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Advances in urinary biomarker discovery in urological research

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A disease-specific biomarker (or biomarkers) is a characteristic reflecting a pathological condition in human body, which can be used as a diagnostic or prognostic tool for the clinical management. A urine-based biomarker(s) may provide a clinical value as attractive tools for clinicians to utilize in the clinical setting in particular to bladder diseases including bladder cancer and other bladder benign dysfunctions. Urine can be easily obtained by patients with no preparation or painful procedures required from patients' side. Currently advanced omics technologies and computational power identified potential omics-based novel biomarkers. An unbiased profiling based on transcriptomics, proteomics, epigenetics, metabolomics approaches et al. found that expression at RNA, protein, and metabolite levels are linked with specific bladder diseases and outcomes. In this review, we will discuss about the urine-based biomarkers reported by many investigators including us and how these biomarkers can be applied as a diagnostic and prognostic tool in clinical trials and patient care to promote bladder health. Furthermore, we will discuss how these promising biomarkers can be developed into a smart medical device and what we should be cautious about toward being used in real clinical setting.

Keywords: Biomarkers; Cystitis, interstitial; Urinary bladder; Urinary bladder neoplasms; Urine

INTRODUCTION

The bladder is a hollow, soft muscular organ located in the lower abdomen, which stores urine until it is ready to excrete. In urological diseases, the incidence of bladder diseases is quite high. The common bladder diseases include bladder cancer (BC), bladder dysfunction (cystitis, urinary incontinence, overactive bladder, etc.) and other bladder problems. BC is the sixth most common cancer in the United States, accounting for 4.7% of cancer cases [1]. About 45,000 men and 17,000 women in the United States are diagnosed as BC every year.

Interstitial cystitis (IC) is the most common disease in bladder dysfunction. According to the International Continence Society, the definition of IC is "the complaint of suprapubic pain related to bladder filling, accompanied by other symptoms such as increased daytime and nighttime frequency, in the absence of proven urinary infection or other obvious pathology." [2]. The morbidity of IC in the general population is 0.26% to 12.6% [3,4]. The estimated morbidity of IC in women is 45/100,000, which is 4 to 5 times than that in men, with the morbidity of 8/100,000 [5]. In the United

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States, 3.3 million women are diagnosed as IC every year [6]. At present, one of the most important methods to diagnose bladder diseases is cystoscopy, but this technique is invasive and may lead to urinary tract infection. Compared to cystoscopy, urine testing is easier to perform in clinical practice. Urine can be obtained non-invasively and shows increased stability over serum or blood, which allows for easy multiple sampling. With the direct contact between urine and bladder diseases, the use of urinary biomarkers detection in bladder diseases becomes more and more important.

WILL IT BE USEFUL THE URINE-BASED DIAGNOSTIC BIOMARKERS TO DETECT AND MONITOR THE BLADDER DISEASES?

Urinary biomarkers are particularly attractive due to the direct contact of the urine with the urothelial tumor cells and the ease of sample collection. Urine-based diagnostic biomarkers are reviewed in our paper from the following aspects: gene mutations and gene expression-based biomarkers, proteomic biomarkers, metabolomic biomarkers, and DNA methylation biomarkers.

1. Gene mutations associated with BC

The exact cause of BC is still unclear. There are several risk factors related to BC, including environment, smoking, toxic industrial chemicals and gases, bladder inflammation, and gene mutations. As a noninvasive method, detecting mutant genes in urine plays an important role in the diagnosis of BC.

A study of Zhu et al. [7] indicated 14 important mutation genes related to BC by searching the Catalogue Of Somatic Mutations In Cancer (COSMIC) database. The mutation genes included P53, fibroblast growth factor receptor 3 (FGFR3), TSC complex subunit 1 (TSC1), stromal antigen 2 (STAG2), HRas proto-oncogene (HRAS), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), Erb-B2 receptor tyrosine kinase 3 (ERBB3), neurofibromatosis type 1 (NF1), ERBB2, FGFR1, cyclin-dependent kinase inhibitor 2A (CDKN2A), AT-rich interaction domain 1A (ARID1A), histone-lysine *N*-methyltransferase 2D (KTM2D), and CREB binding protein (CREBBP). Several studies showed that the development of BC is associated with the mutations of P53 gene [8-11]. Sidransky et al. [12] first described the mutations of P53 gene in the urine of BC patients in 1991. They found that alterations in P53 gene were associated with poor differentiation, advanced urothelial cell carcinoma and poor prognosis [8,9]. Traczyk-Borszynska et al. [13] showed that the mutations of P53 gene were more common in clinically and histologically advanced carcinoma, and were the negative prognostic factor in BC. FGFR3 mutations also participated in the development of BC. A study showed that mutations of the FGFR3 gene were surrogate markers for the detection of genome stable bladder tumors [14]. Another study indicated that FGFR3 mutations were the feature of well-differentiated BC but not the prognostic marker in BC [13]. In other studies, van Rhijn et al. [15] showed that the combination of FGFR3 with MIB-1 (Ki67) had a more accurate prediction of the progression and survival in BC. Ploussard et al. [16] found that the progression and recurrence of FGFR3 mutations in disease depended on allele loss of 9p22. Also, Rebouissou et al. [17] found that the progression of FGFR3 mutations in non-muscle-invasive disease depended on the homozygous deletion of 9p21. HRAS is a proto-oncogene, which may promote tumorigenesis in several organs including the bladder. HRAS gene mutations in bladder cells were associated with BC, but the mutation rate was low. A study showed that the mutation rate of HRAS gene varies greatly in BC (0%–30%) [18]. Beukers et al. [19] indicated that HRAS gene mutations were more likely to occur in young BC patients (<20 years) compared with older patients. It suggested that mosaicism of oncogenic HRAS mutations may increase the risk of developing BC at a young age.

Several studies showed that TSC1 had inactive point mutations on 9q34 in10% to 15% of BC patients, resulting in complete loss of function of TSC1 [20-22]. Also, the deletion of a single TSC1 allele may promote the growth of bladder epithelial cells and therefore promote the development of BC [23]. STAG2 mutations were recently identified in BC patients. However, the significance of STAG2 mutations remains controversial. Solomon et al. [24] showed that loss of STAG2 promoted the lymph node metastases in BC and increased the risk of recurrence and mortality. However, different subtypes of BC may exhibit different mutations [25]. In several studies, loss of STAG2 was reported to be associated with BC in low stage and low grade [26-28]. Lelo et al. [29] found that STAG2 mutations were much more common in non-muscle invasive BC (32%) than in muscle invasion BC (12%). These studies suggested that STAG2 could be a potentially useful biomarker for predicting recurrence and progression in non-muscle invasive BC. BRCA1-associated protein 1 (BAP1) is a nuclear ubiquitin carboxy-terminal hydrolase or deubiquitinating enzyme which can regulate several cellular functions, including cell cycle, differentiation, proliferation, and DNA damage response [30]. The recent research indicated that BAP1 mutations were related to BRCA pathway alterations in BC. Lin et al. [31] indicated that patients carrying BAP1 genetic variant al-

leles of rs12163565 had an increased risk of developing BC, although the increased risk was not statistically significant (odds ratio, 1.17; p=0.070). There were studies showed that PIK3CA gene alterations, including mutations, copy gains or amplifications, were associated with non-muscle invasive BC [32,33]. Dueñas et al. [33] showed that PIK3CA gene alterations were frequent and associated with low recurrence and low progression in non-muscle invasive BC, which indicated that PIK3CA may be a potential biomarker for predicting recurrence and progression in non-muscle invasive BC. Collectively, many genes mutations have been found in BC patients. Further studies are required to discover more gene mutations and new biomarkers in BC before they can be used in clinical practice.

2. Gene expression-based BC biomarkers

Gene expression-based urinary biomarkers have good sensitivity and specificity in the detection of BC. They are less likely to be affected by inflammatory and other benign conditions. Several important genetic changes in BC have been identified in the past two decades. Based on the technology of rapid nucleic acid extraction and the proven stability of DNA and RNA in urine, gene expression-based biomarkers play an important role in the detection of BC.

A study from Beukers et al. [34] showed that FGFR3, telomerase reverse transcriptase (TERT), and orthodenticle homeobox 1 (OTX1) were significant in the diagnosis of BC. They acted as a urinary biomarker combination with a sensitivity of 57% in low grade primary BC patients and 83% in pT1 or muscle invasive BC. In a study of Holyoake et al. [35], the researchers used microarray data from BC patients and healthy controls to generate a panel of genes that were differentially expressed in various stages and grades of BC patients and normal controls. They tested the markers in voided-urine samples to generate an mRNA panel, including cyclin-dependent kinase-1 (CDK1; also known as CDC2), midkine (MDK), insulin like growth factor binding protein 5 (IGFBP5), and homeobox A3 (HOXA3), which could predict the presence of BC with a sensitivity of 48% to 100% and a specificity of 85%. Park et al. [36] examined aurora kinase A (AURKA) gene amplification in exfoliated cells in urine samples. They concluded that AURKA could be a biomarker for the detection of BC with a specificity of 96.6% and a sensitivity of 87%, and the degree of gene amplification was also associated with high grade BC. Urquidi et al. [37] used Affymetrix arrays of 92 patients (52 BC and 40 controls) and derived a 14 gene panel that could predict the presence of BC, with high sensitivity and specificity (90% and 100%, respectively) and AUC (area under the receiver operating curve) of 0.98. The 14 genes were: carbonic anhydrase 9 (CA9), transmembrane protein 45A (TMEM45A), C-C motif chemokine ligand 18 (CCL18), matrix remodeling associated 8 (MXRA8), matrix metallopeptidase 9 (MMP9), semaphorin 3D (SEMA3D), ERBB2, vascular endothelial growth factor A (VEGFA), desmocollin 2 (DSC2), Ras-related protein Rab-1A (RAB1A), angiotensinogen (AGT), synaptogyrin 1 (SYNGR1), deleted in malignant brain tumors 1 (DMBT1), angiogenin (ANG). The first seven genes were upregulated and the last seven genes were down-regulated in the urines of BC patients. Bongiovanni et al. [38] found that the expression levels of septin 4 (SEPT4) were up-regulated in the urine of BC patients, with a sensitivity of 93%, a specificity of 65%, and AUC of 0.798. All these studies have shown promise in the diagnosis of BC. However, the majority of them remain in the discovery phase.

MicroRNAs (miRNAs) are a class of small, endogenous, noncoding RNA. They regulate gene expression by affecting mRNA translation and stability or by modulating promoter activity of their target genes. In oncology, miRNAs are considered as promising biomarkers for early diagnosis, prognosis evaluation and therapeutic response prediction of the tumor. A large number of studies showed that miRNAs acted as diagnostic biomarkers in urine samples of BC patients [36,39-42]. Some miRNAs were down-regulated such as miR-125b, miR-140-5p, miR-141, miR-200a, miR-200c, and others were up-regulated such as miR-18a, miR-92a, miR-96. Other studies indicated that miR-126, miR-152, miR-222, and miR-452 were up-regulated in BC [43-45]. However, miR-200 family, miR-155, miR-192, miR-205, and miR-143 were found to be down-regulated in studies [44,46]. Eissa et al. [47] found that the levels of miR-324-5p, miR-4738-3p, and FOSB mRNA were up-regulated in the urine of BC patients, whereas lncRNA miR-497-HG and RCAN1 mRNA were down-regulated in BC patients, compared with patients with benign lesions and healthy controls. The sensitivities and accuracies of the RNAs were significantly higher than those of cytology. In the urinary ceRNA: lncRNA-miRNA-mRNA network, 2 mRNAs (FOS B and RCAN1) displayed the highest accuracy for the diagnosis of BC. A study of Chen [48] showed that miR-101 was decreased in BC patients, and was negatively associated with aggressive clinical characteristics, with a sensitivity of 82.0% and a specificity of 80.9% in BC.

Most of the studies on miRNAs were different in methodology, with little overlap, and no results were fully validated. At present, there are no valid conclusions about urinary miRNAs in the detection of BC patients. Multicenter prospective validation studies in large clinical settings are needed in the future.

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3. Proteomics profiling revealed urinary biomarkers for BC

The urinary proteome enriched in proteins reflects the development and invasion of the tumor through direct contact with BC. The study of urinary proteomic biomarkers has been mainly used to help diagnose primary and recurrent BC and to assess the aggressiveness of the disease.

Nuclear matrix protein 22 (NMP22) is one urinary biomarker approved by the U.S. Food and Drug Administration (FDA) using enzyme-linked immunosorbent assay (ELISA) test and BladderChek point-of-care test [49,50]. However, in a meta-analysis of 19 studies for the detection of BC, the sensitivity of NMP22 was 52% to 59% and the specificity was 87-89%, with an AUC of 0.83 [51]. Another biomarker approved by FDA is the bladder tumor antigen (BTA), also known as human complement factor H related protein (hCFHrp). In a meta-analysis of 13 studies using BTA STAT test, the sensitivity of BTA was 64% to 69% and the specificity was 73% to 77% [52]. In a meta-analysis of 5 studies using BTA STAT test, the sensitivity of BTA was 62% to 71% and the specificity was 45% to 81% [53]. Both of the two markers above were not good in sensitivities and specificities.

An ideal protein biomarker should be the one with high sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and AUC values [54]. Several studies showed that Apo-A1, BLCA-4, and hyaluronidase in urine were independently validated in BC with high sensitivities and specificities [55-60]. Apo-A1 is the primary protein component of high-density lipoprotein, which may improve tumor angiogenesis through kinase activation [61,62]. But the association between lipoproteins and BC progression is still not very clear. Studies showed that Apo-A1 was independently validated in BC with the sensitivity of 89% to 95% and the specificity of 85% to 92% [55-57]. BLCA-4 is a nuclear transcription factor found in the early stages of BC. Cai et al. [58] found that BLCA-4 was independently validated with the sensitivity of 93% and the specificity of 97% through an analysis of nine studies. Hyaluronidase could improve cellular proliferation and motility through hyaluronic acid [63]. Studies of Eissa et al. [59] and Pham et al. [60] showed the sensitivity and specificity of hyaluronidase ranged from 87% to 100% and 89% to 98% respectively. Besides the three proteins, there were several additional urine proteins that exhibited with high sensitivities and specificities, but they have not yet been independently validated, including ANG, apolipoprotein E (APOE), CA-9, interleukin-8 (IL-8), MMP, MMP10, plasminogen activator inhibitor 1 (PAI-1), VEGF [63,64]. Goodison et al. [63] found that the eight-biomarker panel above achieved a sensitivity of 92% and a specificity of 97%, while the BTA TRAK ELISA test achieved a sensitivity of 78% and a specificity of 83% in the same cohort for BC detection. Another study of Urquidi et al. [65] showed that urine CCL18 achieved a sensitivity of 88% and a specificity of 86%, while BTA TRAK ELISA achieved a sensitivity of 80% and a specificity of 84% in the same cohort for BC detection. All of the biomarkers above had better sensitivities and specificities than BTA. These head-to-head studies compared the biomarkers with the FDA-approved test in the same patient cohort, increasing the validity of the studies.

4. Proteomics profiling revealed urinary biomarkers for IC

IC/bladder pain syndrome (BPS) is the most common disease in bladder dysfunction. At present, the etiology of IC/BPS is still not fully understood. There are several possible mechanisms, including infection, inflammation, toxic substances absorption, mucus layer with deficient glycosaminoglycan, hypoxia, and genetics. So far there are no gold standards in the diagnosis of IC/BPS. Some invasive testing including biopsy, urodynamic, and cystoscopy are applied to help diagnose the disease. However, there is still a lack of tools to facilitate accurate diagnosis and objective follow-up. Therefore, it is significant to investigate urinary biomarkers that can be used in clinical practice.

A study by Magalhaes et al. [66] reviewed the urinary biomarkers associated with IC/BPS. They found potential biomarkers investigated in urine specimens included macrophage inhibitory factor (MIF), nerve growth factor (NGF), methylhistamine, histamine, IL-6, antiproliferative factor (APF), epithelial growth factor (EGF), heparin-binding (HB)-EGF, glycoprotein G5P1, and a chemokine profile. Tonyali et al. [67] detected urinary NGF and nerve density in the bladder mucosa. They found that urinary NGF/Cr was significantly increased in IC/BPS patients comparing to control groups, which was similar to nerve density. Corcoran et al. [68] assessed both urine samples and bladder biopsy samples to determine the profile of 23 chemokines in 10 IC/BPS patients and 10 controls. The results indicated that univariate analysis showed no significant differences in any of the urinary proteins assessed, but multivariate analysis showed that VCAM-1 and ICAM-1 in urine were significantly different between IC/BPS and controls. A study of Vera et al. [69] studied urinary MIF concentrations in subgroups of BPS with and without Hunner lesions and control groups. They verified that urinary MIF was significantly higher in BPS patients with Hunner lesion compared with patients without Hunner and with controls, with a sensitivity of 74.4%, a specificity of 71.8%, and AUC 0.718. For the urinary

MIF/Cr ratio, the sensitivity was 47%, the specificity was 91% and AUC was 0.730 in identifying patients with IC/BPS and Hunner lesions. Lamale et al. [70] investigated urinary histamine, IL-6, and methylhistamine in IC/BPS patients and controls. They found that urinary concentrations of histamine and IL-6 were increased in IC/BPS patients. However, methylhistamine levels had no significant differences between IC/BPS patients and controls. Further logistic regression analysis demonstrated that the best predictor for IC/BPS was a combined model with IL-6 and methylhistamine, with an AUC of 0.788. Furthermore, Keay et al. [71] found that APF was increased in IC/BPS patients compared to controls, but HB-EGF concentrations were decreased in IC/BPS patients. Byrne et al. [72] demonstrated that glycoprotein G5P1 concentration in urine was lower in IC/BPS patients than that in controls.

In general, urine proteomic biomarkers of bladder diseases have great promise, but the best biomarkers with the highest clinical utility remain to be discovered. There is still a need for more comprehensive screening of urine proteomic markers through extensive multi-institution validation. Table 1 shows the urinary biomarkers suggested for BC and IC diagnosis [51-53,55-60,63,65,67-72].

5. Metabolomic biomarkers for BC

At present, urinary metabolomic biomarker studies are primarily conducted either by NMR-based or mass spectrometry (MS)-based identification. Three metabolites (2,5-furandicarboxylic acid, ribitol, and ribonic acid) were found to be lower in the urine of BC patients than in healthy controls [73-75]. Taurine is the metabolite known as a free-radical scavenger that can prevent cell damage. Studies showed that taurine was elevated in the urine of BC patients than in healthy controls [75,76]. Several studies showed that urinary citrate, succinate, and hippurate were reduced in BC patients compared with control groups, which suggested that citrate changes were related to an altered tricarboxylic acid (TCA) cycle in BC metabolism [73-77]. On the study of glycolysis-related metabolites, decreased fructose levels and increased lactate levels were showed in BC patients [73,75]. Urinary acetylcarnitine and adipate in BC patients were elevated, which were the results of disturbed fatty acid transportation, altered mitochondrial TCA cycle, and energy metabolism processes or an excess of acetyl-CoA production [74,75,77].

Wittmann et al. [75] identified between 178 and 233 discriminating metabolites (depending on the respective comparison) in a retrospective MS study. They compared current BC patients with three different control groups: patients with haematuria, controls with BC in the past but without the current disease, and a mixed group of patients with haematuria, those with BC in the past and some healthy subjects. They found that 3-hydroxybutyrate and gluconate were the most highly increased in BC patients, while anserine, 3-hydroxyphenylacetate and pyridoxate showed the lowest values in BC patients. In another high-resolution liquid chromatography (LC)-MS study, glycolysis and acylcarnitines were increased in BC than a combined control group (patients with haematuria and healthy controls) [78]. Besides, amino acid metabolism and fatty acid oxidation were also important factors in BC pathology. A study showed that the acylcarnitines, decanoylcarnitine, decenoylcarnitine, hydroxynonanoylcarnitine and hydroxybutyrylcarnitine were all increased in BC patients [79]. These urinary metabolomic biomarkers may have potential significance in the diagnosis of BC.

6. Metabolomic biomarkers for IC

In the research of IC/BPS, Parker et al. [80] used LC-MS in urine samples of 40 women with IC/BPS and 40 controls to determine metabolomic profiles. They found six metabolites were closely associated with IC/BPS. One of them was etiocholan-3alpha-ol-17-one (Etio-S). The elevated Etio-S was a good predictor of IC/BPS, with a sensitivity of 91.2%, a specificity of 87.4%, and AUC of 0.92. Longitudinal analysis of women in this cohort showed that the differences in Etio-S persisted, indicating that these changes could last long.

The results from these early studies on metabolomic biomarkers suggest that urine may act as a potential tool on screening or monitoring bladder diseases in the clinical field, but it is still in the discovery phase. More large multicenter studies with independent validation cohorts are needed to advance the field. Table 2 shows the urinary biomarkers suggested for IC diagnosis [73-80].

7. DNA methylation biomarkers for BC

DNA methylation has been recognized to be important in developmental biology and cancer etiology [81]. Aberrant DNA methylation is a major characteristic of BC and it plays an important role in tumor occurrence and progression [82-84]. Compared to RNA or protein, DNA is inherently stable, so it is more powerful in cancer detection. Chan et al. [85] examined the DNA methylation of seven genes (the retinoid acid receptor-β [RARβ]), death associated protein kinase 1 (DAPK1), Ecadherin, CDKN2A (p16), $p15^{INK4b}$ (p15), glutathione S-transferase Pi 1 (GSTP1), and O-6-methylguanine-DNA methyltransferase (MGMT) in voided urine of BC patients and age and sexmatched controls. Four biomarkers

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Table 1. Proteomics-based urinary biomarkers

Bladder diseases	Biomarkers	Study	Sample size	Method	Sensitivity (%)	Specificity (%)	AUC	Notes
BC	NMP22	Wang et al., 2017 [51]	5,291 patients total (meta-analysis of 19 studies)	NMP22 BladderChek, ELISA	52–59	87–89	0.83	FDA-approved
	BTA	Guo et al., 2014 [52]	3,462 patients total (meta-analysis of 13 studies)	BTA stat test	64–69	73–77	0.75	FDA-approved
	BTA	Glas et al., 2003 [53]	829 patients total (meta-analysis of 5 studies)	BTA TRAK test	62–71	45–81	NO	FDA-approved
	Apo-A1	Li et al., 2011 [55]	107 BC and 49 OUC	ELISA	83.7–91.6	85.7–89.7	0.875-0.928	
		Li et al., 2014 [56]	223 BC and 153 controls	ELISA	89	85	0.948	
		Chen et al., 2010 [57]	126 specimens	ELISA	95	92	0.982	
	BLCA-4	Cai et al., 2015 [58]	1,119 subjects total (meta-analysis of 9 studies)	ELISA (8 studies), qPCR (1 study)	93	97	0.9607	
	Hyaluronidase	Eissa et al., 2015 [59]	94 BC, 60 OUC, and 56 HC	Zymography	89	91	0.948	PPV=89%
		Pham et al., 1997 [60]	22 G1 BC, 9 G2 BC, 40 G3 BC, 48 OUC, and 20 HC	ELISA-like assay	100	89	NO	
	ANG, APOE, CA9, IL-8, MMP9, MMP10, PAI-1, VEGF	Goodison et al., 2012 [63]	64 BC and 62 HC	ELISA	92	97	NO	
	CCL18	Urquidi et al., 2012 [65]	64 BC and 63 controls	ELISA	88	86	0.919	PPV=86%, NPV=87%
IC/BPS	NGF	Tonyali et al., 2018 [67]	15 women with BPS, 18 male and female controls	ELISA	NO	NO	NO	NGF/Cr was increased ($p<0.001$)
	VCAM-1, ICAM-1 and MCP-3	Corcoran et al., 2013 [68]	10 men and women with BPS, 10 male and female controls	Immuno-assay	NO	NO	NO	VCAM-1 and ICAM-1was increased; MCP-3 was decreased ($p<0.05$)
	MIF	Vera et al., 2018 [69]	55 women with BPS without Hunner lesions, 43 women with BPS with Hunner lesions, and 100 female controls	ELISA	MIF 74.4, MIF/Cr 47	MIF 71.8, MIF/Cr 91	MIF 0.718, MIF/Cr 0.730	MIF, MIF/Cr was increased in BPS with Hunner lesion
	Histamine, IL-6, and methyl-histamine	Lamale et al., 2006 [70]	40 women with BPS, and 29 female controls	RIA, ELISA	70.00	72.40	0.788	Histamine and IL-6 was increased ($p<0.05$). Best preditor: combined model with IL-6 and methylhistamine
	APF and HB-EGF	Keay et al., 2004 [71]	24 men with BPS 36 male controls	3H-thimidine incorporation in cell cultures, ELISA	94	95	NO	APF was increased, HB-EGF was decreased ($p<0.00001$)
	G5P1	Byrne et al., 1999 [72]	36 patients with BPS and 23 controls	ELISA	NO	NO	NO	G5P1/Cr was decreased ($p<0.0001$)

AUC, area under the receiver operating curve; BC, bladder cancer; NMP22, nuclear matrix protein 22; ELISA, enzyme-linked immunosorbent assay; FDA, U.S. Food and Drug Administration; BTA, bladder tumor antigen; NO, not reported; OUC, controls with other urinary conditions; qPCR, quantitative polymerase chain reaction; HC, healthy controls; PPV, positive predictive value; ANG, angiogenin; APOE, apolipoprotein E; CA9, carbonic anhydrase 9; IL, interleukin; MMP, matrix metallopeptidase; PAI-1, plasminogen activator inhibitor 1; VEGF, vascular endothelial growth factor; CCL18, C-C motif chemokine ligand 18; NPV, negative predictive value; IC/BPS, interstitial cystitis/bladder pain syndrome; NGF, nerve growth factor; MIF, macrophage inhibitory factor; APF, antiproliferative factor; HB-EGF, heparin-binding epithelial growth factor.

Table 2. Metabolomics urinary biomarkers

Bladder diseases	Biomarkers	Study	Sample size	Method	Sensitivity (%)	Specificity (%)	AUC	Notes
BC	2,5-furandicarboxylic acid, ribitol, and ribonic acid	Pasikanti et al., 2013 [73]	38 BC and 61 controls	GC×GC–TOFMS	71	100	NO	Decreased
		Pasikanti et al., 2010 [74]	24 BC and 51 controls	GC–TOFMS	100	NO	0.9	Decreased
	Taurine	Wittmann et al., 2014 [75]	95 BC and 345 controls	UHPLC-MS/MS and GC-MS	NO	NO	NO	Increased
		Srivastava et al., 2010 [76]	33 BC and 37 healthy	1H NMR spectroscopy	NO	NO	NO	Increased
	Citrate	Pasikanti et al., 2013 [73]	38 BC and 61 controls	GC×GC–TOFMS	71	100	NO	Decreased
		Pasikanti et al., 2010 [74]	24 BC and 51 controls	GC–TOFMS	100	NO	0.9	Decreased
	Succinate and hippurate	Pasikanti et al., 2010 [74]	24 BC and 51 controls	GC–TOFMS	100	NO	0.9	Decreased
		Huang et al., 2011 [77]	27 BC and 32 controls	LC-MS	92.60	68.80	0.867 (hippurate)	Decreased
	Fructose and lactate	Pasikanti et al., 2013 [73]	38 BC and 61 controls	GC×GC–TOFMS	71	100	NO	Fructose decreased and lactate increased
	Acetylcarnitine and adipate	Pasikanti et al., 2010 [74]	24 BC and 51 controls	GC–TOFMS	100	NO	0.9	Increased
		Huang et al., 2011 [77]	27 BC and 32 controls	LC-MS	NO	NO	0.598 (acetylcarnitine)	Increased
	Component I and Carnitine C9:1	Huang et al., 2011 [77]	27 BC and 32 controls	LC-MS	90.50	96.90	0.9 and 0.88, respectively	Increased
	3-hydroxybutyrate and gluconate	Wittmann et al., 2014 [75]	95 BC and 345 controls	UHPLC-MS/MS and GC-MS	NO	NO	NO	Increased
	Anserine, 3-hydroxyphenylacetate and pyridoxate	Wittmann et al., 2014 [75]	95 BC and 345 controls	UHPLC-MS/MS and GC-MS	NO	NO	NO	Decreased
	Glycolysis and acylcarnitines	Jin et al., 2014 [78]	138 BC, 52 haematuria, and 69 healthy	high-resolution LC-MS	85–91.3	85–92.5	0.937	Increased
	Acylcarnitines, decanoylcarnitine, decenoylcarnitine, hydroxynonanoylcarnitine and hydroxybutyrylcarnitine	Liu et al., 2018 [79]	53 BC, 6 benign lesions, and 203 healthy controls	high-resolution LC-MS	NO	NO	0.8	Increased
IC/BPS	Etio-S	Parker et al., 2016 [80]	40 women with BPS and 40 controls	liquid chromatography -MS	91.20	87.40	0.92	Increased

AUC, area under the receiver operating curve; BC, bladder cancer; GC×GC–TOFMS, two-dimensional gas chromatography time-of-flight mass spectrometry; NO, not reported; UHPLC-MS/MS, ultra-high-performance liquid chromatography/tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; Etio-S, etiocholan-3alpha-ol-17-one.

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Table 3. Epigenetic urinary biomarkers

Bladder diseases	Biomarkers	Study	Sample size	Method	Sensitivity (%)	Specificity (%)	AUC	Notes
BC	A combination of FGFR3, TERT, and OTX1	Beukers et al., 2017 [34]	977 BC	RT-PCR	57–83	59	NO	
	A combination of CDC2, MDK, IGFBP5, and HOXA3	Holyoake et al., 2008 [35]	75 BC and 77 controls	RT-PCR	48–100	85	NO	
	AURKA	Park et al., 2008 [36]	23 BC and 7 controls	FISH	87	96.60	0.939	
	A 14 gene panel: CA9, TMEM45A, CCL18, MXRA8, MMP9, SEMA3D, ERBB2, VEGFA, DSC2, RAB1A, AGT, SYNGR1, DMBT1, ANG	Urquidi et al., 2012 [37]	52 BC and 40 controls	Affymetrix arrays	90	100	0.98	The first 7 genes were upregulated and the last 7 genes were down-regulated
	SEPT4	Bongiovanni et al., 2012 [38]	41 BC and 17 controls	RT-PCR	93	65	0.798	Upregulated
	miR126 and miR152	Hanke et al., 2010 [43]	18 BC and 18 controls	RT-qPCR	72 (the RNA ratio of miR-126:miR-152)	82 (the RNA ratio of miR-126:miR-152)	0.768 (the RNA ratio of miR-126:miR-152)	Upregulated
	miR222 and miR452	Puerta-Gil et al., 2012 [44]	37 BC and 57 controls	RT-qPCR			0.718 and 0.848	Upregulated
	miR96 and miR183	Yamada et al., 2011 [45]	100 BC and 498 controls	RT-qPCR	71 and 74	89.2 and 77.3	0.831 and 0.817	Upregulated
	miR-200 family, miR-155, miR-192, miR-205	Wang et al., 2012 [46]	51 BC and 24 controls	RT-qPCR	NO	NO	NO	Downregulated
	miR-324-5p, miR4738-3p, and FOSB mRNA	Eissa et al., 2019 [47]	98 BC, 48 benign diseases, and 50 controls	RT-qPCR	87.7, 84.7, and 99	86.7, 80.6, and 98.9	NO	Upregulated
	lncRNA miR497-HG and RCAN1 mRNA	Eissa et al., 2019 [47]	98 BC, 48 benign diseases, and 50 controls	RT-qPCR	90.5 and 99	83 and 98.9	NO	Downregulated
	DNA methylation biomarkers							
	DAPK, RARβ, E-cadherin, and p16	Chan et al., 2002 [85]	22 BC and 17 controls	MSP	91	76	NO	
	DAPK, TERT, and BCL2	Friedrich et al., 2004 [86]	37 BC and 20 controls	MSP	78	100	NO	
	CDKN2A, p14ARF, MGMT, and GSTP1	Hoque et al., 2006 [87]	160 BC and 94 controls	qMSP	69	100	NO	
	TWIST1 and NID2	Renard et al., 2010 [88]	496 urine samples	qMSP	90 and 48, respectively	93 and 96, respectively	NO	
		Abern et al., 2014 [89]	111 patients	qMSP	79 (a combination)	63 (a combination)		
		Fantony et al., 2015 [90]	209 patients	qMSP	67 (a combination)	69 (a combination)	NO	
	A 4-marker panel (ZNF154, HOXA9, POU4F2, and EOMES)	Reinert et al., 2011 [91]	119 BC and 59 controls	MSP	84	96	NO	

Table 3. Continued

Bladder diseases	Biomarkers	Study	Sample size	Method	Sensitivity (%)	Specificity (%)	AUC	Notes
	A 6-marker panel (EOMES, HOXA9, POU4F2, TWIST1, VIM, and ZNF154)	Reinert et al., 2012 [92]	184 BC and 35 controls	MS-HRM	82–89	94–100	NO	
	A 3-marker panel (SOX1, IRAK3, and L1-MET)	Su et al., 2014 [93]	90 non-muscle invasive BC	MSP	86 in BC with recurrence and 80 in BC with no recurrence	89 in BC with recurrence and 97 in BC with no recurrence	NO	
	A combination of CFTR, SALL3, and TWIST1	van der Heijden et al., 2018 [96]	111 BC and 57 controls	MSP	85	68	0.874	
IC/BPS	CpG-sites, MCP-3, G5P1, and HB-EGF	Magalhaes et al., 2019 [66]	478 records total	A systematic review	NO	NO	NO	Hypomethylation
	CpG sites	Bradley et al., 2018 [97]	19 IC BPS and 17 controls	Illumina Infinium MethylationEPIC BeadChip	NO	NO	NO	86% of MARK pathway sites with hypomethylation

AUC, area under the receiver operating curve; BC, bladder cancer; FGFR3, fibroblast growth factor receptor 3; TERT, telomerase reverse transcriptase; OTX1, orthodenticle homeobox 1; RT-PCR, reverse transcriptase-polymerase chain reaction; NO, not reported; MDK, midkine; IGFBP5, insulin like growth factor binding protein 5; HOXA3, homeobox A3; AURKA, aurora kinase A; FISH, fluorescence *in situ* hybridization; CA9, carbonic anhydrase 9; TMEM45A, transmembrane protein 45A; CCL18, C-C motif chemokine ligand 18; MXRA8, matrix remodeling associated 8; MMP9, matrix metallopeptidase 9; SEMA3D, semaphorin 3D; ERBB2, Erb-B2 receptor tyrosine kinase 2; VEGFA, vascular endothelial growth factor A; DSC2, desmocollin 2; RAB1A, Ras-related protein Rab-1A; AGT, angiotensinogen; SYNGR1, synaptogyrin 1; DMBT1, deleted in malignant brain tumors 1; ANG, angiogenin; SEPT4, septin 4; RT-qPCR, quantitative reverse transcriptase-polymerase chain reaction; DAPK, death associated protein kinase; RARβ, retinoid acid receptor-β; MSP, methylation-specific polymerase chain reaction; CDKN2A, cyclin dependent kinase inhibitor 2A; MGMT, O-6-methylguanine-DNA methyltransferase; GSTP1, glutathione S-transferase Pi 1; qMSP, specific high-resolution melting; TWIST1, Twist family BHLH transcription factor 1; NID2, nidogen 2; ZNF154, zinc finger protein 154; HOXA9, homeobox protein Hox-A9; POU4F2, POU class 4 homeobox 2; EOMES, eomesodermin; VIM, vimentin; MS-HRM, methylation-specific high-resolution melting; SOX1, SRY-box transcription factor 1; IRAK3, interleukin 1 receptor associated kinase 3; CFTR, cystic fibrosis transmembrane conductance regulator; SALL3, spalt like transcription factor 3; HB-EGF, heparin-binding epithelial growth factor.

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DAPK1, RARβ, E-cadherin, and p16) achieved a sensitivity of 91% and a specificity of 76% for detecting BC. And cytology achieved a sensitivity of 46% and a specificity of 100% by comparison. Friedrich et al. [86] examined DNA methylation of apoptosisassociated genes (DAPK, TERT, and apoptosis regulator [BCL2]) in the urine of BC patients. They found that combined methylation analyses achieved both high sensitivity and specificity (78% and 100%, respectively) for detecting BC. In another study, Hoque et al. [87] examined the DNA methylation of nine genes (adenomatous polyposis coli (APC), ARF tumor suppressor (p14ARF), cadherin-1 (CDH1), GSTP1, MGMT, cyclin dependent kinase inhibitor 2A (CDKN2A), retinoic acid receptor beta (RARβ2), ras association domain family member 1 (RASSF1A), and TIMP metallopeptidase inhibitor 3 (TIMP3). They found that combined methylation analysis based on four genes (CDKN2A, p14ARF, MGMT, and GSTP1) achieved a sensitivity of 69% and a specificity of 100%. Recently there were some studies on Twist family BHLH transcription factor 1 (TWIST1) and nidogen 2 (NID2) genes. Renard et al. [88] reported that TWIST1 and NID2 genes were frequently methylated in BC patients in a total of 496 urine samples collected from three urology clinical sites. The sensitivity of this 2 gene panel was significantly better than that of cytology (90% and 48%, respectively), with the specificity of 93% and 96%, respectively. The PPV and NPV of the 2 gene panel was 86% and 95%, respectively. However, the sensitivities of these two genes were poor in the studies of Abern et al. [89] and Fantony et al. [90]. In other studies, Reinert et al. [91] found a 4marker panel (zinc finger protein 154 [ZNF154], homeobox protein Hox-A9 (HOXA9), POU class 4 homeobox 2 (POU4F2), and eomesodermin (EOMES) achieved a sensitivity of 84% and a specificity of 96% for detecting BC in urine samples from 119 BC patients and 59 controls. Another study of Reinert et al. [92] found a 6-marker panel (EOMES, HOXA9, POU4F2, TWIST1, vimentin (VIM), and ZNF154) had a sensitivity of 82% to 89% and a specificity of 94% to 100% for detecting BC in urine samples from 184 BC patients and 35 controls.

In a study of 368 urine samples collected from 90 non-muscle invasive BC patients, Su et al. [93] reported that a panel of 3 markers (SRY-box transcription factor 1 [SOX1], interleukin 1 receptor associated kinase 3 [IRAK3], and L1-MET) discriminated between patients with recurrence and with no recurrence, with a sensitivity of 86% and a specificity of 89% of patients with recurrence, compared with the sensitivity of 80% and specificity of 97% of patients with no recurrence in validation sets. The results demonstrated that the combination of SOX1, IRAK3, and L1MET could detect disease recurrence with high sensitivity and specificity. Another study selected seven DNA methylation biomarkers (CDH13, cystic fibrosis transmembrane conductance regulator [CFTR], NID2, spalt like transcription factor 3 (SALL3), transmembrane protein with EGF like and two follistatin like domains 2 [TMEFF2], TWIST1, and VIM2) from four recently published BC studies [81,94,95]. They found that the best possible combination to discriminate against BC from controls was the combination CFTR, SALL3, and TWIST1 [96]. The three-gene methylation classifier achieved an AUC of 0.874, with a sensitivity of 85% and a specificity of 68%. The discovery of highly sensitive methylation biomarkers may allow us to lower the number of follow-up cystoscopies in patients with BC, which can improve the life quality of the patients.

8. DNA methylation biomarkers for IC

In the research of IC/BPS, Magalhaes et al. [66] concluded that DNA methylation in urine samples was associated with IC/BPS. Bradley et al. [97] determined DNA methylation profiles in IC/BPS and controls. After Bonferroni correction, there was no genome-scale significantly different methylation in CpG sites. Among the methylated CpG sites, the most prominent enrichment pathway was the mitogen-activated protein kinase (MAPK) pathway. This pathway had 86% of sites with hypomethylation in IC/BPS patients compared to the controls.

There is evidence that DNA methylation biomarkers

Table 4. Commercially available biomarker kits

Biomarker kits	Study	Sensitivity (%)	Specificity (%)	Notes
Cytology	Liou, 2006 [99]	16–89	81–100	FDA-approved
Hematuria dipstick	Liou, 2006 [99]	40–93	51–97	FDA-approved
NMP22	Wang et al., 2017 [51]	52–59	87–89	FDA-approved
BTA stat test	Guo et al., 2014 [52]	64–69	73–77	FDA-approved
BTA TRAK test	Glas et al., 2003 [53]	62–71	45–81	FDA-approved
Immuno Cyt	Liou, 2006 [99]	39–100	73–84	Approved only for BC surveillance
FGFR3	Beukers et al., 2017 [34]	57–83	59–82.7	FDA-approved

FDA, U.S. Food and Drug Administration; NMP22, nuclear matrix protein 22; BTA, bladder tumor antigen; FGFR3, fibroblast growth factor receptor 3.

are more sensitive than cytology although there were biomarkers tested on cohorts that varied between studies. And some markers showed specificity comparable with that of cytology. A highly selective panel of methylation biomarkers may increase the sensitivity and specificity of urine analysis in the clinical studies [98]. Standardized assays and cutoff values should be used in a large and well-designed cohort in future studies. Table 3 summarizes urine epigenetics-based biomarkers for BC and IC [34-38,43-47,66,85-93,96,97].

CONCLUSIONS

Many studies have shown that urinary-based biomarkers have high sensitivity and specificity in the diagnosis of bladder diseases (such as BC and IC), which confirms the feasibility of using urinary exfoliated epithelium as an analyzer to diagnose bladder diseases. As shown in Table 4 [34,51-53,99], commercially available biomarker kits for diagnosis of bladder disease such as BC have been introduced in market. If this method is accurate and reliable enough, it can be used not only for the diagnosis of bladder diseases but also for the screening of diseases in the population. However, further researches are needed to apply urinary biomarkers to clinical practice. More efforts should be made to improve and validate the biomarker panel and promote the progress of urine-based biomarker analysis, which will be applied to clinical work as soon as possible.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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AUTHORS' CONTRIBUTIONS

Research conception and design: Wun-Jae Kim. Data acquisition: Jayoung Kim. Data analysis and interpretation: Jayoung Kim. Drafting of the manuscript: Jayoung Kim. Critical revision of the manuscript: Won Tae Kim. Administrative, technical, or material support: Won Tae Kim. Supervision: Wun-Jae Kim. Approval of the final manuscript: all authors.

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