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Review

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A systematic review on mutation markers for bladder cancer diagnosis in urine

Anouk E. Hentschel[1], Emma E. van der Toom[1], André N. Vis[1], Johannes C.F. Ket[3], Judith Bosschieter[1], Martijn W. Heymans[4], R. Jeroen A. van Moorselaar[1], Renske D.M. Steenbergen[2] and Jakko A. Nieuwenhuijzen[1]

[1]Departments of Urology, [2]Pathology, Amsterdam University Medical Centers, Cancer Center Amsterdam, [3]Medical Library, Vrije Universiteit Amsterdam, and [4]Amsterdam, Epidemiology & Biostatistics, Amsterdam Public Health, Amsterdam, The Netherlands

Objectives

To systematically summarise the available evidence on urinary bladder cancer (BC) mutation markers. Gene mutations are expected to provide novel biomarkers for urinary BC diagnosis. To date, evidence on urinary BC mutation markers has not proven sufficient to be adopted by clinical guidelines. In the present systematic review, diagnostic accuracy of urinary mutation analysis is separately assessed for primary BC diagnosis (BC detection) and for follow-up of BC patients (BC surveillance).

Methods

A literature search (PubMed, Embase.com and Wiley/Cochrane Library) and systematic review was performed up to 31 October 2019. As studies were too heterogeneous, no quantitative analysis could be performed.

Results

In total, 25 studies were summarised by qualitative analysis. For BC detection, diagnostic accuracy differed considerably for single mutation markers (sensitivity 1–85%, specificity 84–100%), and for marker panels (sensitivity 50–94%, specificity 43–97%). Similarly, for BC surveillance, diagnostic accuracy was highly variable for single mutation markers (sensitivity 0–85%, specificity 66–100%), and for marker panels (sensitivity 51–84%, specificity 66–96%).

Conclusion

Urinary mutation analysis showed to be a promising diagnostic tool for non-invasive BC diagnosis. Nonetheless, we observed substantial differences in diagnostic accuracy of urinary BC mutation markers among publications. To translate the data summarised in the present review to future clinical practice, heterogeneity in research design, BC population, mutation analysis technique and urinary DNA should be considered. Eventual clinical implementation of urinary BC mutation markers can only be achieved by collecting more and stronger evidence. Combining different molecular assays might overcome current shortcomings of urinary mutation analysis.

Keywords

biomarkers, mutation, molecular diagnostics, urinary bladder neoplasms, urine analysis, #BladderCancer, #blcsm

Introduction

Bladder cancer (BC) is a worldwide clinical problem, as it is present among the top 10 most commonly diagnosed cancers. A first indication of BC is often painless haematuria without other symptoms. After transurethral resection of the bladder tumour (TURBT), patients are either diagnosed with non-muscle-invasive (NMIBC) or muscle-invasive BC (MIBC). In organ-confined MIBC, radical cystectomy or chemoradiation are indicated for local tumour control, because poor prognosis necessitates a radical approach [1]. As patients with NMIBC have a more favourable prognosis, the bladder can be preserved in these patients. Treatment consists of TURBT, followed by adjuvant instillations dependent on the risk classification: low-, intermediate- or high-risk [2].

The risk of recurrence (31–78%) or progression (1–45%) remains substantial for patients with NMIBC [3]. Therefore,

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follow-up visits are required at regular intervals during a minimum period of 1 year for low-risk and 5 years for intermediate-risk patients, while high-risk patients need lifelong follow-up [2]. Cystoscopy is the 'gold standard' for primary BC diagnosis (BC detection) and for follow-up of BC patients (BC surveillance) [2]. Following cystoscopy, patients can experience irritative urinary symptoms and, although rare, even severe complications (e.g. urosepsis). Besides the fact that cystoscopy is an invasive procedure, it is also expensive and time-consuming [4]. Urinary analysis is considered a non-invasive and affordable alternative for cystoscopy. Urinary cytology has a sensitivity of 48%, but is only part of clinical practice in high-grade (HG) disease, as here sensitivity increases to 84%, while in low-grade (LG) disease sensitivity decreases to 16% [2,5]. In order to replace cystoscopy, urinary analysis should perform well in all grades and stages of disease. The past decade, several potential urinary biomarkers for BC diagnosis have been proposed at DNA, RNA and protein level, such as DNA methylation, microRNA and Survivin protein, respectively [6]. Many studies reported on this topic, but evidence on urinary biomarkers has not proven sufficient to be adopted by clinical guidelines [2,7].

In addition to DNA methylation, which we previously assessed in a systematic review, other urine-based biomarkers at the DNA level include DNA point mutations, as well as copy number and microsatellite changes. In the present review, we focus on BC mutation markers because of the high mutational load in BC and the extensive number of published reports on DNA mutation analysis in urine for BC diagnosis [8]. DNA point mutations linked to development of BC (e.g. in fibroblast growth factor receptor 3 [*FGFR3*], phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit α [*PIK3CA*] and telomerase reverse transcriptase [*TERT*] promoter) can be present at different hotspots; e.g. *FGFR3* hotspot mutations are particularly found at specific positions in exon 7, 10 or 15, and *PIK3CA* hotspot mutations are mainly detected in exon 9 or 20 [9,10]. Some mutation markers are frequently detected in NMIBC (e.g. *FGFR3* hotspot mutations), while others are more often found in MIBC (e.g. tumour protein p53 [*TP53*] hotspot mutations) [8]. Studies have not only evaluated single mutation markers (e.g. *FGFR3* only), but have also investigated panels of two or more mutation markers and have even performed genome-wide screening for mutation markers [6].

To our knowledge, this is the first systematic review that specifically focusses on mutation markers for urine-based BC diagnosis. Diagnostic accuracy of urinary mutation analysis is separately assessed for BC detection and for BC surveillance. We present a detailed insight into the available literature, a critical review of the existing evidence and an overview of the most promising urinary mutation marker(s) for future practice. Furthermore, we explore heterogeneity between studies and make suggestions for future research.

Methods

Search strategy

A systematic review of the literature was conducted according to the Cochrane Methods Group for Systematic Review of Screening and Diagnostic Tests [11,12].

PubMed, Embase.com and Wiley/Cochrane Library were searched from inception up to 31 October 2019, for relevant publications (by J.C.F.K. and A.E.H.) as represented in the Appendix S1. The search included indexed terms and free-text words for 'bladder cancer', and 'urine', and 'DNA' or 'mutation marker', and 'sensitivity' or 'specificity'. Two reviewers independently (A.E.H. and E.E.T.) checked the references of the selected full-text articles for relevant records. Duplicate publications were removed. If a research group published twice on the same population, the latest publication was included.

Study selection

The online tool Rayyan Qatar Computing Research Institute (QCRI) (https://rayyan.qcri.org) was used to manage the selected records from the bibliographic databases [13]. Publications were independently screened on title and abstract by two reviewers (A.E.H. and E.E.T.). If title and abstract were inconclusive, full-texts were screened. Original articles on DNA mutation markers for urine-based BC diagnosis were eligible for inclusion. Authors could use any kind of technique for urinary mutation analysis, but outcomes of urinary mutation analysis had to be compared with the current 'gold standard' for BC diagnosis (cystoscopy/histology). Studies had to be written in English and had to include a minimum of 10 patients with BC. Animal studies and studies without primary data (e.g. reviews, commentaries) were excluded. Studies that focussed on BC caused by occupational exposure or Bilharzia were excluded as well. These in- and exclusion criteria are largely in accordance with an earlier systematic review conducted by our research group on methylation markers for BC diagnosis in urine [14]. Disagreements between the reviewers were discussed in a consensus meeting with an expert (J.A.N.).

Data extraction and quality assessment

If available online, the full-text articles were judged independently by the two reviewers (A.E.H. and E.E.T.). Sensitivities, specificities, negative and positive predictive values were retrieved from the published data on patients with BC and controls. Data on single mutation markers and marker panels were collected.

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Risk of bias (RoB) assessment

Reviewers A.E.H. and E.E.T. independently assessed the RoB of the included articles by using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS)-2 tool [15]. Reproducibility of the QUADAS-2 tool was piloted in two studies. Disagreements were again discussed in a consensus meeting together with an expert (J.A.N.).

Data analysis

Reviewers A.E.H. and E.E.T. independently abstracted data of the included articles. Diagnostic accuracy of urinary mutation analysis was assessed for BC detection and for BC surveillance. Sensitivity was defined as the percentage of true-positive results in the patients with BC. Specificity was defined as the percentage of true-negative results in the controls. Sensitivity was determined at patient level (1 urine sample/patient), whereas specificity could be determined at patient level or at urine sample level (≥1 urine samples/patient). Tumour-informed sensitivity was defined as the percentage of true-positive results in the bladder tumour tissues with mutations. Tumour-informed specificity was defined as the percentage of true-negative results in the bladder tumour tissues without mutations.

For single-mutation markers, sensitivity and specificity were visualised in forest plots if ≥10 studies reported on the same mutation marker (irrespective of the number of patients included). Sensitivity and specificity were described from five or more studies (irrespective of the number of patients included). Tumour-informed sensitivity and specificity were described when ≥100 tumours were included in the study.

For marker panels, sensitivity and specificity, and tumour-informed sensitivity and specificity were presented in tables. The most promising marker panels with a sensitivity and specificity of ≥80% were described.

Review methods were established in a protocol prior to the conduct of the review and no significant deviations from the protocol were made.

As studies were too heterogeneous, we could not perform a meta-analysis of the data. We therefore decided to summarise the data by performing a qualitative analysis.

Review Manager Version 5.3 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) was used for the construction of graphs.

Results

Quantity of evidence identified

We selected 25 studies from the literature, as shown in the Preferred Reporting Items for Systematic and Meta-Analysis (PRISMA) flow diagram (Fig. 1) [16–40]. An overview of the studies included is given in Table 1 [16–40]. Urinary mutation analysis was used for BC detection in 15 studies [19–22,24–29,31,32,34,39,40], for BC surveillance in three studies [18,30,35], and for BC detection and surveillance in seven studies [16,17,23,33,36–38]. Single mutation markers were assessed in 13 studies [18,19,21–27,30,33,36,39] and marker panels in 12 studies [16,17,20,28,29,31,32,34,35,37,38,40]. For BC detection, 27 single-mutation markers were investigated (Table S1). For BC surveillance, 20 single-mutation markers were evaluated (Table S2). The number of mutation markers included in the panels ranged from two to 20. From the publications on multiple mutation markers, data were retrieved on the marker panel and on the single-mutation markers (if data were provided). Tumour-informed sensitivity and specificity were reported in 10 studies [17,19,25–29,33,35,37], including three studies that reported on tumour-informed analysis only [19,27,28].

RoB of included studies

In 20/25 studies, high RoB was scored for patient selection. In most studies, high RoB was introduced by non-consecutive collection of urine samples or by case-control designs (Fig. 2). For eight of the 25 studies, high RoB was scored for index (urine) test, because the threshold of urinary mutation analysis was not specified, or because it was not stated whether reviewers of urinary mutation results were blinded to the gold standard results. In five of the 25 studies, high applicability concerns were scored for patient selection, as studies solely reported on the diagnostic accuracy of tumour-informed analysis, or as studies selected patients according to their tumour mutation status/their urinary cytology status. Lastly, two of the 25 studies scored high applicability concerns for the index test, as positivity of urinary mutation analysis was also considered true-positive if it preceded positivity of the gold standard by several months. Judgements on bias and applicability were all scored low for three of the 25 studies [20,24,30].

BC detection

Single-mutation markers

Among 18 studies, data could be retrieved on 27 single-mutation markers for urine-based BC detection, with sensitivities from 1% to 85%, and specificities from 84% to 100% (Table S1). Three studies investigated several single mutation markers, but only found negative outcomes for one of their single-mutation markers in both patients with BC and controls: NRAS proto-oncogene, GTPase (*NRAS*), lysine demethylase 6A (*KDM6A*) and Erb-B2 receptor tyrosine kinase 3 (*ERBB3*), respectively [32,34,37]. Sensitivity and specificity of *FGFR3* was reported in 10 studies. The results

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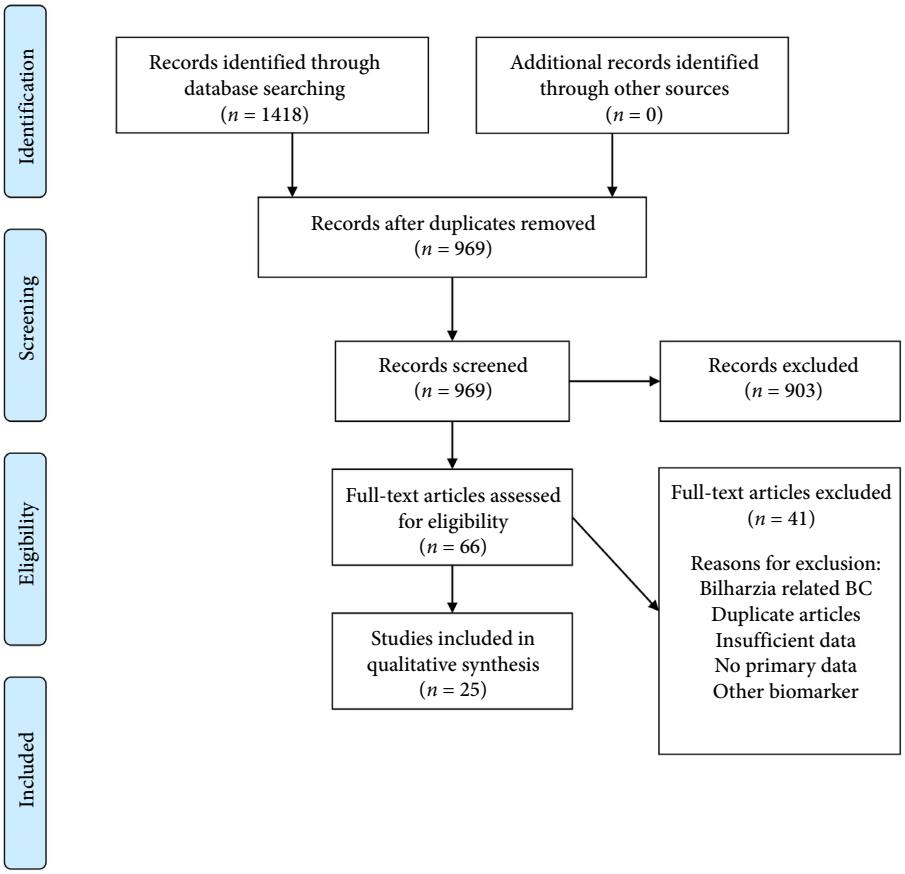
Fig. 1 Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) 2009 flow diagram. BC = Bladder cancer.



are summarised in a forest plot and a receiver operating characteristic (ROC) plot (Fig. 3A,B). Allory *et al.* [16], Beukers *et al.* [17] and Kandimalla *et al.* [23] solely reported on *FGFR3* sensitivity for urinary BC detection and were therefore not included. All three studies showed comparable sensitivities: 36–39%. Diagnostic accuracy of *TERT* was reported in 10 studies, and results are shown in Fig. 4A,B. The study by Beukers *et al.* [17] was not included, because they only assessed *TERT* sensitivity (73%), but no specificity. The total of 11 studies on *TERT* all focussed on mutations in the promoter region of the gene. Sensitivity and specificity of HRas proto-oncogene, GTPase (*HRAS*) was reported in six studies and of *PIK3CA* in five studies (Table S1). *HRAS* sensitivity ranged from 1% to 44% and specificity was 100% in all studies. *PIK3CA* sensitivity varied from 13% to 19% and specificity from 96% to 100%.

Single-mutation markers: tumour-informed analysis

Across six studies, data could be collected on six single-mutation markers for tumour-informed urinary BC detection, with sensitivities from 34% to 100% and specificities from 38% to 100% (Table S1). Noel *et al.* [28] and Serizawa *et al.* [29] investigated the diagnostic accuracy of single-mutation markers in ≥100 tumours. For *FGFR3*, Noel *et al.* [28] found a sensitivity of 43% (95% CI 27–61%) at a specificity of 98% (95% CI 92–100%), whereas Serizawa *et al.* [29] reported a sensitivity of 63% (95% CI 48–77%) at a specificity of 98% (95% CI 90–100%). For *TP53*, Noel *et al.* [28] reported a sensitivity of 34% (95% CI 22–48%) and a specificity of 87% (95% CI 74–95%) compared to Serizawa *et al.* [29] with a sensitivity of 67% (95% CI 41–87%) at a specificity of 99% (95% CI 93–100%). Serizawa *et al.* [29] also investigated *PIK3CA* and found a sensitivity of 53% (95% CI 29–76%) at a specificity of 94% (95% CI 86–98%).

Marker panels

A total of 11 studies reported diagnostic accuracy of eight different marker panels for urinary BC detection (Table 2 [16,17,20,28,29,31,32,34,37,38,40]). Sensitivity ranged from 50% to 94%, whereas specificity ranged from 43% to 97%. Sensitivity and specificity were most promising (≥80%) for urinary marker panels of Dahmcke *et al.* [20], Dudley *et al.* [37] and Rodriguez Pena *et al.* [38]. Dahmcke *et al.* [20] included two mutation markers (*FGFR3* and *TERT*), whereas

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Table 1 Overview of the studies included.

Study ID	First author (year)	BC patients/ controls, *n*	Primary/ recurrent, *n*	Tumour-informed analysis*	Level analysis	Mutation marker (s)	Controls	NMIBC/ MIBC, %	Urinary DNA‡	Mutation analysis technique	Ref.
1.	Allory (2014)	278/124	M (135/143)	No	Both	*FGFR3, TERT*	S	90/8†	Cellular	Sanger Seq, SNaPshot	[16]
2.	Avogbe (2019)	Test: 94/93 Validation: 50/50	M (95/48)	No	Patient	*TERT*	M	81/19	Cellular/ Cell-free	Ultra-deep Seq	[36]
3.	Beukers (2017)	977 BC cases (305 samples of primary BC cases and multiple negative control samples during FU)	M (977/578)	Both	Both	*FGFR3, TERT*	S	100/0 (132 MIBC cases were excluded)	Cellular	SNaPshot	[17]
4.	Couffignal (2015)	74 (multiple negative control samples during FU)	R	No	Urine sample	*FGFR3*	S	100/0	Cellular	allele-specific PCR	[18]
5.	Curigliano (2001)	10/14 (validated in 10 non-BC cases)	P	Yes	Urine sample	*TP53*	BC cases with WT *TP53*	100/0 (T1 only)	Cellular	DGGE	[19]
6.	Dahmcke (2016)	99/376	P	No	Patient	*FGFR3, TERT*	S	85/13†	Cellular	Droplet digital PCR, hydrolysis probe-based assays	[20]
7.	Descotes (2017)	348/167	P	No	Patient	*TERT*	M	82/18	Cellular	nested PCR, Sanger Seq	[21]
8.	Dudley (2019)	91/94	M (54/37)	Both	Patient	*AKT1, ARID1A, BRAF, CDKN1A, CDKN2A, EP300, ERBB2, ERBB3, FBXW7, FGFR3, KDM6A, KRAS, MED12, PIK3CA, PLEKHS1, RB1, STAG2, TERT, TP53, TSC1*	M	85/15	Cellular	NGS	[37]
9.	Fitzgerald (1995)	100/20	P	No	Patient	*HRAS*	H	U	Cellular	SSCP	[22]
10.	Kandimalla (2013)	Test: 140/70 Validation: 95/130	M (39/196)	No	Patient	*FGFR3*	M	93/6†	Cellular	SNaPshot	[23]
11.	Karnes (2012)	Test: 48/240 Validation: 58/690	P	No	Patient	*FGFR3*	S	91/9	Genomic	PCR clamping	[24]
12.	Millholland (2012)	43/24	P	Both	Patient	*FGFR3*	S	84/16	Genomic	NGS	[25]
13.	Miyake (2007)	13/20	P	Both	Patient	*FGFR3*	H	100/0 (MIBC were considered as controls and were therefore excluded)	Cellular	PCR clamping, direct Seq	[26]
14.	Miyake (2010)	24/21 (not validated in non-BC cases)	P	Yes	Patient	*FGFR3*	BC cases with WT *FGFR3*	100/0	Cellular	PCR clamping, direct Seq	[27]

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Table 1 (continued)

Study ID	First author (year)	BC patients/ controls, *n*	Primary/ recurrent, *n*	Tumour-informed analysis*	Level analysis	Mutation marker (s)	Controls	NMIBC/ MIBC, %	Urinary DNA‡	Mutation analysis technique	Ref.
15.	Noel (2015)	76/27 (validated in 5 non-BC cases)	P	Yes	Patient	*FGFR3, TP53*	BC cases with WT *FGFR3* or *TP53*	75/25	Cellular	SNaPshot, functional assay	[28]
16.	Rodriguez Pena (2019)	260/186	M (25/235)	No	Urine sample	*CDK2A, ERBB2, FGFR3, HRAS, KRAS, MET, MLL, PIK3CA, TERT, TP53, VHL*	S	75/10†	Cellular	NGS, SafeSeqS	[38]
17.	Serizawa (2011)	118/33	P	Both	Patient	*FGFR3, HRAS, KRAS, NRAS, PIK3CA, TP53*	H	83/17	Cellular	DGGE, direct Seq	[29]
18.	Shore (2012)	63/670	R	No	Patient	*FGFR3*	S	98/0†	Genomic	PCR clamping	[30]
19.	Stasik (2019)	53/36	P	No	Patient	*TERT*	S	81/19	*Cellular*/ Cell-free	NGS	[39]
20.	van Kessel (2016)	74/80	P	No	Patient	*FGFR3, HRAS, KRAS, NRAS, PIK3CA, TERT*	S	68/32	Cellular	SNapShot	[32]
21.	van Kessel (2017)	97/103	P	No	Patient	*FGFR3, HRAS, TERT*	S	82/8†	Cellular	SNapShot	[31]
22.	van Rhijn (2003)	51/15	M (U)	Both	Patient	*FGFR3*	S	80/19†	Cellular	SSCP, Seq	[33]
23.	Ward (2016)	122/109	P (2/122 were recurrent)	No	Patient	*FGFR3, HRAS, PIK3CA, RXRA, TERT, TP53*	M	66/32†	Cellular	NGS	[34]
24.	Zhu (2019)	95/67	P	No	Patient	*ARID1A, CDKN2A, CREBBP, ERBB2, ERBB3, FGFR1, FGFR3, HRAS, KTM2D, NF1, PIK3CA, STAG2, TP53, TSC1*	S	100/0	Cellular	NGS	[40]
25.	Zuiverloon (2013)	136 BC cases (multiple negative control samples during FU)	R	Both	Urine sample	*FGFR3, HRAS, KRAS, NRAS, PIK3CA*	S	100/0	Cellular	SNapShot	[35]

*ARID1A, AT-rich interaction domain 1A; DGGE, denaturing gradient gel electrophoresis; FU, follow-up; H, healthy controls; M, mixed controls; P, primary; PLEKHS1, leckstrin homology domain-containing family S member 1; R, recurrent; RB1, retinoblastoma; Ref., reference; RXRA, Retinoid X receptor alpha; S, symptomatic patients or patients under surveillance; SSCP, single-strand conformation polymorphism; STAG2, stromal antigen 2; TSC1, TSC complex subunit 1; U, unknown; VHL, Von Hippel–Lindau tumour suppressor; WT, wild-type. *Tumour-informed analysis: tumour-informed sensitivity/specificity represent the % true-positive urines in mutated tumours/the % true-negative urines in non-mutated tumours. †T stage is not known for all BC cases. ‡Cellular, cell-free and genomic refers to DNA isolated from urine pellet, urine supernatant and full void urine, respectively.*

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Fig. 2 Overview of RoB and applicability concerns according to QUADAS-2.

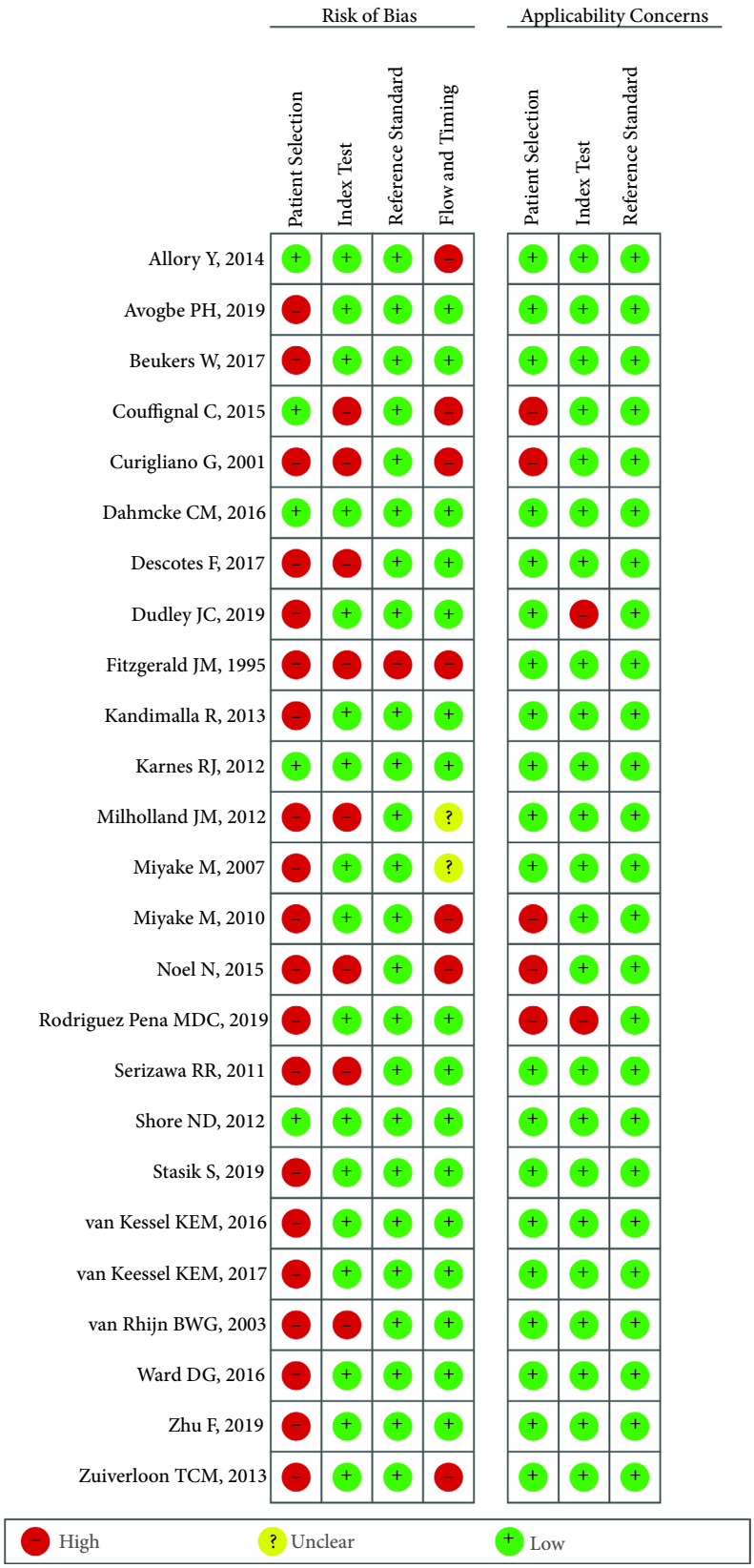

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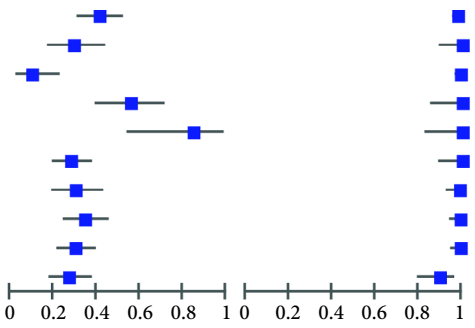
Fig. 3 Forest plot (**A**) and ROC plot (**B**) of the estimated diagnostic accuracy of *FGFR3* for urine-based BC detection. The number of true-positive (TP), false-positive (FP), false-negative (FN) and true-negative (TN) results are provided.

A

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Dahmcke CM, 2016	41	8	58	368	0.41 [0.32, 0.52]	0.98 [0.96, 0.99]
Dudley JC, 2019	16	0	38	34	0.30 [0.18, 0.44]	1.00 [0.90, 1.00]
Karnes RJ, 2012	5	2	43	238	0.10 [0.03, 0.23]	0.99 [0.97, 1.00]
Milholland JM, 2012	24	0	19	24	0.56 [0.40, 0.71]	1.00 [0.86, 1.00]
Miyake M, 2007	11	0	2	20	0.85 [0.55, 0.98]	1.00 [0.83, 1.00]
Serizawa RR, 2011	32	0	81	33	0.28 [0.20, 0.38]	1.00 [0.89, 1.00]
van Kessel KEM, 2016	21	1	48	76	0.30 [0.20, 0.43]	0.99 [0.93, 1.00]
van kessel KEM, 2017	33	1	62	98	0.35 [0.25, 0.45]	0.99 [0.95, 1.00]
Ward DG, 2016	37	1	85	108	0.30 [0.22, 0.39]	0.99 [0.95, 1.00]
Zhu F, 2019	26	7	69	60	0.27 [0.19, 0.37]	0.90 [0.80, 0.96]



B

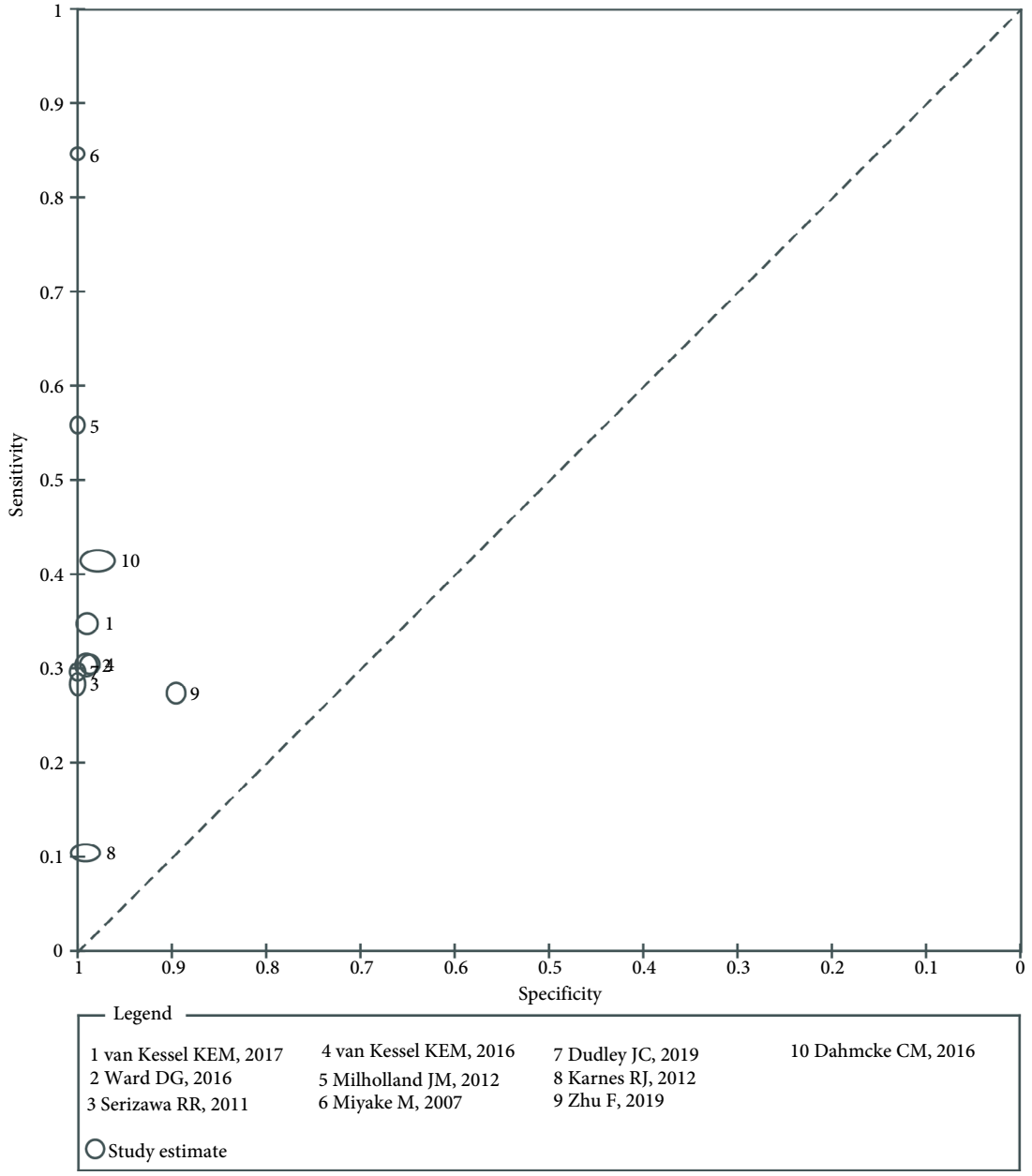


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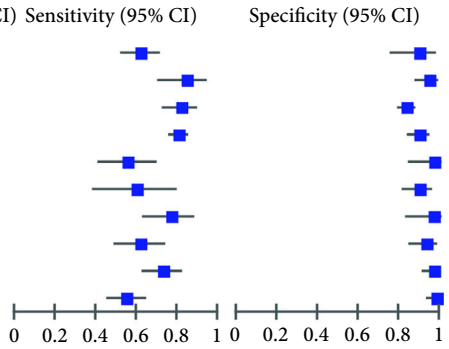
Fig. 4 Forest plot (**A**) and ROC plot (**B**) of the estimated diagnostic accuracy of *TERT* for urine-based BC detection. The number of true-positive (TP), false-positive (FP), false-negative (FN) and true-negative (TN) results are provided.

A

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Allory Y, 2014	73	4	45	35	0.62 [0.52, 0.71]	0.90 [0.76, 0.97]
Avogbe PH, 2019	38	5	7	88	0.84 [0.71, 0.94]	0.95 [0.88, 0.98]
Dahmcke CM, 2016	81	62	18	314	0.82 [0.73, 0.89]	0.84 [0.79, 0.87]
Descotes F, 2017	280	17	68	150	0.80 [0.76, 0.84]	0.90 [0.84, 0.94]
Dudley JC, 2019	30	1	24	33	0.56 [0.41, 0.69]	0.97 [0.85, 1.00]
Rodriguez Pena MDC, 2019	15	9	10	80	0.60 [0.39, 0.79]	0.90 [0.82, 0.95]
Stasik S, 2019	40	1	12	30	0.77 [0.63, 0.87]	0.97 [0.83, 1.00]
van Kessel KEM, 2016	42	5	26	69	0.62 [0.49, 0.73]	0.93 [0.85, 0.98]
van Kessel KEM, 2017	70	3	26	96	0.73 [0.63, 0.81]	0.97 [0.91, 0.99]
Ward DG, 2016	67	2	55	107	0.55 [0.46, 0.64]	0.98 [0.94, 1.00]



B

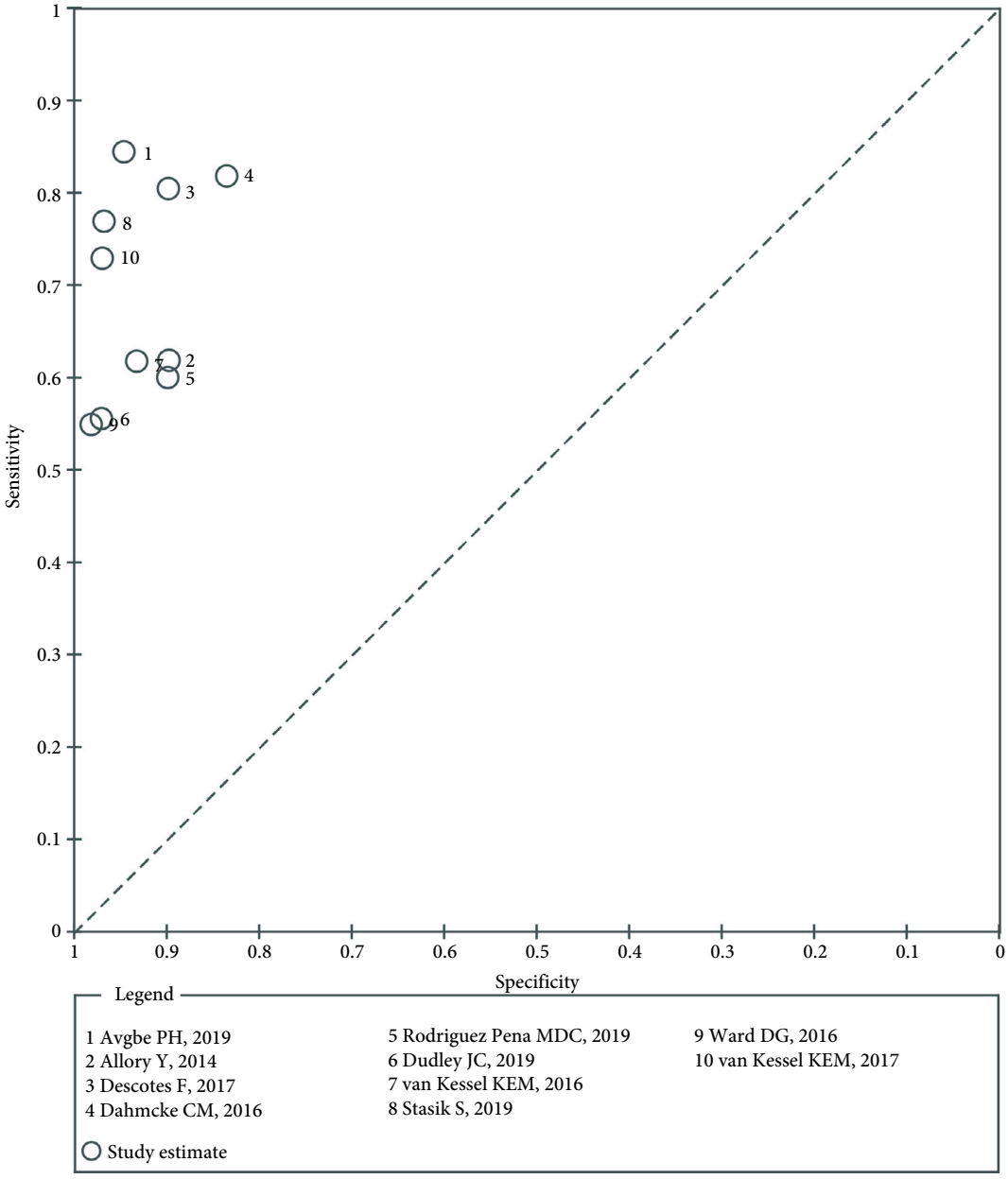


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Table 2 Marker panels of urinary mutation markers for bladder cancer detection.

Marker panels	First author (year)	Ref.	BC patients/ controls, *n*	Sensitivity, %	Specificity, %	Tumour-informed sensitivity, %	Tumour-informed specificity, %
AKT1/ARID1A/BRAF/ CDKN1A/CDKN2A/EP300/ ERBB2/ERBB3/FBXW7/ FGFR3/KDM6A/KRAS/ MED12/PIK3CA/ PLEKHS1/RB1/STAG2/ TERT/TP53/TSC1	Dudley (2019)	[37]	91/94	83.3	97.1	93	96
ARID1A/CDKN2A/CREBBP/ ERBB2/ERBB3/FGFR1/ FGFR3/HRAS/KTM2D/ NF1/PIK3CA/STAG2/ TP53/TSC1	Zhu (2019)	[40]	95/67	93.7	43.3	–	–
CDKN2A/ERBB2/FGFR3/ HRAS/KRAS/MET/MLL/ PIK3CA/TP53/VHL	Rodriguez Pena (2019)	[38]	260/186	88.0	96.6	–	–
FGFR3/HRAS/TERT	van Kessel (2016)	[32]	74/80	72.1	93.2	–	–
FGFR3/HRAS/TERT	van Kessel (2017)	[31]	97/103	77.3	96.9	–	–
FGFR3/PIK3CA/RAS/TP53*	Serizawa (2011)	[29]	118/33	50.4	93.9	71.2	92.9
FGFR3/TERT	Allory (2014)	[16]	278/124	69.5	–	–	–
FGFR3/TERT	Beukers (2017)	[17]	977/multiple negative control samples during FU	78.6	–	–	–
FGFR3/TERT	Dahmcke (2016)	[20]	99/376	88.9	82.2	–	–
FGFR3/TP53	Noel (2015)	[28]	76/27	–	–	46	81
FGFR3/HRAS/PIK3CA/ RXRA/TERT/TP53	Ward (2016)	[34]	122/109	70.5	97.2	–	–

*FU, follow-up; Ref., reference. *RAS = HRAS/KRAS/NRAS.*

Dudley *et al.* [37] and Rodriguez Pena *et al.* [38] included 20 and 10 mutation markers, respectively. The combination *FGFR3/TERT* was reported in three studies with comparable sensitivities in Allory *et al.* [16] (70%) and Beukers *et al.* [17] (79%), and a higher diagnostic accuracy in Dahmcke *et al.* [20] (sensitivity 89% and specificity 82%). Marker panel *FGFR3/HRAS/TERT* was studied by the same research group (van Kessel *et al.* [31,32]) in 2016 and 2017, with similar diagnostic accuracy in both cohorts: sensitivity 72% vs 77% and specificity 93% vs 97%, respectively.

Marker panels: tumour-informed analysis

Three studies described diagnostic accuracy of three different marker panels for tumour-informed urinary BC detection. Sensitivity varied from 46% to 93% and specificity varied from 81% to 96% [28,29,37]. Diagnostic accuracy was most promising for Dudley *et al.* [37], with a sensitivity of 93% and a specificity of 96%.

BC surveillance

Single-mutation markers

Among nine studies, data could be retrieved on 20 single-mutation markers for urinary BC surveillance, with sensitivities from 0% to 85% and specificities from 66% to 100% (Table S2). Dudley *et al.* [37] only found negative results in both patients with BC and controls for six of 20 mutation markers: serine/threonine-protein kinase 1 (*AKT1*), B-Raf proto-oncogene, serine/threonine kinase (*BRAF*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), E1A binding protein P300 (*EP300*), F-Box and WD repeat domain containing 7 (*FBXW7*) and mediator complex subunit 12 (*MED12*) [37]. *FGFR3* and *TERT* were investigated in more than five studies. Across seven studies, *FGFR3* sensitivity differed from 3% to 73% and specificity from 66% to 100%. In five studies, *TERT* sensitivity was 43% to 83% and specificity was 68% to 100%.

Single-mutation markers: tumour-informed analysis

In two studies, data could be collected on two single-mutation markers for tumour-informed urinary BC surveillance in ≥100 tumours [17,35]. For *FGFR3*, Beukers *et al.* [17] found a sensitivity of 44% (95% CI 35–52%) at a specificity of 81% (95% CI 75–87%) and Zuiverloon *et al.* [35] reached a sensitivity of 66% (95% CI 60–72%) at a specificity of 50% (95% CI 40–60%). For *TERT*, Beukers *et al.* [17] found a sensitivity of 71% (95% CI 61–80%) and a specificity of 56% (95% CI 46–66%).

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Table 3 Marker panels of urinary mutation markers for bladder cancer surveillance.

Marker panels	First author (year)	Ref.	BC patients/ controls, *n*	Sensitivity, %	Specificity, %	Tumour-informed sensitivity, %	Tumour-informed specificity, %
AKT1/ARID1A/ BRAF/CDKN1A/ CDKN2A/EP300/ ERBB2/ERBB3/ FBXW7/FGFR3/ KDM6A/KRAS/ MED12/PIK3CA/ PLEKHS1/RB1/ STAG2/TERT/ TP53/TSC1	Dudley (2019)	[37]	91/94	83.8	96.3	91	100
CDKN2A/ERBB2/ FGFR3/HRAS/ KRAS/MET/MLL/ PIK3CA/TP53/ VHL	Rodriguez Pena (2019)	[38]	260/186	53.6	88.7	–	–
FGFR3/TERT	Allory (2014)	[16]	278/124	50.5	70.6	–	–
FGFR3/TERT	Beukers (2017)	[17]	977/multiple negative control samples during FU	54.3	66.1	66.7	55.6
*FGFR3/PIK3CA/ RAS**	Zuiverloon (2013)	[35]	136/multiple negative control samples during FU	–	–	71.4	62.5

*FU, follow-up; Ref., reference. *RAS = HRAS/KRAS/NRAS.*

Marker panels

Four studies investigated diagnostic accuracy of three different marker panels for urinary BC surveillance (Table 3 [16,17,35,37,38]). Sensitivity ranged from 51% to 84% and specificity ranged from 66% to 96%. Sensitivity and specificity were most promising (≥80%) for Dudley *et al.* [37] who described a sensitivity of 84% at a specificity of 96% for their urinary marker panel.

Marker panels: tumour-informed analysis

Three studies evaluated diagnostic accuracy of three different marker panels for tumour-informed urinary BC surveillance. Sensitivity varied from 67% to 91% and specificity varied from 56% to 100%. Dudley *et al.* [37] described the most promising urinary marker panel with a sensitivity of 91% and a specificity of 100%.

BC detection vs BC surveillance

We evaluated seven studies that included both patients with primary and recurrent BC, of which six studies provided separate data for both groups. Allory *et al.* [16], Beukers *et al.* [17] and Kandimalla *et al.* [23] reported higher *FGFR3* sensitivities for BC detection than for BC surveillance: 36% vs 19%, 36% vs 23% and 39% vs 10%, respectively (Tables S1 and S2). Allory *et al.* [16] and Beukers *et al.* [17] also detected higher *TERT* sensitivities for BC detection than for BC surveillance: 62% vs 42% and 73% vs 49%, respectively. However, Avogbe *et al.* [36] and Rodriguez Pena *et al.* [38] found comparable *TERT* sensitivities for BC detection and surveillance: 84% vs 85% and 60% vs 65%, respectively. Dudley *et al.* [37] found that their urinary marker panel had a similar sensitivity in the BC detection and surveillance setting (83% vs 84%), whereas Rodriguez Pena *et al.* [38] reported a higher sensitivity of their urinary marker panel for BC detection than for BC surveillance (88% vs 54%).

Discussion

Urinary mutation analysis seems to provide a promising diagnostic tool for non-invasive BC diagnosis. Systematic review of available literature revealed acceptable diagnostic potential for single-mutation markers and for marker panels. Yet, diagnostic accuracy of urinary BC-mutation markers differed considerably among publications. This was particularly evident for single-mutation markers, not only showing varying diagnostic performance between markers, but even so for the same marker (e.g. *FGFR3*, Fig. 3). For marker panels, differences in diagnostic performance were less evident, but still substantial. The varying results can partly be explained by heterogeneity in study characteristics, e.g. differences in the sample size, the (non-)consecutive/ prospective collection of samples, the proportion of patients with NMIBC/MIBC, the comparison to (non-)matched controls, and the type of mutation analysis technique. Present data do not yet allow for clinical decision making, and

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diagnostic use of urinary BC-mutation markers can only be achieved by gathering more and stronger evidence.

In most studies, diagnostic accuracy of urinary mutation analysis was based on the presence or absence of BC. However, some studies calculated diagnostic accuracy of urinary mutation analysis based on the presence or absence of concordant mutations in the tumour (so-called tumour-informed analysis). We believe that tumour-informed analysis is less likely to be implemented for urine-based BC diagnosis, as this can only be applied in patients with histologically confirmed BC in which the mutational status has been determined. Also, tumour-informed specificities were generally lower. Tumour mutation analysis represents only a small part of the tumour and this may result in more 'false-positive' urines [41]. Therefore, we will discuss the most promising (defined as sensitivity and specificity ≥80%) single-mutation markers and marker panels for urinary mutation analysis based on the presence or absence of BC.

For BC detection, *TERT* reached a sensitivity and specificity of ≥80% across three studies [20,21,36]. Avogbe *et al.* [36] reported the highest values with a *TERT* sensitivity of 84% at a specificity of 95% in the test set (45 patients with BC/93 controls). However, *TERT* sensitivity decreased to 68% in the validation set, while specificity remained high at 98% (50 patients with BC/50 controls) [36]. The test set was prospectively collected in France, while the validation set was retrospectively collected in Portugal. Avogbe *et al.* [36] suggest that differences in countries, sampling procedures or exposures may have caused varying diagnostic accuracy between sets. In addition, the validation set had unfavourable tumour characteristics with 24% LG/76% HG tumours and 64% NMIBC/36% MIBC patients compared to the test set with 40% LG/60% HG tumours and 91% NMIBC/9% MIBC patients. Also, the validation set included young healthy controls (median age 46 years), while the test set included older controls with other urological diseases or with benign colonoscopy (median age 70 years). The other two studies did not report on a validation series [20,21]. The percentage of NMIBC/MIBC patients was comparable between the three studies, but only Dahmcke *et al.* [20] included matched haematuria controls [20]. For BC detection, *FGFR3* reached a diagnostic accuracy of ≥80% in one study: 85% sensitivity and 100% specificity [26]. However, this was a small series of 13 patients with NMIBC and 20 chronic cystitis controls. Given that six patients with MIBC without *FGFR3* mutations were also considered as controls, we excluded these patients from our analyses as they do not represent 'true' controls. For BC detection, *FGFR3*/*TERT* was the only promising marker panel, with a sensitivity of 89% and a specificity of 82% [20]. This study was of high quality, based on the large sample size (99 patients with BC/376 matched controls), which was consecutively collected, a clinically relevant representation of patients with BC (85% NMIBC/13% MIBC), and low concerns regarding bias and applicability.

For BC surveillance, Avogbe *et al.* [36] reported promising results for *TERT*, with a sensitivity of 85% at a specificity of 95% in the test set (47 patients with BC/93 controls), but these results were not evaluated in a validation set [36]. The population of patients with BC consisted of 42% LG/58% HG and 90% NMIBC/10% MIBC, which properly reflects clinical practice of patients with BC under follow-up for recurrence. However, patients with BC were compared to non-matched controls (with other urological diseases or with benign colonoscopy), as of which specificity may be overestimated. No other studies found promising results for BC surveillance using urinary mutation marker(s).

In general, recurrent tumours are discovered earlier than primary tumours. Accordingly, recurrent tumours are smaller and less tumour DNA is expected in urine samples, which may result in lower sensitivity of urinary mutation analysis [42]. Across studies that described primary and recurrent tumours within the same publication, sensitivity was either similar for BC detection and BC surveillance, or sensitivity decreased in the BC surveillance setting. High sensitivity is important for BC detection, because patients with a negative test result will not be monitored afterwards and BC should therefore be ruled out with high certainty. For BC surveillance, requirements of test sensitivity depend on the follow-up strategy that is applied. A follow-up strategy in which cystoscopy is completely replaced by urinary mutation analysis would require high sensitivity. However, a follow-up strategy in which cystoscopy is alternated with urinary mutation analysis would allow for a somewhat lower sensitivity, because false-negative results would be encountered at cystoscopy after an acceptable time-interval. High specificity is important in both diagnostic settings, as false-positive results would lead to unnecessary cystoscopies.

Dudley *et al.* [37] and Rodriguez Pena *et al.* [38] also reported promising results of their urinary BC marker panels, consisting of 20 and 10 mutation markers, respectively. However, they followed patients with repeat diagnostic procedures to verify earlier urinary mutation analysis results, while other studies compared urinary mutation analysis results to cystoscopy/histology at one point in time. It is known that positivity of urinary mutation analysis regularly precedes positivity of standard diagnostic procedures, the so-called 'anticipatory effect' [16,17,35]. In their BC surveillance series, Dudley *et al.* [37] and Rodriguez Pena *et al.* [38] indeed found that positivity of their urine test preceded clinical diagnosis by an average of several months. The 'anticipatory effect' is also illustrated by Beukers *et al.* [17], showing that patients with a primary *FGFR3*-positive tumour and a 'false-positive' follow-up urine were more likely to experience a recurrence after 2 years than patients with a

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primary *FGFR3*-positive tumour and a negative follow-up urine: 73% vs 41% ($P = 0.005$) [17]. In clinical practice, such 'false-positive' urines could induce two possible responses. Firstly, urologists could be more attentive during cystoscopy, resulting in more positive cystoscopies. Secondly, urologists could perform cystoscopies at shorter time-intervals, which for some patients would lead to earlier diagnosis, but for other patients would lead to over-diagnosis. Future studies should pay attention to the clinical effects from these 'false-positive' results.

To translate the data summarised in the present review to future clinical application, heterogeneity in research design, BC population, mutation analysis technique, and urinary DNA should be considered. Firstly, research design should reflect clinical practice by prospective and consecutive collection of patient samples and comparison to matched controls. Diagnostic accuracy may be overestimated by non-consecutive sample collection and use of non-matched controls, in particular healthy individuals [22,26,29]. For example, Descotes *et al.* [21] found promising results for *TERT* (sensitivity 80%, specificity 90%) in a series of 348 patients with BC and 167 controls. Their controls varied from healthy individuals to patients with other cancers, whereas controls with haematuria were excluded, which may have introduced bias. Secondly, the BC population should reflect clinical practice. At initial presentation, ~75% is diagnosed with NMIBC and 25% is diagnosed with MIBC [2]. During follow-up, the percentage of NMIBC is higher. As the percentage of patients with NMIBC/MIBC differed considerably across studies (Table 1), results may not correspond to 'true' diagnostic accuracy. For BC detection, Miyake *et al.* [26] included 13 (100%) patients with NMIBC and found promising results for *FGFR3* (sensitivity 85%, specificity 100%), while Dahmcke *et al.* [20] included 84 (85%) patients with NMIBC and 13 (13%) patients with MIBC and found less promising results for *FGFR3* (sensitivity 41%, specificity 98%) [20,26]. Dahmcke *et al.* [20] showed that *FGFR3* sensitivity decreased with tumour stage in their series (Ta 59%, T1 30% and ≥T2 31%), supporting the notion that *FGFR3* sensitivity is strongly correlated to tumour stage [20,43]. Thirdly, as we did not apply any restrictions regarding mutation analysis technique, diagnostic accuracy will also depend on the type of mutation analysis technique to be eventually used in clinical practice. Millholland *et al.* [25] demonstrated that within the same series of 43 patients with BC, *FGFR3* sensitivity improved from 12% with quantitative PCR to 56% with ultra-deep next-generation sequencing (NGS). Specificity was 100% with the latter technique, but was not assessed by quantitative PCR. Their PCR vs NGS results emphasise that the type of mutation analysis technique can have large impact on diagnostic accuracy. Advantages of PCR are its low costs, its relatively short processing time, and the extensive experience with PCR in routine diagnostic laboratories. The advantages of NGS, on the other hand, include the comprehensive analysis of multiple mutations and the potential to increase sensitivity by increasing sequencing coverage, which however comes at higher costs [44,45]. The method of choice in clinical practice should be based on assay characteristics (e.g. single-mutation marker vs extensive marker panel), clinical feasibility (e.g. time and costs) and diagnostic accuracy. The latter will also rely on the type of urinary DNA analysed [44,46,47]. Most studies used cellular DNA from urine pellet [16–23,26–29,31–40], of which two recent studies also assessed cell-free DNA from urine supernatant (Table 1) [36,39]. Avogbe *et al.* [36] found that diagnostic accuracy of *TERT* was similar for cellular DNA and cell-free DNA analyses, but diagnostic accuracy was highest when both analyses were combined in primary (sensitivity 87%, specificity 95%) and recurrent (sensitivity 88%, specificity 95%) disease, which is consistent with previous work [36,44]. Stasik *et al.* [39] concluded that *TERT* sensitivity was higher for cellular DNA (77%) than for cell-free DNA (63%) at comparable specificities (97% vs 100%, respectively) [39]. In the present review, we only included cellular DNA results from the latter two studies to reduce heterogeneity between studies and because cellular DNA sensitivity (somewhat) outperformed cell-free DNA sensitivity. Three studies used DNA isolated from full void urine, also referred to as genomic DNA [24,25,30]. To date, researchers primarily gained experience with mutated cellular DNA for urine-based BC diagnosis. Focus now seems to have shifted to mutated cell-free DNA, which is released upon apoptosis and necrosis of bladder tumour cells resulting in potential enrichment of tumour DNA and an improved sensitivity [44,46,47]. Analogous to our recent study on urinary methylation analysis [48], future studies should elaborate on which type of urinary DNA is to be preferred for mutation analysis in clinical practice: cellular DNA, cell-free DNA, a combination of these two, or genomic DNA as obtained from full void urine.

Three studies were of highest quality [20,24,30]. Findings by Dahmcke *et al.* [20] were discussed previously. The other studies were performed by the same research group and focussed on *FGFR3* mutations. For BC detection, Karnes *et al.* [24] included patients with primary BC (test 48, validation 58) with 91% NMIBC/9% MIBC, and compared these to matched controls (test 240, validation 690). They found low sensitivity (test 10%, validation 35%), but high specificity (test 99%, validation 100%). For BC surveillance, Shore *et al.* [30] included 63 patients with recurrent NMIBC and 670 patients with previous NMIBC under follow-up for recurrence, and reported 30% sensitivity and 96% specificity in the validation set. They expanded their *FGFR3* assay with methylation and protein markers, and found that sensitivity improved for BC detection and surveillance (88% and 91%, respectively), but this was at the cost of specificity (56% and 35%, respectively).

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An optimal urine test for BC diagnosis should perform adequately across all grades and stages of disease. A number of studies determined sensitivities of their urine test per BC grade and/or stage [17,20–23,25,26,29,34–39], some of which also calculated *P* values between sensitivities per BC grade and stage [20,21,36,37]. In general, *TERT* sensitivity remained comparable across BC grade and stage [20,21,36]. This is in accordance with a landscape study by Hurst *et al.* [43], in which *TERT* is found to be frequently present in patients with BC irrespective of grade and stage. On the other hand, Descotes *et al.* [21] found that *TERT* sensitivity was significantly higher for HG tumours than for LG tumours ($P = 0.015$), while *TERT* sensitivity did not differ across BC stage ($P = 0.498$). Dahmcke *et al.* [20] found that *FGFR3* sensitivity was significantly higher for Ta tumours than for other tumour stages ($P = 0.002$) [20]. This is consistent with earlier studies, which also showed that *FGFR3* mutations most frequently occur in Ta tumours [49]. Dudley *et al.* [37] reported that their marker panel, consisting of 20 mutation markers, had a higher sensitivity for HG tumours than for LG tumours ($P = 0.022$) and a higher sensitivity for ≥T2/Tis tumours than for Ta tumours, although the latter was not statistically significant ($P = 0.054$) [37].

This systematic review has some limitations that need to be considered. Firstly, we could not perform a quantitative analysis of the data, because studies were too heterogeneous for a meta-analysis. As mentioned earlier concerns were raised about heterogeneity in study characteristics. Secondly, we did not collect data on the predictive and/or prognostic potential of urinary mutation analysis (although a number of studies did report on this) [16,18,21,27,37,40]. Thirdly, we did not review the combined performance of urinary mutation analysis and other tests. Yet, papers on combined analysis all reported higher sensitivities after expanding their mutation markers with methylation markers, microsatellite analysis and/or cytology [17,20,22–24,29,33,35,36]. As tumour heterogeneity does not only exist between tumours, but also within tumours, combined molecular analysis may provide the answer in the search for reliable urine-based BC markers. Mutation and methylation markers have already been subject to a large number of publications. An upcoming field of interest involves copy number and microsatellite changes. All together a combination of these four in a combined assay might hold the best promise for reliable urinary BC diagnosis [14,47,50]. Certainly when ongoing technological developments are considered, combined analysis may prove a sensitive method for urinary BC diagnosis in the coming years. Fourthly, we did not consider BC mutation markers in other liquid biopsies (e.g. blood). Nonetheless, recent sequencing data of blood samples from 586 patients with BC revealed a large number of potential gene mutations for BC diagnosis [51]. However, two studies which simultaneously analysed blood and urine samples of patients with BC concluded that the urine samples contained higher amounts of (mutated) tumour DNA [44,52]. This analytical advantage underlines the potential benefit of urine over blood, let alone the fact that urine is a 'true' non-invasive liquid biopsy.

Taken together, we believe that the existing evidence on mutation markers for BC diagnosis in urine does not allow for clinical implementation at present. Combining different molecular markers, such as mutation and methylation markers, may even provide a better option for non-invasive BC diagnosis, and future studies should therefore focus on identifying adequate marker panels. Large, prospective studies with consecutive sampling methods and matched control groups are required to expedite future clinical application.

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Conflict of interest

R.D.M. Steenbergen has a minority share in Self-screen BV, a spin-off company of Amsterdam UMC, location VUmc. J. Bosschieter, R.D.M. Steenbergen and J.A. Nieuwenhuijzen are inventors on patent(s) related to the work.

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Correspondence: Jakko A. Nieuwenhuijzen, Department of Urology, Amsterdam University Medical Centers, Vrije Universiteit Amsterdam, Post box 7057, 1007 MB Amsterdam, The Netherlands.

e-mail: j.nieuwenhuijzen@amsterdamumc.nl

Abbreviations: AKT1, serine/threonine-protein kinase 1; BC, bladder cancer; BRAF, B-Raf proto-oncogene, serine/threonine kinase; CDKN2A, cyclin-dependent kinase inhibitor 2A; EP300, E1A binding protein P300; ERBB3, Erb-B2 receptor tyrosine kinase 3; FBXW7, F-Box and WD repeat domain containing 7; FGFR3, fibroblast growth factor receptor 3; HG, high grade; HRAS, HRas proto-oncogene, GTPase; KDM6A, lysine demethylase 6A; LG, low grade; MED12, mediator complex subunit 12; (N)MIBC, (non-) muscle-invasive BC; NGS, next-generation sequencing; NRAS, NRAS proto-oncogene, GTPase; PIK3CA, phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit α; PRISMA, Preferred Reporting Items for Systematic and Meta-Analysis; QUADAS, Quality Assessment of Diagnostic Accuracy Studies; RoB, risk of bias; ROC, receiver operating characteristic; TERT, telomerase reverse transcriptase; TP53, tumour protein p53; TURBT, transurethral resection of the bladder tumour.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Overview of single-mutation markers for urine-based BC detection.
Table S2. Overview of single-mutation markers for urine-based BC surveillance.
Appendix S1. Search strategy.

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