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Research Progress of New Urine Markers in the Diagnosis of Bladder Cancer

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Abstract

Bladder cancer is the tenth most commonly diagnosed cancer worldwide and poses a great threat to human health. It has a high recurrence rate and requires long-term close monitoring and follow-up after surgery. At present, the most reliable method for the clinical diagnosis of bladder cancer is still cystoscopy and urine exfoliative cytology. However, cystoscopy is an invasive examination, which is often accompanied by complications such as infection, bleeding, pain and discomfort, and is cost ineffective. At the same time, the sensitivity of urine cytology for low-grade tumors is low, and the subjective factors of the examiners have a great impact on the test results. Urinary biomarkers have the advantages of non-invasive, safe, and simple detection, possessing clinical diagnostic value. At present, it has been found that many urine markers show higher sensitivity than urine exfoliative cytology in the detection of bladder cancer, but due to their poor specificity, they are not widely used in clinical practice. Therefore, there is an urgent need to find novel noninvasive and reliable method for the diagnosis of bladder cancer with high specificity and sensitivity. This article reviews the recent research progress of some new urine biomarkers in the diagnosis of bladder cancer.

Key words urine markers, bladder cancer, new type, diagnosis, research progress

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Introduction

Bladder cancer (BCa) is the tenth most frequently diagnosed cancer in the world and the most common urological tumor. In recent years, it has shown a high incidence worldwide, with about 573,000 new cases and 213,000 deaths in 2020 [1, 2]. At the same time, the incidence of bladder cancer in China is on the rise year by year. BCa has the characteristics of rapid progression and high recurrence rate, so the early diagnosis and postoperative recurrence monitoring of BCa patients is clinically important. Number of methods are pursued for detection of bladder cancer, but cystoscopy is still the gold standard in the diagnosis of BCa. However, it is an invasive surgery with pain, discomfort and infection complications, high cost and poor follow-up compliance [3-6]. Urine expiratory cytology is also commonly used to diagnose Bca and is a non-invasive test compared to cystoscopy. More importantly, its specificity for diagnosing Bca is as high as 99% (95%CI: 83% ~ 99.7%) [7], and its sensitivity varies greatly with tumor grade, ranging from 84% for high-grade tumors to as low as 16% for low-grade tumors [8, 9]. Therefore, in order to improve the monitoring level and quality of life of Bca patients, many researchers are looking for a non-invasive, sensitive, and specific biomarker(s) for BCa diagnosis. Urine is a liquid that is in direct contact with bladder tumors, which can be obtained in large quantities and non-invasively, and it is convenient to obtain, so urine-based BCa biomarker detection could be a useful future research trend. Currently, bladder cancer antigen (BTA), and various urine biomarkers such as NMP22 and fibrin/fibrinogen degradation products (FDP) have been applied to clinically assist diagnosis of BCa. It has been approved by the US Food and Drug Administration (FDA). These urine biomarkers are more sensitive to BCa than urine exfoliation cytology but have not been widely used in the clinic due to their low specificity. Therefore, it is urgent to discover new urine biomarker with high sensitivity and specificity for the early diagnosis and postoperative recurrence monitoring of BCa.

Microchromosome maintenance protein 5

Microchromosome maintenance 5 protein (MCM5) is a new biomarker recently discovered as a marker of DNA replication, and its expression is significantly increased in BCa [2]. All proliferating cells express MCM5, whereas in normal bladder tissue, MCM5 is only expressed in actively proliferating basal cell layer cells, and may serve as an important biomarker. However, cells in the basal cell layer do not usually shed into the urine, and the cells that shed into the urine in a normal bladder are MCM5-negative. However, in urothelial bladder cancer (UBC), cell proliferation is unlimited, and MCM5 expression is spread throughout all layers of the urothelium, causing cells to shed from the bladder surface into the urine with MCM5 expression, and the presence of MCM5-positive cells in the urine indicates the presence of a tumor (**Figure 1** shows the schematic diagram for various urine-based biomarkers).

Previous tests for MCM5 in urine were complex, expensive, and impractical. However, the recently developed adx bladder (Arquer Diagnostics, Sunderland) test, a commercial MCM5 enzyme-linked immunosorbent test, is capable of detecting MCM5 in urine mud in BCa hematuria patients [10]. A meta-analysis of 5,114 patients showed that MCM5 predicted BCa with an overall sensitivity and specificity of 66% and 72%, respectively. Among them, subgroup analysis using adx bladder detection technology showed that the sensitivity and specificity of the diagnosis of BCa were 61% and 67%, respectively [11]. Therefore, the urine MCM5 test has moderate diagnostic accuracy in diagnosing BCa. ADX bladder is a simpler ELISA based method for detecting MCM5 in urine and may provide a new urine marker detection method for the initial diagnosis of BCa, but more clinical studies with big datasets are needed to further determine its diagnostic value in BCa.

Cytokeratin 20

Cytokeratin (CK) is a broad family of intermediate filament polypeptides expressed by human skin cells and epithelial cells. cytokeratin 20 (CK20) is a low molecular weight cytokeratin encoded by the KRT20 gene and located on chromosome 17q21.2. The expression pattern of CK20 in normal tissues is limited. CK20 expression in urothelial cells is limited to bladder surface umbrella cells, even in the presence of severe inflammation [12]. Only malignant tumors can induce changes in CK20 expression pattern, resulting in abnormal increase of CK20 expression [13, 14]. By detecting CK20-mRNA in urine through polymerase chain reaction (PCR), BCa can be noninvasively detected [15].

A meta-analysis of 27 studies showed an overall sensitivity of 79% and an overall specificity of 90% for urine CK20 detection of BCa. Urinary CK20 was more sensitive to the diagnosis of UBC than all other types of BCa (83% vs. 75%). In addition, the diagnostic accuracy of urinary CK20 improves with the progression of tumor stage and grade [16]. Therefore, urine CK20 may be a potential non-invasive biomarker for detecting BCa, specifically UBC. However, larger clinical studies are required to further validate urinary CK20 testing. The actual clinical value of BCa and the diagnostic significance of urinary CK20 testing for BCa remain controversial. This is an important reason restricting its application in the diagnosis of bladder cancer. **Table 1** is a summary on urine markers in bladder cancer.

Abnormal glycosylated integrin α3β1

Integrins are a large family of cell membrane receptors that play an important role in the development and development of tumors and are involved in various processes, including cell proliferation, migration and extracellular matrix adhesion [17]. Integrin α3β1 is a high-affinity receptor for collagen, laminin and fifientin, and its interaction plays a key role in epithelial tissue maintenance and organogenesis [18]. Glycosylation refers to the process of forming glycosidic bonds with amino acid residues on proteins under the action of a series of glycosidases and glycosyltransferases [19]. It is important to note that abnormal glycosylation is associated with the occurrence and development of certain tumor types. Li et al. [20] developed a monoclonal antibody BCMab1 targeting BCa, which can specifically identify the abnormal glycogenated abnormal glycogenated integrin α3β1 (AG31) epitope on the cell membrane of bladder tumors. They found that AG31 was specifically expressed in BCa tissue, but not in normal bladder tissue. In addition, the AG31-mediated signaling pathway activates the FAK pathway and is involved in the development of bladder tumors. In addition, the expression level of AG31 in tumor tissues is positively correlated with the severity and prognosis of BCa patients [20].

A recent study showed that urine AG31 detection of BCa has a sensitivity of about 90.8% and a specificity of approximately 91.5%, and urine AG31 detection can also distinguish patients with BCa from patients with other tumors of the urinary system and benign inflammatory diseases. The study also found that urine AG31 level was positively correlated with tumor stage and grade. In addition, the sensitivity and specificity of urine AG31 for the diagnosis of BCa were higher than those of NMP22 (90.6% vs. 47.2% vs. 98.2% vs. 87%). More importantly, the diagnostic accuracy of urine AG31 testing in BCa patients is not affected by hematuria, age, and gender [19]. Therefore, the detection of urine AG31 may become a promising new urine marker in the diagnosis

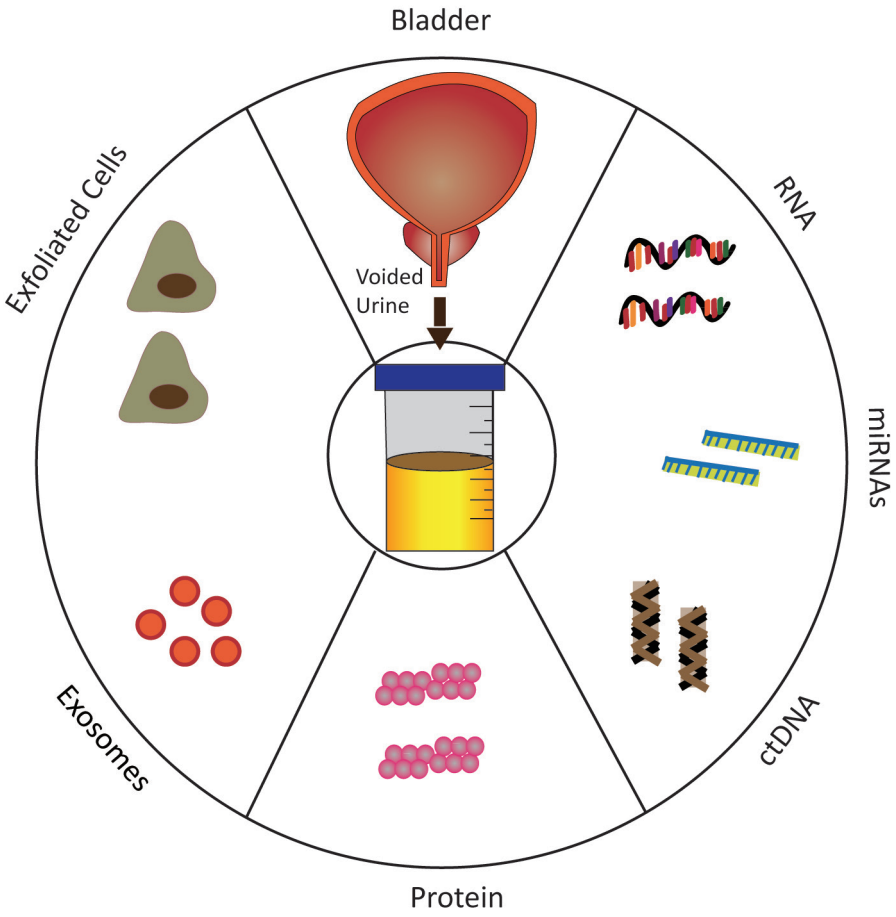

Figure 1. Schematic diagram for various urine-based biomarkers.

of BCa, and it is worth further improving its clinical value in the diagnosis of BCa. However, a large number of studies are still needed to prove it in the future.

Tumor M2-PK

M2-pk is a dimer of pyruvate kinase muscle isozyme M2 (PKM2). PKM2 is a glycolytic rate-limiting enzyme that plays an important role in tumor growth and metabolism [20-23]. Recent studies have shown that PKM2 is highly expressed in a variety of cancer types, and the poor prognosis of tumor patients is also related to the high expression of PKM2 [24-26]. However, the specific role of PKM2 in cancer development remains controversial. PKM1 and PKM2 are encoded by *PKM* gene and are different splicing products of M gene. When examining BCa sample data from the Cancer Genome Atlas (TCGA) registry, RNA sequencing data showed that 97% of 131 aggressive tumors expressed PKM2 transcripts, while PKM1 was only 3% [27-29]. In addition, PKM2 expression measured by immunohistochemistry was associated with increased tumor grade in BCa compared to normal urothelium [30]. PKM2 comes in two different forms: the highly active tetramer form and the inactive dimer form (i.e., tumor M2-PK). PKM2 switches between inactive dimers and active tetramers, and the dimerization of PKM2 in tumor cells is induced by the direct interaction of PKM2 with different cancer proteins [31].

More and more studies proved that BCa increased the expression of PKM2. The content of tumor M2-PK in the urine of BCa patients was increased, and statistical score analysis showed that the sensitivity of this method was 82% [32]. Therefore, PKM2, especially tumor M2-PK, has shown potential as a urine biomarker, but its specificity in the diagnosis of BCa still needs more clinical evaluation.

MicroRNAs

MicroRNAs (miRNAs) are small, 18-22 nucleotides long non-coding rna transcripts that play a very important role in the regulation of life activities, regulating the expression of target

Table1. Summary on urine markers in bladder cancer.

Urine markers	Definition	Principle	Current detection method	References
Microchromosome maintenance protein 5	A marker of DNA replication	Only expressed in actively proliferating basal cell layer cells and in urothelial bladder cancer (UBC), cell proliferation is unlimited and spread throughout all layers of the urothelium, causing cells to shed from the bladder surface into the urine with MCM5 expression	ADXBLADDER (Arquer Diagnostics, Sunderland) test	[2], [10], [11]
Cytokeratin 20	A low molecular weight cytokeratin encoded by the KRT20 gene	The expression of CK20 in urothelial cells is limited to bladder surface umbrella cell and malignancies resulting in abnormally elevated CK20 expression; The diagnostic accuracy of urinary CK20 improves with the progression of tumor stage and grade, especially for UBC	PCR	[12-15]
Abnormal glycosylated integrin α3β1	A high-affinity receptor for collagen, laminin and fifientin	Abnormal glycosylation is associated with the occurrence and development of certain tumor types and the expression level of AG31 in tumor tissues is positively correlated with the severity and prognosis of BCa patients	-	[17-20]
Tumor M2-PK	A dimer of pyruvate kinase muscle isozyme M2 (PKM2)	PKM2 is highly expressed in a variety of cancer types, and the poor prognosis of tumor patients is also related to the high expression of PKM2 and PKM2 expression measured by immunohistochemistry was associated with increased tumor grade in BCa compared to normal urothelium	-	[20-26]
MicroRNAs	Small, 18-22 nucleotides long non-coding rna transcripts	The expression levels of let-7b-5p, miR-149-5p, miR-146a-5p and miR-423-5p in the urine of BCa patients are significantly increased and high expression of miR-149-5p and miR-193a-5p was significantly associated with lower overall survival in BCa patients	The combined detection of BCa by miR-125b, miR-145, miR-183 and miR-221	[33-35]
DNA methylation	The covalent binding of methyl groups to cytosine residues and pyrimidine rings	It affects the genomic stability and gene expression of bladder cancer	utMeMA, the combination of GHSR/MAL genes	[44-47]
Exosomal CA9 mRNA and lncRNAs	A class of nanoscale extracellular vesicles (EVs) that carry cell-specific proteins, lipids, and nucleic acids	The urine of BCa patients is rich in urinary exosomes, and CA9 mRNA can be detected in urinary exosomes	Urinary exosome CA9mR NA, exosomal long non-coding Rnas (LNcrnas)	[52-54]
Telomerase reverse transcriptase promoter mutation	One of the most frequently mutated genomic regions in urothelial carcinoma (UC) tissues	TERT promoter mutations can be detected in urine 10 years before clinical diagnosis of BCa	-	[60-64]
Urine free DNA	Urothelial cells shed and undergo apoptosis or necrosis after releasing DNA from the cell	Unlike normal cells, tumor cells release longer stretches of DNA with higher DNA integrity and the urine cfDNA integrity of BCa patients is much higher than that of normal individuals	PCR, gene combinations	[69-73]

genes mainly by binding to the 3' untranslated region of target genes, and are considered to be important regulators of related molecular mechanisms, including carcinogenesis. In recent years, more and more studies have shown that microRNAs (miRNAs or miRs) play an extremely important role in the diagnosis of tumors. Due to their tissue-specific and tumor-specific expression, microRNAs (miRNAs or MIRs) have become potential diagnostic and pre - and post- biomarkers of BCa. Studies have shown that the expression levels of let-7b-5p, miR-149-5p, miR-146a-5p and miR-423-5p in the urine of BCa patients are significantly increased. In addition, in BCa patients, high expression of miR-149-5p and miR-193a-5p was significantly associated with lower overall survival [33].

Currently, relevant studies have shown that the sensitivity, specificity, positive predictive value, and negative predictive value of the combined detection of BCa by miR-125b, miR-145, miR-183 and miR-221 are 73.1%, 95.7%, 97.4% and 61.1%, respectively. The sensitivity can be increased by nearly 8% [34, 35]. Cytological examination of excess urine exfoliation alone confirms this finding. This shows a very important significance compared with other detection methods, and urine mirna has great potential as a novel biomarker for detecting BCa. However, prospective studies with more samples are needed to further validate the value of urine mirnas in the diagnosis of BCa. There are many mirnas closely related to BCa, and which miRNA combinations have the greatest diagnostic value for BCa still need to be further explored.

DNA methylation

DNA methylation is one of the most important forms of regulation in living organisms. DNA methylation usually refers to the covalent binding of methyl groups to cytosine residues and pyrimidine rings. DNA methylation is one of the key forms of epigenetic regulation of gene expression, affecting genome stability and gene expression, and playing an important role in the occurrence and development of tumors. Many studies have focused on potential urine DNA methylation markers for BCa detection, and their diagnostic performance varies widely [36-39].

At present, there are many studies on DNA methylation in the field of urinary tumor detection, among which Chen et al. [40] developed an effective DNA methylation detection method utMeMA for urinary tumors. A comprehensive analysis of BCa sequencing data from 3 cohorts identified 26 BCa specific methylation sites with sensitivity and specificity of 90% and 83.1%, respectively. In addition, compared with urine flow cytology and FISH, UTMEMa-based assays significantly increased susceptibility to early BCa (Ta stage and low-grade BCa), small residual tumors, and recurrent tumors. In addition, another bladder methylation assay can detect urine tumor DNA methylation, with a sensitivity and specificity of 74% and 84%, respectively, for the diagnosis of noninvasive BCa [41-43]. Notably, the combination of *GHSR*/*MAL* genes in urine DNA methylation markers was the best for the diagnosis of BCa, outperforming single DNA methylation markers and other combinations, achieving 92% sensitivity and 85% specificity [44-47]. Therefore, urine tumor DNA methylation assessment is a rapid, high-throughput, non-invasive and promising method for early diagnosis, small residual tumor detection and BCa monitoring. In particular, the urine *GHSR/MAL* gene combination has shown greater accuracy in detecting BCa, which can greatly benefit patients by reducing the burden of cystoscopy and blind secondary surgery.

Exosomal CA9 mRNA and lncRNAs

Exosomes are a class of nanoscale extracellular vesicles (EVs) that carry cell-specific proteins, lipids and nucleic acids and are present in almost all body fluids, formed by various types of cytoplasmic membranes through exocytosis [48-50]. In recent years, researchers have gradually found that exosomes are also involved in many biological processes such as nucleic acid and protein transport, antigen presentation, intercellular communication, tumor invasion and metastasis, and are important tools for intercellular information and substance exchange [51]. A large number of studies have shown that exosomes are involved in the occurrence and development of cancer. In recent years, exosomes are a hot spot in the field of biomarker research, because exosomes carry specific proteins, lipids, and nucleic acids of their origin. Exosomes are abundant in body fluids, so exosomes are often considered the primary source of liquid biopsies.

Carbonic anhydrase 9 (CA9) is a transmembrane member of the carbonic anhydrase family. It catalyzes reversible hydration of carbon dioxide with bicarbonate and protons, thereby maintaining a neutral pH of tumor cells in an acidic microenvironment, which plays an important role in the development of tumors. A large number of studies have found that the urine of BCa patients is rich in urinary exosomes, and CA9 mRNA can be detected in urinary exosomes, and the sensitivity and specificity of urinary exosome CA9 mRNA in the diagnosis of BCa are 85.2% and 83.2%, respectively [52]. Therefore, urinary exosome CA9mRNA may be a reliable non-invasive diagnostic biomarker for BCa. However, the separation and characterization methods of exosomes are complex and varied, and a unified and simple technique has not yet been formed. With the continuous development of smart technology, it is possible to develop more precise technology in the future that can significantly improve the detection rate of urine exosome CA9 mRNA.

More and more studies have proved that exosomal long non-coding Rnas (LncRNAs) play an important clinical role in the early diagnosis and prognosis of some cancers. Abbastabar et al. [53] found that urinary exosomal lncrnas carrying prostate cancer-associated transcript 1 (prostate cancer-associated transcript 1), antisense RNA at INK4 site (ANRIL) and PCAT-1 expression levels in BCa patients were significantly higher than those in normal subjects. The diagnostic accuracy of urinary exosome lncRNA *PCAT-1* and *ANRIL* for BCa were 0.73 (sensitivity 43.33%, specificity 87.5%) and 0.72 (sensitivity 46.67%, specificity 87.5%), respectively. Another meta-analysis subgroup discussed the studies on exosome derived lncRNAs in urine and blood, and finally concluded that exosome derived lncRNAs have high accuracy in the diagnosis of BCa [54]. This means that exosome lncrnas in urine and blood have great potential as biomarkers for BCa diagnosis. However, due to the large heterogeneity of this study, further multicenter prospective studies are needed to verify its clinical value. However, urinary exosomal lncRNAs are promising as reliable non-invasive diagnostic biomarkers for BCa.

Telomerase reverse transcriptase promoter mutation

Telomeres refer to special structures at the ends of chromosomes in which DNA interacts with specific proteins to form "caps", which prevent the ends of chromosomes from degrading and maintain their integrity [55, 56]. Telomerase is an RNA-dependent DNA polymerase that lengthens telomeres, and its activation is critical for malignant transformation in human cells [57-59]. The core enzyme of telomerase consists only of the subunit that catalyzes the synthesis of telomere DNA, telomerase RNA that contains an internal template, and the catalytic component telomerase reverse transcriptase (TERT) [60]. Studies have shown that *TERT* promoters are one of the most frequently mutated genomic regions in urothelial carcinoma (UC) tissues [61, 62]. The biological function of *TERT* promoter mutation in UC is becoming more and more clear. There is increasing evidence that genomic instability

after *TERT* promoter mutation is a key step in UC tumorigenesis [63].

Current studies have shown that *TERT* promoter mutations can be detected in urine 10 years before clinical diagnosis of BCa, with a specificity of 100% and a sensitivity of 46.7% [64]. This means that urine *TERT* promoter mutations have great potential as a non-invasive biomarker for the early detection of BCa, although further studies are needed to confirm this finding and evaluate its clinical value in other longitudinal cohorts. Pakmanesh et al. [65] showed that the overall specificity and sensitivity of urinary *TERT* promoter mutation to detect BCa were 88.0% and 67.7%, respectively. Urinary cytology showed similar sensitivity (67.7%) but lower specificity (62.0%) for *TERT* promoter detection of BCa. The combination of urinary *TERT* promoter mutation with urinary cytology increased the sensitivity to 83.8% and reduced the specificity to 52.0%. This suggests that urine *TERT* promoter mutations have good diagnostic accuracy for BCa as a non-invasive urine biomarker. Another study found that BCa patients with *TERT* promoter mutations had a higher risk of recurrence [66, 67]. This suggests that *TERT* promoter mutations may also be potential predictors of BCa recurrence.

Urine free DNA

There are many important biological tests in the human body, one of which is urine free DNA. Urine cell-free DNA (cfDNA) comes from a variety of sources, including transrenal circulating DNA, bacteria, and urothelial cells [68]. Most cfDNA in urine comes from urothelial cells in the urinary system, which can shed and undergo apoptosis or necrosis, releasing DNA from the cells. Unlike normal cells, tumor cells release longer stretches of DNA with higher DNA integrity [69]. A recent study confirmed that a high proportion of urine cell DNA and cfDNA can reflect the presence of tumor cells [70-73]. Detection of urine cfDNA integrity and mutations can be used to predict BCa. The urine cfDNA integrity of BCa patients is much higher than that of normal individuals [71, 72]. This provides a promising therapeutic approach for non-invasive diagnosis of bladder cancer.

A large number of studies have proved that the specificity and sensitivity of detecting urine cfDNA for the diagnosis of BCa are 72%-84% and 57%-86% [74]. Urine cfDNA sequencing identified valuable genetic mutations that could be used to detect BCa. For example, mutations that are frequently detected in BCa, such as *TERT*, *TP53*, *PIK3CA*, *KRAS*, and *FGFR3* genes are significantly altered in urine cfDNA, and the diagnostic accuracy of BCa using these five gene combinations is high (AUC of 0.94) [70]. In another study, researchers analyzed thermal gene mutations in urine cfDNA (*TERT* promoter and *FGFR3*) by drop digital PCR and showed a specificity of 100% and a sensitivity of 68.9% for urine cfDNA detection of UBC. When combined with urine cytology, the sensitivity was increased to 85.9% [75]. Therefore, the detection of urine cfDNA, especially the detection of heat gene mutations in urine cfDNA, has great potential for the diagnosis and prognosis prediction of BCa, and should be focused on in the future. Another structural feature of urine cfDNA, called "zigzag ends," can inform the diagnosis of BCa. Zhou et al. [76] evaluated single-strand terminus with 5' nucleosome prominence and noted that patients with BCa had a lower zigzag terminus index than healthy controls (AUC of 0.83). Therefore, the jagged ends of urinary cfDNA are also highly likely to serve as new urine diagnostic markers for BCa.

Conclusion and perspective

For many years, cystoscopy and urinary abstinence cytology have been the gold standard for the diagnosis of bladder cancer and remain the most reliable methods for diagnosing BCa in the clinic, but cystoscopy is an invasive, expensive method that is difficult to detect small tumor lesions. The sensitivity of urine stripping cytology to the diagnosis of BCa is poor. In recent years, the field of BCa urine biomarker detection has developed rapidly, and it has been found that some urine markers can effectively improve the detection rate of BCa as an auxiliary test for BCa diagnosis, but there is no urine biomarker that can completely replace cystoscopy and urinary ablation cytology. The newly discovered series of urine epigenetic, genetic and protein biomarkers urgently requires validation through clinical studies with multicenter big datasets. Identifying different positive thresholds, lack of actual clinical trials, and limited confirmatory studies are a series of major challenges for urine biomarker validation. With the continuous progress of science and technology, in the future, the application of advanced artificial intelligence and novel urine biomarker detection methods can continue to provide great help for the development of urine biomarkers. It is believed that it is possible to find a new urine marker with high sensitivity and specificity in the future to provide a non-invasive, safe, and simple means for the early diagnosis and postoperative recurrence monitoring of BCa.

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None.

Ethical policy

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. Approval from institutional ethical committee was taken.

Availability of data and materials

All data generated or analysed during this study are included in this publication.

Author contributions

RL: Conception, design of study, literature search and review, manuscript writting, approval for the final version of the manuscript and funding supports.

Competing interests

The authors have no competing interest.

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