Modern Pathology (2019) 32:1544–1550
https://doi.org/10.1038/s41379-019-0276-y

ARTICLE

Incidence and distribution of UroSEEK gene panel in a multi-institutional cohort of bladder urothelial carcinoma

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Received: 29 January 2019 / Revised: 22 March 2019 / Accepted: 23 March 2019 / Published online: 25 April 2019

Abstract

Noninvasive approaches for early detection of bladder cancer are actively being investigated. We recently developed a urine-based molecular assay for the detection and surveillance of bladder neoplasms (UroSEEK). UroSEEK is designed to detect alterations in 11 genes that include most common genetic alterations in bladder cancer. In this study, we analyzed 527 cases, including 373 noninvasive and 154 invasive urothelial carcinomas of bladder from transurethral resections or cystectomies performed at four institutions (1991–2016). Two different mutational analysis assays of a representative tumor area were performed: first, a singleplex PCR assay for evaluation of the *TERT* promoter region (TERTSeqS) and second, a multiplex PCR assay using primers designed to amplify regions of interest of 10 (*FGFR3, PIK3CA*, *TP53*, *HRAS*, *KRAS*, *ERBB2*, *CDKN2A*, *MET*, *MLL*, and *VHL*) genes (UroSeqS). Overall, 92% of all bladder tumors were positive for at least one genetic alteration in the UroSEEK panel. We found *TERT* promoter mutations in 77% of low-grade noninvasive papillary carcinomas, with a relatively lower incidence of 65% in high-grade noninvasive papillary carcinomas and carcinomas in situ; $p = 0.017$. Seventy-two percent of pT1 and 63% of muscle-invasive bladder tumors harbored *TERT* promoter mutations with g.1295228C>T alteration being the most common in all groups. *FGFR3* and *PIK3CA* mutations were more frequent in low-grade noninvasive papillary carcinomas compared with high-grade noninvasive papillary carcinomas and carcinomas in situ ($p < 0.0001$), while the opposite was true for *TP53* ($p < 0.0001$). Significantly higher rates of *TP53* and *CDKN2A* mutation rates ($p = 0.005$ and 0.035, respectively) were encountered in muscle-invasive bladder tumors compared with those of pT1 stage. The overwhelming majority of all investigated tumors showed at least one mutation among UroSEEK assay genes, confirming the comprehensive coverage of the panel and supporting its potential utility as a noninvasive urine-based assay.

Supplementary information The online version of this article (https://doi.org/10.1038/s41379-019-0276-y) contains supplementary material, which is available to authorized users.

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Introduction

Bladder cancer is the fourth most common cancer in men and the most common malignancy of the urinary tract in both male and female. In 2019, 80,470 new cases are being estimated in the Unites States, leading to 17,670 deaths [1].

Invasive urothelial carcinoma evolves through two distinct pathways: low- and high-grade noninvasive papillary urothelial carcinoma and "flat" carcinoma in situ [2]. Due to high recurrence rates and likelihood of progression to muscle-invasive bladder cancer, periodical follow-up with cystoscopy and cytology for patients diagnosed with noninvasive tumors is required [3–6]. These procedures entail an estimated $3 billion burden to the health care system every year [7]. Alternative approaches for surveillance are therefore needed.

Early detection of bladder cancer remains a challenge in clinical practice. Hematuria, with or without lower urinary tract symptoms, is the most common presenting symptom. While asymptomatic microscopic hematuria is prevalent (up to 31%) [8, 9], only a small fraction of patients will ultimately be diagnosed with bladder cancer (3–5%) [10]. Therefore, risk stratification with a noninvasive method to avoid unneeded cystoscopy is of great utility. Desquamated urothelial cells in urine have long been used as a valuable source for noninvasive detection of bladder cancer. Beside cystoscopy, urine cytology remains the gold standard for bladder cancer detection. However, its overall low sensitivity (11–76%), especially in low-grade tumors, tampers its utility. Several FDA- approved urine-based noninvasive assays are currently available (e.g., UroVysion, ImmunoCyt/uCyt+™, NMP22®, and BTA®) for both early detection and surveillance of bladder cancer. Sensitivity and specificity for these assays range from 56 to78% and 74 to 88%, respectively [11–18]. We recently developed a noninvasive bladder cancer assay with promising performance characteristics in both early detection and surveillance setting. When combined with cytology, a sensitivity of 95% and a specificity of 93% were reached in the early detection cohort [19]. The assay, termed "UroSEEK", consists of three components (TERTSeqS, UroSeqS, and FastSeqS). It covers molecular alterations that are frequently encountered in bladder cancer, in addition to aneuploidy (FastSeqS). The alterations include *TERT* promoter mutations (TERTSeqS) that occur in up to 80% of bladder cancers [20, 21] and 10 additional genes: *FGFR3, PIK3CA, HRAS, KRAS, TP53, CDKN2A, ERBB2, MLL, MET*, and *VHL* (UroSeqS) [22–24].

Evidently, a noninvasive mutation-based approach can only be effective if its genes are closely matched to the tumors it aims to detect. Therefore, in this study, we sought to identify the distribution of the UroSEEK gene panel in archival tumor tissues from a multi-institutional international cohort of bladder urothelial carcinoma. The relationship with tumor grade and stage was also assessed.

Materials and methods

Patient samples and clinical data

The study was approved by the Institutional Board Review of participating institutions. The required material transfer agreements were obtained. The 527 formalin-fixed, paraffin-embedded bladder urothelial carcinoma specimens were collected between 1991 and 2016 from four international academic institutions (Johns Hopkins Hospital, Baltimore, MD, United States; A.C. Camargo Cancer Center, Sao Paulo, Brazil; Osaka University Hospital, Osaka, Japan; and Hacettepe University Hospital, Ankara, Turkey). The mutational findings of the UroSEEK gene panel in a subset (102 tumors) were described in our previously reported study, describing the noninvasive multigene assay (UroSEEK) [19]. All histologic sections from transurethral resection of bladder tumor and cystectomy specimens were reviewed by a genitourinary pathologist, to confirm the diagnosis and select a representative tumor area. The corresponding formalin-fixed, paraffin-embedded blocks were cored for DNA purification as previously described [20]. Clinicopathologic data were obtained from electronic medical records. Only cases with a minimum follow-up time of 3 months were included in outcome analysis. Disease recurrence was defined as the development of histologically documented tumor occurrence. Progression was defined as the occurrence of histologically documented upgrade or upstage of disease.

Mutation analysis

Mutation and data analysis were performed as previously described [19, 20, 25, 26]. In brief, purified DNA was submitted for SafeSeqS analysis, a sequencing error-reduction technique capable of discriminating mutations from artifactual sequencing variants introduced during the sequencing process [27, 28]. Two different mutational analysis assays were performed: first, a singleplex PCR assay for evaluation of *TERT* promoter region (TERTSeqS) and a second multiplex PCR assay [20] using primers designed to amplify regions of interest of 10 (*FGFR3*, *PIK3CA*, *TP53*, *HRAS*, *KRAS*, *ERBB2*, *CDKN2A*, *MET*, *MLL*, and *VHL)* genes (UroSeqS). Primers are listed in Supplementary Table S1 [19].

To evaluate the statistical significance of observed mutations, DNA from white blood cells of 188 unrelated healthy individuals was also assessed. A variant was scored as a mutation only if the mutant allele frequency was much

higher than that observed in normal white blood cells. As previously described [19], the classification of a sample's DNA status was based on two complementary criteria applied to each mutation: 1) the difference between the average mutant allele frequency in the sample of interest and the corresponding maximum mutant allele frequency observed for that same mutation in a set of controls; and 2) the Stouffer's Z-score obtained by comparing the mutant allele frequency in the sample of interest to a distribution of normal controls.

Statistical analysis

Statistical analysis was performed using R version 3.5.1 (07-02-2018) from the R Foundation for Statistical Computing (Vienna, Austria). For hypothesis testing, statistical significance was established at $p < 0.05$ for two tails of distribution. The relationship between mutation status and pathological and outcome variables was analyzed by a Chi-square test with Yates' continuity correction.

Results

Clinicopathologic features

Five hundred and twenty-seven tumors from 484 patients were included in the study. One-hundred and thirteen patients were female and 371 were male. The median age was 68 years (range 28–96). Three hundred and twenty-nine patients were Caucasian, 68 were Asian, and 46 were African-American with the race in the remaining 41 patients being undetermined or from other category. The tumor included 188 low-grade noninvasive papillary carcinomas, 129 high-grade noninvasive papillary carcinomas, 56 carcinomas in situ, and 154 high-grade invasive urothelial carcinomas, including 111 pT1 and 43 ≥ pT2 (muscle-invasive) tumors.

Mutation analysis

The frequency of mutations of all genes of the UroSEEK gene panel across histopathological categories is summarized in Fig. 1. The mutation variants of analyzed genes are listed in Supplementary Table S2. Overall, 93% of non-invasive and 92% of invasive tumors were positive for at least one mutation in genes included in UroSEEK assay.

TERT promoter mutations were identified in 70% of all cases, with the most common alteration being g.1295228C>T, followed by g.1295250C>T. A previously unreported variant (g.1295223G>T, in one case) was detected. A higher incidence of *TERT* promoter mutation was identified in low-grade noninvasive papillary carcinomas compared with high-grade noninvasive papillary carcinomas and carcinomas in situ (77% vs. 65%; $p = 0.017$; see Table 1). Although a higher frequency of *TERT* promoter mutations occurred in pT1 cases (72%) compared with muscle-invasive bladder cancer (63%), the difference was not statistically significant.

Among the 10 genes included in the UroSeqS assay, *FGFR3* and *PIK3CA* mutations occurred significantly more often in low-grade noninvasive papillary carcinoma tumors compared with high-grade noninvasive papillary carcinomas and carcinomas in situ ($p < 0.0001$), while the reverse was true for *TP53* ($p < 0.0001$; see Table 1). In invasive bladder cancer, *CDKN2A* and *TP53* mutations were more commonly observed in muscle-invasive bladder cancer compared with pT1 tumors ($P = 0.035$ and 0.005, respectively; see Table 2). Regarding mutation variants, p.S249C was the most frequent *FGFR3* mutation (67%), while p.E545K and p.H66P were the most common *PIK3CA* and *CDKN2A* mutations (49 and 79%, respectively; see Supplementary Table S2).

Two hundred and nineteen tumors demonstrated the co-occurrence of mutations in both assay components, with 59% of tumors harboring *TERT* promoter mutation, also showing a mutation in at least one UroSeqS gene ($p = 0.001$). Finally, occasional cases demonstrated multiple variants of *TERT* promoter (13 tumors) and *TP53* (14 tumors) mutations, and one case displayed two *FGFR3* variant mutations.

Mutational analysis in patients with sequential tumors

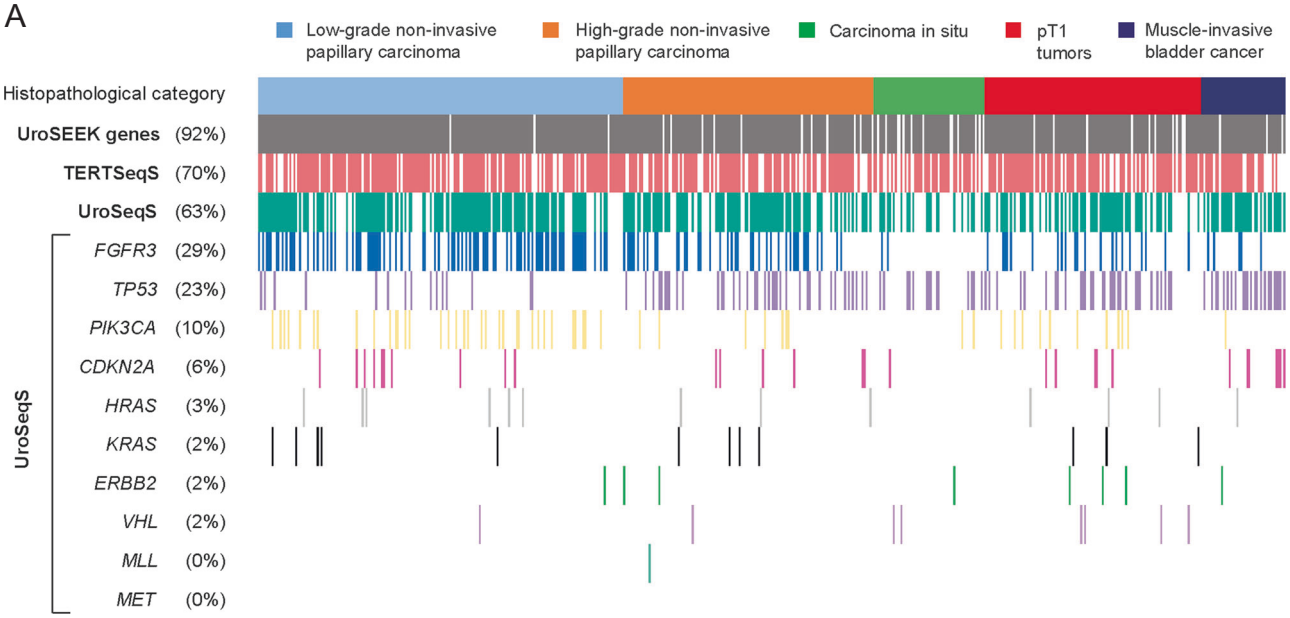
Tissues from sequential tumors were available for mutational analysis in 36 patients. As illustrated in Fig. 2, *TERT* promoter mutations were absent in all samples in 7 of the 36 patients. In the remaining 29 patients, the same *TERT* promoter mutation was persistently present across tumors in 22 and variably found in 7 patients. Only 2 of 15 patients with observed *FGFR3* mutations had the same mutation present across tumors.

Association of UroSEEK assay with outcome

The distribution of the three hundred and three cases that met the minimum follow-up requirement for outcome analysis was as follows: 124 low-grade noninvasive papillary carcinomas, 78 high-grade noninvasive papillary carcinomas, 24 carcinomas in situ, 58 pT1, and 19 muscle-invasive bladder tumors.

The association of tumor UroSEEK, TERTSeqS, and UroSeqS findings with recurrence and progression are summarized in Table 3. As shown, 94% of tumors with subsequent recurrence had mutation in at least one of the 11 genes included in UroSEEK. A comparable rate of 95% was found in tumors without recurrence ($p = 0.784$). Only 21

A



B

	Low-grade non-invasive papillary carcinoma		High-grade non-invasive papillary carcinoma		Carcinoma in situ		pT1 tumors		Muscle-invasive bladder cancer		Total cases	
UroSEEK genes	185/188	(98%)	118/129	(92%)	43/56	(77%)	101/111	(91%)	40/43	(93%)	487/527	(92%)
TERTSeqS	145/188	(77%)	87/129	(67%)	34/56	(61%)	80/111	(72%)	27/43	(63%)	370/527	(70%)
UroSeqS	131/188	(70%)	87/129	(67%)	21/56	(38%)	62/111	(56%)	32/43	(74%)	333/527	(63%)
FGFR3	87/188	(46%)	40/129	(31%)	2/56	(4%)	21/111	(19%)	5/43	(12%)	155/527	(29%)
TP53	15/188	(8%)	37/129	(29%)	14/56	(25%)	31/111	(28%)	23/43	(54%)	120/527	(23%)
PIK3CA	35/188	(19%)	7/129	(5%)	2/56	(4%)	10/111	(9%)	1/43	(2%)	55/527	(10%)
CDKN2A	10/188	(5%)	6/129	(5%)	1/56	(2%)	5/111	(5%)	7/43	(16%)	29/527	(6%)
HRAS	6/188	(3%)	3/129	(2%)	0/56	(0%)	3/111	(3%)	1/43	(2%)	13/527	(3%)
KRAS	5/188	(3%)	4/129	(3%)	0/56	(0%)	3/111	(3%)	0/43	(0%)	12/527	(2%)
ERBB2	1/188	(1%)	2/129	(2%)	1/56	(2%)	3/111	(3%)	1/43	(2%)	8/527	(2%)
VHL	1/188	(1%)	1/129	(1%)	2/56	(4%)	4/111	(4%)	0/43	(0%)	8/527	(2%)
MLL	0/188	(0%)	1/129	(1%)	0/56	(0%)	0/111	(0%)	0/43	(0%)	1/527	(0%)
MET	0/188	(0%)	0/129	(0%)	0/56	(0%)	0/111	(0%)	0/43	(0%)	0/527	(0%)

Fig. 1 Distribution of mutations in the UroSEEK gene panel and its TERTSeqS and UroSeqS components across 527 bladder carcinomas. **a** Oncoplot graphical representation of data. Each column represents one tumor. The top line colored boxes indicate the histopathological category. Rows indicate affected gene(s) and their rate of mutations. Detailed listing of the absolute numbers of mutations is shown in **b**

patients developed progression during follow-up. Tumors with progression were less likely to harbor a mutation in one or more of the UroSEEK assay genes (81% vs. 96%; $p = 0.016$).

Discussion

In our current multicenter study, we investigated the comprehensive coverage of the UroSEEK gene assay in archival bladder cancer tissues. The TERTSeqS component detected mutations in the *TERT* promoter region in 70% of all tumors. Mutations in the UroSeqS gene panel were found in 63% of all cases. Combining these two components led to a capture rate for alteration of the UroSEEK assay in 92% of all tumors. More specifically, in the subset of low-grade noninvasive papillary carcinoma cases, we found at least one gene alteration in 98% of the cases, with 77% being positive for TERTSeqS and 70% positive for UroSeqS. This is especially important, given the low sensitivity of routine

Table 1 Distribution of mutations of the UroSEEK gene panel in noninvasive bladder cancer

	Low-grade noninvasive papillary carcinoma	High-grade noninvasive papillary carcinoma + carcinoma in situ	*p*-value
UroSEEK genes	185/188 (98%)	161/185 (87%)	**<0.0001**
TERTSeqS	145/188 (77%)	121/185 (65%)	**0.017**
UroSeqS	131/188 (70%)	108/185 (58%)	**0.030**
FGFR3	87/188 (46%)	42/185 (23%)	**<0.0001**
TP53	15/188 (8%)	51/185 (28%)	**<0.0001**
PIK3CA	35/188 (19%)	9/185 (5%)	**<0.0001**
CDKN2A	10/188 (5%)	7/185 (4%)	0.644
HRAS	6/188 (3%)	3/185 (2%)	0.515
KRAS	5/188 (3%)	4/185 (2%)	1.0
ERBB2	1/188 (1%)	3/185 (2%)	0.604
VHL	1/188 (1%)	3/185 (2%)	0.604
MLL	0/188 (0%)	1/185 (1%)	0.994
MET	0/188 (0%)	0/185 (0.0%)	.

Assay categories (UroSEEK and its components) and significant *p*-value are listed in bold

Table 2 Distribution of mutations of the UroSEEK gene panel in invasive bladder cancer

	pT1 tumors	Muscle-invasive bladder cancer	*p*-value
UroSEEK genes	101/111 (91%)	40/43 (93%)	0.933
TERTSeqS	80/111 (72%)	27/43 (63%)	0.354
UroSeqS	62/111 (56%)	32/43 (74%)	0.053
FGFR3	21/111 (19%)	5/43 (12%)	0.399
TP53	31/111 (28%)	23/43 (54%)	**0.005**
PIK3CA	10/111 (9%)	1/43 (2%)	0.273
CDKN2A	5/111 (5%)	7/43 (16%)	**0.035**
HRAS	3/111 (3%)	1/43 (2%)	1.0
KRAS	3/111 (3%)	0/43 (0%)	0.661
ERBB2	3/111 (3%)	1/43 (2%)	1.0
VHL	4/111 (4%)	0/43 (0%)	0.486
MLL	0/111 (0%)	0/43 (0%)	.
MET	0/111 (0%)	0/43 (0%)	.

Assay categories (UroSEEK and its components) and significant *p*-value are listed in bold

cytology for the diagnosis of low-grade noninvasive tumors. As evidenced in our recent study, urine cytology was negative in all 49 low-grade noninvasive papillary carcinoma cases, while urine UroSEEK was positive in 2/3 of these cases [19].

The current observation of 54% of *TP53* mutation in muscle-invasive bladder cancer is in line with findings of recent studies, such as The Cancer Genome Atlas [23] and Kim et al. [29], where *TP53* was altered in 48 and 57% of cases, respectively. We observed a lower frequency for *PIK3CA*, *ERBB2*, and *MLL* in muscle-invasive bladder cancer compared with these two studies.

Our study represents one of the largest assessments of a multigene assay in low-grade noninvasive papillary carcinoma tumors to date, where 188 cases were analyzed. In this group, *FGFR3* mutations were the most frequent (46%) among the 10 genes included in UroSeqS, followed by *PIK3CA* mutation in 19%. These rates are lower than those obtained by Hurst et al. [30] in their study of whole-exome and targeted sequencing of 82 Ta tumors (79% and 54% for *FGFR3* and *PIK3CA* mutations, respectively). This could be due to our hotspot-focused mutational analysis approach, given that Hurst et al. have identified many non-hotspot mutations in their analysis.

TERT promoter mutations are one of the most frequent alterations in bladder cancer and its variants [25, 26, 31–33]. In this analysis, we found *TERT* promoter mutations in 70% of all cases, with the highest rate observed in low-grade noninvasive papillary carcinomas (77%). The here-found distribution rates of *TERT* promoter mutations in noninvasive lesions are in line with our originally reported rates in conventional urothelial carcinoma in Kinde et al., where the low-grade noninvasive papillary carcinoma group had the highest rate of *TERT* promoter mutations of 86% [20]. In contrast, Pietzak et al. found *TERT* promoter mutations to be more frequent in their high-grade non-invasive papillary carcinoma cases compared with low-grade noninvasive papillary carcinomas (88% and 61%, respectively) [34].

Our analysis of sequential tumors in 36 patients unveiled an identical *TERT* promoter mutation across tumors in 22 of the 29 patients harboring *TERT* promoter mutations. In surveillance setting, UroSEEK is envisioned to be used for follow-up in patients, whose index tumor is positive for at least one alteration in the panel. This consistency of *TERT* promoter alteration across tumors, if proven in a larger cohort of sequential tumors, suggests that assessment of the index tumor might be sufficient and could obviate the need for repeat sequential tumor testing. The same approach would not apply to cases where the index tumor is only positive for one of the ten genes in UroSeqS, where we found inconsistency in mutation detection across tumors from the same patient. This might in part be due to tumor molecular heterogeneity that was not captured by our adopted technique.

Given the nature of the cohort in the original study describing the noninvasive multigene assay (UroSEEK) [19], analysis of association with outcome could only be performed in a subset of patients. This is due to the fact that in a proportion of cases, tumors that are contemporaneous

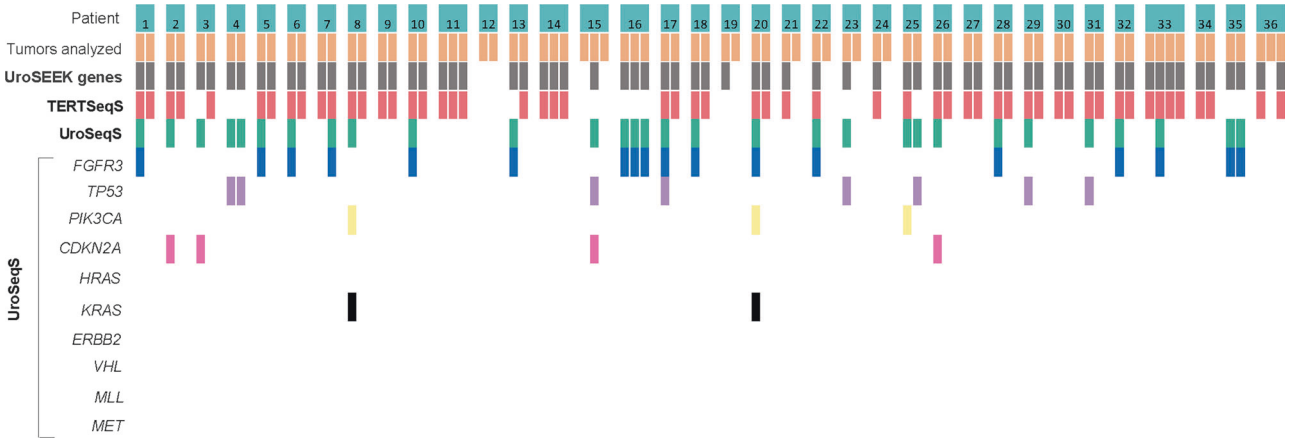


Fig. 2 Oncoplot representation of mutations in the UroSEEK gene panel and its TERTSeqS and UroSeqS components in 36 patients with sequential tumors. Each broad column represents one patient. The individual tumor per patient (orange box) and their mutated gene(s) are indicated in each row

Table 3 Association of the UroSEEK gene panel with outcome

	Recurrence		*P*-value	Progression		*p*-value
	Yes	No		Yes	No	
	$N = 132$	$N = 171$		$N = 21$	$N = 282$	
Mutation in **UroSEEK** genes	124/132 (94%)	163/171 (95%)	0.784	17/21 (81%)	270/282 (96%)	**0.016**
Mutation in **TERTSeqS**	101/132 (77%)	115/171 (67%)	0.101	12/21 (57%)	204/282 (72%)	0.217
Mutation in **UroSeqS**	87/132 (66%)	120/171 (70%)	0.505	12/21 (57%)	195/282 (69%)	0.369

Assay categories (UroSEEK and its components) and significant *p*-value are listed in bold

with analyzed urine samples were selected. The strengths of the study include the large number of cases in the multi-institutional cohort (527 tumors) and the advantageous error-proof nature of the SafeSeqS technique.

In conclusion, the overwhelming majority of all investigated tumors showed at least one mutation among genes included in the recently reported UroSEEK assay, across tumor grade and stage. This confirms the comprehensive coverage of the UroSEEK panel and supports its potential utility as a noninvasive urine-based assay. Our findings are especially reassuring in the subset of low-grade non-invasive papillary carcinoma, where routine cytology lacks sensitivity.

Acknowledgements We are grateful for the generous support provided by the Johns Hopkins Greenberg Bladder Cancer Institute, Henry and Marsha Laufer, the Virginia and DK Ludwig Fund for Cancer Research, the Commonwealth Foundation, the John Templeton Foundation, and the Conrad R Hilton Foundation. All sequencing was performed at the Sol Goldman Sequencing Facility at Johns Hopkins. This work was also supported by grants from the NIH (Grants CA-77598, CA 06973, GM 07309, and ES019564).

Compliance with ethical standards

Conflict of interest NP, KWK, and BV: founders of Personal Genome Diagnostics and PapGene and advisors to Sysmex-Inostics. KWK and BV also advise Eisai. BV is also an advisor to Camden Partners. These companies and others have licensed technologies from Johns Hopkins that are related to the work described in this paper. These licenses are associated with equity or royalty payments to NP, KWK, GJN, and BV. Additional patent applications on the work described in this paper may be filed by Johns Hopkins University. The terms of these arrangements are managed by the university in accordance with its conflict of interest policies. The remaining authors declare that they have no conflict of interest.

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