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Liquid Biopsy Approach In The Management of Prostate Cancer

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Abstract

Liquid biopsies examine tumor cells or tumor genomic content in circulating fluids. In advanced prostate cancer which metastasizes frequently to the bone, it is difficult to evaluate underlying and evolving genomic heterogeneity of skeletal metastases for effecting clinical care for which reason liquid biopsies offer an alternate approach. In this review, we will summarize the current state of a wide variety of liquid biopsy-based biomarker assays currently being investigated and developed for managing prostate cancer. We will also highlight technical and clinical challenges and opportunities for translating liquid biopsies into clinical applications.

Overview of liquid biopsy and its components

Liquid biopsy is a generic term applied to the study of tumor cells and tumor attributable genetic nucleic acids in body fluids of cancer patients. It relies on the principle that in cancer patients tumor cells and genetic fragments from cancer cells are shed in body fluids which can be detected and used as biomarkers for clinical applications (1). These shed cells or circulating tumor cells (CTCs) or tumor cell genetic fragments include as cell-free circulating tumor DNA (ctDNA), cell-free micro RNA (miRNA), and extracellular micro-vehicles including exosome-based miRNAs can be purified from body fluids and analyzed for determining tumor-specific mutations, copy number variations, gene rearrangements and other tumor-attributable genetic abnormalities. This offers an opportunity to develop potential clinical applications of circulating tumor-associated abnormalities in biological fluids which can range from diagnostic, prognostic, predictive biomarkers of treatment responses to monitoring disease.

For the isolation of CTCs the two main enrichment approaches used are based on physical characteristics or on marker based selection of CTCs. Detection of CTCs based on size and other biophysical properties takes advantage of fact that CTCs are usually larger than normal blood cells (2, 3). On the other hand a marker based identification of CTCs uses markers commonly expressed on the surface of these cells, such as EpCAM (4). The advantage of

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analyzing shed CTCs is that DNA and RNA from such cells may potentially provide complete genomic landscape of tumor cells to gain insights into tumor biology and host-tumor and drug interactions. However, the challenge remains to correctly identify CTCs using either of these enrichment resources. There is also the observation that tumors don't release intact cells into the bloodstream until relatively late in the disease and so there are limited number of CTCs in circulation in early cancer stages making this approach difficult for clinical applications.

For exosome separation from body fluids various methods including ultracentrifugation, polymer-based precipitation, and membrane proteins such as the CD63, CD9, and CD81 (5, 6) have been performed. Exosomes are small (30–100 nm) membrane vesicles released into the extracellular environment on fusion of multivesicular bodies with the plasma membrane (7) and contain RNAs selectively enriched from parent cells. Because they contain proteins as well as a range of nucleic acids, including DNA, mRNAs, and miRNAs, exosomes have been increasingly used as source materials for cancer biomarkers. A number of plasma exosomal RNAs are reported as diagnostic, prognostic, or even therapeutic biomarkers in cancer patients (6). In addition to CTCs and exosomes, circulating tumor-derived DNA (ctDNA) and cell free DNA (cfDNA) in bodily fluids are a focus area of liquid biopsy development. ctDNA is typically meant to indicate tumor derived fragmented DNA in bloodstream, while cfDNA is a more broader term applied to DNA that is freely circulating in body fluids (blood/urine) but not necessarily from tumor origin. It is believed that ctDNA are mostly derived from apoptotic tumor cells, as evidenced by an enrichment of DNA fragment size of single or multiple nucleoprotein complexes (8). After being shed in the circulation due to lack of protection circulating DNAs can be severely degraded reducing the concentration in plasma. Because of relatively low content in cancer patients, traditional Sanger sequencing is insufficient to detect low somatic mutations in plasma ctDNA. To overcome this limitation, next generation sequencing (NGS) and digital-PCR-based technologies with a high level of analytical sensitivity and specificity have been developed enabling high-throughput, targeted examination of the mutant genes of interest on high background of wild-type alleles. The digital-PCR approach enables dynamic profiling of individual mutations, but requires *a priori* knowledge of the mutant allele, whereas the NGS technique enables the discovery of novel mutated variants, but has higher costs and cannot be readily applied to monitor patients longitudinally (9).

Liquid biopsies in prostate cancer

Solid tumor biopsy-based biomarker development especially in advanced prostate cancer poses several unique challenges for which reason liquid biopsy has emerged as a promising option for biomarker development. The natural history of progression of prostate cancer from local, organ confined to initially non metastatic biochemically progressive and then to a clinically metastatic stage is prolonged. Clonal evolution at each stage of progression is shaped by selective pressures of prior treatments and increasing “bystander” genomic heterogeneity. For example in advanced stage the mutational rate substantially increases from 0.94 per megabase of the genome by a factor of three-fold (10). Genomic analysis of advanced castrate resistant tumors also reveal a qualitative difference in the genomic landscape including more frequent abnormalities in the *AR* signaling, *PTEN* and *TP53*

among others (10, 11). There is a high rate of abnormalities in PI3K pathway, DNA repair pathway, WNT pathway, RAF kinases or cell cycle abnormalities (10). On the other hand 19–23% of tumors with alterations on the DNA repair pathways (10, 12) occur both in primary and advanced prostate cancer while in organ confined stages fusion of the oncogenic E26 transformation-specific (ETS) family of transcription factors (more commonly ERG) with the androgen regulated TMPRSS2, resulting in androgen regulation of the oncogenic transcription factor (13) are frequently observed apart from mutations in *SPOP*, *TP53*, *FOXA 1* and *IDH1*, and also *PTEN* loss, PI3K pathway activation and *MYC* amplification (14, 15).

To capture this heterogeneity in the evolving genomic landscape in prostate cancer serial tissue biopsies are needed, which is difficult to execute in clinical practice given that the time period of progression is often measured in years. Another challenge in prostate cancer is that at the time of clinical metastasis the overwhelming site of spread is skeletal, which is difficult to sample and technically challenging to obtain tumor tissue-based nucleic acids for genetic testing. Finally, intra-tumor heterogeneity at different sites within the same patient can lead to underestimation of tumor genomic landscape portrayed from a single representative tumor-biopsy sample (16).

Liquid biopsies with CTC and nucleic acids-based genomic profiling in this clinical context may provide a potential solution to overcome the technical and practical challenges in solid tumor biopsies (17). Sampling tumor-associated genetic material in bodily fluids may also potentially capture a more “complete” repertoire which encompasses heterogeneous tumor biology. This potential of liquid biopsy has therefore been explored in earnest by academia and industry despite the clinical challenges mentioned for translating liquid biopsies into a practical tool for clinical applications. In this review, we will examine the current state of evidence for the investigative and clinical use of a variety of liquid biopsy-based biomarker assays for managing prostate cancer.

Cell-free DNA-based liquid biopsies

Current studies in cell-free DNA (cfDNA)-based assays have focused on clinical applications of diagnosis, screening, prognostication after treatment of organ confined disease and for identifying actionable genomic targets for drug therapy for advanced stage patients. cfDNA investigations have examined for both quantitative and qualitative changes in plasma cfDNA. While higher amounts are non-diagnostic, a number of studies have shown that patients with PCa have a higher level of plasma circulating cfDNA as compared to controls. In addition, amount of circulating cfDNA in advanced stage can also vary with the burden (high versus low) of metastatic disease (18). Qualitative assays measure the genomic abnormalities including single-gene methylation alterations and mutations, DNA integrity, loss of heterozygosity (LOH), and circulating nucleosomes. Studies have investigated epigenetic changes such as hypermethylated promoters in cfDNA as makers for diagnosis and screening of prostate cancer (19). Among the genes of interest hypermethylated markers in cfDNA for *GADD45a*, *GSTP1*, *CDH13*, *MDR1*, *RASSF1*, *APC*, *PTGS2*, *RARB2* and *TIG1* have been investigated for gene panel development to increase the diagnostic yield in prostate cancer. Single gene focus such as hypermethylated *GSTP1*

has also been evaluated in plasma cfDNA (20) as it is the most frequently observed somatic genome alteration in prostate cancer with a relatively high specificity (21). Wu et al showed in a meta-analysis that *GSTPI* promoter methylation measured in plasma, serum, or urine samples, in combination with PSA screening, would significantly enhance the diagnosis accuracy for prostate cancer (22). Another meta-analysis of 19 studies published from 2001 to 2015, with a total of 2239 cases was performed to assess the diagnostic capability of cfDNA. Four studies only evaluated abnormal levels of cfDNA in plasma/serum/blood (quantitative analysis group) and 12 assessed single-gene methylation alterations (qualitative analysis group), while three studies conducted quantitative and qualitative analysis simultaneously. A separate analysis was performed for studies testing *GSTPI* promoter methylation. Pooled specificity values in each subgroup of quantitative analysis, qualitative analysis, and *GSTPI* were 0.80 (95% CI, 0.70–0.87), 0.99 (95% CI, 0.97–1.00), and 0.98 (95% CI, 0.94–1.00) respectively, which were all much higher than the specificity of PSA, although the sensitivity of each subgroup was lower than PSA screening (23). Interestingly it was noted almost a decade ago that predictive value for the diagnosis of prostate cancer can be increased with the addition of cfDNA to standard diagnostic procedures (24). A recent effort confirmed that a combination of PSA with cfDNA concentration (cfDNA 188 ng/mL) and *GADD45a* methylation can increase the specificity of 87.5% and sensitivity of 94.1% (25). These results are interesting but are yet to establish cfDNA for PCa screening.

Several studies have demonstrated that ctDNA fraction in metastatic hormone sensitive prostate cancer (18) and in metastatic castrate-resistant prostate cancer (mCRPC) patients correlate with poor prognosis (26, 27). Similarly, the degree of ctDNA decline in response to systemic therapy has also been evaluated and has shown correlation with treatment response. The levels of ctDNA were found to be lower while responding to AR-directed therapy (such as with abiraterone acetate and enzalutamide) as compared to baseline or clinical progression (26, 28). Further evidence for prognostic significance of cfDNA comes from TOPARP-A trial of the PARP inhibitor olaparib in mCRPC where a greater than 50% decline in total plasma cfDNA after 4 weeks of treatment was independently associated with longer progression-free survival (29).

Beyond the yield, amount and decrease of cfDNA levels before and after treatment in advanced prostate cancer patients, several studies have utilized ctDNA to identify AR receptor amplification and certain mutations as a resistance maker to anti-androgen therapy with abiraterone (T878A and L702H) and enzalutamide (F877L). In a study of 62 patients with mCRPC progressing on systemic therapy, copy number analysis and androgen receptor (*AR*) exon 8 sequencing of plasma cfDNA showed that *AR* amplification was linked to enzalutamide resistance (30). This was followed by integrated genomic profiling on serially collected cfDNA from 65 mCRPC patients treated with enzalutamide. This analysis identified aberrations associated with both primary resistance (*AR* amplification, multiple *AR* mutations, *RB1* loss) and acquired resistance (*AR*-L702H and *AR*-T878A mutations, PI3K pathway alterations, *CTNNB1* mutations). At the time of progression, cfDNA sequencing revealed mutations or copy number changes in all patients tested including clinically actionable alterations in DNA damage repair genes and PI3K pathway genes, and a high frequency (4 of 14) of activating *CTNNB1* mutations (28). Another study analyzing 274 plasma samples from 94 patients with mCRPC identified two *AR* point mutations

(L702H and T878A) as associating with resistance to abiraterone on sequential plasma samples. This study concurrently evaluated both copy number and somatic point mutations in cfDNA of the *AR* coding regions from plasma of CRPC patients immediately before starting abiraterone, on treatment, and after progression. Patients with *AR* copy number gain or L702H and T878A mutations had a significantly worse decline in prostate specific antigen (PSA), progression free survival (PFS) and overall survival (OS) (26). A current effort is now focused on optimizing a droplet digital PCR (ddPCR)-based assay to identify *AR* copy number and mutations (T878A and L702H, F877L) for use in clinical practice (31).

There is also some early evidence for the urine-based copy number variation in cfDNA as a biomarker in advanced stage prostate cancer. In a small study performed of metastatic hormone sensitive prostate cancer patients before and after initiating androgen deprivation therapy and in metastatic castration-resistant state patients before and after initiating docetaxel chemotherapy whole genome sequencing of urine cfDNAs to identify tumor-associated copy number variations was performed (32). The copy number analysis was able to detect common genomic abnormalities in prostate cancer including *AR* amplification in 5/10 CRPC patients. Other genomic aberrations identified included *TMPRSS2-ERG* fusion, *PTEN* gene deletion, *NOTCH1* locus amplification along with amplifications at 8q24.3, 9q34.3, 11p15.5 and 14q11.2, and deletions at 4q35.2, 5q31.3, 7q36.3, 12q24.33, and 16p11.2. By comparing copy number between pre- and post-treatment significant copy number changes in 34 genomic loci were detected. While interesting, these initial efforts will need validation.

CTCs-based liquid biopsies

To evaluate the underlying heterogeneity of prostate cancer which is constantly evolving as a result of a therapeutic selection pressure CTCs have been suggested as a non-invasive way to interrogate the molecular characteristics of advanced prostate cancer (33). Currently, CTC enumeration has been FDA cleared in mCRPC as a prognostic marker, which has been confirmed in well conducted prospective trials (34, 35). While efforts in ctDNA investigations continue to focus on *AR* aberrations, CTC-based liquid biomarker studies have focused on the detection of *AR* splice variants (*AR*-Vs). These *AR*-Vs result from alternate splicing of full length *AR* mRNA. The truncated receptor isoforms can be constitutively active, thereby impacting tumor progression and therapeutic response. Among 15 different splice variants, AR-V7, AR-V9 and AR-V567es are suggested as the most clinically relevant isoforms (36, 37). The AR-V7 lacks the ligand-binding domain (LBD) but it is constitutively active. This results in an increased expression of *AR* full length (AR-FL) protein. Importantly, this overexpression of a constitutively active receptor without LBD (a binding site for some anti-androgen drugs) confers resistance to anti-androgen treatment (abiraterone and enzalutamide) and has therefore been evaluated as a predictive biomarker in mCRPC.

AR-FL and AR-V7 mRNAs in CTCs from prospectively enrolled men with mCRPC has been reported in the context of response and resistance to abiraterone and enzalutamide. CTCs captured from 7.5 ml blood using CTC AdnaTest Prostate Cancer Select platform for

CTC isolation and enrichment was followed by qRT-PCR to assess expression of *AR-FL* and *AR-V7* from these cells. Patient with non-detectable *AR-V7* in CTCs was observed to have a response to either enzalutamide or abiraterone, as determined by a reduction in serum PSA levels of 50% or more. Based on these initial results a follow up study using the same CTC platform in a larger prospective study of 202 patients with mCRPC starting abiraterone or enzalutamide confirmed these initial findings (38). In this study the prognostic value of CTC detection (positive versus negative) and *AR-V7* detection (positive versus negative) using a CTC-based *AR-V7* mRNA assay was assessed. Outcomes were observed to be best for CTC negative patients, intermediate for CTC positive/*AR-V7* negative patients, and worse for CTC positive/*AR-V7* positive patients. Results were similar for PFS and OS. Of note, 26% of the patients with mCRPC in this study did not have detectable CTCs. Responses to abiraterone or enzalutamide were observed in 40 of 53 men with non-detectable CTCs, in 59 of 113 men with detectable CTCs but negative for *AR-V7*, and in five of 36 men with detectable CTCs and positive for *AR-V7* in a multivariable analysis which considered initial Gleason score, metastatic disease at presentation, higher PSA levels and previous treatments. Based on these observations CTC-based *AR-V7* expression appears to be a potential predictive biomarker associated with resistance to abiraterone and enzalutamide (38).

In a subsequent study *AR-V7* expression in CTCs was also observed to be associated with resistance to enzalutamide or abiraterone utilizing a different method for detection of *AR-Vs*. In this study a single-tube assay was used to detect *AR-V7* splice variants and *AR* point mutations in CTCs using immunomagnetic cell isolation, followed by quantitative real-time PCR and DNA pyrosequencing. A positive predictive value for response and non-response to therapy by *AR* status in CTCs was observed (39).

A more recent study evaluated detection of CTC *AR-V7* protein while providing additional evidence for the role of *AR-V7* as a treatment response biomarker. This study examined the clinical utility of CTCs with *AR-V7* versus a broader definition of *AR-V7* positive CTCs “agnostic” to protein localization in the cytoplasm or nucleus (39). A different platform for CTC detection (Epic Sciences platform for non- EpCAM based automated immunofluorescent staining of DNA, CKs, CD45 and *AR-V7*) was used in this study as compared to previous studies which allowed isolation of CTCs independent of cell-surface markers (eg, EpCam, cytokeratins). It was observed that the presence of *AR-V7* in the nucleus (but not elsewhere) was a strong predictor of resistance to *AR* signaling inhibitor (ARSIs), and patients with nuclear-specific *AR-V7* CTCs had better survival with taxanes versus ARSIs. The results also pointed to heterogeneous resistance mechanisms to ARSIs (20% of resistant patients had nuclear-specific *AR-V7* staining. While it is not yet clear if nuclear-specific protein localization is required to reliably inform treatment selection between ARSI and taxanes using a CTC *AR-V7* biomarker (40), the suggestion that limits “*AR-V7* positivity” to only cases with nuclear expression is particularly relevant. This study provides evidence that men with nonnuclear *AR-V7* still exhibit some PSA responses to ARSIs (39). The predictive capacity of an isolated CTC versus *AR-V7* mRNA transcripts in a pooled sample are under evaluation in two independent validation studies for which reports are expected in near future (41).

The role of AR-V7 as a treatment response biomarker is not only limited to ARSIs. It is known that taxanes work as microtubule stabilizers and induce mitotic arrest. They may also function in prostate cancer by preventing microtubule-dependent translocation of AR into the nucleus.

Conversely, the nuclear transport of AR-V7 appears to be intact in taxane resistant patients, suggesting that AR-V7 expression may also predict resistance to taxanes (42). Using previously validated assays AR-V7 in CTCs from mCRPC patients has been observed not to be associated with primary resistance to taxane chemotherapy. In AR-V7–positive men, taxanes appear to be more efficacious than enzalutamide or abiraterone therapy, whereas in AR-V7–negative men, taxanes and enzalutamide or abiraterone may have comparable efficacy (43). The results of a follow-up study validate CTC nuclear expression of AR-V7 protein using a different assay in men with mCRPC as a treatment-specific biomarker that is associated with superior survival on taxanes therapy over ARS-directed therapy in a clinical practice setting (44).

AR-V7 is a dynamic marker and repeated measurements may capture transitions in AR-V7 status as a result of different treatments. Conversions from AR-V7 negative to positive most often occur in patients undergoing AR-directed therapies, whereas reversions from AR-V7 positive to negative seem to occur only with taxane chemotherapies. Sequential CTC sampling could provide insights into disease evolution, and therefore, the authors suggested follow-up studies to further determine the clinical significance of these AR-V7 transitions (45).

From a clinical perspective the application of molecular (not enumeration) of CTC-based prognosis and prediction appears to be limited by the ability to detect CTCs, which in turn depends on the stage of prostate cancer progression with earlier stage patients having a lower frequency for detecting CTCs. Table 1 summarizes several cfDNA and CTC-based studies with clinical application in advanced stage prostate cancer. Since the detection rates of CTC in whole blood are extremely low in organ confined and non-metastatic states the application of CTC-based assays are of limited value as liquid biopsies in these states. A further refining of the assay platforms therefore is needed to increase assay sensitivity and determine the most appropriate detection parameters and molecular markers that may be employed in clinical practice.

Extracellular RNA-based liquid biopsies

MicroRNAs (miRNAs) are a class of endogenous 19– 22 nucleotides long, single-stranded non-coding RNA molecules. miRNA expression profiles have been found to be tissue type-specific and frequently dysregulated in various cancers. Several groups have identified miRNA signatures by performing miRNA profiling in plasma or serum of patients with localized or metastatic prostate cancer, BPH and healthy individuals and claimed high diagnostic accuracy (46, 47). However, such claims are based on very small studies with technical challenges and methodological constraints. Such clinical assays will need to be optimized technically and validated in prospective studies.

Urine RNA expression biomarkers.

Prostate Cancer Antigen 3 (PCA 3) is a prostate cancer-specific antigen with high specificity for cancer. It is a non-coding-RNA encoded on chromosome 9q21–22(48) and is not expressed in prostate tissue, lowly expressed in hyperplastic prostate tissue but highly expressed in prostate cancer (49). Urinary PCA3 has been evaluated in men with an elevated serum PSA and prior negative biopsy for diagnosis of cancer (50). A urine based assay development collected after a prostate massage and before transrectal biopsy in patients with elevated PSA (above 2.5 ng/ml) has shown to yield a sensitivity of 69% and specificity of 79% for predicting cancer on biopsy. An optimal cutoff for urinary PCA3 levels has not been established as a liquid biopsy biomarker for screening since urinary PCA3 based cancer diagnosis has not yet demonstrated reduction in prostate cancer specific mortality.

Since gene fusions involving *ERG* are frequent observed molecular aberrations in prostate cancer, development of *TMPRSS2-ERG* fusion assay in post digital rectal examination (DRE) urine has been investigated to determine its diagnostic utility. This assay has a specificity of up to 93%, with a positive predictive value of 94%, but at the cost of a low sensitivity of 37%. This has led to combining assays of urinary PCA 3 and *TMPRSS2-ERG* RNA fusion for detecting high Gleason score tumors (51, 52). Recently in a study with a test and validation cohort the combined detection of both at thresholds that preserved 95% sensitivity for detecting aggressive prostate cancer improved specificity from 18% to 39% (53). It was calculated that the use of this combination assay decreased by forty-two percent prostate biopsies to select men for repeat biopsy. Further prospective longitudinal trials are now evaluating the reproducibility and validity of this assay for clinical practice. Differentially expressed miRNA profiles across different risk features in untreated early and advanced stages of the disease have been reported including in patients with biochemical recurrence, patients developing clinical progression and metastases (54, 55). miRNA-based molecular characterization, however, is limited to research at present due to the lack of well-powered studies with clinically relevant outcomes.

Plasma-derived exosomal RNA biomarkers.

Plasma-derived exosomal RNA has been suggested as an alternative source to analyze AR-V7. The utility of AR-V7 detection in RNA extracted from cancer cell vesicles released into blood was first demonstrated in 2016. In a small study of 26 patients receiving abiraterone and enzalutamide, 39% of patients were found to be AR-V7 positive. Median progression-free survival was significantly longer in AR-V7 negative versus AR-V7 positive patients (20 vs 3 months; $p < 0.001$). Overall survival was significantly shorter in AR-V7 positive participants at baseline compared with AR-V7 negative participants (8 months vs not reached; $p < 0.001$). The results confirmed the microvesicles as a reliable source of AR-V7 RNA and the authors claimed that method is fast, highly sensitive, and affordable (56), but validation studies remain pending.

Plasma exosomal miRNAs have also been evaluated for clinical utility in advanced prostate cancer including prognosis of castration-resistant prostate cancer. In one study (57) with a screening and validation cohort of mCRPC patients RNA sequencing of plasma exosomal miRNAs was performed to identify candidate miRNAs associated with overall survival.

Candidate miRNAs were further evaluated for prognosis using quantitative real-time polymerase chain reaction in the follow-up cohort. In this to date only study of its kind with adequate longitudinal follow up, RNA sequencing in screening cohort reported a total of 375 known and 57 novel miRNAs. Cox regression analysis identified an association of miR-1290, -1246, and -375 with OS and higher levels of miR-1290 and -375 were subsequently confirmed to be significantly associated with poor OS. Interestingly, miR-375 may also have a role as a predictive biomarker of docetaxel resistance as it appears that miR-375 induces docetaxel resistance in prostate cancer by targeting *SEC23A* and *YAP1* genes (58).

Liquid biopsy-based predictive biomarkers

The assays based on cfDNA, miRNA and CTCs have consistently shown prognostic implications. Similarly, several studies suggest that presence of AR V-7 positivity in CTCs reflects aggressive tumor biology. Not surprisingly, AR-V7 expression in tumor tissues or CTCs was correlated with a significantly shorter OS when compared with those with lower levels of AR-V7 in tissue or when absent in CTCs (43, 44, 59, 60). There are several FDA approved treatment options for the management of advanced stage states, hormone sensitive prostate cancer and castration resistant prostate cancer such as taxane chemotherapies (Docetaxol, Cabazitaxol), second generation anti-androgens (Abiraterone, Enzalutamide and Apalutamide), Immunotherapeutic options (Sipuleucel-T) and Radium-223. Multiple trials have shown improvement in overall survival with the use of various agents, but only one large trial to date has compared two life-prolonging therapies (FIRSTANA trial). It is not clear, however, how to tailor a particular therapeutic choice based on the tumor or host genome so that there is a maximum benefit to prolong survival with minimum possible toxicity. In the absence of head to head trials, the choice of frontline therapy both in mCRPC or in hormone sensitive prostate cancer state is based on empiric clinical expertise mostly. Sequential liquid biopsies focusing on treatment-response biomarkers have the potential to predict frontline treatment and inform prompt switching when the resistance occurs. Therefore, development and validation of surrogate biomarkers are of a high priority in research on prostate cancer.

Challenges and future directions

While early studies offer definite targets at the mutational and copy number variations and gene expression levels for developing liquid biopsy-based biomarkers, several challenges remain.

Liquid biopsy biomarker techniques, including CTC counts, cfDNA and exosomal RNA, have shown promise to guide the treatment of mCRPC. While several biomarker candidate targets have been explored, there is increasing evidence that *AR* is the key mediator of resistance to androgen deprivation and therefore a potential biomarker to guide therapy. A recent review showed inconsistency between results with liquid biopsies and findings obtained from tumor specimens (61). Lack of consistency between different tests was borne out in a [recent independent comparison](#) of two commercially available liquid biopsies for evaluation of patients with prostate cancer (62). While liquid biopsy has significant potential

there is not enough evidence at this time to support the routine use of ctDNA tests for early-stage cancer, making treatment decisions, monitoring predicting response or for cancer screening. A systematic approach for developing these assays from discovery to clinical validation and utility has been recommended recently by the American Society of Clinical Oncology and the College of American Pathologists (61), which informs researchers a uniform pathway of rigorous scientific approach. Despite these challenges the promise of developing a variety of liquid biopsy markers in prostate cancer has entered an exponential level. Supplementary Table 1 summarizes several on-going observational and interventional studies for a variety of clinical applications that are likely to impact prostate cancer management in future. It is clear that pre-analytic specimen processing and analytic reproduction, validity and clinical context for application will need further attention as has been recommended recently (61) for the successful adoption of these technologies in clinical practice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ARS	Androgen Receptor Signaling
ARSI	Androgen Receptor Signaling Inhibitors
AA	Abiraterone Acetate
AR	Androgen Receptor
AR-FL	Androgen Receptor Full length
AR-V	Androgen Receptor splice Variant
BPH	Benign Prostatic Hyperplasia
CNVs	Copy Number Variation
CRPC	Castration Resistant Prostate Cancer
CTC	Circulating Tumor Cells
ctDNA	circulating tumor DNA
cfDNA	cell free DNA
ddPCR	Droplet digital Polymerase Chain Reaction

DNA	DeoxyriboNucleic Acid
DRE	Digital Rectal Examination
E	Enzalutamide
EpCAM	Epithelial Cell Adhesion Molecule
HSPC	Hormone sensitive prostate cancer
LBD	Ligand-Binding Domain
mCRPC	Metastatic Castration Resistant Prostate Cancer
mRNAs	messenger ribonucleic acid
miRNAs	micro ribonucleic acid
NGS	Next Generation Sequencing
NHT	Novel Hormonal Therapy
PARP	poly ADP ribose polymerase
PCa	Prostate Cancer
PCR	Polymerase Chain Reaction
PSA	Prostate Specific Antigen
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic Acid
RAF	Rapidly Accelerated Fibrosarcoma

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Table 1: Summary of CTC and cfDNA based liquid biopsy based studies in advanced stage prostate cancer

Study	N	Biomarker	Study Population/Method	State of prostate cancer	Prognostic/Predictive/Monitoring	Comments
Antonarakis et al. (2014)	62	CTC	mCRPC patients initiating treatment with AA(n=31) or E(n=31) were evaluated by qRT-PCR for AR-V7 in CTCs	mCRPC	Monitoring, Predictive	CTCs AR V7 mRNA may be associated with resistant to NHT
Antonarakis et al.(2015)	37	CTC	mCRPC patients initiating taxane chemotherapy were evaluated using qRT-PCR for AR-V7 in CTCs	mCRPC	Predictive	CTCs AR V7 mRNA is not associated with resistant to taxanes
Azad et al. (2015)	62	cfDNA	Patients ceasing AA (n = 29), E (n= 19), or other agents (n= 14) due to disease progression were evaluated	mCRPC	Predictive	AR gene CNVs and Missense exon 8 mutations (F876L, H874Y,T877A) are associated with resistance to ARSIs
Nakazawa et al. (2015)	14	CTC	Serial liquid biopsies for CTC based AR-V7 analysis at baseline, during therapy and at progression to assess whether AR-V7 is a dynamic marker	CRPC	Monitoring	Conversions from AR-V (-) to (+) were seen both with NHT and taxanes while AR-V (+) to (-) is only seen with taxanes/reversions from AR-V7 positive to negative = 6 (all during docetaxel or cabazitaxel treatment)
Romanel et al. (2015)	97	cfDNA	CRPC patients treated with AA were evaluated	CRPC	Predictive	AR gain or T878A or L702 are associated with inferior PSA decline, OS and PFS
Steinestel et al.(2015)	47	CTC	PC patients with PSA progression awaiting therapy switch were evaluated using single tube assay for AR-V7 splice variants and mutations	CSPC or CRPC	Monitoring, Predictive	Positive Predictive value of response and non-response to by AR status in CTCs is 94%
Del Re et al. (2016)	36	Exosomal RNA	CRPC patients at progression before they began 2nd line NHT	CRPC	Predictive	CTC ARV7 exosomal RNA by ddPCR may predict response to NHT
Scher et al. (2016)	161	CTC	mCRPC patient at progression were evaluate before next line therapy using nuclear AR-V7 in CTCs	mCRPC	Predictive	Pre-therapy CTC nuclear ARV7 expression predicts superior OS for taxanes over NHT
Wyatt et al. (2016)	65	cfDNA	mCRPC patients receiving E were evaluated	mCRPC	Monitoring	Alterations in AR gene (CNVs, AR-L702H, AR-T878A)PI3K pathway,CTNNB1 mutations were identified
Antonarakis et al. (2017)	202	CTC	CRPC patients starting AA or E were prospectively evaluated using qRT-PCR for AR-V7 in CTCs	CRPC	Predictive	CTCs AR V7 mRNA may predict outcomes in CRPC patients receiving 1st and 2nd line NHT
Bernemann et al. (2017)	21	CTC	Assessment of response in AR-V7 positive CRPC patients who had received AA or E.	CRPC	Predictive	Subgroup of AR-V7 positive may benefit from NHT

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Study	N	Biomarker	Study Population/Method	State of prostate cancer	Prognostic/Predictive/Monitoring	Comments
Conteduca et al. (2017)	171	cfDNA	CRPC patients enrolled in any of 3 biomarker protocols with prospective clinical data were evaluated	CRPC	Monitoring, Predictive	AR status assessment using ddPCR identifies CRPC patients with worse outcomes to ARSIs
Scher et al. (2017)	161	CTC	mCRPC patients at disease progression were evaluated to expand the AR-V7 positivity criteria	mCRPC	Predictive, Prognostic	Nuclear localization of CTC AR-V7 protein is associated with resistance to 2nd line NHL and reduced survival
Annala et al. (2018)	202	cfDNA	Whole exome sequencing in Treatment-naïve mCRPC patient randomized to AA(n = 101) or E (n = 101)	mCRPC	Predictive	Liquid Biopsy testing can guide use of AR directed therapy in clinical practice

Abbreviations: ARSIs=Androgen receptor signaling Inhibitors, AA=Abitraterone Acetate, E=Enzalutamide, mCRPC=Metastatic Castration Resistant Prostate Cancer, CRPC=Castration Resistant Prostate Cancer, PSA=Prostate Specific Antigen, AR=Androgen Receptor, CNVs=Copy number variation, CTC=Circulating Tumor Cells, qRT-PCR= Quantitative Reverse Transcriptase Polymerase Chain Reaction, NHT=Novel Hormonal Therapy