98% of the mutations were at hg1295228 (79%) and hg1295250 (19%), which are 66 and 88 bp upstream of the transcription start site, respectively. These positions have been previously shown to be critical for the appropriate transcriptional regulation of TERT. In particular, the mutant alleles recruit the GABPA/B1 transcription factor, resulting in the H3K4me2/3 mark of active chromatin and reversing the epigenetic silencing present in normal cells [47]. 4% of the 395 patients in this cohort who did not develop BC during the course of the study had a detectable mutation in the TERT promoter. Only one of the 188 urinary samples from healthy individuals harbored a TERT promoter mutation.

Aneuploidy was detected in 46% (95% CI 39% to 54%) of the 175 urinary cell samples from the patients that developed BC during the course of the study, Supplementary Table 1 and Supplementary File 4. The most commonly altered arms were 5q, 8q, and 9p. All three of these arms harbor well-known oncogenes and tumor suppressor genes which have been shown to undergo copy number alterations in many cancers, including BC [48]. 1.5% of the urinary cell samples from the 395 patients who did not develop BC during the course of the study exhibited aneuploidy. None of the 188 urinary samples from healthy individuals exhibited aneuploidy when assessed with the same technology.

**Comparison with primary tumors.** Tumor samples from 102 of the patients enrolled in this cohort were available for comparison and were studied with the same three assays used to study the urinary cell samples (Supplementary File 3). In 91 (89%) of these 102 cancers, at least one mutation in the eleven genes studied were mutated (in the 10-gene panel or in the TERT promoter). Moreover, at least one of the mutations identified in the urine samples from these 102 patients was also identified in 83% of the corresponding BC (Supplementary Files 2 and 3). Analysis of the BCs also shed light...
on the basis for "false negatives", i.e., the reason that 21% of urine samples from patients who developed BC had no detectable mutations in the 11 genes tested. The reason could either have been that the corresponding BC did not harbor a mutation in these 11 genes or that it did, but the fraction of neoplastic cells in the urine sample was not high enough to allow its detection with the assays we used. We could identify a mutation in at least one of the 11 genes in 62% of the primary tumors from patients with false negative urine tests for mutations (Supplementary File 5 and 6). We conclude that 38% of the 29 false negative tests for mutations were due to the fact that none of the queried mutations were present in the tumor and that the other 62% of the false negatives were due to insufficient amounts of cancer cells in the urine.

**UroSEEK: biomarkers in combination.** As noted above, the ten-gene multiplex assay, the TERT singleplex assay, and the aneuploidy assays yielded 68%, 57%, and 46% sensitivities, respectively, when used separately (Supplementary Table 1 and Supplementary Files 2, 3, and 4). 45 samples without TERT promoter mutations could be detected by mutations in one of the other ten genes (Figure 3A and Supplementary File 2). Conversely, 35 samples without detectable mutations in the multiplex assay could be detected by virtue of TERT promoter mutations (Figure 3A and Supplementary File 3). Ten of the urinary cell samples without any detectable mutations in the 11 genes could be detected by the assay for aneuploidy (Figure 3A and Supplementary Table 4). Thus, when the three assays were used together (test termed "UroSEEK"), and a positive result in either assay was sufficient to score a sample as positive, the sensitivity rose to 83% (95% CI 76% to 88%). Only one of the 188 samples from healthy individuals was scored positive by UroSEEK (specificity 99.5%, CI 97% to 100%). Twenty-six (6.5%) of the 395 patients in this cohort who did not develop BC during the course of the study scored positive by the UroSEEK test (specificity 93%, CI 91% to 96%). On average, UroSEEK positivity preceded the diagnosis of BC by 2.3 months, and in eight cases by more than a year (Figure 4A and Supplementary File 1).
Figure 3. Venn Diagram of the distribution of samples that were positive by each of the three assays for the (A) early detection and (B) surveillance cohorts. URO = Ten gene panel, TERT = TERT promoter region, ANEU = Aneuploidy test.

Figure 4. Bar graphs of the lead time between a positive UroSEEK test and the detection of disease at the clinical level in the (A) Early Detection and (B) Surveillance cohorts.
**UroSEEK plus cytology.** As both cytology and UroSEEK tests are non-invasive and can be performed on the same urine sample, we assessed their performance in combination. There were 347 patients in the Early Detection cohort in whom cytology was available (Supplementary Table 1 and Supplementary File 1). Among the 40 patients who developed biopsy-proven cancer in this cohort, 17 were positive by cytology (43% sensitivity). None of the 299 patients that did not develop cancer were positive by cytology (100% specificity). UroSEEK was positive in 100% of the 17 cancer patients whose urines were positive by cytology and in 95% of the 23 cancer patients whose urines were negative by cytology. Thus, in combination, UroSEEK plus cytology afforded 95% (95% CI 83% to 99%) sensitivity, a 12% increase over UroSEEK and a 52% increase over cytology. Among the 299 patients in the early detection cohort who did not develop BC during the course of the study, 20 (6.6%) were positive by UroSEEK or cytology, giving the combination of UroSEEK and cytology a specificity of 93% (95% CI 90% to 96%).

**Surveillance Cohort**

**Cohort characteristics.** Our strategy for surveillance was different than the one we used for early detection. Patients in whom a BC was surgically excised for treatment and diagnosis generally have tumor tissue available, and in most such tumors, a mutation can be identified. For example, we found during the course of this study that a mutation in at least one of the 11 queried genes was present in 95.2% of BCs evaluated. All patients selected for the surveillance study had biopsy confirmed BC and had a urine sample collected 0 - 5 years after surgery. We were to evaluate a total of 322 patients that donated urines and whose BC contained a mutation in at least one of the 11 genes evaluated. We determined whether a single urine sample taken relatively short time following surgical excision of the BC could reveal residual disease in these 322 patients, as evidenced by later recurrence. 187 (58%) of the 322 patients developed clinically evident BC after a median follow-up period of 10.7 months (range 0 to 51 months). The histopathologic types and tumor stages of these patients are summarized in Supplementary Table 2 and detailed Supplementary File 7. The median age of the participants was 62 (range 20 to 93). As expected from the demographics of BC, 75% of the patients were male.

**Genetic analysis.** The multiplex assay in urinary cells detected mutations in 49% of the urinary cell samples from patients that developed recurrent BC during the study interval (95% CI 45% to 60%; Supplementary File 7 and Supplementary File 8). The mean mutant allele frequency in the urinary cells with detectable mutations was 16% and ranged from 0.08% to 93%. The most commonly altered genes were FGFR3 (43% of the 134 mutations) and TP53 (30% of the 134 mutations; Figure 2B). Seven percent of the 135 patients who did not develop recurrent BC during the course of the study had a detectable mutation in their urinary cell sample (these are considered to be false positives; see Discussion). The mean interval between a positive multiplex assay test and the diagnosis of recurrent BC was 7 months (range 0 to 51 months).
Mutations in the TERT promoter were detected in 51% of the urinary cell samples from patients that developed recurrent BC during the study interval (95% CI 44% to 58%; Supplementary Table 2 and Supplementary File 9). The mean TERT mutant allele frequency in the urinary cells with detectable mutations was 6% and ranged from 0.23% to 43%. Mutations were detected in the same three positions observed in the urinary cells of the Early Detection cohort. 10% (95% CI 83% to 94%) of the 135 patients who did not develop recurrent BC during the course of the study had a detectable TERT promoter mutation in their urine sample (false positives). The mean interval between a positive TERT test and the diagnosis of recurrent BC was 7 months (range 0 to 40 months).

Aneuploidy was detected in 30% (95% CI 24% to 37%) of the urinary cell samples from patients that developed recurrent BC during the course of the study (Supplementary Table 2 and Supplementary File 10). The most commonly altered arms were 8p, 8q, and 9p, as in the Early Detection cohort. Two percent of the 135 patients who did not develop recurrent BC during the course of the study exhibited aneuploidy in at least one of their urinary cell samples.

**Markers in combination.** As noted above, the ten-gene multiplex assay, the TERT singleplex assay, and the aneuploidy assays yielded 49%, 51%, and 30% sensitivities, respectively, when used separately (Supplementary Table 2 and Supplementary Files 8, 9, and 10). Thirty-two samples without TERT promoter mutations could be detected by mutations in one of the other ten genes (Figure 3B and Supplementary File 8). Conversely, 41 samples without detectable mutations in the multiplex assay could be detected by virtue of TERT promoter mutations. Three of the urinary cell samples without any detectable mutations could be detected by the assay for aneuploidy. Thus, the sensitivity of UroSEEK was 66% (95% CI 59% to 73%) Supplementary Table 2). Fourteen percent of the 135 patients in this cohort who did not develop BC during the course of the study scored positive by the UroSEEK test, yielding a specificity of 86% (95% CI 77% to 91%). On average, UroSEEK positivity preceded the diagnosis of BC by 7 months, and in 47 cases by more than one year (Figure 4B and Supplementary File 7).

There were 196 patients in the Surveillance cohort in whom cytology was available (Supplementary File 7). Among the 120 patients who developed recurrent BC in this cohort, 30 (25%) were positive by cytology. Conversely, no positive cytology results were observed in patients whose tumors did not recur. UroSEEK was positive in 90% of the recurrent BC patients whose urines were positive by cytology and in 61% of the 90 recurrent BC patients whose urines were negative by cytology. Thus in combination, UroSEEK plus cytology afforded 71% sensitivity (95% CI 61.84% to 78.77) (Figure 3D and Supplementary File 5). Among the 76 patients who did not develop recurrent BC during the course of the study and in whom cytology was available, 18% scored as positive by either cytology or UroSEEK, affording a specificity of 82% (95% CI 71% to 90%; see Discussion).
Low vs. high grade urothelial neoplasms (both cohorts)

The advantage of UroSEEK over cytology was particularly evident in low-grade tumors (Papillary urothelial neoplasms of low malignant potential and non-invasive low grade papillary urothelial carcinomas). There were a total of 49 low-grade tumors evaluated in this study in whom cytology was available (six from the Early Detection cohort and 43 from the Surveillance cohort). None of these low-grade tumors were detected by cytology (0% sensitivity; 95% CI 0.0% to 6.7%) In contrast, UroSEEK detected 67% (95% CI 51% to 81%) of the low-grade tumors (identical rate of 67% in both cohorts; Supplementary Table 3 and Figure 5). Analogously, there were a total of 102 high-grade tumors (in-situ urothelial carcinoma, non-invasive high grade papillary urothelial carcinoma or infiltrating high grade urothelial carcinoma) evaluated in this study in whom cytology was available (34 from the Early Detection cohort and 68 in the Surveillance cohort). Cytology was positive in 45% of these patients (50% and 41% in the Early Detection and Surveillance cohorts, respectively) while UroSEEK was positive in 80% of them (100% and 71% in the Early Detection and Surveillance cohorts, respectively; Supplementary Table 3).

Figure 5. Bar graphs showing the performance of Cytology Vs. UroSEEK in diagnosis of low and high grade urothelial neoplasms in the Early Detection and Surveillance cohorts.
Discussion

Our purpose for developing UroSEEK was not to replace cytology but rather to augment it. Cytology is a non-invasive test that is highly specific, and in expert hands nearly always indicates the presence of a BC when positive. This specificity was verified in our study: all of the 51 patients whose urine samples were positive by cytology developed biopsy-proven cancer. However, cytology is not particularly sensitive. UroSEEK adds considerably to sensitivity, as it raised the sensitivity of cytology from 43% to 95% in the Early Detection Cohort and from 25% to 71% in the Surveillance cohort. This sensitivity was highlighted by the fact that UroSEEK positive results preceded clinical diagnosis or positive cytology by months to years.

The advantage of using UroSEEK in addition to cytology was particularly evident for low-grade tumors. Cytology was negative in all 49 patients with such tumors, while 2/3 were positive with UroSEEK. Another example of the utility of the combination of UroSEEK plus cytology was evident in patients who had an equivocal cytology reading. A relatively large number of urine samples receive such an equivocal cytologic reading, even in the hands of a sub-specialized, board-certified cytopathology expert such as employed in the current study [49]. In the Early Detection Cohort, for example, 105 urine samples were scored as "atypical", and of these cases, 19% developed recurrence while the other 81% did not. UroSEEK was positive in 95% of the atypical cases that developed BC, but only in 13% of the atypical cases that did not develop cancer. These results demonstrate that UroSEEK can be used to more confidently interpret atypical cytology results.

Although UroSEEK is more sensitive than cytology, it is less specific. In this study, we were able to judge specificity in several independent ways. The first, and in some ways, most straightforward, was in a collection of urine samples from healthy individuals. In 188 such individuals, we found only one instance of a positive test, yielding a specificity of 99.5% (CI 97% to 100%). That high specificity can be considered the technical specificity of the test, but biological specificities are also important. In the Early Detection cohort, 26 of the 395 patients who did not develop BC scored positive, yielding a specificity of 93% (CI 90.50% to 96%), i.e., 6.5% false positives.

These "false positives" detected by UroSEEK could result from several factors. First, we cannot be certain that the patients whose urinary cells harbored genetic alterations did not have cancer. The follow-up period for many of patients was only one year, and cystoscopy was not generally performed. Second, it is possible that there are clonal proliferations in the bladder epithelium that increase with age. The patients in the Early Detection cohort were on average older than those in the 188 healthy individuals used as controls (40 years vs. 58 years). Though this explanation is speculative, clonal proliferations that are not considered neoplastic have been described in the bone, skin, and other tissues [50, 51]. This speculation may also explain some of the reason that mutations identified in urinary cells were not always identified in the primary tumors of the same patients. Though in the majority of cases, at least one of the mutations
identified in the urine was also present in the primary tumor, this was not true in 22% of the cases in the Early Detection Cohort. In these cases, UroSEEK could be detecting clonal proliferations in the bladder epithelium that did not progress to cancer, and such proliferations may be more common in patients with BC than in the general population [50, 51]. Because only one biopsy from the primary tumor was available for comparison, it is also possible that intra-tumoral heterogeneity explains part of the discrepancies. False positives in the Surveillance Cohort could be explained in similar ways. False positives are not unique to our study; they have been observed in all other molecular assays for bladder cancer, including those that are FDA-approved [52-54]. Whether the false positives in these other assays have the same biological basis is an important area for future research.

Our study lays the conceptual and practical framework for a novel test that could inform the managements of patients with bladder cancer. Large prospective trials will be required to demonstrate the ability of UroSEEK to improve the management of patients with hematuria or dysuria or patients at risk for recurrence of BC. Before carrying out large-scale trials to evaluate such clinical utility, it is informative to predict what the performance characteristics of such a test might be. As one example, consider the use of UroSEEK plus cytology in patients presenting to their physician with microscopic hematuria or dysuria. This is a commonly encountered situation. For example, in large population-based studies involving over 80,000 individuals that participated in health screening, the fraction of individuals with micro-hematuria ranged from 2.4% to 31.1% [43, 55]. It has been estimated that 5% of such patients actually have bladder cancer [56]. In the current study, UroSEEK plus Cytology had a sensitivity of 95% and a specificity of 93% in patients of this type. This extrapolates to a positive predictive value (PPV) of 66% (95% CI 55.3 to 74%) and a negative predictive value (NPV) of 99.3% (95% CI 97.3% to 99.8%). These values are well above those generally considered to be diagnostically helpful and is considerably higher than achieved in FDA-approved tests for this indication [53, 54]. The cost of a UroSEEK test is estimated to be $1000, which is comparable to that of cystoscopy, but UroSEEK is non-invasive.

Materials and Methods

Patients and Samples

Urine samples were collected prospectively from patients in four participating institutions including Johns Hopkins Hospital, Baltimore, MD, USA; A.C. Camargo Cancer Center, Sao Paulo, Brazil; Osaka University Hospital, Osaka, Japan; and Hacettepe University Hospital, Ankara, Turkey. The study was approved by the institutional Review Boards of Johns Hopkins Hospital and all other participating institutions. Proper material transfer agreements were obtained. Patients with a known history of malignancy other than bladder cancer were excluded from the study. The study included two cohorts of patients. The Early Detection cohort comprised patients who were referred to a urology clinic in one of the above hospitals because of hematuria or lower urinary tract symptoms (Supplementary File 1) (570 Patients). The second cohort (322 patients) represented patients with prior established diagnosis of BC who are on surveillance for disease recurrence (Surveillance Cohort). As noted in the main text, these patients'
primary tumors harbored mutations in at least one of the 11 genes assessed through the multiplex or singleplex assays. A minimum follow-up of 12 months was from date of urine collection was required for cases with no evidence of incident or recurrent tumors in the Early Detection or Surveillance cohorts, respectively. Urine samples were collected prior to any procedures, such as cystoscopy, performed during the patients’ visits. A total of 892 urine samples were analyzed in the study, composed of two type of samples. The first was residual urinary cells after processing with standard BD SurePath™ liquid-based cytology protocols (Becton Dickinson and Company; Franklin Lakes, NJ, USA). To allow for standard-of-care, residual SurePath® fluids were kept refrigerated for 6-8 weeks prior to submission for DNA purification to allow for any potential need for repeat cytology processing of the same sample. The second sample type was composed of bio-banked fresh urine samples in which 15 - 25 mL of voided urine samples were stored at 4°C for up to 60 min prior to centrifugation (10 min at 500 g) and the pellets stored at minus 80°C prior to DNA purification. Urines from 188 healthy individuals of average age 26 were also obtained and processed identically to the bio-banked fresh urine samples.

Formalin-fixed paraffin-embedded (FFPE) tumor tissue samples from trans-urethral resections (TURB) or cystectomies were collected in 413 of the 892 cases. When several different tumors from the same patient were available (because of recurrences), the earliest tumor tissue obtained following the donation of the urine sample was used in the Early Detection Cohort. In the surveillance cohort, the tumors preceding the donation of the urine sample was used in 146 of the 322 patients. In the other 176 Surveillance cases, the earliest tissue obtained following the donation of the urine sample was used. A genitourinary pathologist reviewed all histologic slides to confirm the diagnosis and select a representative tumor area with as high tumor cellularity as possible for that case. Corresponding FFPE blocks were cored with a sterile 16-gauge needle. One to three cores were obtained per tumor and placed in 1.5-mL sterile tubes for DNA purification, as previously described [14]. Electronic medical records were reviewed to obtain medical history and follow up data in all patients.

**Mutation analysis**

Three separate PCR assays were used to search for abnormalities in urinary cell DNA. First, a multiplex PCR was used to detect mutations in regions of ten genes commonly mutated in urologic malignancies CDKN2A, ERBB2, FGFR3, HRAS, KRAS, MET, MLL, PIK3CA, TP53, and VHL [33-40]. The 57 primer pairs used for this multiplex PCR were divided in a total of three multiplex reactions, each containing non-overlapping amplicons (Supplementary Table 13) These primers were used to amplify DNA in 25 uL reactions as previously described [42] except that 15 cycles was used for the initial amplification. Second, the TERT promoter region was evaluated. A single amplification primer was used to amplify a 73-bp segment containing the region of the TERT promoter known to harbor mutations in BC [14]. The conditions used to amplify it were the same as used in the multiplex reactions described above except that Phusion GC Buffer (Thermo-Fisher) instead of HF buffer was used and 20 cycles were used for the initial amplification. Note that the TERT promoter region could not be included in the multiplex PCR because of the high GC content of the former. PCR products were
puriﬁed with AMPure XP beads (Beckman Coulter, PA, USA) and 0.25% of the puriﬁed PCR products (multiplex) or 0.0125% of the PCR products (TERT singleplex) were then amplified in a second round of PCR, as described in [57]. The PCR products from the second round of ampliﬁcation were then puriﬁed with AMPure and sequenced on an Illumina instrument. For each mutation identiﬁed, the mutant allele frequency (MAF) was determined by dividing the number of uniquely identiﬁed reads with mutations [42] by the number of total uniquely identiﬁed reads. Each DNA sample was assessed in two independent PCRs, for both the TERT promoter and multiplex assays, and samples were scored as positive only if both PCRs showed the same mutation. The mutant allele frequencies and number of UIDs listed in the Supplementary Tables refer to the average of the two independent assays.

To evaluate the statistical signiﬁcance of putative mutations, we assessed DNA from white blood cells of 188 unrelated normal individuals. A variant observed in the samples from cancer patient was only scored as a mutation if it was observed at a much higher MAF than observed in normal WBCs. Speciﬁcally, the classiﬁcation of a sample’s ctDNA status was based on two complementary criteria applied to each mutation: 1) the difference between the average MAF in the sample of interest and the corresponding maximum MAF observed for that same mutation in a set of controls, and 2) the Stouffer’s Z-score obtained by comparing the MAF in the sample of interest to a distribution of normal controls. To calculate the Z-score, the MAF in the sample of interest was ﬁrst normalized based on the mutation-speciﬁc distributions of MAFs observed among all controls. Following this mutation-speciﬁc normalization, a P-value was obtained by comparing the MAF of each mutation in each well with a reference distribution of MAFs built from normal controls where all mutations were included. The Stouffer’s Z-score was then calculated from the p-values of two wells, weighted by their number of UIDs. The sample was classiﬁed as positive if either the difference or the Stouffer’s Z-score of its mutations was above the thresholds determined from the normal WBCs. The threshold for the difference parameter was deﬁned by the highest MAF observed in any normal WBCs. The threshold for the Stouffer’s Z-score was chosen to allow one false positive among the 188 normal urine samples studied.

Analysis of aneuploidy. Aneuploidy was assessed with FastSeqS, which uses a single primer pair to amplify ~38,000 loci scattered throughout the genome [41]. After massively parallel sequencing, gains or losses of each of the 39 chromosome arms covered by the assay were determined using a bespoke statistical learning method described elsewhere (Deauville et al., in preperation). A Support vector machine (SVM) was used to discriminate between aneuploid and euploid samples. The SVM was trained using 3150 low neoplastic cell fraction synthetic aneuploid samples and 677 euploid peripheral white blood cell (WBC) samples. Samples were scored as positive when the genome-wide aneuploidy score was >0.7 and there was at least one gain or loss of a chromosome arm.

Identity checks. A multiplex reaction containing 26 primers detecting 31 common SNPs on chromosomes 10 and 20 was performed using the ampliﬁcation conditions
described above for the multiplex PCR. The 26 primers used for this identity evaluation are listed in Supplementary Table 14.

Statistical Analysis

Performance characteristics of urine cytology, UroSEEK and its three components was calculated using using MedCalc statistical software, online version (https://www.medcalc.org/calc/diagnostic_test.php).

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