Novel urinary biomarkers for the detection of bladder cancer: A systematic review

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Abstract

Background

Urinary biomarkers for the diagnosis of bladder cancer represents an area of considerable research which has been tested in both patients presenting with haematuria and non-muscle invasive bladder cancer patients requiring surveillance cystoscopy. In this systematic review, we identify and appraise the diagnostic sensitive and specificity of reported novel biomarkers of different 'omic' class and highlight promising biomarkers investigated to date.

Methods

A MEDLINE/ Pubmed systematic search was performed between January 2013 and July 2017 using the following keywords: (bladder cancer OR transitional cell carcinoma OR urothelial cell carcinoma) AND (detection OR diagnosis) AND urine AND (biomarker OR assay). All studies had a minimum of 20 patients in both bladder cancer and control arms and reported sensitivity and/ or specificity and/ or receiver operating characteristics (ROC) curve. QUADAS-2 tool was used to assess risk of bias and applicability of studies. The search protocol was registered in the PROSPERO database (CRD42016049918).

Results

Systematic search yielded 115 reports were included for analysis. In single target biomarkers had a sensitivity of 2-94%, specificity of 46-100%, positive predictive value (PPV) of 47-100% and negative predictive value (NPV) of 21-94%. Multi-target biomarkers achieved a sensitivity of 24-100%, specificity of 48-100%, PPV of 42-95% and NPV of 32-100%. 50 studies achieved a sensitivity and specificity of ≥ 80%. Protein (n=59) and transcriptomic (n=21) biomarkers represents the most studied biomarkers. Multi-target biomarker panels had a better diagnostic accuracy compared to single biomarker targets. Urinary cytology with urinary biomarkers improved the diagnostic ability of the biomarker. The sensitivity and specificity of biomarkers were higher for primary diagnosis compared to patients in the surveillance setting. Most studies were case control studies and did not have a

predefined threshold to determine a positive test result indicating a possible risk of bias.

Conclusion

This comprehensive systematic review provides an update on urinary biomarkers of different 'omic' class and highlights promising biomarkers. Few biomarkers achieve a high sensitivity and negative predictive value. Such biomarkers will require external validation in a prospective observational setting before adoption in clinical practice.

Keywords: Bladder cancer; Biomarker, Diagnosis, Systematic review, Urine

Highlights:

- Multi-target biomarker panels had a better diagnostic accuracy compared to single biomarker targets
- The sensitivity and specificity of biomarkers were higher for primary diagnosis compared to patients in the surveillance setting
- Most studies were case control studies and did not have a predefined threshold to determine a positive test result indicating a possible risk of bias
- Prospectively field tested to validate biomarkers for the detection of bladder cancer are required
- Utilization of next generation sequencing with machine learning represents a promising approach for biomarker discovery

Introduction

Bladder cancer is the eight most common cancer and ranks 13th in terms of cancer associated mortality[1]. Haematuria, a cardinal symptom for bladder cancer, has a positive predictive value of 8% and this rises to as high as 18.7% in men ≥ 70 years [2]. Patients presenting with haematuria undergo investigations including cystoscopy and upper tract imaging. Eighty percent of patients with bladder cancer have non-muscle invasive bladder cancer (NMIBC) at presentation. While this is favorable compared to muscle invasive bladder cancer (MIBC), up to 50% of NMIBC cases recur and 20% will progress within 5 years[3]. Due to this high recurrence rate, regular surveillance cystoscopy is recommended, and the surveillance interval can be as frequent as three monthly in high risk disease[4].

Cystoscopy remains the gold standard for the detection of bladder cancer in patients investigated following haematuria and in patients requiring surveillance for recurrent disease following resection of the initial tumour. However, it is not without morbidity and up to 5.5% of patients may develop a urinary tract infection[5]. The requirement for life long surveillance in high risk patients have significant healthcare cost implications. Hence, there is an urgent need to develop a highly specific and sensitive urinary biomarker for the detection of bladder cancer.

Currently the US Food and Drug Administration has approved six urinary assays for clinical use; BTA stat (Polymedco), BTA TRAK (Polymedco), NMP22 (Matritech), NMP22 BladderCheck Test (Alere), uCyt (Scimedx) and UroVysion (Abbott Molecular). The tests performe with overall sensitivity between 57-82% and specificity between 74-88%[6]. Although sensitivity is higher in high grade and stage tumours, cystoscopy remains the gold standard for detection of bladder cancer, with a sensitivity as high as 98%[7]. Thus, none of these assays are approved to be used without cystoscopy.

There has been considerable interest in the development in urinary biomarkers as evident by the large number of published reports. While many show promising results, few have been reproduced in subsequent independent validation studies. Traditional assays have been designed for single targets or small panel assays restrained by the technology and assay performance. More recently, next generation sequencing and advancements in bioinformatics has enabled a paradigm shift whereby biomarker panels comprise multiple targets has been utilised using small quantities of input DNA.

In this systematic review, a literature search between January 2013 to July 2017 was performed to provide an update of urinary biomarkers for the detection of bladder cancer across the spectrum of protein, genomic, epigenetic and transcriptomic biomarkers. The purpose of this study is to highlight promising biomarkers which may have clinical utility in the future.

Methods

Literature search

A systematic search of the literature was performed using MEDLINE/PubMed to identify articles evaluating novel urine biomarkers for the detection of bladder cancer. A comprehensive literature search was performed between 1st January 2013 and 31st July 2017 using the following keywords and MeSH terms: (bladder cancer OR transitional cell carcinoma OR urothelial cell carcinoma) AND (detection OR diagnosis) AND urine AND (biomarker OR assay). The search protocol was registered in the PROSPERO database (CRD42016049918).

Study selection

Article selected were written in English and reported the diagnostic characteristics of novel urinary biomarkers for the detection of bladder cancer. Following screening of abstracts to exclude review articles, comments and letters to the editor or non-relevant articles, each manuscript was reviewed and data was extracted and its references searched for relevant missing manuscripts.

All studies required a minimum of ≥ 20 patients in both bladder cancer and control arm to be included and report both sensitivity and/ or specificity and/ or receiver operating characteristics (ROC) curve. The presence of bladder cancer was defined as the presence of cancer at histopathological examination following transurethral resection of bladder cancer. Biomarkers were classified to protein, genomic, epigenetic, transcriptomic and combination of different ‘omic’ biomarkers.

All abstracts and full text were independently screened by two investigators. Where there were disagreements, this was discussed with a third investigator and resolved by a consensus view. Cohort and cross-sectional studies were included.

Data extraction and quality assessment

Data was extracted from selected studies about type and biomarker used, assay used, study design, percentage of low grade cancer assayed, urine collection details and number of patients with bladder cancer and controls (WST, WPT, MYT, PK). Where more than one patient cohort were described, the final validation patient group was used. Low grade tumours were defined according to EAU risk classification[8]. A 2 X 2 table with number of true-positive, false-positive, true-negative, and false-negative results from published sample sizes was constructed to determine the sensitivity, specificity, positive (PPV) and negative predictive value (NPV) where available. ROC curve where reported was included. A second investigator confirmed data were extracted accurately. QUADAS-2 tool was used to assess risk of bias and concerns about applicability of studies[9].

Results

Characterization of studies

The PRISMA flowchart is shown in Figure 1. The database search identified 646 articles and after the addition of other relevant articles, a total of 656 abstracts were screened. Dual review of abstracts and titles excluded 377 studies which were not original research, not in English or unrelated articles. A further 164 studies were excluded after full text review as they did not meet the inclusion criteria leaving 115 articles which were included for analysis.

Articles were then classified to the following biomarkers: protein (n=59), genomic (n=7), epigenetic (n=19), transcriptomic (n=21) or combination of different 'omic' biomarkers (n=10). Twenty five protein[10-34], 1 genomic[35], 8 epigenetic[36-43], 10 transcriptomic[44-53] and 6 combination of different 'omic'[54-59] biomarkers had a sensitivity and specificity ≥ 80%. Studies with a sensitivity and specificity of < 80% are shown in the Appendix A2-A6).

Of the studies with a sensitivity and specificity ≥ 80%, most of these studies were designed as case control with selected groups comprising of urine from bladder cancer and control cases indicating selection bias (Appendix A1). Four prospective observational studies with some incorporating sequential urine sampling with surveillance cystoscopy although none had pre-planned statistical power calculations[41, 50, 51, 56]. Twenty three studies had a low risk of bias in determining the characteristics of the index test according to the QUADAS-2 tool[18, 20, 21, 23, 24, 26, 27, 30, 31, 33,

[36, 38-43, 45, 50-52, 55, 56]. Quality assessment using the QUADAS-2 tool for individual studies are summarized in Appendix A1.

Protein biomarkers

Protein based biomarkers were the most commonly tested biomarker for the detection of bladder cancer and used either immunoassays (n=35) or spectrometry (n=9) for protein quantification. Multiple protein targets were tested in 14 studies using multiplex immunoassay platforms interrogating between 3-10 biomarkers (Table 1 & A2).

Fourteen tests which tested an individual protein biomarker reporting a sensitivity and specificity ≥ 80%[10-18, 20, 21, 27, 30, 34] (Table 1). Of these, Orosomucoid 1 (ORM1), an acute phase transport protein, identified using mass spectrometry was quantified using ELISA of urine with a sensitivity of 92%, specificity of 94% and an ROC of 0.965[10]. A separate study of 152 patients reported good diagnostic accuracy using the serine protease, HtrA1, and achieved a sensitivity of 93% and specificity of 96%[14].

Survivin is a protein which is implicated in the inhibition of apoptosis, has been investigated by a number of studies[12, 13, 60]. Quantification of survivin using ELISA reports a sensitivity of 71-85% with a specificity of 81-95%[12, 13, 60]. Soluble Fas was reported by two studies and showed varying sensitivity of 51% and 88% which suggesting a lack of reproducibility[16, 61].

Amplified in breast cancer 1 (AIB1) which has been shown to promote cell proliferation via AKT pathway had a sensitivity and specificity of 80% and 86% respectively[62]. When combined with eukaryotic initiation factor 2 (EIF5A2) and nuclear matrix protein (NMP22) this increased to a sensitivity of 89%, specificity of 91% and ROC of 0.898[18]. Other reports on single protein biomarkers include apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE1/Ref-1), apolipoprotein A-I (Apo-A1), calprotectin, and NMP52 reporting sensitivity and specificity ranging from 82-94% and 80-93% respectively[11, 15, 17, 20].

Four studies reported the diagnostic ability of proteins cytokeratin 8 and 18 using the UBC Rapid point of care Omega 100 reader[63-66]. Cytokeratin are constituents of intermediate filaments of epithelial cells. This point of care test, requires three drops of urine and results from a photometric reader is available within 10 minutes. The sensitivity of the assay ranges from 30-87 % with carcinoma *in situ* (CIS) patients having the highest sensitivity and a specificity of between 63- 91% and a ROC of up to 0.750 suggesting a limited diagnostic performance. One study investigated the role of Ubiquitin 2 immunocytological staining reporting a sensitivity and specificity of 88% and 98% respectively although results for cytological based test were operator dependent [34].

A combination of urinary cytology, midkine (NEGF2) and gamma synuclein quantification using ELISA reported a ROC of 0.949 with a sensitivity and specificity of 91.8% and 97.5% respectively[27]. The nonsulfated glycosaminoglycan hyaluronic acid (HA) quantified by ELISA reported a sensitivity and specificity of 88% and 82% increasing to 90% and 84% respectively when combined with hyaluronidase, a catalytic enzyme that degrades HA[21]. Another 5-panel biomarker using gamma synuclein with Coronin-1A, Apolipoprotein A4, Semenogelin-2 and DJ-1/PARK7 compared ELISA to Western blot[26]. Western blot achieved higher sensitivity (93.9% vs 79.2%) and a similar specificity (97% vs 100%) compared to ELISA in pTa/ pT1 cancers[26]. However, western blot for protein quantification would not be practical in a large scale setting. Rosser et al. reported a RC of 0.948 using a multiplex ELISA system when combining three biomarkers: Interleukin 8 (Il-8), Matrix metallopeptidase 9 (MMP9) and vascular endothelial growth factor A (VEGFA)[27]. However, further studies incorporating the same three biomarkers and with the addition of between a further 4-7 markers have yielded an ROC of between 0.878-0.926 on validation studies [22-25, 67].

Six studies utilised spectroscopy or chromatography to determine a metabolic signature or a molecular compound with a sensitivity and specificity of ≥ 80%[28-31 32, 33]. Several of these assays achieve sensitivty and specificities of ≥ 90% and while promising would require external validation [30, 31, 33].

Genomic biomarkers

Seven studies investigated the role of genomic biomarkers for the detection of bladder cancer. Four were based on analysis of mutations and included in Table A3. Telomerase reverse transcriptase (*TERT*) mutation represents the most common bladder cancer mutation present in > 70% of all bladder cancers[68]. One study by Descotes and colleagues reported a sensitivity and specificity of 81% and 90% respectively for *TERT* although others have reported a lower sensitivity of 62%[35, 68, 69]. *TERT* mutation was also associated with a > 5-fold increase relative risk of recurrence (p=0.0004)[35].

FGFR3 achieved a sensitivity of 39% as a standalone test for bladder cancer[70]. *FGFR3* mutation is more common in low grade disease (p=0.02) and significantly associated with shorter time to recurrence (45% mutant vs 27% wild type, p=0.02)[70, 71]. Other mutations such as *TP53, PIK3CA* and *RAS* have reported limited performance because of the low frequency of mutations and variability of genomic alterations between individual tumours. Sensitivity for TP53 of 12-13%, PIK3CA 13-14% and RAS 4.8% have been reported [69, 71]. The diagnostic performance of the combination of *FGFR3* and *TERT* with *PIK3CA, RAS* and *TP53* improved bladder cancer detection but only achieved a sensitivity of 73%[69]. Of note, it has been demonstrated that following complete resection of tumour, 20.7% of patients will continue to test positive for *FGFR3* and *TERT* mutation despite no cystoscopic detectable tumour in patients followed up for 3 years[71]. In addition to targeted mutation analysis, the quantitative cell-free DNA analysis has been explored as a marker for the presence of bladder cancer as well as analysis of the integrity of cell-free DNA. To date studies are preliminary and report limited diagnostic performance with ROC of 0.725-0.834[72, 73].

Epigenetic biomarkers

Twelve studies reported the diagnostic performance of microRNA (miRNA) and 8 studies investigated the role of DNA methylation as biomarkers for the detection of bladder cancer (Table 2 & A4). No studies investigated the role of histone modifications. Single target epigenetic biomarkers have a poor diagnostic performance overall and epigenetic biomarker panels with a sensitivity and

specificity of ≥ 80% are set out in Table 2. Of note, biomarker panels include between 2-150 targets to determine the presence of bladder cancer.

Of the miRNA panels, four have a sensitivity and specificity of ≥ 80% (Table 2) and employed miRNA arrays or next generation sequencing (NGS) to identify targets[36, 38-40]. MiRNA was then quantified by real-time qPCR[36-38, 40]. M*iRNA-125b* was used in two diagnostic panels although its sensitivity and specificity as a single biomarker varies between 59-85 and 76-96% respectively[36, 74]. The combination of two miRNAs, *miRNA-99a* and *miRNA-125b,* had a sensitivity and specificity of 87% and 81% respectively[36]. Using multivariable modeling Urquidi and colleagues determined the top 25 miRNA targets and determined the diagnostic ability of the top 10, 15, 20 and 25 targets using the LASSO approach to model the performance of each biomarker[39]. Their results suggest that incorporating increasing number of biomarkers can increase both sensitivity and specificity with marginal gains with each increase.

Only three of the 8 DNA methylation studies reported sensitivity and specificity ≥ 80% (Table 2). All studies included ≥3 DNA methylation targets and all report a ROC of >0.9. Methylation status was determined by quantitative methylation specific PCR (qMS-PCR)[42], pyrosequencing[41] and next generation sequencing[43]. Su and colleagues interrogated three methylated targets and deduced that the combination of *SOX1, IRAK3*, *L1-MET* methylation had sensitivity and specificity of 80% and 97% respectively[41]. The three-target methylation panel of *POU4F2* + *PCDH17* + *GDF15* showed sensitivity and specificity of 91% and 88% respectively[42]. Feber and colleagues derived a methylation signature of 150 loci incorporating a machine learning algorithm[43]. The assay, UroMark, used a targeted bisulphite sequencing approach and was validated with two independent sets of urine samples comprising of bladder cancer and control samples reporting a sensitivity of 98%, specificity of 97% and ROC of 0.97[43].

Transcriptomic biomarkers

All studies used RT-PCR to determine expression of target genes (Table 3 & A5). Four studies report single target gene expression[44-46, 53] and four studies combined

transcriptomic markers with urine cytology[47, 48, 52, 53] to achieve a sensitivity and specificity of ≥ 80% (Table 3). Of the four studies reporting a single biomarker, sensitivity ranges from 45-92% and specificity of between 65-96% and ROC of 0.741- 0.966. Studies reporting combination biomarkers achieved a sensitivity of 36-97%, specificity of 82-100% and a ROC of 0.860-0.949.

S100A4, carbonic anhydrase IX (CAIX) and hepatoma upregulated protein RNA (HURP) and long non-coding RNA urothelial carcinoma associated-1 (lncRNA-UCA1) represent single biomarker targets which have sensitivity and specificity of ≥ 80%[44 45 46 53]. De Martino and colleagues quantified CAIX in paired tumour and urine and validated their results in an independent cohort comprising 155 urine samples reporting sensitivity, specificity and ROC of 81%, 96% and 0.883 respectively[45]. Analysing six cytoplasmic calcium binding protein, S100A4 had the highest diagnostic accuracy with sensitivity of 90%, specificity of 92% and ROC of 0.978[44]. Eissa and colleagues used gold nanoparticle based RT-PCR and reported a sensitivity of 89% and specificity of 94% for the presence of Hepatoma upregulated protein RNA (HURP)[46]. The technology performed better than conventional HURP RT-PCR, suggesting significant variation in results from different platforms[47]. Another novel hybridization assay, nanoparticle RT-PCR of long non-coding RNA urothelial carcinoma associated-1 (lncRNA-UCA1) reported sensitivity and specificity of ≥ 90% and ROC of 0.966[53]. UCA1 has been implicated in bladder cancer progression through PI3K-AKT dependent pathways and the development of cisplatin resistance via Wnt signaling[75, 76]. However, conventional RT-PCR of lncRNA-UCA1 has not reproduced these results[77].

Cytokeratin 20 (CK20) was used as part of two multiplex assays[49, 52]. In contrast to CK8 and 18, CK20 is expressed on urothelium but not epithelial cells, and has a reported diagnostic sensitivity, specificity and ROC of 76-85%, 86% and 0.82-0.87 respectively[49, 52]. CK20 overexpression in combination with p53 and Ki-67 have been shown by immunohistochemistry to suggest urothelial dysplasia[78]. The combination of cytology with CK20 has a sensitivity and specificity of ≥ 90% which has a higher diagnostic accuracy compared to other combinations such as Ki-67 with survivin, Ki-67 with CK20 and survivin with CK20[49]. When CK20 is used in combination with

insulin like growth factor (IGF2), the sensitive and specificity increases to 90% and 84% respectively[52].

The most promising transcriptomic panel that has been validated and tested in a prospective observational study is based on a combination of two genes IGF2 and Melanoma-associated antigen 3 (MAGE-A3)[50, 51]. Both IGF2 and MAGE-A3 were selected from a panel of 12 genes and this two gene combination has a sensitivity of 81%, specificity of 91%, PPV of 87%, NPV of 88% and ROC of 0.944 in a prospective blinded validation study[50]. The initial 12 gene expression targets were selected following screening using gene expression microarrays[50, 51]. IGF2 represents glycoprotein receptors on the cell membrane IGF2 promotes tumorigenesis via the PI3K-AKT pathway which is implicated in most bladder cancer[79]. MAGE-A3 which has been shown to be expressed in 43% of bladder cancer and in various tumour types but not in healthy tissue with the exception of testis and placenta[80, 81].

Combination of different 'omic' biomarkers

Ten studies used a combination of difference 'omic' biomarkers with the aim to identify bladder cancer from exfoliated urinary bladder cells (Table 4 and Table A6). Six studies combined genomic with epigenetic biomarkers including one with microsatellite analysis[54, 56, 82-84]. The other three studies used a transcriptomic and protein combination panel[57, 58, 85]. One study utilised a protein (HYAL1), epigenetic (*miR-210, miR-96*) and transcriptomic (lncRNA-UCA1) combination. *TERT* and *FGFR3* mutation were used in most combination markers incorporating genomic biomarkers[54, 56, 82, 83].

In a retrospective analysis of case control study of 74 bladder cancer and 80 controls presenting with haematuria, a combination of *FGFR3*, *TERT* and *HRAS* mutation in combination with twist-related protein (*TWIST*), *OTX1* and *ONECUT2* methylation, reported sensitivity of 97% and specificity of 83%[54]. The authors modelled the PPV of 39% and NPV of 99.6% assuming a 10% prevalence of bladder cancer[54]. This six gene panel of epigenetic and genomic targets, was subsequently validated in a prospective case control study with 97 bladder cancer and 103 controls presenting with haematuria with a sensitivity of 93% and ROC of 0.96[55]. This assay builds on a

previously reported assay comprising of *FGFR3* mutation in combination with *OTX1, ONECUT2* and odd-skipped-related 1 (*OSR1*) methylation profile in a patient cohort of 95 cancer and 40 controls[82]. This assay panel achieved a sensitivity of 79%, PPV of 92%, NPV of 76% and ROC of 0.864.

The other study by Dahmcke and colleagues was a prospective study with utilized a biomarker panel comprising of *FGFR3* and *TERT* mutation with 6 methylated genes namely *ONECUT2*, Cyclin-A1 (*CCNA1*), *BCL2, EOMES* and vimentin (*VIM*)[56]. This 8-biomarker combination had sensitivity of 97%, specificity of 76.9%, NPV of 99% and ROC of 0.963[56]. Beukers and colleagues tested a three-panel biomarker comprising of *FGFR3* and *TERT* mutation with *OTX1* methylation and in pre-TURBT urine collection from 305 patients, achieving a sensitivity of 81-94% depending on tumour grade[83]. However, in patients undergoing surveillance cystoscopy, the sensitivity and specificity of identifying tumour recurrence was much lower at 57-72% and 55-59% respectively[83].

A four-panel biomarker of *FGFR3* mutation with Heparan sulfate glucosamine 3-O-sulfotransferase 2 (*HS3ST2*), *SLIT2* or *SEPTIN9* methylation was tested in a cohort of patients for the identification of NMIBC recurrence with surveillance cystoscopy[86]. Roperch and colleagues incorporated clinical features such as age and smoking which improved the diagnostic accuracy of the assay from a sensitivity of 67-89% depending on tumour grade to 98% with an ROC of 0.96[86]. However, when used in the surveillance setting, consistent with results from Beukers and colleagues, the sensitivity fell to 95% with an ROC of 0.82. Similarly, Zuiverloon and colleagues also observed that the diagnostic ability of urinary biomarkers to identify tumour recurrence during surveillance cystoscopy was poor[84].

The other three studies by Eissa et al. used combinations of protein and transcriptomics[57-59]. Survivin involved in the EMT pathway was tested in combination with Matrix metalloproteinase (MMP) 2 & 9 and hyalurodinase. Survivin with MMP 2 & 9 had a sensitivity and specificity of 91% and 85% which increased to 96% and 85% when urinary cytology has been incorporated[57]. Sensitivity and specificity of survivin with hyalurodinase was 95% and 90% respectively[58]. The protein-epigenetic

combination of HYAL1, lncRNA-UCA1, *miR-210* with *miR-96* had a sensitivity of 100%, specificity of 89% and ROC of 0.981[59].

Discussion

This study highlights that single target assays have limited value regardless of 'omic' class. Performance is uniformly below that of multi-target biomarker panels. Only 4 single target urinary biomarkers achieved a sensitivity and specificity of ≥ 90% (Table 5). Across the studies none had a pre-planned statistical power calculation performed with only four non-case controlled prospective observational studies[41, 50, 51, 56]. Independent validation cohorts were reported in six studies interrogating two biomarker panels. The first, a 10 protein based multiplex assay (IL8 + SERPINA1 + ANG + VEGF-A + CA9 + MMP 9 & 10 + APOE + PAI-1 + SDC1) and the second, a two panel gene expression assay (IGF2, MAGEA3)[22-25, 50, 51]. Both assays reported a sensitivity and specificity of < 90% and ROC of <0.95. One panel comprising of 6 DNA methylation *(SALL3* + *ONECUT2* + *CCNA1* + *BCL2* + *EOMES* + *VIM*) and two mutation (*TERT* & *FGFR3*) was field tested in a prospective blinded patient cohort of haematuria patients reporting a sensitivity, specificity and ROC of 97%, 77% and

554 0.963 respectively but panel has not been validated in an independent patient
555 cohort[56]. A significant number of studies on urinary biomarkers had a poor diagnostic
556 ability and require validation in a prospective clinical setting. Single and combination
557 biomarkers with sensitive and specificity ≥ 80% are shown in Table 5.
558
559 This study highlights that there is considerable interest in the use of urinary
560 biomarkers to diagnose bladder cancer. This applies to both in the screening of the
561 haematuria patient cohort as well as in patients with NMIBC who require surveillance
562 cystoscopy. The requirement for cystoscopy represents a significant cost to health
563 care services in diagnosing bladder cancer[87]. Traditional imaging modalities with or
564 without urine cytology does not have the necessary sensitivity to replace cystoscopy
565 for the detection of bladder cancer [88]. Cystoscopy requires a hospital visit and is an
566 invasive procedure which is associated with a risk of urinary tract infection[5]. A highly
567 sensitive and specific non-invasive urinary assay will revolutionise both the
568 haematuria and NMIBC surveillance pathway and is urgently needed.
569
570 In this study, we report that the diagnostic accuracy of urinary biomarkers varies
571 considerably. In single target biomarkers had a sensitivity of 2-94%, specificity of 46-
572 100%, PPV of 47-100% and NPV of 21-94%. Multi-target biomarkers achieved a
573 sensitivity of 24-100%, specificity of 48-100%, PPV of 42-95% and NPV of 32-100%.
574 Such variation in diagnostic accuracy can be explained by combination of patient
575 factors and assay factors. The diagnostic ability of urinary biomarkers was
576 considerably better in identifying high grade tumours as well as CIS. This is constant
577 with urinary cytology which has an overall 34% sensitivity and 99% specificity but the
578 sensitivity increases to 63% in CIS and high grade tumours[89]. This is due to increase
579 cell exfoliation in tumour cells and might in fact reflect why novel urinary biomarkers
580 also detect high grade disease with a higher sensitivity and specificity. In fact
581 advanced bladder cancer is often associated with a high mutational burden and
582 hypermethylation[90].
583
584 Beside patient specific variables, reproducibility of biomarkers to allow highly
585 accurate results is an issue. While efforts are made by the implementation of Good
586 Laboratory Practice to uphold the quality of management controls to ensure

consistent and reliability of results, there are other sources of variation for the same biomarker. The variations in evaluating the same target protein, epigenetic change or gene expression makes it different to compare studies due to the lack of standardization of methodology[91]. NGS performed in 5 different centers of the International Cancer Genome Consortium (IGGC) suggest that difference in variant calling and complete sequencing pipelines can result in a difference in identified mutation of ≥ 75%[92]. Further, variation in genetic differences such as mutation, post transcription modifications, gene expression and epigenetic changes are complex and is difficult to elucidate. Additionally, the threshold used to define a positive result may differ between studies making comparison difficult.

A significant number of biomarkers reported did not have external validation in prospective field testing. For reasons described above, diagnostic accuracy of initial reports is often not reproducible. Where validation was performed, it was typically performed using selected patient cohort which is not representative of 'real world practice' of haematuria patients or NMIBC patients having surveillance cystoscopy. Majority of studies were based on retrospective patient cohorts comprising of selected bladder cancer and control patient groups. Hence, accurate PPV and NPV is not accurate or are based on assumptions as they are dependent on prevalence of disease in the patient cohort.

This study shows that the use of multi-target biomarkers is increasing and these biomarker panel have higher accuracy (Table 5). Traditionally, the number of biomarkers incorporated in an assay was limited by DNA yield from urinary cells. Female patients have a higher DNA yield compared to male patients[93]. In addition, DNA extraction kit used and sampling time can also affect the DNA quality and yield [93]. Particularly in methylation based assays which requires DNA bisulphite conversion, a loss of DNA yield of 70-90% is common [94]. Fluorometer quantification of urinary DNA suggest that between 2 to 440 ng/ ml of DNA can be retrieved from urinary cell pellet[93]. In the studies reviewed, the limit on biomarker targets interrogated for protein, genomic, epigenetic, transcriptomic and combination biomarkers are 10, 5, 150, 12 and 8 respectively. The utility of NGS has allowed the development of highly multiplex assays, for genomic, epigenomic or transcriptomic

biomarkers. The first to utilize this technology used multiplex biomarker panel of 150 loc[43].

The use of multi-target biomarkers is supported by seminal studies suggesting that there is significant intra-tumour heterogeneity within the same primary tumour[95]. Hence, the diagnostic accuracy of biomarkers can be improved by a multitarget approach and it is unlikely that a single biomarker will be able to achieve a high diagnostic accuracy which meets the expectations of patients[96]. While it is established that common mutations such as *FGFR3* and *TERT* are common in NMIBC, even in combination, a *FGFR3-TERT* mutation assay will miss > 20% of bladder cancers[69].

Currently, multi-panel biomarkers are often identified using next generation sequencing or arrays followed by a validation cohort of patients. However, incorporating more biomarkers may not improve diagnostic accuracy[30, 50, 51]. The traditional methods such as defining a positive test using by a score and benchmarking it against an arbitrary threshold when evaluating multiple biomarkers is not ideal. Additionally, the choice of biomarkers to be incorporated is key. Using multiple biomarkers with a high sensitivity and specificity with significant overlap may risk poorer results. Hence, modern approaches incorporating complex bioinformatics and machine learning approaches using big data analysis represents a step change approach[97]. Mathematical models such as random forest classifier or network models allows for the aggregation of higher sensitive and specific biomarkers with those of poorer accuracy that do not overlap resulting in a more robust test. In addition, considering KEGG pathways to determine truncal biological pathways implicated in bladder cancer carcinogenesis may allow for better biomarker selection which reflects functional biology[98]. Further, aggregating different 'omic' biomarkers such as simultaneous analysis of DNA methylation, mutation, gene expression and copy number alterations has been hypothesized to improve biomarker accuracy[99]. This approach has been utilised by two groups combining genomic with DNA methylation targets to achieve an ROC of 0.96[55, 56]. Several studies also incorporated urinary cytology in addition to other biomarkers which resulted in improved biomarker performance[20, 48, 52, 53]. Combining standard radiological images

with genetic analysis has also proven to be an effective strategy in biomarker development[100].

The acceptable threshold of a urinary biomarker is dependent on its use as a companion test or a definitive test to replace cystoscopy. The NPV expected in a urinary assay used to replace cystoscopy in the hematuria setting is high given the devastating consequences in missing a bladder cancer particularly high-risk disease. In patient surveys, patients would only consider a urinary test with a diagnostic accuracy of ≥ 95%[96]. However, when used as a companion test, currently available urinary biomarkers have been shown to increase the accuracy of cystoscopy which is operator dependent[101].

We acknowledge that there are limitations to our study. In our systematic review, we reviewed the published literature since 2013 hence reported markers with a high diagnostic accuracy published before 2013 will not be captured. However, given that no urinary biomarker still has the diagnostic ability to replace cystoscopy, we would expect that validation studies of promising biomarkers would continue to be reported. As with most studies, positive results are often reported, and negative results remain unpublished hence there might be more biomarkers investigated but they are likely to be of limited value.

The field of urinary biomarkers for the detection of bladder cancer is rapidly developing. However, no biomarkers reported today can replace cystoscopy. The lack of field testing, validation studies, use of different threshold to determine a positive test, tumour heterogeneity and complex interplay of different ‘omics’ represents challenges in in biomarker development and validation. However, NGS with the use of complex machine learning and mathematical modeling may represent a promising approach for biomarker discovery and promising biomarkers should be field tested to validate them.

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Table 1: Study characteristics and diagnostic accuracy of urinary protein biomarkers for the diagnosis of bladder cancer with sensitivity and specificity ≥ 80%.

Title	Type of marker	Marker	Test platform	Study design	Urine collection	Country; % TCC	Low Grade (%)	Tumour arm	Control arm	Sensitivity	Specificity	PPV	NPV	ROC
Li et al. 2016 [10]	transport protein	ORM1	ELISA	Case control	20 ml morning void	China; 100%	35	112	53	92	94			0.965
Abd El-Hakim et al. 2014[12]	Inhibitor of apoptosis protein	Survivin	ELISA	Case control	Not specified	Egypt; 85%	25	40	20	85	95	94	86	0.95
Srivastava et al. 2013[13]	Inhibitor of apoptosis protein	Survivin	ELISA	Case control	50 ml void	India; 100%	41	117	74	83	81			0.881
Choi et al. 2016 [11]	DNA repair protein	APE1/Ref-1	ELISA	Case control	Not specified	Korea; 100%	58	169	108	82	80	86	73	0.83
Srivastava et al. 2016 [16]	cell-surface receptor for apoptosis	Soluble FAS	ELISA	Case control	50 ml void	India; 100%	25	117	74	88	89			0.912
Zhou et al. 2016 [18]	Transcription coactivator (AIB1), transcription kinase (EIF4A2),	AIB1	ELISA	Case control	50 ml midstream fist void	China; Not specified	42	134	76	80	86	91	71	0.827
		Combination of AIB1 + EIF5A2 + NMP22								89	91	94	82	0.898
Lorenzi et al. 2013 [14]	serine protease	HtrA1	ELISA	Case control	First void	Italy; 100%	Not specified	68	84	93	96	95	93	0.984
Li et al. 2014 [20]	HDL related protein	Apo-A1	ELISA	Case control	50 ml midstream first void	China; Not specified	Not specified	223	156	89	85			0.948
		Apo-A1 + cytology								94	84			
Ebbing et al. 2014 [17]	Inflammation related protein	calprotectin	ELISA	Case control	10 ml void	Germany; 100%	54	46	40	80	93	93	80	0.88
Attallah et al. 2015[15]	Nuclear matrix protein	NMP52	ELISA	Case control	Not specified	Egypt; Not specified	19	62	94	94	80			0.91
Shimada et al. 2016 [34]	Regulatory protein	Ubiquitin 2	Immunocytology	Case control	Not specified	Japan; 100%	29	102	143	88	99	98	93	
Soukup et al. 2015 [19] .	Heparin binding growth factor (midkine), peripheral nervous system protein (gamma synclein)	cytology+ midkine + gamma synuclein	ELISA	Case control	Second morning void	Czech Republic; 100%	27	70	49	92	98	98	89	0.9486
Jamshidian et al. 2014[21]	Glycoaminoglycan (hyaluronic acid), Hydrolytic enzyme (hyaluronidase)	Hyaluronidase	ELISA	Case control	Not specified	Iran; 100%	47	97	97	88	82			
		Hyaluronic acid								83	90			
		Hyaluronidase + hyaluronic acid								90	84			
Kumar et al. 2015 [26]	Actin binding protein (Coronin-1A), Apolipoprotein (Apo-A4),Gell matrix protein (Semenogelin-2), transmembrane (type I) heparan sulfate proteoglycan (Gamma synuclein), Peptidase (PARK7/ DJ-1)	DJ-1/PARK7	ELISA & Western	Case control	20 ml void	Singapore, France, Germany, South Korea; Not specified	All pTa/pT1	173	66	83-96	100	100	71-91	
		Coronin-1A + Apo-A4 + Semenogelin-2 + Gamma synuclein + DJ-1/PARK7								79 (ELISA)/ 94 (western)	100 (ELISA)/ 97 (western)			0.92 (ELISA)/ 0.98 (western)
Rosser et al. 2014 [27]	Chemokine (IL8), Protease (MMP9), Growth factor (VEGF-A)	IL-8	ELISA	Case control	50 ml void	USA, Not specified	45	31	42	90	86	82	92	0.907
		IL8+ MMP9 + VEGFA								93	81	78	94	0.9476
Goodison et al. 2016 [22]	Chemokine (IL-8), Protease (MMP9, MMP10), Inhibitor of serine proteases (SERPINA1), Hydrolyzes cellular RNA and promotes angiogenesis (Angiogenin), Growth factor (VEGF-A), zinc metalloenzymes (Carbonic anhydrase 9), Apolipoprotein (APOE), Serine protease inhibitor (PAI-1), transmembrane (type I) heparan sulfate proteoglycan (SDC1)	10 biomarker panel: IL8, MMP9 & 10, SERPINA1, Angiogenin, VEGF-A, Carbonic anhydrase 9, APOE, PAI-1, SDC1 Matrix metallopeptidase 9 (MMP9)	MULTI-ARRAY technology- custom multiplex immunoassay	Retrospective case control	Not specified	Japan, Not specified	38	211	67	85	81	93	63	0.8925
Shimizu et al. 2016 [23]			multiplex array compared to ELISA	Case control	Not specified	USA, Not specified	17	100	100	85	81	82	84	0.9258
Chen et al. 2014 [24]			ELISA	Case control	>3 ml void	Denmark, Spain, Germany, Portugal, USA, Netherlands; Not specified	32	183	137	79	79	73	84	0.8475
Rosser et al. 2014 [25]			ELISA	Case control	50 ml void	USA, Spain; Not	57	53	72	79	88	82	85	0.904

						specified								
Gok et al. 2016 [28]	Molecule signature	Reflection mode: Spectral range- 1500-1340, 1100-900, 900-800	Infrared spectroscopy	Case control	10 ml bladder wash	Turkey; Not specified	Not specified	40	21	82	81	90	81	
Nakai et al. 2015 [29]	porphyrin	difference between ALA treated and ALA untreated samples at 635 nm	spectrophotometry	Case control	150 ml void	Japan; Not specified	46	61	50	82	80			0.84
Inoue et al 2014 [30]	porphyrin	uroporphyrin I (UPI)	Florescence spectroscopy	Case control	15 ml void	Japan; Not specified	n/a	66	20	100	96			0.994
		coproporphyrin I (CPI)								100	92			0.978
		coproporphyrin III (CPIII)								80	82			0.828
		total porphyrins								80	94			0.827
Jin et al. 2014[31]	Metabolic signature	OPLAS-DA model: 12 peaks corresponding to. succinate, pyruvate, oxoglutarate, carnitine, phosphoenolpyruvate, trimethyllysine, melatonin, isavalsrylcarnitine, glytarylcarnitine, octenoylcarnitine, decanoylcarnitine, acetyl-coA	Mass spectroscopy	Case control	Morning void	Korea; Not specified	23	138	121	91	93			0.937
Shen et al. 2015 [32] .	Metabolic signature	MixModel1: GlyCysAlaLys, Inosinic acid, Trehalose, Nicotinuric acid, Asp Asp Gly Trp, Ureidosuccinic acid	Mass spectroscopy	Case control	Morning void	China; Not specified	Not specified	23	21	91	81			0.934
Aggio et al. 2016 [33]	Metabolic signature	Principal component analysis	gas chromatography	Case control	0.75 ml of morning void	UK; Not specified	Not specified	24	73	96	100			0.99

AIB1: amplified in breast cancer 1; APE1/Ref-1: apurinic/apyrimidinic endonuclease 1/redox factor-1; I Apo-A1: apolipoprotein A1; Apo-A4: apolipoprotein A4; Apo-E: Apolipoprotein E; EIF5A2: eukaryotic initiation factor 2; NPV: negative predictive value; NMP22: nuclear matrix protein 22; NMP52: nuclear matrix protein 52; ORM1: orosomucoid 1; SDC1: Syndecan; IL8: Interleukin 8, MMP9: Matrix metallopeptidase 9; MMP10: Matrix metallopeptidase 10; PPV: Positive predictive value; PAI-1: Plasminogen activator inhibitor-1; TCC: transitional cell carcinoma; VEGF-A: Vascular endothelial growth factor A;

Table 2: Study characteristics and diagnostic accuracy of urinary epigenetic for the diagnosis of bladder cancer with sensitivity and specificity ≥ 80%.

Title	Type of marker	Marker	Test platform	Study design	Urine collection	Country; % TCC	Low Grade (%)	Tumour arm	Control arm	Sensitivity	Specificity	PPV	NPV	ROC
Zhang et al. 2014 [36]	miRNA	miR-99a +miR-125b	RT-qPCR	Case control	Not specified. Urine supernatant	China; Not specified	30	50	21	87	81	92	71	0.876
Eissa et al. 2015 [37]	miRNA	MiR-96+ cytology	RT-qPCR	Case control	30-60 ml void	Egypt; 55.3%	G1/2=73	94	60	80	87	86	80	
Mengual et al. 2013 [38]	miRNA	6 miRNAs: miR-187 + miR-18a + miR-25 + miR-142-3p + miR-140-5p + miR-204	RT-qPCR	Case control	Not specified	Spain; 100%	38	151	126	85	87	88	83	0.921
Urquidi et al. 2016 [39]	miRNA	25 panel	RT-qPCR	Case control	30-50 ml midstream void	USA; Not specified	16	61	60	87	100			0.982
		10 panel								84	87			0.902
Du et al. 2017[40]	Cell free microRNA	7 cell-free miRNA: miR-7-5p, miR-22-3p, miR-29a-3p, miR-126-5p, miR-200a-3p, miR-375, and miR-423-5p	RT-qPCR	Case control	15 ml midstream urine. Urine supernatant	China; Not specified	38	120	120	85	87			0.916
Su et al. 2014 [41]	DNA methylation	SOX1 + IRAK3 + L1-MET	pyrosequencing	Prospective cohort	50 ml void/ bladder wash	USA; 100%	41	34 recurrences from 90 patients between 5-89 months follow up		89	97			0.95
Wang et al. 2016 [42]	DNA methylation	POU4F2	qMS-PCR	Case control	Morning void	China; 100%	Not specified	72	92	91	92	88	94	0.921
		TCF21								86	82	76	90	0.910
		POU4F2 + EOMES								88	91	86	92	0.930
		POU4F2 + PCDH17								91	93	90	94	0.923
		POU4F2 + PCDH17 + GDF15								91	88	83	94	0.914
Feber et al. 2017 [43]	DNA methylation	150 CpG	RainDance microdroplet PCR, NGS	Case control	Voided urine	UK; Not specified	38	107	167	98	97		97	0.97

EOMES: Eomesodermin; GDF15: Growth/differentiation factor 15; IRAK3: Interleukin 1 Receptor Associated Kinase 3; L1-MET: Line 1 MET; NPV: negative predictive value; PPV: positive predictive value; PCDH17: Protocadherin-17; POU4F2: POU Class 4 Homeobox 2; TCC: transitional cell carcinoma; TCF21: Transcription factor 21

Table 3: Study characteristics and diagnostic accuracy of urinary transcriptomic biomarkers for the diagnosis of bladder cancer with sensitivity and specificity ≥ 80%.

Title	type of marker	marker	test platform	Study design	Urine collection	Country; % TCC	Low Grade (%)	tumour arm	control arm	sensitivity	specificity	PPV	NPV	ROC
Ismail et al. 2016 [44]	Cytoplasmic calcium binding protein	S100A4	RT-qPCR	Case control	10ml void	Egypt; 68.3%	16	120	30	90	92	89	93	0.978
De Martino et al. 2015 [45]	zinc metalloenzyme	carbonic anhydrase IX	RT-qPCR	Case control	Not specified	Austria; Not specified	56	83	72	81	96	96	81	0.883
Eissa et al. 2014 [46]	Cell-cycle regulating protein	hepatoma upregulated protein RNA	gold nanoparticles RT-PCR	Case control	Voided urine	Egypt; 84%	16	50	50	89	94			
Eissa et al. 2014 [47]	Cell-cycle regulating protein	hepatoma upregulated protein (HURP) + cytology	RT-qPCR	Case control	30-60 ml void	Egypt; 87.7%	18	211	133	91	94	96	87	
Srivastava et al. 2014 [48]	Inhibitor of apoptosis protein	X-linked inhibitor of apoptosis protein (XIAP) + cytology	RT-qPCR	Case control	50 ml urine	India; 100%	25	117	74	98	93			
Schmidt et al. 2016 [49]	Inhibitor of apoptosis protein (surviving), Nuclear protein for cellular proliferation (Ki-67), Intermediate filament of urothelial cells (CK20)	CK20	RT-qPCR	Case control	50-200 ml urine	Germany; 100%	29	105	156	85	87			0.87
		Cytology + survivin								91	97			
		Cytology + CK20								97	90			
		ki67+ CK20								85	87			
Ribal et al. 2016 [50]	growth factor (IGF2), melanoma-associated antigen (MAGE-A3), zinc finger transcription factor (KLF9), hormone (CRH), glutamate transporter (SLC1S6), POSTN-ligand to support cell adhesion and migration (POSTN), Catalytic subunit of telomerase enzyme (TERT), nuclear protein (AHNAK2), cel lular protein providing membrane scaffold (ANXAA10), protease (CTSE), protein for cellular structural integrity (KRT20); cellular protein that reverses serine/ threonine phosphorylation (PPP1R14D)	12 genes: IGF2, MAGEA3, KLF9, CRH, SLC1A6, POSTN, TERT, AHNAK2, ANXA10, CTSE, KRT20, PPP1R14D	RT-qPCR	Prospective consecutive observational	50-100 ml void	Spain; Not specified	41	216	309	79	93	89	86	0.905
		10 genes: IGF2, MAGEA3, KLF9, CRH, SLC1A6, POSTN, EBF1, CFH, MCM10, MMP12								80	94	90	87	0.908
		5 genes: IGF2, MAGEA3, KLF9, CRH, SLC1A6								79	92	87	86	0.903
		2 genes: GF2, MAGEA3								81	91	87	88	0.918
Mengual et al. 2014 [51]		12 genes: IGF2, MAGEA3, KLF9, CRH, SLC1A6, POSTN, TERT, AHNAK2, ANXA10, CTSE, KRT20, PPP1R14D	RT-qPCR	Prospective consecutive observational	50-100 ml void	Spain; 100%	Not specified	96	111	86	90	89	88	0.944
		10 genes: IGF2, MAGEA3, KLF9, CRH, SLC1A6, POSTN, EBF1, CFH, MCM10, MMP12								86	90	89	88	0.949
		5 genes: IGF2, MAGEA3, KLF9, CRH, SLC1A6								84	91	89	87	0.941
		2 genes: IGF2, MAGEA3								79	91	88	83	0.913
Salomo et al. 2017 [52]	growth factor (IGF2), Intermediate filament of urothelial cells (CK20)	IGF2 + CK20	RT-qPCR	Case control	Voided urine	Germany; Not specified	18	103	50	90	84	92	81	
		IGF2 + CK20 + cytology								93	82	91	85	
Eissa et al. 2015 [53]	Oncogenic long-non-coding RNA	long non-coding RNA urothelial carcinoma associated-1 (lncRNA-UCA1)	nano assay RT-PCR	Case control	40 ml void	Egypt; 80.6%	17	139	81	92	96	88	98	0.966
		lncRNA-UCA1 + cytology								97	96	95	98	

AHNAK2: AHNAK nucleoprotein 2; ANXA10: Annexin A10; CK20: cytokeratin 20; CRH: cortisol releasing hormone; CTSE: Cathepsin E; IGF2: insulin like growth factor; KLF9: Krueppel-like factor 9; KRT20: Keratin 20; MAGE-A3: Melanoma-associated antigen 3; MCM10: minichromosome maintenance complex component 10; MMP12: matrix metalloprotease 12; NPV: negative predictive value; POSTN: Periostin; PPV: positive predictive value; PPP1R14D: Protein phosphatase 1, regulatory (inhibitor) subunit 14D; SLC1A6: solute carrier family 1 member 6; TCC: transitional cell carcinoma; TERT: Telomerase reverse transcriptase

Table 4: Study characteristics and diagnostic accuracy of different combination 'omic' urinary biomarkers for the diagnosis of bladder cancer with sensitivity and specificity ≥ 80%.

Title	type of marker	marker	test platform	Study design	Urine collection	Country; % TCC	Low Grade (%)	tumour arm	control arm	sensitivity	specificity	PPV	NPV	ROC
Van Kessel et al. 2016 [54]	Epigenetic + genomic	Methylation: TWIST1, ONECUT2 and OTX1 Mutation analyses: FGFR3, TERT and HRAS	TWIST1- qMS-PCR OTX1 & ONECUT2- SNaPshot methylation assay, TERT, FGFR3, HRAS mutation- PCR	Case control	Not specified	Netherlands; Not specified	20	74	80	97	83	23-39	100	0.93
Van Kessel et al. 2017 [55]	Epigenetic + genomic	Methylation: TWIST1, ONECUT2 and OTX1 Mutation analyses: FGFR3, TERT and HRAS	TWIST1- qMS-PCR OTX1 & ONECUT2- SNaPshot methylation assay, TERT, FGFR3, HRAS mutation- PCR	Prospective case control	Not specified	Netherlands, Spain, Sweden; Not specified	26	97	103	93	86		99	0.96
Dahmcke et al. 2016 [56]	Epigenetic + genomic	Methylation: SALL3, ONECUT2, CCNA1, BCL2, EOMES, VIM Mutation: TERT, FGFR3	SALL3, ONECUT2, CCNA1, BCL2, EOMES, VIM- methyl light TERT, FGFR3- Droplet digital PCR	Prospective observational consecutive blinded	Not specified	Denmark; 100%	34	99	376	97	77	53	99	0.963
Eissa et al. 2013 [57]	Transcriptomic + protein	Survivin +MMP2&9	Survivin- RT-PCR MMP 2 & 9- zymography	Case control	30-60 ml void	Egypt; 60%	G1/2: 76	46	20	91	85	88	89	
		Cytology + survivin								85	95	95	84	
		Cytology +MMP2&9								85	90	91	84	
		Cytology + survivin + MMP2&9								95	85	88	94	
Eissa et al. 2013 [58]	Protein + transcriptomic Protein: survivin Transcriptomic: Hyaluronidase	hyaluronidase	Survivin- ELISA Hyaluronidase- RT-PCR	Case control	30-60 ml void	Egypt; 70%	G1/2: 79	60	40	87	98	83	98	
		Survivin + cytology								83	83	77	88	
		Hyaluronidase + cytology								90	98	87	98	
		Survivin + hyaluronidase								93	90	90	93	
		Survivin + hyaluronidase + cytology								95	90	92	93	
Eissa et al. 2015 [59]	Protein + epigenetic + transcriptomic Protein: HYAL1 Transcriptomic: lncRNA-UCA1 Epigenetic: miR-210, miR-96	HYAL1	HYAL1- zymography miR-210 + miR96- RT-qPCR lncRNA-UCA1- RT-qPCR	Case control	40-60 ml void	Egypt; 78.7%	17	94	116	89	91	89	91	0.948
		lncRNA-UCA1								92	97	96	93	0.975
		HYAL1 + miR-210+ miR96+ LucRNA-UCA1+ cytology								100	90	88.7	100	0.981

BCL2: B-cell lymphoma 2; CCNA1: Cyclin A1; EOMES: Eomesodermin; FGFR3: fibroblast growth factor receptor 3; HYAL1: Hyaluronoglucosaminidase 1; lncRNA-UCA1: long non-coding RNA-urothelial cancer associated 1; MMP2: matrix metalloproteinase-2; MMP9: matrix metalloproteinase-9; NPV: negative predictive value; ONECUT 2: One Cut Homeobox 2; OTX1: orthodenticle homeobox 1; PPV: positive predictive value; SALL3: spalt-like transcription factor 3; TCC: transitional cell carcinoma; TERT: Telomerase reverse transcriptase; TWIST1: Twist Family BHLH Transcription Factor 1; VIM: Vimentin

1 Table 5: Urinary biomarkers stratified according to 'omic' class and single vs multiple target biomarker with a sensitivity and specificity of ≥ 80%.

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Promising single biomarker	
Protein	• orosomucoid 1 (ORM1)* • Survivin • APE1/Ref-1 • Soluble FAS • HtrA1* • Apo-A1 • Calprotectin • Nuclear matrix protein 52 • Ubiquitin 2 • Hyaluronidase • Hyaluronic acid • DJ-1/PARK7 • Interleukin-8 • uroporphyrin I • coproporphyrin • AIB1
Genomic	• TERT
Epigenetic	• POU Class 4 Homeobox 2* • Transcription factor 21
Transcriptomic	• S100A4 • carbonic anhydrase IX • hepatoma upregulated protein RNA • Cytokeratin 20 • long non-coding RNA urothelial carcinoma associated-1*
Promising biomarker combination	
Protein	• Amplified in breast cancer 1 + eukaryotic initiation factor 2 + Nuclear

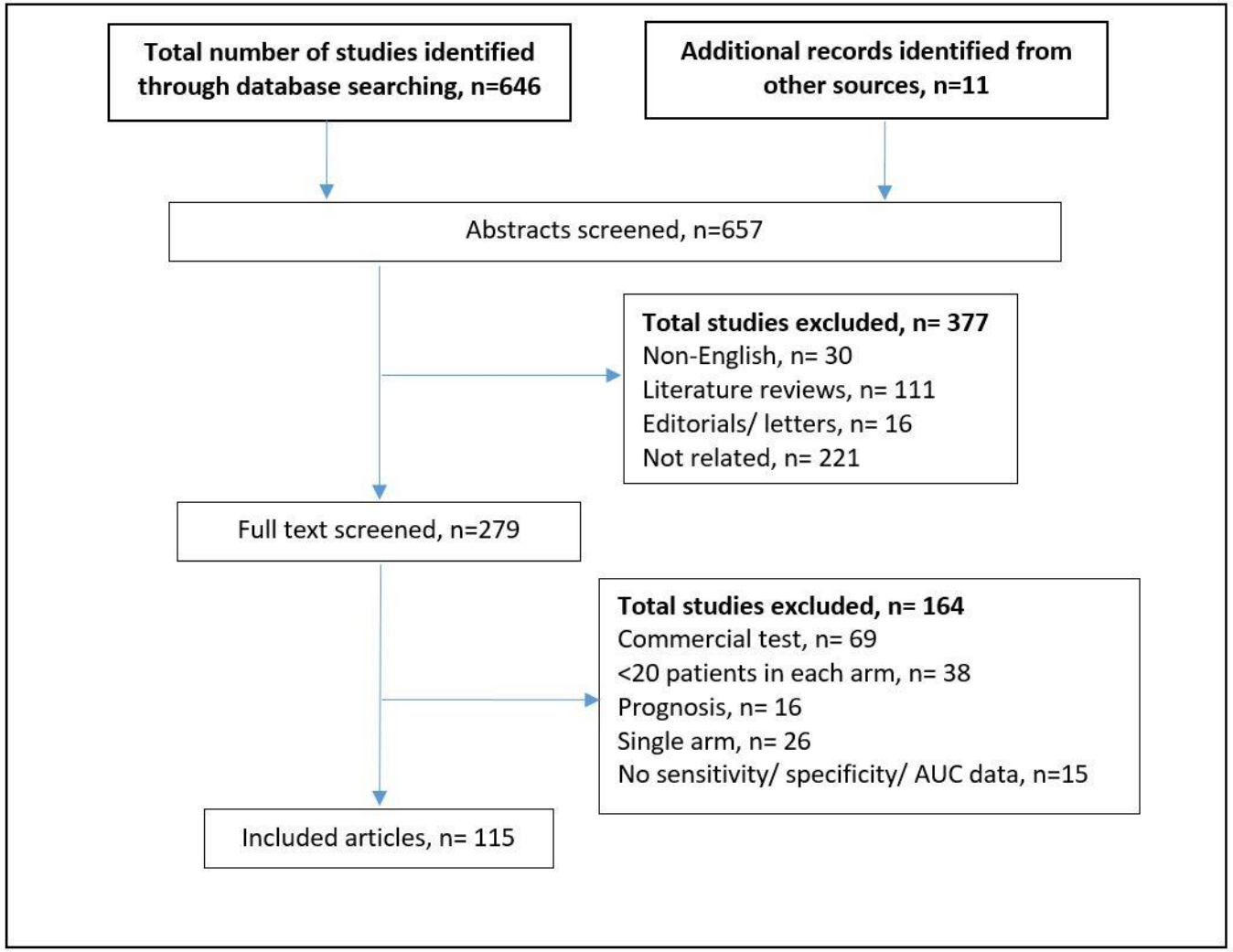
	matrix protein 22 • Apolipoprotein A1 + cytology • Cytology+ midkine + gamma synuclein* • Hyaluronic acid + hyaluronidase • Coronin-1A + Apolipoprotein A4 + Semenogelin-2 + synuclein-g + PARK7/ DJ-1* • Interleukin 8+ Matrix metallopeptidase 9 + Vascular endothelial growth factor A • Interleukin 8 + SERPINA1 + ANG + Vascular endothelial growth factor A • + CA9 + Matrix metallopeptidase 9 & 10 + Apolipoprotein E + Plasminogen activator inhibitor-1+ Syndecan[ŧ] • Spectral range- 1500-1340, 1100-900, 900-800 • Metabolic signature- succinate, pyruvate, oxoglutarate, carnitine, phosphoenolpyruvate, trimethyllysine, melatonin, isavalsrylcarnitine, glytarylcarnitine, octenoylcarnitine, decanoylcarnitine, acetyl-coA* • Metabolic signature- GlyCysAlaLys, Inosinic acid, Trehalose, Nicotinuric acid, Asp Asp Gly Trp, Ureidosuccinic acid • Principal component analysis*
Epigenetic	• mRNA-99a +mRNA-125b • MiR-96+ cytology • miR-187 + miR-18a + miR-25 + miR-142-3p + miR-140-5p + miR-204 • 10 and 25 panel miR • Cell free: miR-7-5p + miR-22-3p + miR-29a-3p + miR-126-5p + miR-200a-3p + miR-375 + miR-423-5p • methylation: SOX1 + Interleukin 1 Receptor Associated Kinase 3 + Line 1 MET • methylation: POU Class 4 Homeobox 2 + Protocadherin-17* • methylation: 150 CpG sites*
Transcriptomic	• hepatoma upregulated protein + cytology* • X-linked inhibitor of apoptosis protein + cytology*

	• Cytokeratin 20 + cytology* • Survivin + cytology* • Ki67 + Cytokeratin 20 • Insulin like growth factor 2, Melanoma-associated antigen 3 ǂ • Cytokeratin 20 + Insulin like growth factor 2 • long non-coding RNA urothelial carcinoma associated-1 + cytology*
Multi 'omic' biomolecule	• Methylation: Twist Family BHLH Transcription Factor 1, One Cut Homeobox 2 + orthodenticle homeobox 1. Mutation: Fibroblast growth factor receptor 3, Telomerase reverse transcriptase and HRAS ǂ • Methylation: Spalt-like transcription factor 3 + One Cut Homeobox 2 + Cyclin A1 + B-cell lymphoma 2 + Eomesodermin + Vimentin. Mutation: Telomerase reverse transcriptase + Fibroblast growth factor receptor 3 • Matrix metalloproteinase 2 & 9 (protein) + survivin (mRNA) + cytology* • Survivin (protein) + hyaluronidase (mRNA) + cytology* • HYAL1 (protein) + miR-210 + miR96+ long non-coding RNA-urothelial cancer associated 1 (mRNA) + cytology*

*≥90% sensitivity and specificity

ǂ independent cohort validation studies

Figure 1: Flow chart of studies identified, excluded and included.



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