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].
States, 33 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), FGFR1, cyclin-dependent kinase inhibitor 2A (CDKN2A), AT-rich interaction domain 1A (ARID1A), histone-lysine N-methyltransferase 2D (KMT2D), 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, Rebuissou 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 9p24 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, 117; 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), CC motif chemokine ligand 18 (CCL18), matrix remodeling associated 8 (MRXRA8), matrix metalloproteinase 9 (MMP9), somatostatin 3D (SEMA3D), ERBB2, vascular endothelial growth factor A (VEGFA), desmocollin 2 (DSC2), Ras-related protein Rab-1A (RAB1A), angiotensigen (AGT), syntapogyrin 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 urine 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 IncRNA 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 miRNAs (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 820% and a specificity of 80% 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.
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 US Food and Drug Administration (FDA) using enzyme-linked immunosorbent assay (ELISA) test and BladderChek point-of-care test [49,60]. 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, urodynamics, 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 glucanote 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, decanoylcarntine, deconoylcarntine, hydroxynonanoylcarntine and hydroxybutyrylcarntine 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 (Eto-S). The elevated Eto-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 Eto-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), p15INK4b (p15), glutathione S-transferase Pi 1 (GSTPi), and O6-methylguanine-DNA methyltransferase (MGMT) in voided urine of BC patients and age and sexmatched controls. Four biomarkers...
## 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 |
|                  |            | 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 | PPV=89% |
|                  |            | 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 | |
|                  |            | 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 | |
| IC/BPS           | CCL18      | Urquidi et al., 2012 [65] | 64 BC and 63 controls | ELISA | 88 | 86 | 0.919 | PPV=86%, NPV=87% |
|                  | NGF        | Tonyali et al., 2018 [67] | 15 women with BPS, 18 male and female controls | ELISA | NO | NO | NO | |
|                  | 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 | |
| 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 predictor: 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) |
| GSP1             | Byrne et al., 1999 [72] | 36 patients with BPS and 23 controls | ELISA | NO | NO | NO | GSP1/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 |
| Taurine          | Pasikanti et al., 2010 [74] | 24 BC and 51 controls | GC–TOFMS | 100 | NO | 0.9 | Decreased |
|                   | Wittmann et al., 2014 [75] | 95 BC and 345 controls | UHPLC-MS/MS and GC-MS | NO | NO | 0.9 | 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 |
| 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 |
| 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, deconoylcarnitine, 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.
| 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 |       |
|                  |             | 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 downregulated |
|                  | BC         | 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 | NO | 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 markers | DAPK, RARβ2, 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 |       |
|                  | A 4-marker panel (ZF154, HOXA9, POU4F2, and EOMES) | Abem et al., 2014 [89] | 111 patients | qMSP | 79 (a combination) | 63 (a combination) | NO |       |
|                  | | Fantony et al., 2015 [90] | 209 patients | qMSP | 67 (a combination) | 69 (a combination) | NO |       |
|                  | | 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; IGFbps, 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 2; DSC2, desmocollin 2; RAB1A, Ras-related protein Rab-1A; AGT, angiotensinogen; SYNGR1, synaptogyrin 1; DMBT1, deleted in malignant brain tumors 1; ANG, angiotenin; SEPT4, septin 4; RT-qPCR, quantitative reverse transcriptase-polymerase chain reaction; DAPK, death associated protein kinase; RARβ, retinoic 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 Hoe-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.
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 apoptosis-associated 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 (RARβ), 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 4-marker 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 Li-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 LiMET 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 [34-38,43-47,66,85-96,97].

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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