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Review Article

Clinical applications of urinary cell-free DNA in cancer: current insights and promising future

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Abstract: Liquid biopsy is gaining significant attention as a tool for unveiling the molecular landscape of tumor and holds great promise for individualized medicine for cancer. Cell-free DNA serves as an extremely important component of liquid biopsy for cancer, and cell-free DNA in urine is even promising due to the remarkable advantage of urine as an ultra-noninvasive sample source over tissue and blood. Compared with the widely studied cell-free DNA in blood, less is known about the role of urinary cell-free DNA. Urinary cell-free DNA has the ability to give comprehensive and crucial information on cancer as it carries genetic messages from cells shedding directly into urine as well as transporting from circulation. As an indispensable component of liquid biopsy, urinary cell-free DNA is believed to have the potential of being a useful and ultra-noninvasive tool for cancer screening, diagnosis, prognosis, and monitoring of cancer progression and therapeutic effect. In this review, we provide the current insights into the clinical applications of urinary cell-free DNA in cancer. We also introduce the basic biological significance and some technical issues in the detection of urinary cell-free DNA.

Keywords: Liquid biopsy, urinary cell-free DNA, cancer, clinical application

Introduction

Cancer is a serious public health threat worldwide [1]. Diagnosis and treatment towards cancer mainly depend on the genomic profiles of tumors [2]. Since molecular profiling based on tissue samples is limited by tumor heterogeneity and difficulties of repeated sampling, liquid biopsy based on various circulating molecules emerges at the right moment [3-6]. Liquid biopsy is gaining significant attention as a tool for unveiling the molecular landscape of tumor and holds great promise for individualized medicine for cancer [7, 8]. Cell-free DNA (cfDNA) exists as fragmented nucleic acids in various extracellular body fluids in both healthy individuals and people with diseases, serving as an extremely important tool of liquid biopsy [9]. In recent years, cfDNA in blood circulation has become a topic of interest in the field of cancer, and developed as novel biomarkers in cancer clinical management [3, 10, 11].

Apart from blood, cfDNA could also be detected in other body fluids, the most intriguing among which is urine. The presence of genetic materials in urine has long been established, though initially from urinary cells [12-15]. CfDNA in urine is later discovered with the ability to give comprehensive and crucial information on cancer as it carries genetic messages releasing from cells shedding directly into urine as well as transporting from circulation. Genomic aberrations detected in urinary cell-free DNA (ucfDNA) have been demonstrated to be the same as those in primary tumors. Therefore, urine could be a novel source of genetic material that reflects the genomic aberrations of cancers [16]. More importantly, urine has a remarkable advantage as an ultra-noninvasive sample source over tissue and blood [16], especially for patients who need repeated sampling to monitor cancer progression and therapeutic effect. Furthermore, unlike serum or plasma collection which requires specialized facility or equip-

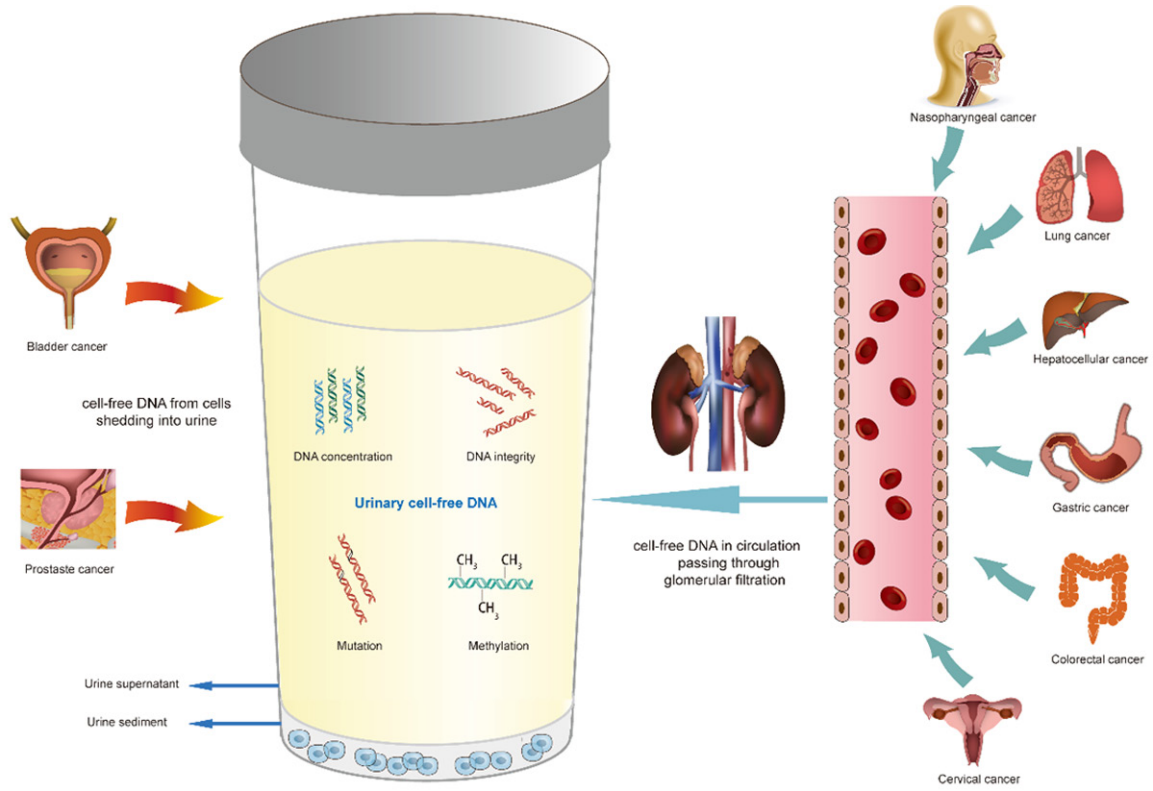

Figure 1. Origins of urinary cell-free DNA. Urinary cell-free DNA originates either from cells shedding into urine from genitourinary tract, or from cell-free DNA in circulation passing through glomerular filtration. Genetic alterations including DNA concentrations, integrity, mutations and methylation status are examined to evaluate the potential clinical utility of urinary cell-free DNA for both urological and non-urological cancers.

ment, the collection of urine that requires only sterile collection containers is implementable even in remote areas and is relatively time and cost efficient. As urine contains lower levels of protein than blood, the isolation of DNA fragments could therefore be technically easier [17].

Compared with the widely studied cfDNA in blood, less is known about the role of ucfDNA. UcfDNA is an important component of liquid biopsy in clinical oncology, containing a wide range of genetic information. More and more attention has been paid for the role of ucfDNA as a novel ultra-noninvasive biomarker in the clinical management of both urological and non-urological cancers, exploring the potential clinical utility of ucfDNA in screening, diagnosis, surveillance and prognosis. In this review, we put an emphasis on the clinical applications of ucfDNA in cancer. At the same time, the origin and biological characteristics of ucfDNA, as well as some important technical issues in the process of ucfDNA detection are also discussed.

Origin and characteristics of ucfDNA

The origins of ucfDNA are shown in **Figure 1**. UcfDNA originates either from cells shedding into urine from genitourinary tract, or from cell-free DNA in circulation passing through glomerular filtration. The latter is also known as "transrenal DNA" [18, 19].

UcfDNA fragments can be classified into two categories according to their size: high-molecular-weight DNA and low-molecular-weight DNA [17, 18, 20]. High-molecular-weight ucfDNA fragments are usually 1 kbp or longer, deriving from the necrotic cells along the urogenital tract falling into urine or from lymphocytes that normally exist in urine [17, 21]. DNA fragments up to 1.3 kbp and 19 kbp were found in male and female urine, respectively [20]. Low-molecular-weight DNA can originate either from circulation or apoptotic cells in contact with urine [16, 17, 22]. The size of low-molecular-weight DNA fragments in urine has been evaluated in various studies. Su et al. recovered DNA fragments between 150 and 250 bp from urine

supernatant [17]. Another study drew the similar conclusion that the main part of ucfDNA ranged from 150 to 400 bp [20]. In 2008, Melkonyan et al. improved the isolation and detection methods of ucfDNA, and observed a group of shorter ucfDNA fragments of 10-150 bp, in addition to the previously discovered 150-200 bp DNA fragments [23]. Furthermore, a recent study evaluating fetal DNA in the urine of pregnant women revealed that fetal ucfDNA fragments were even shorter, with a peak of 29 to 45 bp in length [24].

UcfDNA in urological diseases has been studied more extensively, since the majority of ucfDNA originates from apoptosis or necrosis of cells exfoliated from urogenital system. It has been estimated that more than 3 x 10^6 epithelial cells from urogenital tract can fall into urine within one day under normal conditions [22] and may partially undergo apoptosis to release DNA fragments into urine [18]. Urological diseases, especially tumors, which can be in direct contact with urine, can also release tumor cells into urine. Genetic information in ucfDNA has therefore been widely studied in urological cancers, predominantly in bladder cancers [25] and prostate cancers [26]. Donor-derived DNA sequences were detected for the first time in 1999, in the urine of renal transplantation recipients [27]. Subsequent research uncovered that graft-derived ucfDNA in transplant recipients increased significantly during acute rejection and returned to normal after anti-rejection therapy [28, 29].

Transrenal DNA, another indispensable source of ucfDNA, is gaining significant attention in recent years. Originating from blood circulation, transrenal DNA contains important genomic information from various positions all over the body. The existence of transrenal DNA has been verified in various studies. In 2000, Botezatu et al. confirmed, for the first time, from several experiments on animals and humans, the existence of ucfDNA and its ability to traverse the kidney barrier from blood [18]. Purified ^{32}P-labeled DNA was injected subcutaneously into the peritoneal cavity of mice, and 150-160 bp fragments of this labeled DNA were found in the urine of mice within 3 days [18]. Human Raji cells were also injected into mice and urinary DNA was isolated for PCR analysis. The results showed that human–specific *Alu* sequences were detected in mice urine [18]. They also detected Y chromosome-specific DNA sequences in the urine of women pregnant with male fetuses and females transfused with male blood [18]. Subsequent studies also confirmed the presence of fetal cell-free DNA in maternal urine [30-32]. *KRAS* mutations were discovered in ucfDNA in the urine of patients with colorectal [17, 18, 21, 33] and pancreatic cancer [18]. UcfDNA has also been evaluated in various kinds of non-urological cancers as a potential biomarker for diagnosis, treatment monitoring, and prognosis [3, 16, 34-36].

Some technical issues in isolation and detection of ucfDNA

Isolation and detection of ucfDNA are of vital importance in the study of clinical value of ucfDNA in cancer. The reliability of clinical application of ucfDNA is, to a great extent, dependent on the sensitivity and reproducibility of assays in the process of isolation and detection of ucfDNA [19]. Since there are no standard protocols for isolation and detection of cfDNA in urine, multiple methods have been introduced in research papers. The concentration of cfDNA in urine is relatively low, and the fragments of ucfDNA are mostly short [23]. The development of techniques mainly focused on improving the isolation and detection sensitivity of short DNA fragments in urine [19].

CfDNA in urine could be isolated using either commercial kits or classical laboratory techniques [16, 17]. Since circulating DNA fragments are usually short, the choice of commercial DNA isolation kits that are able to isolate low-molecular-weight DNA fragments should be taken into consideration. As for the quantification of ucfDNA, the most commonly used approaches include the spectrophotometric method, the fluorimetric method, as well as the amplification method [37].

All this time, methods for detection of genomic alterations in ucfDNA are mainly PCR-based assays [38, 39]. To gain better detection sensitivity, short amplicons are designed and applied in both conventional and real-time PCR assays [23]. More recently, the rapid development of new molecular assays such as droplet digital PCR (ddPCR) technology and the next-generation sequencing (NGS) has largely improved the sensitivity of ucfDNA detection [40, 41]. These

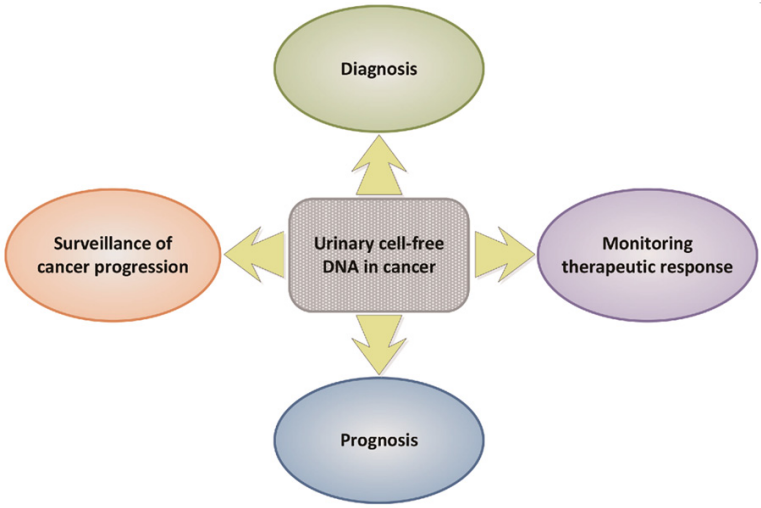

Figure 2. Clinical applications of urinary cell-free DNA in cancer.

newly developed assays have been proven to offer a deeper insight into the clinical utility of ucfDNA in cancer [42].

Clinical applications of ucfDNA in cancer

UcfDNA analysis holds promise in the clinical management of cancer. Potential applications of ucfDNA analysis include cancer detection and diagnosis, surveillance of tumor progression, monitoring of treatment response and predicting tumor prognosis (**Figure 2**). A summary of literature focusing on the potential clinical applications of ucfDNA in cancer is shown in **Table 1**.

Detection and diagnosis of cancer

The most studied area of the clinical applications of ucfDNA could be referred to the detection and diagnosis of cancer. A variety of DNA alterations including DNA quantification, DNA integrity, mutations and methylation status have been investigated in urine of both urological and non-urological cancer to evaluate the diagnostic utility of ucfDNA [7, 16, 42, 43].

Urological cancer markers: Since urological cancer is in direct contact with urine flowing through the urogenital tract, urine may contain cell-free DNA sequences derived from apoptotic or necrotic tumor cells shedding into urine. Thus, urine has great potential as a desirable source of diagnostic biomarkers because of the genetic information storing in the urinary cell-free tumor DNA. Previously, genetic and epigenetic alterations in urological cancer, including renal cancer [14, 44, 45], bladder cancer [46-49] and prostate cancer [50-52] have been studied extensively, using DNA from urine sediment. However, ucfDNA from urine supernatant has become a more preferable target for investigating in urological cancer, and it has been considered to be superior to urine sediment because a mass of normal DNA existing in urine sediment could interfere the analytical results [13]. UcfDNA analysis in urological cancer mainly focuses on bladder cancer and prostate cancer.

Various types of genetic alterations were studied using ucfDNA to evaluate the possible diagnostic value of ucfDNA for bladder cancer. In 2005, Zancan et al. first investigated the ucfDNA concentrations in 35 suspected bladder cancer patients before cystoscopy [53]. UcfDNA concentrations higher than 250 ng/mL were detected in all bladder cancer patients (16/16) confirmed by cystoscopy, whereas only 36.8% patients (7/19) with negative cystoscopy had ucfDNA higher than 250 ng/mL. Thus, a higher concentration of ucfDNA in bladder cancer patients, compared to that in healthy subjects, was confirmed, indicating the promising diagnostic utility of ucfDNA concentration in bladder cancer. Subsequently, the same research team conducted a further study in 2009, on a larger cohort, including 45 bladder cancer patients and 87 healthy individuals [54]. UcfDNA concentrations were quantified using four different methods, and were found, by each of the methods, not to differ significantly between bladder cancer patients and healthy individuals. As a result, ucfDNA concentration was not considered a reliable diagnostic marker for bladder cancer. In another study performed by Chang et al., ucfDNA concentration was evaluated adjusting to urine creatinine in 46 bladder cancer patients and 98 controls [25]. The mean ucfDNA/UCr concentration in bladder cancer patients was significantly higher than that in controls, indicating that ucfDNA/UCr had the

Table 1. Summary of potential clinical applications of ucfDNA in cancer

Clinical application	Markers	Types of cancer	Detection methods	References
Detection/Diagnosis	UcfDNA concentration	Bladder cancer	GeneQuant Pro	Zancan et al. [54]
			Quant-iT DNA high-sensitivity assay kit	
			Real-time PCR	
			NanoDrop 1000	
	UcfDNA/UCr concentration and ucfDNA integrity	Bladder cancer	PicoGreen 400-bp real-time PCR	Chang et al. [25]
	UcfDNA quantification	Bladder cancer	Real-time PCR	Brisuda et al. [55]
	TopoIIA levels	NMIBC	Real-time PCR	Kim et al. [60]
	UcfDNA integrity	Bladder cancer	Real-time PCR using IQ SYBR Green	Casadio et al. [38]
	Six microsatellite markers on chromosomes 4, 9, and 17	Bladder cancer	PCR	Utting et al. [66]
	Twelve microsatellite markers on 6 chromosomes	Bladder cancer	PCR	Szarvas et al. [13]
	TSPAN13-to-S100A9 ratio	Prostate cancer	Real-time PCR	Yan et al. [68]
	UcfDNA integrity (c-Myc, BCAS1, and HER2)	Prostate cancer	Real-time PCR	Casadio et al. [39]
	UcfDNA integrity (c-MYC, HER2, and AR)	Prostate cancer	Real-time PCR	Salvi et al. [69]
	GSTP1 gene promoter hypermethylation	Prostate cancer	Methylation-specific PCR	Bryzgunova et al. [72]
	KRAS mutations	Advanced colorectal adenocarcinoma and advanced pancreatic cancer	PCR	Botezatu et al. [18]
	KRAS mutations	Colorectal cancer	Restriction-enriched PCR	Su et al. [17, 33, 35]
	mVIM	Colorectal cancer	Quantitative MethyLight PCR-based assay	Song et al. [73]
	TP53 mutation	Hepatocellular carcinoma	Locked nucleic acid clamp-mediated PCR assay	Lin et al. [74]
	HCC-associated HBV mutation	HBV-associated hepatocellular carcinoma	Real-time PCR	Lin et al. [75]
	HPV DNA	Cervical cancer	NGS	Guerrero-Preston et al. [80]
Surveillance of cancer progression	Somatic variants	Bladder cancer	ddPCR	Birkenkamp-Demtröder et al. [81]
	Somatic variants	UBC	OncoScan assay	Togneri et al. [82]
	FGFR3 and *PIK3CA* mutations	Bladder cancer	ddPCR	Christensen et al. [83]
	EGFR mutations	NSCLC	ddPCR	Li et al. [88]
	KRAS mutations	NSCLC	ddPCR	Wang et al. [85]
Monitoring treatment response	Copy number variations	Prostate cancer	Whole genome sequencing	Xia et al. [86]
	EGFR mutations	NSCLC	Short footprint mutation enrichment NGS	Reckamp et al. [40]
	EGFR mutations	NSCLC	ddPCR	Li et al. [84]
				Chen et al. [89]
				Husain et al. [96]
				Tchekmedyian et al. [91]
	EGFR mutations	Gastric cancer	ddPCR	Shi et al. [36]
	CAD-ALK gene rearrangement	Colorectal cancer	NGS	Siravegna et al. [92]
	BRAF V600E mutations	Colorectal neuroendocrine cancer	PCR	Klempner et al. [93]
	KRAS G12/G13 mutations	Advanced cancers	Mutation-enrichment NGS	Fujii et al. [41]
Prognosis	EBV DNA	Nasopharyngeal carcinoma	Real-time PCR	Chan et al. [94]
				Sengar et al. [95]
	EGFR mutations	NSCLC	ddPCR	Li et al. [84]
	KRAS mutations	NSCLC	ddPCR	Wang et al. [85]

Abbreviations: ucfDNA, urinary cell-free DNA; NMIBC, non-muscle-invasive bladder cancer; GSTP1, glutathione S-transferase P1 gene; mVIM, hypermethylated vimentin gene; NGS, next-generation sequencing; ddPCR, droplet digital PCR; UBC, urothelial bladder cancer; NSCLC, non-small cell lung cancer.

potential for diagnosing bladder cancer. Brisuda and co-workers proposed that the inconsistent results about the role of ucfDNA concentration in bladder cancer could be attributed to the use of various non-standardized methodologies [55]. They standardized the methodology and quantified the ucfDNA concentrations in 66 bladder cancer patients and 34 controls using the optimized method. According to the results shown in this study, ucfDNA levels had the ability to discriminate between the presence and absence of bladder cancer, exhibiting a potential diagnostic utility of ucfDNA concentrations in bladder cancer patients. Topoisomerase-II alpha (*TopoIIA*) is an isoform of DNA gyrase, and altered expression of *TopoIIA* exists in various cancers as well as normal tissues [56-58]. *TopoIIA* was reported to be associated with the progression and recurrence of primary non-muscle-invasive bladder cancer (NMIBC) [59]. The level of *TopoIIA* cell-free DNA in the urine of bladder cancer patients was also examined for potential diagnostic value [60]. The results showed that urinary *TopoIIA* cfDNA was significantly higher in bladder cancer patients compared to that in non-cancer patient controls and hematuria patients. In addition, urinary *TopoIIA* cfDNA level was also able to discriminate between muscle-invasive bladder cancer (MIBC) and NMIBC. This study provided the evidence for the potential diagnostic value of urinary *TopoIIA* cfDNA for bladder cancer [60].

It has been reported that DNA from normal cells is mainly through apoptosis, resulting in short and uniform fragments, while for tumor cells, released DNA fragments are relatively long as a result of necrosis [61, 62]. Therefore, the integrity of ucfDNA in urological cancers was widely studied. DNA integrity was also examined as a diagnostic marker of bladder cancer. Chang et al. discovered that ucfDNA concentration was more reliable and accurate for diagnosis of bladder cancer when detecting longer DNA fragments using the 400-bp real-time PCR-based detection method [25]. Another study drew a similar conclusion by verifying sequences longer than 250 bp (c-Myc, BCAS1, and HER2) in ucfDNA from 51 bladder cancer patients, 46 symptomatic patients, and 32 healthy controls [38]. UcfDNA integrity analysis showed a satisfactory sensitivity and specificity in early diagnosis of bladder cancer.

Microsatellite alterations are integral and valuable markers for human cancer detection [63-65]. Six microsatellite markers on chromosomes 4, 9, and 17 were analyzed in cfDNA in urine and blood from patients with conspicuous bladder lesions [66]. It turned out that 88% of the microsatellite changes could be detected in at least one of the body fluids, thus indicating the clinical utility of ucfDNA for diagnosis and screening of bladder cancer. Similar conclusion was also obtained in a study carried out by Szarvas and co-workers [13].

UcfDNA levels, integrity, and methylation status were also extensively investigated as potential diagnostic markers of prostate cancer.

Yan et al. explored the diagnostic value of ucfDNA levels in prostate cancers using a previously described two-gene expression ratio method [67]. TSPAN13 and S100A9 were selected as candidate genes and the TSPAN13-to-S100A9 ratio was evaluated in 95 urine specimens from prostate cancer patients and benign prostatic hyperplasia controls (BPH) [68]. The TSPAN13-to-S100A9 ratio in ucfDNA was significantly higher in prostate cancer than in BPH, demonstrating the excellent potential of ucfDNA as a diagnostic biomarker for prostate cancer patients.

UcfDNA integrity was evaluated for early diagnosis of prostate cancer. In 2013, Casadio et al. examined ucfDNA integrity in 29 prostate cancer patients and 25 healthy volunteers by quantifying sequences longer than 250 bp corresponding to 3 genes (c-Myc, BCAS1, and HER2) [39]. Showing a sensitivity of 0.79 and a specificity of 0.84, ucfDNA integrity was considered to be a promising biomarker for early diagnosis of prostate cancer. After the preliminary study mentioned above, researchers subsequently performed further study of ucfDNA integrity in prostate cancer in a larger cohort, and compared the diagnostic value of ucfDNA with the traditional biomarker prostate-specific antigen (PSA) [69]. Using the same methods, the researchers evaluated DNA fragments longer than 250 bp of 3 frequently amplified genes (c-MYC, HER2, and AR) in 67 prostate cancer patients and 64 patients with benign diseases of the urogenital tract. The results showed that the sensitivity and specificity of ucfDNA analysis were lower than that of PSA, indicating that ucfDNA integrity might not be a reliable bio-

marker for early diagnosis of prostate cancer. The contradictory results of the two studies may be explained as follows [69]. The study cohort recruited in the pilot study was much smaller than that of the confirmatory study. Besides, patients in the confirmatory study included some with inflammation, calculi and cysts, leading to the exfoliation of inflammatory cells into urine, increasing the amount of cfDNA released and thus leading to false positive results. Despite these contradictory results of ucfDNA integrity analysis, ucfDNA could still be a potential source of other biomarkers and enable the detection of gene alterations in prostate cancer.

Promoter hypermethylation of the glutathione S-transferase P1 gene (GSTP1) is the most frequent DNA aberration observed in prostate cancer [70, 71]. Methylation profile of *GSTP1* gene promoter in ucfDNA was evaluated in patients with prostate cancer, patients with benign prostatic hyperplasia and healthy controls [72]. The methylation status of *GSTP1* gene detected in urine was the same as that of the gene in the blood of the same patients. *GSTP1* gene methylation status in ucfDNA of prostate cancer patients was significantly different from that of BPH patients and healthy controls, indicating the potential diagnostic value of ucfDNA.

Non-urological cancer markers: As early as 2000, Botezatu et al. conducted a series of experiments, which demonstrated, for the first time, the existence of transrenal cell-free DNA in animal and human models [18]. In this study, *KRAS* mutations were detected in the urine of four out of five advanced colorectal adenocarcinoma patients and in five out of eight patients with advanced pancreatic cancer [18]. Since then, an increasing number of ucfDNA alterations in various non-urological cancers have been discovered and evaluated as potential biomarkers for cancer diagnosis.

Since the detection of *KRAS* mutations in ucfDNA in colorectal cancer (CRC) [18], further studies were carried out to evaluate the potential clinical utility of *KRAS* mutations in the urine of CRC patients. A study carried out by Su et al. showed that mutated *KRAS* sequences could be identified in the urine derived from *KRAS* proto-oncogene mutation-positive CRC patients, and that mutated *KRAS* sequences were far more abundant in low molecular weight (150 to 250 bp) than in high molecular weight (greater than 1 kb) fragments. Remarkably, *KRAS* mutations were analyzed in paired urine and tissue samples from 20 patients with either CRC or adenomatous polyps, and a 83% concurrence was observed in urine and tissue samples from the same individuals [17]. Subsequently, Su and co-workers conducted two more studies to detect *KRAS* mutations in ucfDNA in the urine of CRC patients. They further examined *KRAS* mutations in urine, plasma and serum of CRC or adenomatous polyps patients. The results showed that the incidence of *KRAS* mutations in ucfDNA was significantly higher than that in serum or plasma. As a result, urine seemed to be a better source for detecting *KRAS* mutations in CRC, compared to serum or plasma, indicating the potential clinical practicality of ucfDNA for the detection and diagnosis of CRC or adenomatous polyps [33, 35]. In addition to *KRAS* mutations, epigenetic DNA markers could also be detected in the urine of CRC patients. In a study conducted by Song et al., hypermethylated vimentin gene (*mVIM*) was evaluated in the urine from 20 CRC patients and 20 control subjects with no known neoplasia. *mVIM* was detected in 75% CRC patients, demonstrating significant association with CRC. In contrast, only 10% of the controls contained *mVIM* in their urine samples. Moreover, *mVIM* was mostly detected in low-molecular-weight urine DNA, confirming its origin from circulation and existence in urine in the form of cell-free DNA. Therefore, it seemed that *mVIM* detection in ucfDNA held promise for CRC screening and diagnosis [73].

In hepatocellular carcinoma (HCC), *TP53* mutation and HCC-associated HBV mutation were detected in ucfDNA, indicating its potential as a diagnostic biomarker for HCC screening. Lin and co-workers investigated an HCC-associated mutation, *TP53* 249T hotspot mutation, in the urine of patients with HCC. It was the first report of detection of *TP53* 249T mutation in the urine of HCC patients. *TP53* mutations were successfully detected in 9 out of 17 samples, indicating urine as a potential non-invasive sample source for HCC screening [74]. In a small pilot study on HBV-associated HCCs, HBV 1762T/1764A double mutation was detected in both circulation and urine. The results of this study showed potential clinical utility of urine for non-invasive HCC screening, although further studies with larger sample size were essential for validating the potential [75].

Molecular detection of human papillomavirus (HPV) DNA has been of considerable interest in risk assessment and screening of cervical cancer [76, 77]. Urine-based HPV DNA testing has been investigated as a non-invasive approach complementary to cytology test [78]. Discovery of cell-free HPV DNA in urine could be traced back to 1999, though sensitivity was relatively low [79]. Recently, new technology such as NGS was applied to cell-free HPV DNA detection in urine, indicating that urine could be a promising material for personalized cervical cancer screening and diagnosis [80].

Surveillance of cancer progression

Cancer surveillance is important for early diagnosis of disease progression and metastasis, as well as for optimizing treatment. Various studies have explored the potential utility of ucfDNA for surveillance of cancer progression by monitoring genomic variants in urine.

A retrospective pilot study was conducted in 12 patients with recurrent or progressive/metastatic NMIBC during a 20-year follow-up from 1994 to 2015 [81]. Somatic variants in cfDNA from urine, blood and tumor were detected using NGS and ddPCR assays. Higher levels of neoplastic ucfDNA were discovered before cancer progression, in patients with progressive disease than those with recurrent disease, and tumor cfDNA could be detected in urine and blood in both early and advanced stages of bladder cancer. Therefore, disease progression in bladder cancer can be monitored by detecting genomic variants in ucfDNA. In urothelial bladder cancers (UBCs), genomic profiles were also performed in ucfDNA [82]. The data demonstrated that high sensitivity was achieved for genomic aberrations detection using ucfDNA and ucfDNA was highly representative in detecting recurrent genomic aberrations. The results were suggestive of the potential utility of ucfDNA in monitoring UBC progression. UcfDNA analysis for *FGFR3* or *PIK3CA* mutations using ddPCR assays also demonstrated its utility in the disease surveillance for bladder cancer [83]. UcfDNA at different time points from NMIBC patient cohort were analyzed for *FGFR3* or *PIK3CA* mutations and showed that higher levels of *FGFR3* or *PIK3CA* mutations in ucfDNA were associated with the progression of NMIBC.

Similar utility of ucfDNA had also been demonstrated in non-small cell lung cancer (NSCLC). A total of 160 NSCLC patients at various stages of disease participated and samples were collected prospectively at 2-month intervals. A baseline sample was taken before treatment commencement. Transrenal DNA was compared with plasma DNA to ascertain the sensitivity. DdPCR was used to profile the urine and blood samples for key *EGFR* mutations. Serial monitoring of NSCLC patients with different disease stages showed stable molecular signatures and correlated to different treatments [84].

Another study also focused on ucfDNA alterations in NSCLC patients. DdPCR was used to detect mutant DNA in 200 NSCLC patients. Transrenal DNA was successfully detected in all the patients (100%). Overall concordance rate for mutant *KRAS* DNA within urine specimens and primary tissue biopsies was 95%. More importantly, longitudinal monitoring of urine specimens showed an increase in the quantity of transrenal DNA, which was highly associated with disease progression and outcome. The study indicated that urinary specimens that can be extracted non-invasively present new opportunities to track patients with *KRAS* mutation, who were undergoing therapy [85].

Monitoring therapeutic response

Targeted therapy for cancer is now common in the context of precision medicine. Monitoring response and resistance to targeted therapy could be indispensable during the course of treatment of cancer patients. Since tumor biopsy is constrained by disadvantages such as tumor heterogeneity and invasive repeated samplings, non-invasive specimens are gaining in importance. UcfDNA had been detected in diversified cohorts receiving certain antitumor therapies, and the dynamic tracking of genetic abnormalities in ucfDNA was therefore evaluated for monitoring the efficacy of the therapeutic process.

Whole genome sequencing was used for the first time in 2016 to evaluate copy number variations (CNV) in ucfDNA from advanced prostate cancer patients [86]. In this study, a series of tumor-associated CNVs were detected in ucfDNA before and after androgen deprivation therapy in hormone sensitive prostate cancer (HSPC) and docetaxel chemotherapy in castrate resistant prostate cancer (CRPC).

Significant CNVs in 34 genomic loci were discovered during the course of therapy. In addition, a urine genomic abnormality (UGA) score algorithm was established to evaluate the ten most significant segments with CNVs. The UGA scores could reflect cancer progression status and overall survival during therapy, indicating that ucfDNA has potential clinical utility in monitoring and predicting treatment response in advanced prostate cancer.

A study carried out by Reckamp and co-workers evaluated the *EGFR* mutations in matched urine and plasma samples from *EGFR* mutant-positive advanced NSCLC patients under rociletinib treatment using a short footprint mutation enrichment NGS assay. The results showed that the sensitivity of *EGFR* mutation detection in urine was 72% (34 of 47 specimens) for T790M, 75% (12 of 16) for L858R and 67% (28 of 42) for exon 19 deletions, using tissue samples as a reference. Furthermore, when the specimens met the recommended volume requirement, the sensitivity increased to 93% (13 of 14 specimens) for T790M, 80% (4 of 5) for L858R, and 83% (10 of 12) for exon 19 deletions, which were comparable to plasma findings. Twelve additional T790M-positive cases with undetectable T790M mutations in tissue sample analysis were identified by testing both urine and plasma. UcfDNA in NSCLC exhibited high sensitivity and complementarity in *EGFR* mutation detection, and showed great potential in the diagnosis and monitoring of treatment response of NSCLC [40]. Similar results have also been obtained in other studies [87, 88]. *EGFR* mutation status was also identified using ddPCR in ucfDNA from patients in different stages of NSCLC. It turned out that ucfDNA showed good concordance with results derived from plasma DNA in both early- and late-stage NSCLC patients, highlighting the potential clinical utility of ucfDNA in continual monitoring of NSCLC [84]. Chen et al. performed similar analysis of ucfDNA at different time points in NSCLC patients, who received EGFR tyrosine kinase inhibitor (TKI) treatment. The results showed that ucfDNA mutation status correlated closely with treatment efficacy, indicating the potential utility of urine for monitoring EGFR TKI treatment [89]. In another study carried out by Husain and co-workers, they evaluated dynamic changes in *EGFR* activating (exon 19del and L858R) and resistance (T790M) mutation levels in advanced NSCLC patients receiving osimertinib, and found that eight out of nine evaluable NSCLC patients had detectable T790M-mutant DNA fragments in pre-treatment baseline samples. In addition, daily monitoring of *EGFR* mutations in urine of NSCLC patients showed that surveillance of ucfDNA may enable early assessment of patient response and proof-of-concept studies for drug development [90]. Tchekmedyian et al. also performed ultrasensitive detection and longitudinal monitoring of *EGFR* mutations using non-invasive urinary circulating tumor DNA (ctDNA) liquid biopsies in five patients with NSCLC treated with EGFR TKIs. The results verified the diagnostic potential of urinary ctDNA as a non-invasive molecular diagnostic tool to assess tumor burden and response to therapy [91].

In addition to the *EGFR* mutations detected in the urine of lung cancer patients, *EGFR* mutations in ucfDNA were also studied in gastric cancer [36]. Urine *EGFR* mutation status was examined in 120 gastric cancer patients with *EGFR* mutations and 100 healthy controls. During the course of EGFR TKI treatment of gastric cancer, *EGFR* mutations were monitored serially for 12 months. The concordance rate of EGFR mutation status between ucfDNA and primary tissue samples was 92% at baseline and 99% at different time points in gastric cancer patients. The results suggested that ucfDNA may serve as a reliable marker for treatment monitoring in primary gastric cancer [36].

In a study performed by Siravegna et al., researchers detected a *CAD-ALK* gene rearrangement in a metastatic colorectal cancer patient undergoing ALK inhibitor treatment. *CAD-ALK* gene rearrangement in urine was tracked during the course of therapy and found to be concordant with cancer progression, which was also confirmed by radiological test. Thus, urine was believed to have the potential of monitoring tumor progression in a non-invasive way during target therapy and could be further used to monitor minimal residual disease in patients carrying gene fusions [92].

Oncogenic *BRAF* V600E substitutions are observed primarily in melanoma, colon cancer, and non-small cell lung cancer, and have been identified in multiple tumor types [93].

Klempner et al. reported for the first time about the recurrent *BRAF* V600E mutations in advanced high-grade colorectal neuroendocrine tumors and determined the *BRAF* alteration frequency to be 9% in 108 cases. Among these *BRAF* alterations, 80% were *BRAF* V600E. Dramatic response to BRAF-MEK combination occurred in two cases of metastatic high-grade rectal neuroendocrine carcinoma refractory to standard therapy. Following initiation of therapy, there was a rapid decrease in urinary *BRAF* V600E ctDNA, along with serum chromogranin A levels that paralleled clinical resolution of symptoms and preceded radiologic response. The study indicated that urinary *BRAF* V600E circulating tumor DNA had the promising ability to monitor paralleled disease response of colorectal neuroendocrine cancers [93].

Fujii et al. developed a quantitative, mutation-enrichment NGS test for detecting *KRAS* G12/G13 mutations in ucfDNA and the results were compared with those from the clinical testing of archived tumor tissue and plasma cfDNA samples from patients with advanced cancer. In 71 patients, the concordance between ucfDNA and tumor DNA was 73% (sensitivity, 63%; specificity, 96%) for all patients and 89% (sensitivity, 80%; specificity, 100%) for patients with urine samples of 90 to 110 mL. Patients had significantly fewer *KRAS* G12/G13 copies in ucfDNA during systemic therapy than at baseline or during disease progression. Compared with no changes or increases in ucfDNA *KRAS* G12/G13 copies during therapy, decreases in these measures were associated with longer median time to treatment failure [41].

Biomarkers for cancer prognosis

Cancer prognosis, including metastasis and relapse, is of vital importance to cancer patients. Non-invasive methods to determine cancer prognosis are therefore urgently required.

Circulating Epstein-Barr virus (EBV) DNA could be excreted transrenally into urine in nasopharyngeal carcinoma (NPC) patients, and was quantitatively detected in the urine of 42 out of 74 NPC patients using a 59-bp real-time PCR assay. Remarkably, the concentration of EBV DNA in plasma from patients with detectable EBV DNA in urine was also significantly higher, showing a positive correlation between plasma and urine. Therefore, urine EBV DNA analysis may be used as a non-invasive test for NPC monitoring and prognosis [94]. After the discovery of EBV DNA in urine, further study was carried out by Sengar et al. to explore the clinical utility of urine EBV DNA in NPC. It was found that EBV DNA in urine of NPC patients had high diagnostic sensitivity and correlated well with plasma EBV DNA before and after therapy, as well as predicting therapy response and survival of NPC patients. Although studies on larger cohorts are needed, urine EBV DNA is believed to be a promising prognostic biomarker in NPC [95].

In a study involving 160 patients at various stages of NSCLC, researchers detected key *EGFR* mutations in both urine and blood samples. Survival analysis showed good prognostic utility in late-stage patients with high ucfDNA variations and in patients that acquired T790M mutation [84]. In addition to *EGFR* mutations, *KRAS* mutations were also evaluated as a potential prognostic marker in NSCLC patients. Serial samplings were performed during different treatment cycles to gauge the predictive value. Patients with positive results at baseline had lower median overall survival than those with wildtype. Longitudinal monitoring of urine specimens showed an increase in the quantity of ucfDNA, which was closely associated with disease outcome [85].

Conclusions

Although the number of studies concerning the clinical applications of ucfDNA in cancer are still limited and mostly carried out on relatively small patient cohorts, the promising future of the potential clinical value of ucfDNA is worth expecting. As an ultra-noninvasive tool for liquid biopsy, ucfDNA has unique advantages on molecular profiling of tumor, and is believed to have a complementary and synergistic effect on serum and plasma in diagnosis, progression surveillance, treatment monitoring and prognosis for both urological and non-urological cancers. In future, we believed that the advancement of molecular assays (such as NGS and ddPCR) and the development of larger validation and prospective studies could offer a deeper insight into the clinical applications of ucfDNA in cancer.

Disclosure of conflict of interest

None.

Abbreviations

cfDNA, cell-free DNA; ucfDNA, urinary cell-free DNA; ddPCR, droplet digital PCR; NGS, next-generation sequencing; TopoIIA, topoisomerase-II alpha; NMIBC, non-muscle-invasive bladder cancer; MIBC, muscle-invasive bladder cancer; BPH, benign prostatic hyperplasia; PSA, prostate-specific antigen; GSTP1, glutathione S-transferase P1 gene; CRC, colorectal cancer; mVIM, hypermethylated vimentin gene; HCC, hepatocellular carcinoma; HPV, human papillomavirus; UBC, urothelial bladder cancer; NSCLC, non-small cell lung cancer; CNV, copy number variation; HSPC, hormone sensitive prostate cancer; CRPC, castrate resistant prostate cancer; UGA, urine genomic abnormality; TKI, tyrosine kinase inhibitor; ctDNA, circulating tumor DNA; EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma.

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