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Review Article

Novel Urinary Liquid Biopsy Biomarkers and Their Role in Detecting Genitourinary Cancers

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Abstract

As liquid biopsy attracts more attention for the clinical detection and diagnosis of cancer, the need to establish reliable biomarkers has emerged. Plasma has received extensive study. However, for genitourinary (GU) cancers, urine can be the ideal body fluid. Urine can be collected in large quantities for frequent biomarker analysis and disease monitoring with relative ease. New biomarker studies are of great importance due to the limitations of the present diagnostic tests used for cancer detection and monitoring. Recently, many promising studies have investigated the role of cell-free DNA, DNA methylation, extracellular RNAs, and exosome cargos as biomarkers for GU cancer detection. This review explores the recent literature on the discovery of novel urinary biomarkers and their utility in detecting GU cancers. In small-scale studies, several novel biomarkers have shown preliminary evidence of superior clinical sensitivity and specificity compared to conventional GU cancer screening methods. With the use of these new urinary biomarkers, routine non-invasive screening and tumor monitoring may be possible.

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Introduction

A number of studies have searched for reliable biomarkers from human body fluids to replace invasive and underperforming diagnostic tests. Liquid biopsy testing offers numerous advantages over conventional tissue biopsy testing for cancer diagnosis and monitoring. In addition to decreasing unnecessary invasive biopsies, liquid biopsy can provide a better representation of tumor heterogeneity and might prove to be easier to screen for and detect tumors before metastasis.[1,2] For genitourinary (GU) cancers, such as bladder and kidney cancer, urine production and storage are adjacent to the tumor microenvironment. For this reason, urine has received increasing attention for the diagnosis and monitoring of bladder, renal, and prostate cancers. From the urine, both the sediment and supernatant can be analyzed for biomarker discovery. The urine sediment contains various cell types, salts, microorganisms, and others, while the supernatant holds numerous types of circulating nucleic acids.[3] By utilizing both fractions, researchers have identified and continue to search for new biomarkers that are applicable for GU cancer diagnosis and monitoring. Bladder cancer (BC), which is a common cancer type, is expensive to manage, due to the high frequency of recurrence and the need for multiple cystoscopies. Using cystoscopy and urine cytology, BC can be diagnosed and monitored after treatment. Cystoscopy, the use of a scope to view the bladder, is an invasive procedure with subpar patient compliance, which can lead to infection. Although this test has reported sensitivities that range from 90–97%, numerous unnecessary cystoscopies have been performed. Furthermore, roughly 10% of patients with hematuria, who underwent cystoscopy, are diagnosed with BC.[4] In addition, urine cytology can be highly insensitive, especially for low-grade BC. Combined with the high recurrence rate of BC, these tests are expensive, and patients can greatly benefit from new biomarkers and novel screening tests. For prostate cancer (PC), prostate-specific antigen (PSA) testing is commonly used for screening, to detect cancer that may be at high risk for metastasis if untreated, and detect it early before spreading. However, this test has relatively low sensitivity and specificity. Suspected PC due to elevated PSA levels can result in regular prostate biopsies. These procedures are costly and invasive for patients.[5] Lastly, renal cell cancer (RCC) is highly lethal, and lacks a gold-standard diagnostic test.[6] The lack of screening ability inevitably increases the lethality due to late-stage cancer diagnosis. There are clear benefits that urinary biomarkers can impose for GU cancer detection, diagnosis, prognosis, and treatment monitoring. A literature review was conducted using PubMed, and articles that pertain to biomarker discovery and analysis from

Keywords: Genitourinary cancers; Molecular diagnostics; Urinary liquid biopsy; Circulating nucleic acids; DNA methylation; Exosomes.
Abbreviations: GU, genitourinary; BC, bladder cancer; PC, prostate cancer; PSA, prostate-specific antigen; RCC, renal cell cancer; ctDNA, circulating tumor DNA; cfDNA, cell-free DNA; AUROC, area under the receiver operating characteristic; ddPCR, digital droplet polymerase chain reaction; ucfDNA, urinary cell-free DNA; cf-mtDNA, cell-free mitochondrial DNA; miRNAs, micro RNAs; exRNA, extracellular RNA; EVs, extracellular vesicles; EPI, ExoDx Prostate Intelliscore.
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the last five years were selected (2017–2022).

Mutational and fragmentation analysis of ctDNA/cfDNA

In recent years, circulating tumor DNA (ctDNA) has demonstrated new opportunities for cancer diagnosis, the evaluation of response to therapy, and the monitoring of resistance mutations and patients at risk of relapse.[1,2] Furthermore, the mutational analysis of ctDNA and fragmentation analysis of cell-free DNA (cfDNA) have yielded promising biomarker candidates for early cancer detection.[7–10] The cfDNA enters bodily fluids via cellular secretion, and from apoptotic and necrotic cells. Dying cancer cells release ctDNA into the blood, urine, and saliva, which can be extracted and further analyzed to detect diseases.[11] In the present study, focus was given to urine ctDNA studies for GU cancer detection. Ou *et al*. established a five-gene panel using cfDNA obtained from the urine supernatant for BC detection. For the 125 patients (92 BC patients and 33 controls), an area under the receiver operating characteristic (AUROC) curve of 0.94 was calculated, indicating accurate diagnostic capability. Although there were 12 false negative cases, this test can be used for the surveillance screening of patients with hematuria, especially if additional biomarkers are integrated (Table 1).[4,6,12–27] Another study conducted by Russo *et al*. used digital droplet PCR (ddPCR) to search for a specific *TERT* promoter mutation in the urinary cfDNA of 77 BC patients. This mutation is common in BC patients and was detected with 92% sensitivity and 96% specificity.[13] Other studies have focused on creating novel sequencing methods to analyze the unique mutation profiles of urinary cell-free DNA (ucfDNA). The study conducted by Zhao *et al*. revealed promising results for the accurate diagnosis and surveillance of BC using ucfDNA.[14] Dudley *et al*. also focused on early detection, and reported that the high-throughput sequencing method could detect 100% of BC cases identified by cytology and 82% of cases missed by cytology.[15] Another recent study took the sequencing of ucfDNA even further, analyzing the fragmentomics and mutation profiles of cell-free mitochondrial DNA (cf-mtDNA). Although this study did not solely focus on GU cancers, the researchers found that renal cell carcinoma patients presented with an enhanced fragmentation (more fragments <150 bp) of urine cf-mtDNA compared to healthy controls and patients with benign adenomas. In addition, RCC patients had an increased proportion of these fragments ending with thymine and a decreased proportion of fragments ending with adenosine. However, these differences were not detectable in plasma cf-mtDNA. Consequently, the urine cf-mtDNA fragment size and T-end-to-A-end ratio may serve as new biomarkers that can aid in distinguishing between patients with RCC and healthy or benign adenoma patients.[16]

DNA methylation

In addition to cfDNA mutation biomarkers and fragmentomics, researchers have begun to study epigenetic alterations, such as DNA methylation. These methylation signatures play a role in the progression and regulation of cancer.[6] The hypermethylation of DNA can lead to the silencing of tumor suppressor genes, prompting unregulated cell growth and division.[28] The comparison of cfDNA methylation patterns of healthy vs. cancer patients has become a hotspot for new biomarker research in recent years. Nuzzo *et al*. established 300 specific methylated regions of plasma cfDNA to train a novel analytical model. They subsequently performed the same analysis on healthy controls and RCC patients using extracted urine cfDNA, and obtained a mean AUROC curve of 0.858. Although this was not as significant as the mean AUROC for plasma (0.990), the results were promising, and 2/3 of the urine samples were early-stage RCC patients.[17] A similar study used the genome-wide DNA methylation profiling of RCC tissue and healthy renal tissue to determine a highly methylated set of genes. Kubiliute *et al*. identified six genes from the urinary DNA of RCC patients with higher methylation signatures. This gene panel helped to validate their approach as one of the most sensitive urine epigenetic biomarkers, with a sensitivity ranging from 69–78%, depending on the combination of methylated genes used in the analysis.[6] These findings aim to increase the noninvasive detection of RCC and fill the testing void for this GU cancer. In addition, BC has received extensive attention due to the downfalls of present detection methods, and a few recent studies have been conducted to identify methylation biomarkers. Feber *et al*. created the UroMark assay, which is a targeted bisulfite next-generation sequencing method to detect differences in urine sediment DNA methylation between BC patients and non-cancer controls. Using 150 highly methylated, BC-specific CpG loci and high-throughput microdroplet-based PCR amplification, the assay achieved 96% sensitivity and 97% specificity in a validation cohort of 55 BC patients and 133 cancer-free patients.[4] With a higher sensitivity, when compared to traditional cystoscopy, this assay can lower healthcare costs and patient discomfort. The implications include eliminating unnecessary procedures for patients with hematuria and BC patients undergoing treatment. For BC, using urine sediment DNA for methylation is possible because several epithelial bladder cells and bladder cancer cells are directly shed into the urine.[11] Hentschel *et al*. analyzed the potential of different urine fractions (full void, supernatant, and pellet) for detecting methylation signatures and diagnosing BC. They used seven methylated protein-coding genes and two promoter regions of microRNAs (miRNAs) that were previously shown to be hypermethylated in BC patients. Their results revealed that all three urine fractions are useful for methylation analysis and BC diagnosis, but there are minor detection differences between fractions. However, the urine pellet best matches the tumor and is more time and cost-effective to process, making the urine pellet these researchers recommended medium.[18] Similarly, a recent study used genome-wide bisulfite sequencing of BC tumor tissue DNA and urine cfDNA samples to create a targeted assay of 17 differentially methylated regions. This assay can differentiate between high-grade and low-grade tumors, and non-muscle invasive bladder cancer and muscle-invasive bladder cancer. In addition, this can differentiate between the BC type and normal urine obtained from healthy patients. The assay had 100% sensitivity and 100% specificity for high-grade tumors. However, for low-grade tumors, the sensitivity dropped to 62%, but the specificity remained at 100%. In addition, they compared post-surgery follow-up urine samples to pre-surgery urine samples, and it was revealed that DNA methylation can be used to accurately categorize patients at high risk for metastasis from disease-free patients, limiting the need for unnecessary procedures. Following surgery, DNA methylation signals can be used to accurately detect residual disease and group patients accordingly.[19] This study requires further validation through a broader patient population. However, the results were promising, and a urine-based DNA methylation assay like this could replace the presently underperforming tests and biomarkers. With the creation of highly sensitive methylation-based assays for RCC and BC, prostate cancer has also received increasing attention. In a study conducted by Nekrasov *et al*., the methylation signatures of 17 PC-as-

Table 1. Urine biomarker studies

Cancer type	Analyte	Cancer patients enrolled	Analytical technique	Sensitivity	Specificity	AUROC	Year	Reference
BC	cfDNA	77	Digital droplet PCR	92%	96%	NA	2018	Russo *et al*.[13]
BC	cfDNA	54	Novel high-throughput sequencing method (uCAPP-Seq)	84%	96%	NA	2019	Dudley *et al*.[15]
BC	cfDNA	47	Cell-free single-molecule unique primer extension resequencing (cf-SUPER)	82.7%	89.6%	NA	2020	Zhao *et al*.[14]
BC	cfDNA	92	Next-generation sequencing	NA	NA	0.94	2020	Ou *et al*.[12]
RCC	cf-mtDNA	20	Low-depth whole-genome sequencing, capture-based mtDNA sequencing	NA	NA	0.87	2022	Zhou *et al*.[16]
RCC	cfDNA Methylation	120	Cell-free methylated DNA immunoprecipitation and high-throughput sequencing (cfMeDIP-seq)	NA	NA	0.858	2020	Nuzzo *et al*.[17]
RCC	DNA Methylation	123	Qualitative and quantitative methylation-specific PCR	69–78%	69–80%	0.78	2022	Kubiliute *et al*.[6]
BC	DNA Methylation	55	Bisulfite next-generation sequencing	96%	97%	NA	2017	Feber *et al*.[4]
BC	DNA, cfDNA, miRNA Methylation	14	Quantitative methylation specific PCR	78.6%	91.7%	0.87	2020	Hentschel *et al*.[18]
BC	cfDNA Methylation	135	Genome-wide bisulfite sequencing	100% for high-grade tumors, 62% for low-grade tumors	100% for high-grade tumors, 100% for low-grade tumors	NA	2022	Xiao *et al*.[19]
PC	cfDNA Methylation	31	Methylation-specific real-time polymerase chain reaction	78%	100%	NA	2019	Nekrasov *et al*.[20]
PC	Pellet/exosomal miRNA expression	60	Quantitative real-time PCR Reaction	NA	NA	0.872	2017	Foj *et al*.[21]
PC	miRNA	47	Next-generation sequencing, quantitative real-time PCR	91%	89%	0.979	2021	Li *et al*.[22]
PC	Exosomal miRNA expression	149	miRNA-specific quantitative reverse transcription PCR	86.5%	85.3%	0.925	2021	Shin *et al*.[23]
PC	Extracellular vesicle miRNA expression	14	Quantitative real-time PCR	miR-30b-3p: 46.4%, miR-126-3p: 60.7%	miR-30b-3p: 88.0%, miR-126-3p: 80.0%	Combined: 0.667	2021	Matsuzaki *et al*.[24]
PC	Exosomal RNA expression	1,212	Reverse transcriptase polymerase chain reaction	92.3%	30.1%	0.70	2022	Margolis *et al*.[25]
RCC	miR-200a concentration	27	Quantitative real-time PCR	NA	NA	0.826	2019	Wang *et al*.[26]
BC	miR-203 expression	25	Quantitative real-time PCR	miR-203: 93.3%	miR-203: 80.0%	miR-203: 0.929	2022	Singh *et al*.[27]

BC, bladder cancer; cfDNA, cell-free DNA; PCR, polymerase chain reaction; RCC, renal cell cancer; cf-mtDNA, cell-free mitochondrial DNA; mtDNA, mitochondrial DNA; miRNA, microRNA; PC, prostate cancer.

sociated gene promoters were analyzed, and ultimately, a six-gene panel of PC predictive genes was constructed. For PC patients and control subjects, the number of methylated genes (from 0 to 6) was the deterministic factor for PC diagnosis, with greater than two methylated genes indicating the prevalence of PC. The approach of utilizing methylation-specific polymerase chain reaction achieved 78% sensitivity and 100% specificity for ucfDNA.[20] Methylation signatures may be one of the most promising biomarkers for diagnosing GU cancers. A number of studies have highlighted the excellent sensitivity and specificity in detecting the respective cancers they are investigating and the ability to solve problems associated with present testing methods. Larger validation cohorts would help confirm the utility of urinary methylation biomarkers and hopefully bring these novel tests to clinical settings.

exRNA/miRNA/exosomes

Another class of nucleic acids that can be detected in urine are circulating extracellular RNAs (exRNA). These molecules, especially miRNA, play a regulatory role in post-transcriptional gene expression, making miRNA the most common exRNA target for cancer biomarker development. Along with other circulating nucleic acids, miRNAs are often associated with protein complexes or extracellular vesicles in the extracellular domain.[29] Exosomes, a subclass of extracellular vesicles (EVs), are small (approximately 30–140 nm) particles released by cells.[30] Researchers have indicated that these molecules participate in intercellular communication and induce tumorigenesis, impacting cancer progression. In recent years, exosomes have become a major target in liquid biopsy biomarker development. Their stability, relative abundance in body fluids, and representativeness of the cells of their origin make exosomes appealing.[31] It has been speculated that urinary exosomes come from the epithelial cells of the GU system, giving these vesicles the potential to provide pertinent information regarding these organs.[32] Thus, researchers are heavily pursuing novel urinary miRNA and exosomal biomarkers for GU cancers, especially prostate cancer. Foj *et al*. examined five miRNAs that were previously known to be deregulated in tissue and blood obtained from PC patients. They isolated miRNAs from the urine pellet and exosomes, and measured the expression using quantitative real-time PCR. Three of the miRNAs (miR-21, miR-141 and miR-375) detected from 60 PC patients were upregulated when compared to 10 healthy controls, and one miRNA (miR-214) was downregulated. Using miR-21 and miR-375, the researchers achieved an AUC of 0.872, which is the highest of all tested combinations. Although a larger cohort with healthy control tissue samples is necessary, this study revealed the diagnostic and prognostic value of a handful of urinary miRNAs for PC, leading the researchers to consider that miRNAs can be used to distinguish the different stages of PC.[21] Conversely, Li *et al*. more recently concluded that urinary exosomal miR-375 was significantly downregulated. Although this study also utilized quantitative real-time PCR in the validation protocol, next-generation sequencing was used to determine the miRNA expression profiles. The difference in analytical methods and patient cohorts used in these studies may account for the discrepancy.[22] These confounding results illustrate the need for the standardization of exosome/miRNA isolation, miRNA expression analysis, and the pre-analytical factors of sample collection and processing. Nonetheless, Li *et al*. reported that miR-375, miR451a, miR-486-3p and miR-486-5p can be applied to distinguish between PC patients and healthy controls, with 91% sensitivity and 89% specificity. In addition, miR-375 exhibited prognostic capabilities, successfully distinguishing localized and metastatic PC patients.[22] Another study also analyzed the prognostic capabilities of miRNAs as PC biomarkers. Shin *et al*. analyzed 381 urinary exosomal miRNAs. Six of these had statistically significant expression profiles between localized and metastatic PC. Using these miRNA expression levels and clinical variables (age, body mass index [BMI], preoperative PSA, and others), the researchers created a PC metastasis risk-scoring model that could accurately predict the biochemical recurrence-free survival of PC patients. This model offers a novel approach to predict the outcomes for PC patients with better performance, when compared to conventional methods, such as PSA testing.[23] Similar studies have reported that miRNA biomarkers have greater sensitivity and specificity, when compared to serum PSA. In a pilot study, Matsuzaki *et al*. reported the significant elevation of miR-30b-3p and miR-126-3p detected from urinary EVs in PC patients. These researchers took a novel approach to identify miRNAs by examining EVs from patients with moderately elevated serum PSA (4–25 ng/mL). By individually analyzing each miRNA biomarker, PC was predicted with an AUC of 0.663 when miR-30b-3p was used, and an AUC of 0.664 when miR-126-3p was used. Compared to the AUC of 0.525 for serum PSA, these new miRNA biomarkers were more effective in detecting PC.[24] Unlike the previously mentioned PC studies, the ExoDx Prostate Intelliscore (EPI) test utilizes the exosomal RNA expression levels of three genes (*PCA3, ERG* and *SPDEF*). The clinical performance was analyzed, and it was revealed that in three independent clinical trials this test can better distinguish between high-grade and low-grade PC, and benign disease, when compared to PSA levels and other routine screening measures. The EPI test thereby decreases the need for invasive biopsies, and limits the delayed detection of high-grade PC.[25] Each of these studies that researched exosomes and circulating RNAs offer diagnostic or prognostic advantages for PC screening, which could be utilized in the clinic through further validation and larger studies. Future studies would benefit from the combination of exosomal and RNA-based biomarkers, in terms of creating a test with increased sensitivity and specificity. However, it may be challenging to determine the right balance, optimize the sensitivity, and reduce the false positive rate. For BC and RCC, miRNA biomarkers have also been identified. Wang *et al*. assessed the miR-200a concentrations detected from urine samples obtained from 27 healthy controls and 27 RCC patients. Using quantitative real-time PCR, miR-200a was significantly downregulated in RCC patients. These patterns matched the findings for miR-200a extracted from plasma, resulting in an even higher AUC value than plasma.[26] If validated within a larger study, urinary miR-200a could be more useful for RCC diagnosis and early detection, when compared to serum miR-200a, with the added benefit of being truly non-invasive. Singh *et al*. aimed to identify miRNAs linked to urothelial BC. In urine samples obtained from Indian BC patients, the miR-203 expression was significantly higher, when compared to that in benign prostatic hyperplasia and healthy controls. In urine, the expression of this miRNA was also positively correlated with the BC tumor tissue expression, indicating its possible diagnostic potential.[27] Most of the studies conducted on GU cancers, and urinary exosomes or circulating RNA biomarkers are relatively small scale, and in the preliminary stages. However, the results remain encouraging, and may lead to more accurate diagnosis and monitoring of prostate, bladder and renal cancers.

Conclusions

Various novel urinary biomarkers have exhibited increasingly encouraging results as alternative diagnostic methods to replace present lackluster procedures and screening tests. Most of these have exhibited superior sensitivity and specificity when compared to conventional tests, which can improve the early detection and monitoring of GU cancers. However, the clinical utility of these new biomarkers must be determined in a large population. It is noteworthy that more work is needed to validate the results of these studies and with this validation, urinary biomarkers may likely become more commonly used in the routine testing of GU cancers. The new frontier of liquid biopsy biomarkers, which expands beyond cfDNA, is here. Determining the clinical utility of these novel biomarkers would be possible through new analytical technologies, the standardization of pre-analytical factors, and more data. There may not be one specific biomarker that is best for detecting all GU cancers but designing a test with multiple classes of biomarkers can solve this problem. As more knowledge is obtained on urinary exosomes, DNA methylation, and circulating nucleic acids, a multi-cancer, early-detection urine liquid biopsy test can be developed. With this test, the routine administration of GU cancer screening and tumor monitoring would be feasible, with accuracy.

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Conflict of interest

DW is the co-founder of RNAmeTRIX, has equity in Liquid Diagnostics, and is a consultant for Colgate-Palmolive and Spectrum Solutions. LZ has family members who hold leadership positions and ownership interests in Decipher Medicine. FMD has been an editorial board member of JCTP since 2021. The authors have no other conflict of interests to declare.

Author contributions

LZ and FL: study concept and design; BS: drafting of the manuscript; LZ, DTW, FMD, YZ, FL and BS: critical revision of the manuscript; LZ: study supervision. All authors have made a significant contribution to the study, and approved the final manuscript.

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