Liquid biopsy in prostate cancer: current status and future challenges of clinical application

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ABSTRACT

Purpose: Liquid biopsy refers to the detection and analysis of the components from biological fluids non-invasively, including circulating tumor cells, nucleic acids, and extracellular vesicles (EVs). It is necessary to review the clinical value of liquid biopsy assays in PC and explore its potential application.

Materials and methods: We systematically reviewed of PubMed was performed to identify relevant literature on potential clinical applications of circulating tumor cells, circulating nucleic acids, and EVs in prostate cancer (PC).

Results: Liquid biopsy has emerged as a powerful tool to elucidate dynamic genomic, transcriptomic, and epigenomic tumor profiling in real-time. Here, the potential clinical applications of liquid biopsy include early detection, prognosis of survival, assessment of treatment response, and mechanisms of drug resistance in PC.

Conclusions: Liquid biopsy provides great value in diagnosis, prognosis, and treatment response in PC. Characterization of liquid biopsy components provides benefits both to unravel underlying resistance mechanisms and to exploit novel clinically actionable targets in PC. In addition, we suggest that analysis of multiparametric liquid biopsies should be analyzed comprehensively, assisting in monitoring tumor characteristics in real-time, guiding therapeutic selection, and early therapeutic switching during disease progression.

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Introduction

Prostate cancer (PC) is one of the four leading causes of cancer-related deaths in men worldwide [1]. PC is a heterogeneous disease with various clinical outcomes; most PCs are characterized by an indolent course [2]. However, traditional risk stratification based on prostate specific antigen (PSA) levels and prostate biopsy results is often inaccurate, which leads to overtreatment of patients with indolent PC and results in substantial economic burden to the health care system [3]. Dynamic assessment of disease status requires continuous sampling; however, tissue-based molecular characterization provides an incomplete snapshot profile at one-time point [4,5]. Furthermore, prostate biopsies may lead to the release of circulating tumor cells (CTCs) into the circulation, potentially resulting in tumor dissemination [6]. Therefore, a non-invasive approach is urgently needed for the comprehensive evaluation of the biology of primary and metastatic tumors.

Liquid biopsies have emerged as a powerful strategy for cancer research. This technology complements the traditional tissue biopsies in various types of cancer [7]. The components of liquid biopsies include CTCs, circulating tumor DNA (ctDNA), circulating tumor RNA (ctRNA), and extracellular vesicles (EVs), which incorporate abundant genomic, transcriptomic, and epigenomic information of tumors (Figure 1). Liquid biopsies provide a tool for the comprehensive assessment of tumor characteristics in real-time, and offer insights into early diagnosis, prognosis of overall survival (OS), treatment efficacy, and resistance mechanisms [8]. This review summarizes major advances in clinical application of liquid biopsy (Table 1) and discusses the necessity of combined analyses of multiple liquid biopsies in PC.
CTCs in PC

**CTC identification and enrichment**

CTCs, first reported in the blood of a man with metastatic cancer in 1869 [9], are shed from primary and metastatic tumors into the peripheral blood and lymph system [10]. Compared to other liquid biopsy components, CTCs are intact cells that offer comprehensive information. There are two major types of CTC enrichment: epithelial cell adhesion molecule (EpCAM)-dependent and EpCAM-independent assays [11]. The CellSearch system, the only FDA-approved platform to count CTCs using whole blood, identifies CTCs that are EpCAM-positive and CD45 negative nucleated cells [10]. However, the EpCAM-based platform misses a subgroup of CTCs undergoing epithelial-mesenchymal transition (EMT) [10]. It is crucial to capture these CTCs because they are more aggressive and promote the invasion and metastasis of PC [12].

Due to the limitations of the EpCAM-dependent assays, many EpCAM-independent assays have been developed, such as the AdnaTest platform and the Epic Science platform. These two assays and the CellSearch system remain the most used technologies in CTC-related clinical trials. The AdnaTest performs immunomagnetic-based enrichment of CTCs and detects PC-related mRNA of selected cells by reverse transcription-polymerase chain reaction (RT-PCR). This technology is widely used for the analysis of the expression status of the androgen receptor (AR) splice variant (AR-V) mRNA of CTCs [13]. Epic Science, an imaging-based system independent of EpCAM, allows downstream genetic and protein analyses; after label-based enrichment, CTCs are identified as 4',6-diamidino-2-phenylindole+, CD45+, and cytokeratin+ (CK+) cells [14]. In addition to label-based separation, label-free technologies based on particular physical traits of CTCs have been developed, such as microfluidic technologies and size-based methods with high throughput [15,16].

**Clinical applications of CTCs in PC**

**Early diagnosis of PC**

CTC-based early diagnosis of non-metastatic and metastatic PC relies on sufficient sensitivity and high
| Study (if it is a clinical trial, the trial number is below) | Subjects | Sample type | Technique | Analysis | Roles | Treatment type | Main results |
|---|---|---|---|---|---|---|---|
| Nimgaonkar et al. | 41 PC patients and 20 healthy controls | Blood | CMxTM CTC platform | CTC number | Early detection | N/A | All of 25 patients with Stages I–III prostate cancer and 20 healthy controls were accurately identified based on CTC counting. |
| Pang et al. | 84 high-risk men within PSA gray zone | Blood | CMxTM CTC platform | CTC number | Early detection | N/A | CMxTM CTC assay had 80.0% sensitivity and 93.8% specificity in PC diagnosis. |
| Ried et al. | 277 PC patients and 265 asymptomatic men with risk factors | Blood | ISET-Rarecells | CTC number | Early detection | N/A | CTCs were present in all 277 PC patients and in 132 (50% of 265) asymptomatic men. |
| De Bono JS et al. | 276 mCRPC patients | Blood | CellSearch | CTC number | Prognosis | N/A | Patients with ≥ five CTCs/7.5 ml had shorter OS than patients with < five CTCs/7.5 ml (median OS, 11.5 versus 21.7 months; Cox HR, 3.3; p<.0001) |
| Goldkorn et al., NCT00134056 | 264 mCRPC patients | Blood | CellSearch | CTC number | Prognosis | ARSIs | Median OS was 26 months for patients with <5 CTCs/7.5 ml; 13 months for patients with ≥5 CTCs/7.5 ml at baseline (HR, 2.74 [adjusting for covariates]). Rising CTC count during therapy was associated with shorter OS (HR, 2.55) |
| Kozminsky et al. | 41 mCRPC patients | Blood | GO Chip | Gene expression signature of CTCs and CTC clusters | Prognosis | Taxane or ARSIs | The five-gene predictive score obtained by significantly changed RNA expression related to CTC differentiation was associated with PSA progression, radio clinical progression and OS. |
| Antonarakis et al. | 62 mCRPC patients | Blood | AdnaTest platform | AR-V7 mRNA of CTCs | Treatment response | ARSIs | Patients with AR-V7 positive CTCs had significantly shorter PFS and OS, and no PSA response (PSA decline more than 50% from baseline) were observed, compared with patients without AR-V7 positive CTCs. |
| Antonarakis et al. | 37 mCRPC patients | Blood | AdnaTest platform | AR-V7 mRNA of CTCs | Treatment response | Taxane | AR-V7-positive patients treated with taxanes had higher PSA response rates (41% versus 0%; p<.001), longer PFS (HR, 0.21 [95% CI, 0.07–0.59]) for PFS, and OS (HR, 1.6; 95% CI, 0.6–4.4) when treated with cabazitaxel. |
| Onstenk et al. | 29 mCRPC patients | Blood | CellSearch system | AR-V7 mRNA of CTCs | Treatment response | Cabazitaxel | AR-V7 in CTCs was not associated with PFS (HR: 0.895% CI, 0.4–1.9) or OS (HR: 1.6; 95% CI, 0.6–4.4) when treated with cabazitaxel. |
| Lu et al. | 32 mCRPC patients | Blood | Epic Sciences | Nuclear-localized AR-V7 protein of CTCs | Treatment response | ARSIs | Six subjects with nuclear-agnostic AR-V7 CTCs had PSA response after 12 weeks of ARS inhibitors. None subjects with nuclear-specific AR-V7 CTCs PSA response. |
| Scher et al. | 161 mCRPC patients | Blood | Epic Sciences | Nuclear-localized AR-V7 protein of CTCs | Treatment response | Taxane or ARSIs | Patients who were AR-V7 positive had longer OS with taxanes relative to ARS inhibitors (HR, 0.24; 95% CI, 0.10–0.57; p=.035), while patients who were AR-V7 negative did not (HR, 0.92; 95% CI, 0.44–1.95) |

(continued)
| Study (If it is a clinical trial, the trial number is below) | Subjects | Sample type | Technique | Analysis | Roles | Treatment type | Main results |
|----------------------------------------------------------|----------|-------------|-----------|----------|-------|----------------|--------------|
| Scher et al. 142 mCRPC patients Blood The Epic Sciences Nuclear-localized AR-V7 protein of CTCs Treatment response Taxane or ARSIs | Among 70 high-risk patients, patients who were AR-V7 positive treated with ARSIs had a superior OS outcome in comparison to those who were treated with taxanes (median OS, 14.3 versus 7.3 months; HR, 0.62; 95% CI, 0.28–1.39; p=.25). Patients who were AR-V7 negative treated with ARS inhibitors had a superior OS outcome in comparison to those who were treated with taxanes (median OS, 19.8 versus 12.8 months; HR, 1.67; 95% CI, 1.00–2.81; p=.05) |
| Armstrong et al. NCT02269982 118 mCRPC patients Blood AdnaTest platform or the Epic Sciences Nuclear-localized AR-V7 protein of CTCs Treatment response Taxane or ARSIs | Patients who were AR-V7 positive using AdnaTest or the Epic Sciences independently correlated with worse OS (HR, 4.2 [95% CI, 2.1–8.5] and 3.5 [95% CI, 1.6–8.1], respectively) and shorter PFS (HR, 1.9 [95% CI, 1.1–3.3]; p=.032) and 2.4 [95% CI, 1.1–5.1; p=.020], respectively |
| Taplin et al. 38 mCRPC patients Blood AdnaTest platform AR-V7 mRNA of CTCs Treatment response Galaterone or enzalutamide | Patients with AR-V7+ CTCs were randomized to either galaterone (n = 19) or enzalutamide (n = 19). PSA50 values of patients receiving galetorene were lower than those of patients receiving enzalutamide, 2/16 (13%), and 8/19 (42%), respectively |
| Wise et al. 54 mCRPC patients Blood The Epic Sciences GR expression of CTCs Treatment response ARSIs | When treated with ARSIs, patients who were GR positive had a significantly worse OS than those who were GR negative (11.4 months versus not reached, p<.01) |
| Wark et al 20 patients with locally high-risk PC Blood ScreenCell 3 D quantitative telomere profiling of CTCs Treatment response ARSIs and radiation therapy | Patients could be stratified into three subgroups with distinct response to the therapy. These subgroups were consistent with baseline and subsequent changes of CTCs 3D telomere profiles |
| Ye et al. 108 mCRPC patients Blood CanPatrol Epithelial-to-mesenchymal transition (EMT) of CTCs Treatment response ARSIs | For 58 patients with mesenchymal CTCs, median PFS were significantly shorter than patients without mesenchymal CTCs (10.5 months versus 18.0 months, p=.003) |
| McKernan et al. 1022 PC patients Urine ExoDx Prostate (IntelliScore) (EPI) assay Three gene expression of exosomes Early detection N/A | For patients with a PSA level of 2.0–10.0 ng/ml, the urinary assay plus SOC improve the distinguishing power between PC patients with a grade group ≥ 2 and those with grade group of 1 and BPH (AUC = 0.70), helping to avoid 26% unnecessary tissue biopsies |
| Del Re et al. 36 mCRPC patients Plasma Droplet digital PCR AR-V7 mRNA of exosomes Treatment response ARSIs | Patients with plasma-derived exosomal AR-V7 mRNA expression had significantly decreased PFS and OS (PFS 20 months versus 3 months; p<.001; OS 8 months versus not reached; p=.001) |
| De Laere et al. 168 mCRPC patients Blood | ARSIs | (continued) |
The detection rates of the CTC capture system; owing to different enrichment methods, existing CTC capture platforms have various CTC detection rates [17]. EpCAM-dependent technologies are not suitable for early detection of patients with localized PC due to the low detection rate, in some cases as low as 37% [17]. The combination of multiple methods may improve the cumulative detection rate; for example, the combination of two different EpCAM-dependent CTC assays improves the cumulative detection rate to 68.7%, whereas the CTC detection rates for each of them alone are less than 60% in non-metastatic high-risk patients with PC [17].

Recent data suggest that some EpCAM-independent CTC enrichment platforms are capable of identifying patients with PC at early stages. A recent study by De Laere et al. [18] shows that the CMx™ CTC platform, which uses affinity-based microfluidics that captures CTCs expressing CK18 and prostate membrane antigen, is capable of identifying patients with PC at early stages [19]. All patients with PC and healthy controls were accurately identified using this platform [19]. Moreover, the CMx™ CTC assay increases both sensitivity (80.0%) and specificity (93.8%) when identifying patients with PC and avoiding unnecessary tissue biopsies in clinical practice [19].

Advancements in CTC enrichment technologies have increased CTC detection sensitivity, highlighting the great value of CTCs in cancer diagnosis. In addition, some CTC assays enable the identification of patients early-stage PC due to its low sensitivity. The applicability of CTC detection in the early stages of PC remains limited owing to the asymptomatic high-risk populations, contributing to the increasing sensitivity of CTCs for cancer screening [21]. However, in some specific groups, CTCs may serve as a prognostic biomarker. For example, in patients with TP53 wild-type mCRPC, the combination of CTC and PSA predicts clinical benefit [19].

Table 1. Continued.

| Study (If it is a clinical trial, the trial number is below) | Subjects | Sample type | Technique | Analysis | Roles | Treatment type | Main results |
|------------------------------------------------------------|----------|-------------|-----------|----------|-------|----------------|-------------|
| De Laere et al. [18]                                        | mCRPC patients with TP53 wild-type | Blood     | CellSearch; targeted RNA-seq of CTCs, low-pass whole-genome and targeted sequencing of plasma ctDNA | Genomic alterations of cfDNA and CTCs | Treatment response | ARSIs | AR aberrations had independent prognostic value when 3 or more AR perturbations were detected (HR, 2.97, p = 0.02) |

PC: prostate cancer; CTC: circulating tumor cell; cfDNA: cell-free DNA; cfRNA: cell-free RNA; PSA: prostate-specific antigen; AR: androgen receptor; ARSIs: androgen receptor signaling inhibitors; OS: overall survival; PFS: progression-free survival; HR: hazard ratio; AUC: area under curve; SOC: standard of care; N/A: not applicable.
significant PC (AUC = 0.896) [22]. Additionally, patients with localized PC with high CTC counts and copy number variations (CNVs) have a shorter biochemical recurrence time [23]. Even high CTC counts may contribute to the identification of high-risk patients with PC with occult metastases [24]. In metastatic PC, CTC enumeration by CellSearch has been validated as a prognostic biomarker [25,26]. In the IMMC8 study with 231 patients with metastatic castration-resistant PC (mCRPC), subjects with more than five CTCs in 7.5 ml blood have unfavorable outcomes, whereas those with less than four CTCs in 7.5 ml blood have favorable outcomes [26]. Moreover, re-analyses of related clinical trials reveal the added value of CTC counts to standard prognostic parameters, enhancing the accuracy of OS prediction for advanced PC [27].

**Treatment efficacy monitoring of PC**

Monitoring treatment efficacy remains problematic in clinical practice; currently, serum PSA and imaging are the primary strategies used to monitor the treatment efficacy of patients with PC [28]. However, the evaluation of early bone metastasis using imaging methods remains challenging, and PSA levels may be affected by AR signaling inhibitors (ARSIs) [29,30].

Dynamic changes in CTC counts are a promising surrogate indicator of treatment efficacy and early progression [28]. In a pooled analysis integrating 6081 patients with mCRPC from five randomized clinical trials, eight different defined endpoints based on CTC enumeration and PSA level were compared to explore the association with OS. The eight end points considered were CTC0 (CTCs were absent after 12 weeks), CTC conversion (detectable CTCs were more than five at baseline and dropped below the cutoff value after 12 weeks); CTC count and PSA levels decreased by 30%, 50%, and 70% from baseline to week 13, respectively. The CTC0 and CTC conversion had the highest discrimination power for OS. Thus, dynamic changes in CTC counts at multiple time points are significant for assessing therapeutic efficacy [28].

**Resistance mechanisms identified in CTCs**

Tumor development is an evolutionary process involving constant mutations and selection [31]. In this process, different clone populations coexist, which may act competitively or synergistically [31]. Under the pressure of treatment selection, drug-resistant subclones may dominate [31]. The genomic and phenotypic changes in tumor cells may be observed by characterizing CTCs [5]. CTCs are potentially useful to help unravel pre-existing tumor heterogeneity and monitor dynamic clonal selection in real-time, facilitating the discontinuation of ineffective therapy and early therapeutic switching ahead of clinical progression [30].

**AR-dependent characterization of CTCs.** In general, resistance profiling in PC is divided into AR-dependent and AR-independent types. Integrative genomic analysis shows that the AR axis is the most frequently altered pathway in PC [5]. AR is activated and translates to the nucleus after binding to androgens, and then acts as a transcription factor to activate or suppress gene expression to maintain tumor growth and progression in PC [32]. The underlying AR-dependent resistant mechanisms identified include increased synthesis of androgen, AR amplification, AR mutations, and AR-Vs [33]. Tumor-derived CTCs may be utilized to mirror these variations, with the ability to predict drug resistance to ARSIs in patients with mCRPC [34].

Currently, an increasing number of studies have focused on AR-Vs in PC; AR-Vs are alternatively spliced isoforms of AR mRNA, leading to truncated AR proteins [35]. These abnormal proteins lack the ligand-binding domain for AR-targeted drugs to bind, circumventing these inhibitory drugs, thus keeping the signaling pathway constitutively activated independent of androgen [36,37]. Numerous AR-Vs have been characterized, including AR-V1, AR-V2, AR-V3, AR-V4, AR-V5, AR-V7, AR-V9, and AR-V12 [38]. AR-V7 is more prevalent than point mutations and CNVs in PC [39]. Other AR-Vs are frequently co-expressed with AR-V7 [40]. Therefore, AR-V7 is a promising predictive biomarker for drug resistance and treatment selection [41]. AR-V7 is associated with resistance to abiraterone and enzalutamide [42], but is unrelated to taxane-based therapy [35,43,44]. Patients with AR-V7+ CRPC treated with taxanes have longer OS, whereas clinical outcomes in patients with CRPC without AR-V7 are independent of the therapy type [35,45]. Notably, the nuclear localization of the AR-V7 protein is required for treatment selection in patients with mCRPC [43]. The treatment response to ARSIs was compared between patients with mCRPC expressing only nuclear-specific AR-V7 CTCs and patients with nuclear-agnostic (nuclear and cytoplasmic localization) AR-V7 CTCs. PSA response to ARSIs is observed in the latter, whereas there is no PSA response in patients with CTCs exclusively expressing nuclear-specific AR-V7 [43]. AR-Vs are expected to be used as novel therapeutic targets for PC. Galeterone inhibits the AR signaling pathway through a variety of mechanisms, including inhibition of CYP17, competitive inhibition of AR, and...
degradation of full-length AR and AR-V7 proteins [46]. In this multicenter phase 3 clinical trial, 953 patients with mCRPC were recruited to screen for AR-V7 status using the AdnaTest for CTC mRNA. A total of 34% of patients were CTC-positive and the prevalence of AR-V7+ was 8%. Patients with AR-V7+ CTCs were randomized to either galeterone (n = 19) or enzalutamide (n = 19) [47]. However, during treatment, PSA50 values of patients receiving galeterone were lower than those of patients receiving enzalutamide, 2/16 (13%), and 8/19 (42%), respectively. These results indicate that enzalutamide is effective in some mCRPC patients with AR-V7+ CTCs, and galeterone is a weak signal antagonist of the AR signaling pathway [47]. However, mCRPC patients with AR-V7+ CTCs had more metastases and a higher malignancy. It is hoped that AR-Vs will present an effective therapeutic target.

Glucocorticoid receptor (GR) signaling is an alternative pathway that activates similar AR target genes, thus contributing to ARSI resistance [48]. It has been shown that 75.7% of patients with mCRPC have upregulated GR expression in CTCs, suggesting that dysregulated GR expression is prevalent [49]. GR expression levels also undergo dynamic changes, which are reduced in primary tumors, but are increased in metastatic tumors [48]. Significantly upregulated GR expression in CTCs in metastatic tumors is linked to shorter progression-free survival and poorer outcomes following ARSI therapy [49]. Therefore, the evaluation of GR expression levels in CTCs may assist in the prediction of ARSI efficacy and treatment selection [49].

**AR-independent characterization of CTCs.** Lineage plasticity is considered a crucial resistance mechanism that allows tumors to evade detection or treatment. The dynamics of phenotypes and differentiation status of tumor cells may be mirrored by CTCs, which are potentially able to predict resistance and monitor disease progression during therapy [50].

The EMT is a typical type of lineage plasticity in PC, leading to epithelial-origin tumor cells displaying mesenchymal markers, which confers invasiveness [12,51]. Studies on human cell lines and animal models have shown that androgen deprivation induces the EMT, resulting in stem cell-like characteristics of tumor cells, which are associated with resistance to ARSIs. Mesenchymal CTCs are CTCs that completely lose epithelial markers and are more aggressive. Approximately 50% of patients with mCRPC are positive for mesenchymal CTCs, coinciding with worse clinical outcomes compared to those without mesenchymal CTCs [12,51]. In addition, undifferentiated CTCs with low expression levels of epithelial and mesenchymal genes, as well as high expression levels of stemness genes, are linked to rapid progression and poor OS in patients with mCRPC [52].

Treatment interferes with the biological characteristics of PC, including heterogeneity, and further affects treatment efficacy and drug resistance [50]. In PC, the heterogeneity of CTC morphology is linked to drug resistance. High heterogeneity of CTC morphology at baseline is associated with poor treatment efficacy of ARSIs, but is not related to taxane-based efficacy in PC. Quantification of phenotypic heterogeneity in CTCs at baseline may potentially guide treatment selection [53].

During tumor progression and subclonal evolution, prostate adenocarcinoma may transition toward a neuroendocrine phenotype (NEPC), an AR-independent subtype with low AR signaling and resistance to ARSIs [54]. In fact, CTCs of patients with NEPC also have unique characteristics; compared to typical PC, CTCs of patients with NEPC exhibit more neuroendocrine characteristics, including more frequent clusters, lower or absent AR expression, lower CK expression, and smaller morphology [55].

Interestingly, CTC morphology also contains chromosome information and may be used to predict chromosome instability (CIN). Large-scale transitions (LSTs) are biomarkers of CIN, defined as chromosomal breakages that generate chromosomal gains or losses greater than or equal to 10 Mb [56]. The abnormal morphological characteristics of CTCs, including the size and morphology of the cytoplasm and nucleus, and AR and CK expression, are associated with the number of LSTs (determined by single CTC sequencing). Schonhoft et al. have developed an algorithm based on machine learning and digital pathology to analyze the morphological characteristics of a single CTC to predict the number of LSTs, a biomarker of CIN [56]. In a clinical cohort of 294 patients with mCRPC with 10,240 CTCs, a CIN biomarker based on morphological analysis of single CTCs is associated with poor OS in patients with mCRPC receiving ARSI and taxane therapy [56]. Therefore, CIN information contained in CTC morphology might assist in analyzing drug resistance and monitoring treatment response.

In addition, telomere abnormalities persist during the progression of PC; the three-dimensional (3D) telomere profile of CTCs in patients with local high-risk PC presents obvious heterogeneity, reflecting the genomic instability of tumor cells, and is linked to progression and drug resistance [57,58].

The 3D telomere
profile of CTCs is characterized by the size, number, and distribution of nuclear telomeres and the presence or absence of telomere aggregates [57,58]. Wark et al. have shown that 3D telomere profiling of CTCs identifies patients who are sensitive or resistant to androgen deprivation therapy (ADT) or radiation therapy (RT). Subjects were stratified into three types at baseline, according to their 3D telomere profiles [57]. During ADT and RT, each patient group shows distinct changes in 3D telomere profiles, as well as different responses to both treatments [57]. Moreover, a special subgroup of patients was identified after long-term dynamic monitoring of patients with localized high-risk PC. In these patients, the number of short telomeres of CTCs decreases first and then increases during treatment, which may represent the screening of drug-resistant tumor subclones by ADT and RT [58]. This finding indicates that long-term monitoring of genomic instability determined by 3D quantitative telomeric architectures in CTCs may be predictive of therapeutic response and disease progression, and may assist in monitoring treatment response [57,58].

Single-cell analysis has emerged as a powerful technology with a profound influence on the knowledge of tumor biology [59]. Compared to bulk analysis, the analysis of the genome and transcriptome of single cells might reveal more details on the genetic heterogeneity of primary and metastatic tumors, evolutionary principles, and underlying mechanisms leading to resistance. The implementation of single CTC sequencing relies on the capture of single cells and amplification of single-cell nucleic acids [60]. However, allelic dropout events and amplification errors, as well as limited coverage of sequencing, are obstacles that need to be resolved. Undoubtedly, with the increasing output and decreasing cost of sequencing, single CTC analysis is likely to become routine in clinical practice [60].

**Cell-free nucleic acids in PC**

**Cell-free DNAs (cfDNA) in PC**

Cell-free DNA (cfDNA) in the bloodstream is composed of tumor-derived DNA and normal DNA, which mainly emanates from leukocytes [61]. CfDNA is released by cells undergoing necrosis or apoptosis, or is actively secreted by viable cells as 150–200 base-pair fragments [62]. Since ctDNA originates from primary tumors and metastatic tumors, ctDNA analysis detects and analyzes tumor genomes and epigenomes serially. The half-life of cfDNA ranges from several minutes to several hours, providing an opportunity for real-time monitoring [63]. Pre-analytical variables, such as extraction procedure, sample type, tube type, storage time, and temperature impact cfDNA purification approaches and contribute to the optimization of analytical efficacy [64]. For example, cfDNA yields vary greatly with different extraction procedures [64]. Standardization of pre-analytical variables is urgent for the comparison of different studies [65]. There are two types of assays available: PCR-based methods and target next-generation sequencing (NGS) assays [66]. PCR-based methods are attractive for the clinic because of their low cost, ease of performance, and data analysis, but are restricted to known genetic changes [67]. NGS is exploratory, offers broader coverage, and may uncover multiple genetic aberrations; however, this method is time-consuming and requires powerful bioinformatics analysis. Some new NGS technologies, such as amplicon-based NGS combined with molecular barcoding technologies, improve the sensitivity, accuracy, and analytical efficacy of ctDNA [68].

Quantification of cfDNA concentration and tumor fraction may serve as prognostic biomarkers in PC [69,70]. During cancer progression, the ctDNA and cfDNA concentrations increase; the cfDNA concentration and fraction of ctDNA in cancer patients are significantly higher than those in healthy individuals and are associated with poor prognosis of OS in PC [69,70]. Although the detection rate of ctDNA is low in localized PC, the presence of ctDNA may indicate that the disease will progress more rapidly [71].

CtDNA analysis has been employed to unravel the genomic profiling of tumors in various types of cancer [72]. Most of the plasma ctDNA alterations are concordant with tissue DNA in PC [65,73]. Target ctDNA sequencing of patients with mCRPC identifies all somatic mutations and is highly correlated with CNVs that are present in paired metastatic lesions [74]. More importantly, ctDNA analysis has identified clinically actionable changes, including the AR and phosphoinositide 3-kinase pathways. Partial alterations are not shared by liquid or tissue biopsies [74]. According to a joint expert review, discordant results are mainly due to the low sensitivity of ctDNA assays or low shedding of ctDNA by some tumors [65].

The analysis of dynamic genomic changes in ctDNA in response to treatment regimens may potentially assist in monitoring treatment efficacy and early progression [70]. Serial ctDNA genomic analysis non-invasively identifies multiple drug-resistant subclones, as well as clinically actionable targets, presenting potential early signals for treatment conversion [75,76]. Aberrant AR signaling pathways may be revealed by
ctDNA analysis, including somatic mutations in the AR gene, CNVs, and structural rearrangements of the AR gene [76,77]. The frequency of AR point mutations and CNV increase from baseline to progression [76]. In addition, subclonal selection has been uncovered by ctDNA analysis; for example, the number of detected glucocorticoid-sensitive subclones (AR-L702H) increases during enzalutamide therapy [76]. Patients with PC with defects in DNA repair genes account for approximately 25–30% of all sporadic, castration-resistant PC [5].

Poly ADP-ribose polymerase (PARP) inhibitors are effective in patients with mCRPC with defects in DNA damage repair genes; however, some patients gradually lose sensitivity to PARP inhibitors [78]. CtDNA analysis reveals that some patients with mCRPC with defects in DNA repair genes have an initial response to PARP inhibitors; however, during disease progression, additional mutations in DNA repair genes lead to an open reading frame, subsequently restoring the functions of DNA-repair genes, and thereby obtaining resistance to PARP inhibitors [78]. These results indicate that ctDNA analysis enables the identification of events associated with resistance to PARP inhibitors in patients with PC, assisting in predicting the response or resistance to PARP inhibitors and facilitating early treatment conversion [78].

Recent preclinical models have shown that tumor protein 53 (TP53) and retinoblastoma protein (RB1) coordinate to inhibit lineage plasticity in PC [79,80]. Loss of TP53 and RB1 promotes lineage plasticity to an NEPC in PC, accompanied by an increase in expression of epigenetic reprogramming factors [79]. The use of epigenetic reprogramming factor inhibitors may reverse lineage switching and restore AR expression, subsequently improving sensitivity to ARSIs [79]. CtDNA analysis assists in evaluating the lineage plasticity status of patients with NEPC by detecting TP53 and RB1, which is conducive to clinical decision-making and improves clinical efficacy [81].

Epigenetic variations play an important role in the evolution of the tumor phenotype. Abnormal methylation is also a key factor in the insensitivity of patients with CRPC to targeted androgen therapy; the low methylation level of ctDNA reflects the abnormal methylation pattern of PC. In PC, the ratio of ctDNA methylation is highly correlated with the tumor fraction, and the ratio of AR hypomethylation is closely linked to an increase in AR copy number [82]. Beltran has also found that ctDNA helps to track the dynamics of tumor methylation maps, and that there are significant differences in ctDNA methylation maps between NEPC and CRPC. It is of great clinical significance to identify patients with NSPC in time because AR-targeted therapy is ineffective in these patients [83].

**Cell-free RNAs in PC**

Noncoding RNAs, such as long noncoding RNAs and microRNAs (miRNAs) are responsible for the post-transcriptional regulation of gene expression. Noncoding RNAs play crucial roles in oncogenesis and metastasis, rendering the characterization of cell-free RNAs (cfRNA) as a target that carries transcriptomic information of cancer cells [84]. Numerous studies have demonstrated the value of noncoding RNAs and tumor-derived messenger RNAs (mRNAs) as diagnostic, prognostic, and predictive biomarkers in PC [85]. Inconsistencies in published data call for protocol standardization of experimental approaches and the size of patient cohorts [86]. In general, the analytical technologies for the detection and analysis of cfRNA include RT-PCR approaches, microarray analysis, and NGS-based technologies. In contrast to whole-transcriptome NGS-based technologies, PCR-based approaches and microarray analyses are restricted to known genetic changes; however, NGS-based technologies require complex bioinformatics analyses. Preanalytical parameters and analytical technologies, for example, data normalization, profoundly influence the results [86].

Aberrant RNA signatures may pinpoint activated or inhibited key signaling pathways and networks, thus serving as prognostic and predictive biomarkers in the identification of aggressive cancer types, as well as potential therapeutic targets [87].

PC antigen 3 (PCA3), a noncoding RNA, is overexpressed in 95% of primary and metastatic tumors in PC [88]; PCA3 detection in urine samples improves the diagnostic performance in PC [88]. However, the relationship between PCA3 levels and PC aggressiveness is controversial [89,90]. TMPRSS2-ERG, a PC-specific fusion gene derived from chromosomal 21 rearrangement, accounts for approximately 50% of PCs [91]. The urinary test for TMPRSS2-ERG and PCA3 transcripts contributes to the detection of aggressive PC with a sensitivity of 93% and specificity of 33%, avoiding 33% supererogatory biopsies (subjects without PC or with GS of ≤ 6). These results indicate that the use of liquid biopsy is of great value both for detecting aggressive PC and for reducing overdiagnosis of indolent PC [91].

A comprehensive microarray profiling of 2588 circulating miRNAs indicates that dysregulated expression
of circulating miRNAs aids in the identification of patients with PC. A combined analysis of miR-17-3p and miR-1185-2-3p results in a sensitivity of 91% and specificity of 97% [92]. Additionally, five circulating miRNA pairs achieve higher sensitivity (99%) and specificity (100%), which is beneficial for the early diagnosis of PC [93].

Apart from the early detection of aggressive PC, plasma miRNAs are capable of improving risk stratification. Al-Qatati et al. have shown that the upregulation of plasma miR-148a, miR-16, and miR-195 helps to distinguish between high-risk and immediate-risk patients [87]. In addition to CTC-based technology, cfRNA detects AR-V7 transcripts using droplet digital PCR or RT-PCR in whole blood, which are also associated with resistance to ARSIs in patients with mCRPC [94]. Notably, the data indicate that AR-V7 mRNA is expressed in some hematopoietic cells, therefore, the non-tumor-specific AR-V7 mRNA may give false-positive results [95]. Overall, cfRNA is a potential biomarker for the detection and characterization of aggressive PC.

**EVs in PC**

EVs are lipid bilayer-delimited particles, including exosomes, microvesicles, and apoptotic bodies, which play important roles in the mediation of cell-to-cell communication and interaction between PC cells and their adjacent stromal cells [96]. Exosomes are small vesicles with sizes ranging from 30 to 100 nm that originate from the exocytosis of multivesicular bodies. Microvesicles, also called euctosomes, bud from the plasma membrane with a size ranging from 100 to 1000 nm, and apoptotic bodies are derived from cell lysis with sizes of 0.5–5 mm [96]. Because of their protective membranes, EVs stably carry and deliver active biological molecules, such as DNA, RNA, proteins, and lipids, exchange genetic materials to target cells, and modify the microenvironment to facilitate tumorigenesis, metastasis, and invasion [97,98]. To date, the potential applications of EVs in diagnostic, prognostic, and therapeutic strategies have attracted increasing attention in PC; for example, EV cargo originating from PC cells assists in the discrimination of patients with aggressive PC and the prediction of treatment resistance [99–101]. A urinary exosomal assay may identify aggressive PC in patients within the PSA gray zone at the initial biopsy [102]. A combination of urinary exosomal assay and standard of care (PSA level, age, race, and family history) is able to distinguish patients with PC with a grade group (GG) ≥ 2 from those with GG = 1, avoiding 26% of unnecessary tissue biopsies. Only 7% of patients with GG ≥ 2 tumors are missed [102]. In a followed randomized double-blind clinical trial, this urinary exosomal assay avoids the 30% missed diagnosis of advanced PC in patients within the PSA gray zone [103].

**Comprehensive analysis of liquid biopsies in PC**

Multiple biomarkers based on liquid biopsy are potentially capable of providing diagnostic, prognostic, and predictive information for PC. A single type of liquid biopsy exposes certain characteristics of cancer biology. The feasibility and necessity of integrating multiple blood-based biomarkers in PC has been demonstrated [104]. A multiparametric liquid biopsy analysis shows that only 13.8% of somatic single nucleotide variants are shared by CTCs and matched plasma cfDNA, indicating that data derived using only one liquid biopsy strategy is insufficient [104]. Therefore, multiple liquid biopsies complement each other and provide new insights into cancer [105]. A recent combined analysis of CTC and ctDNA showed that TP53-loss is superior to AR aberrations as a predictive biomarker for the treatment response of ARSIs [106]. AR aberrations lose statistical prognostic value after multivariable analysis, whereas TP53 inactivation remains an independent prognostic biomarker [106]. Based on the analysis of CTCs and ctDNA in TP53 wild-type patients, AR aberrations represent an independent prognostic value if three or more AR perturbations are detected [107]. It remains controversial whether a single AR perturbation is sufficient to predict the outcome of AR-targeted therapy. At present “AR burden” has emerged as a more powerful variable of prediction, indicating the necessity of joint applications of various liquid biopsies for the comprehensive evaluation of AR profiling and other genetic characterization. Additionally, a novel liquid biopsy assay has been developed to simultaneously analyze AR genomic changes in cfDNA and cfRNA; concurrent AR gain and AR-V expression are associated with rapid progression [108]. Furthermore, EVs isolated from blood also contain AR-V7 mRNA, potentially assisting in the prediction of resistance to ARSIs [109].

In summary, multiparametric liquid biopsy analysis provides more comprehensive information for diagnosis, prognosis, and clinical decision-making in PC. More comparative and combined clinical studies are required to elucidate the clinical utility of integrated analysis in PC.
Conclusion

Liquid biopsy, a non-invasive strategy, provides an opportunity to comprehensively evaluate genomic, transcriptomic, and epigenomic alterations of primary and metastatic tumors in real-time. Current strategies for liquid biopsy include CTC-based, cfDNA-based, cfRNA-based, and EV-based assays, allowing the analysis of germline and somatic mutations, dysregulated gene expression, and signaling pathways. Comprehensive evaluation of heterogeneity and subclonal evolution through the integration of multiple liquid biopsies may direct individualized therapeutic options. These powerful tools will assist in the discovery of clinically operable targets and new treatment strategies, which will benefit patient management and enhance the understanding of tumor biology. However, there are several limitations and challenges that need to be addressed, such as the standardization of sample preparation, data processing, analysis, and interpretation.

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