Circulating cell-free nucleic acids as prognostic and therapy predictive tools for metastatic castrate-resistant prostate cancer

Navid Sobhani, Marianna Sirico, Daniele Generali, Fabrizio Zanconati, Bruna Scaggiante

Abstract
Metastatic castrate-resistant prostate cancer remains a disease hard to cure, and for this reason predictive tools to monitor disease progression and therapy response are an urgent need. In this respect, liquid biopsy on circulating cell-free nucleic acids represents an interesting strategy based on robust data. The low invasiveness and the possibility to target circulating cell-free tumor deoxyribonucleic acid underline the high specificity, sensitivity and clinical usability of the technique. Moreover, it has been observed that the cell-free tumor deoxyribonucleic acid of metastatic castrate-resistant prostate cancer patients can be representative of the tumor heterogeneity. Cell-free tumor deoxyribonucleic acids express the same behaviors as mutations: Variation in gene copy number or the methylation rate of the tumor tissue. Recently, circulating cell-free ribonucleic acid molecules have emerged as interesting markers to stratify the disease. Due to high-throughput technologies, liquid biopsy on circulating cell-free nucleic acids will soon be utilized in the clinical management of metastatic castrate-resistant prostate cancer patients.

Key words: Metastatic castrate-resistant prostate cancer; Circulating free deoxyribonucleic acid; Cell-free tumor deoxyribonucleic acid; Circulating free ribonucleic acid; Liquid biopsy; Prostate cancer
Core tip: Among men in industrialized countries, prostate cancer is the most frequent occurring type of cancer and the leading cause of cancer-related deaths. To assure an optimal management of metastatic castrate-resistant prostate cancer patients, specific markers to monitor response to therapies and to predict the clinical outcomes are an urgent need. Liquid biopsy on circulating cell-free deoxyribonucleic acid is able to give useful information about the genetic status of the tumor and the prognosis. Liquid biopsy on circulating cell-free nucleic acids has the potential to integrate clinical data for a personalized management of patients.

INTRODUCTION

Prostate cancer is the most frequently occurring type of cancer among men in over one-half (105 of 185) of world’s countries, and it is the leading cause of cancer-related deaths among men in 46 countries[1]. Although inhibition of androgen receptor (AR) signaling with antiandrogens and conventional chemotherapeutic molecules in metastatic castrate-resistant prostate cancer (mCRPC) patients has increased the 5-year survival rate to 29%, it still remains difficult to cure[2]. In mCRPC, cancer cells adapt to live with very low levels of androgens. Such cells try to use different strategies to independently promote the AR pathway.

After developing the first antiandrogens (flutamide, nilutamide and bicalutamide), there was an urgent need for impeding any agonist activity against wild-type AR. There was a necessity to inhibit wild-type AR recruitment of co-activators and to block the consequent AR binding to deoxyribonucleic acid (DNA), which is a transcription factor for androgen-dependent genes that leads to tumor proliferation[3]. This brought the development of second-generation antiandrogens, which showed a significant improvement in clinical treatment. In fact, in 2012 the Food and Drug Administration approved enzalutamide and abiraterone acetate as second-generation antiandrogens for the treatment of mCRPC, while other second-generation antiandrogens are at different stages of pre-clinical and clinical development[4]. Although these two drugs act on different levels of the AR pathway, the cross-resistance is a common event; resistance to abiraterone and enzalutamide was found to be associated with splicing variants of AR without the ligand-binding domain. Such splice variants encode for a receptor incapable of binding to ligands. It is constitutively active as a transcription factor and capable of promoting the activation of target genes. Because enzalutamide exerts its antitumor activity by binding to the ligand-binding domain of the receptor, the splice variants cannot be targeted by the drug, which generates drug-resistance. Moreover, as the receptor is constitutively activated, it will not be influenced by abiraterone, which inhibits the synthesis of androgens. In this scenario, androgens are not required to activate the pathway, as it is already active[5]. Interestingly, 20%-40% of mCRPC patients not previously treated with chemotherapy are intrinsically resistant to abiraterone acetate or enzalutamide[6].

Thus mCRPC cells evolving into an AR-independent type escape targeted therapies against androgens. Sometimes they can acquire characteristics of neuroendocrine prostate cancer, which is a transformation that occurs in the clinical context of a moderate increment of blood prostate specific antigen (PSA) levels[6]. It is clear that mCRPC progression can have different therapeutic options, but the monitoring of mCRPC is an urgent task for patients’ surveillance to anticipate relapse of the disease or to evaluate the efficacy of the therapies. In this respect, we will discuss the emerging role of liquid biopsy for metastatic castrate-resistant prostate cancer patients with a focus on nucleic acids to predict disease progression or therapy efficacy.

Citation: Sobhani N, Sirico M, Generali D, Zanconati F, Scaggiante B. Circulating cell-free nucleic acids as prognostic and therapy predictive tools for metastatic castrate-resistant prostate cancer. World J Clin Oncol 2020; 11(7): 450-463
URL: https://www.wjgnet.com/2218-4333/full/v11/i7/450.htm
DOI: https://dx.doi.org/10.5306/wjco.v11.i7.450
LIQUID BIOPSY IN METASTATIC CASTRATE-RESISTANT PROSTATE CANCER

In contrast to traditional tissue biopsy, liquid biopsy involves the analysis of different biological fluids. As technologies advance, the amplification and detection methods have become more and more efficient and sensitive in the target identification, using minute quantities of nucleic acids. As a proof of principle, liquid biopsy can detect various tumor-specific macromolecules to profile and stratify the pathology. Longitudinal monitoring by liquid biopsy could give critical support to clinical management of patients because specific molecular changes are correlated with tumor progression and response to therapies.

Di NUNNO et al. summarized in six points the advantages of liquid biopsy as an innovative approach to prostate cancer: (1) Low invasiveness; (2) Early detection of most aggressive tumors; (3) Early diagnosis of residual tumors or micrometastasis after surgery; (4) Monitoring patient response and/or progression of the disease after therapy, particularly for mCRPC; (5) Prognostication of the response to therapy; and (6) possibility to delineate an accurate genetic profile of the disease directing the analysis towards key mutations correlated with tumor resistance.

Many molecular alterations (mutations, amplifications, deletions, alterations in the expression of specific genes or in non-coding RNAs transcription levels) have been correlated with resistance or sensibility against specific treatments. Targets in liquid biopsy can be circulating cell-free nucleic acids. Circulating cell-free tumor DNA (ctDNA) analysis includes detection of circulating free DNA (cfDNA) levels, circulating cell-free DNA integrity (cfDI), methylation rate, mutations and/or aberrations in the copy number of specific genes. For example, amplification of AR and/or the detection of certain driver mutations, e.g. deletion of PTEN, can be useful to anticipate an unfavorable clinical outcome. Of interest are the AR mutations, particularly of certain splicing variants (AR-V7), correlating with resistance against second-generation hormone treatments, such as abiraterone acetate and enzalutamide. Moreover, inherited gene mutations that have been found significantly correlated with the prognosis of the disease are eligible for liquid biopsy analysis.

Other valuable emerging tumor biomarkers in liquid biopsy are represented by different classes of RNAs, including microRNAs (miRNAs), long non-coding RNA and messenger RNA (mRNA).

cfDNA analysis in mCRPC patients

The total cfDNA concentration was increased in cancer patients and may be a useful tool. However, more information for patient management is derived from the analysis of cfDNA. Table 1 summarizes the main studies investigating cfDNA in mCRPC patients by liquid biopsy. A strategy is to calculate the circulating cfDI, which is the evaluation of the cfDNA fragmentations of repetitive elements such as ALU and LINE-1. It has been observed that in tumors the cfDI value can increase or decrease. The concentration and integrity of circulating cfDNA have been proposed as a clinical tool for the diagnosis of prostate cancer. Khani et al. proved in Iranian patients that the values of cfDI were significantly higher in patients with prostate cancer vs those with benign hyperplasia and healthy individuals. They measured a region of ALU repetitive elements with shorter fragments of 115 bp nested in longer 247 bp ones by quantitative polymerase chain reaction (qPCR); the ratio of 247 bp over the 115 bp gave the integrity DNA index. Additionally, both the DNA concentration and cfDI were found to be elevated in the metastatic conditions. These results were confirmed by Arko-Boham et al. Interestingly, cfDI of ALU247/115 had already been found to be higher in metastatic prostate cancer patients versus non metastatic ones.

In the amount of purified cfDNA, cfDNA can be analyzed by searching gene aberrations such as copy number variations or mutations in target genes. The analysis of genetic alterations of AR, identified through cfDNA in mCRPC patients, that circulating molecules have a significant potential to guide the use of therapies against this receptor. Furthermore, the monitoring of cfDNA levels could result in a powerful way to trace response of tumors and detect the uprising of resistant subclones. In addition to alterations of the AR pathway, other modifications in TP53, WNT, PI3K and cell cycle pathway (RB1 and CCND1) genes are important for prognosis and drug-development. It should be noted that the deletion of PTEN is found in 40% of patients with mCRPC and loss-of-function mutations in genes of the DNA repair pathway (homologous recombination and mismatch) are expressed in approximately 20% of metastatic prostate cancer. TP53 gene is very frequently mutated in mCRPC. In particular, TP53 gain-of-function mutations have been associated with cancer cell
## Table 1 Diagnostic and prognostic methods and outcomes of studies investigating cell-free tumor deoxyribonucleic acid in metastatic castrate-resistant prostate cancer by liquid biopsy

| Ref. | Methods and patients | Prognostic or predictive outcomes |
|------|----------------------|----------------------------------|
| Ritch et al\(^\text{[19]}\), 2019 | Plasma, whole exome sequencing, Intron and exon sequencing and copy numbers of selected genes. Mismatched repair deficiency, MMRd mPCa (mostly mCRPC) patients (n = 433) | cfDNA analysis of hypermutations and MMR gene alterations in MSH2, MSH6, MLH1 marked ctDNA and can identify aggressive disease. Mutations in ctDNA were found in PTEN, RB1, TP53 (and, interestingly, no copy number loss) and in AKT1, PIK3CA, CTNNB1, AR-LBD. Compared with a control cohort, ctDNA hypermutation and MMRd correlated with a poor response to AR inhibition and to a shorter survival |
| Chapman et al\(^\text{[24]}\), 2019 | Plasma, whole-genome sequencing, Somatic gain of function mutations of TP53. mCRPC patients (n = 143) | In cfDNA somatic GOF mutations of TP53 at codons R175, R248, R273, R282 and G245 were positively and significantly associated with abiraterone and/or enzalutamide progression |
| Gupta et al\(^\text{[14]}\), 2019 | Plasma, low pass whole-genome sequencing ULP-WGS. Somatic copy number alteration, mCRPC patients (n = 93) | The SCNA in ctDNA and CTC were mostly concordant (gain in copy number of FOXA1, AR, and MYC, and loss in BRCA1, PTEN, and RB1). Interestingly, some discordant genomic alterations rarely detected in cfDNA (gain in MYC and BRCA2) were associated to a worse outcome, i.e. MYCN copy number gain correlated with a worse outcome in AR-V7 negative patients and BRCA2 copy number gain correlated with a worse outcome in AR-V7 negative patients treated with abiraterone/ enzalutamide |
| Patsch et al\(^\text{[13]}\), 2019 | Plasma, qPCR of LINE-1 297 bp, mCRPC patients before and after docetaxel treatment (n = 15) | In cfDNA, LINE-1 quantity decreased after chemotherapy but without statistical significance |
| Hahn et al\(^\text{[20]}\), 2019 | Serum, next generation sequencing, somatic copy number alteration of 69 targeted genes, Metastatic PCa patients (n = 101) | In cfDNA, SCNA significantly correlated with the number of new treatments demonstrating changes of tumor genomic profile after therapies. Interestingly, SCNA did not correlate with time of progression |
| Qiu et al\(^\text{[21]}\), 2019 | Plasma, tissue tumor mutational burden by next generation sequencing, targeted gene panel, mCRPC (n = 20) | The ctDNA was identified by two TMB assays: guardian health and the foundation medicine. Between these two assays used to detect ctDNA the results of SNP, Indel, CNV and fusions were concordant. The study evinced a high correlation between ctDNA TMB and the whole exome sequencing of the corresponding tumor tissues. Additionally, there was a good correlation between ctDNA TMB and tissue TMB having high TMB samples; however, the same was not observed for low/medium TMB samples |
| Torquato et al\(^\text{[19]}\), 2019 | Plasma, AR alterations, whole genome sequencing, Somatic copy number alteration, mCRPC patients (n = 62) | In cfDNA the SCNA significantly associated with worse survival outcomes of mCRPC patients. At multivariable analysis, missense mutations in AR ligand-binding domain correlated with shorter DFS and PP53 loss. PIK3CA copy number gain or pathogenic missense mutations correlated with shorter OS |
| De Laere et al\(^\text{[23]}\), 2019 | Plasma, low pass whole-genome sequencing, and targeted gene-body sequencing. Somatic alterations in AR and TP53. mCRPC patients (n = 168) | The presence of AR and TP53 alterations in ctDNA was used to determine tumor burden. AR and TP53 alterations were associated with a worse DFS. TP53 inactivation was an independent prognostic factor outperforming AR alterations and ARV7 expressions was found be useful to stratify patients’ risk |
| Sonpavde et al\(^\text{[3]}\), 2019 | Plasma, somatic mutations by NGS, mCRPC patients (n = 514) | One or more alterations in ctDNA were found in 94% of patients. Mutations were detected in TP53 (36%), AR (22%), APC (10%), NFI (9%), EGFR, CTNNB1 and ARID1A (6%), BRCA1, BRCA2, PIK3CA (5%). The increase in copy number was found for AR (50%), MYC (20%), BRAF (18%). At multivariate analysis, increase in MYC copy number was associated with worse OS |
| Vandekerkhove et al\(^\text{[2]}\), 2019 | Plasma, targeted genome, sequencing, somatic copy number alteration, metastatic castrate-sensitive patients (n = 53) | The ctDNA and tissue biopsy showed 80% of concordance for somatic mutations. TP53 was mutated in 50% of patients and truncated mutations in DNA damage repair genes were found in 21%. The ctDNA SCNA was higher in untreated patients than in patients treated with abiraterone acetate, enzalutamide or apalutamide |
| Mayrhofer et al\(^\text{[7]}\), 2018 | Plasma, low pass whole-genome sequencing and hybridization-capture targeted sequencing. Somatic copy number alteration, mPCa patients (n = 217) | The SCNA found in ctDNA was used to determine tumor burden and to compare lines of therapies. AR variants, microsatellite instability and PTEN, RB1 and TP53 inactivation were found in ctDNA demonstrating mirroring of the genetic alterations that mark metastatic cancer |
| Choudhury et al\(^\text{[2]}\), 2018 | Plasma, ultra-low pass whole-genome sequencing. Tumor fractions, TFi, measured by computational tool ichorCNA, CRPC patients (n = 140) | TFx was determined in ctDNA. TFx positively correlated with PSA and alkaline phosphatase and significantly correlated with the presence and numbers of bone metastases |
| Annala et al\(^\text{[6]}\), 2018 | Plasma, whole-exome sequencing and target capture and | The alterations in TP53 and AR gene truncations measured in ctDNA correlated to resistance to abiraterone and enzalutamide. |
| Study                        | Methodology                                                                                                                                   | Findings                                                                                                                                                                                                 |
|------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Kohli et al (2018)           | Plasma, dPCR for copy number gain of AR, mCRPC patients (n = 92)                                                                              | A poor clinical outcome was associated with alterations in ctDNA of BRCA2 and ATM genes                                                                                                                    |
| Mehrza et al (2018)          | Plasma, cfDNA quantity quant-IT picogreen HS DNA kit and a BioTek microplate spectrophotometer (480ex/520em), mCRPC patients (n = 571)     | In pre chemotherapy patients, the AR copy number gain found in ctDNA was associated with a worse outcome                                                                                                  |
| Seyedolmohadessein et al (2018) | Plasma, cfDNA quantity NanoDrop. Localized PCa (n = 50), metastatic PCa (n = 26) and healthy controls (n = 10)                         | The cfDNA level was significantly higher in metastatic PCa patients (19.62 ng/μL) with respect to localized PCa (15.4 ng/μL) and BPH patients (9.5 ng/μL); healthy controls had the lowest value (8.7 ng/μL) |
| Belic et al (2018)           | Plasma, deep AR sequencing, Illumina MiSeq; whole genome sequencing and targeted sequencing, Somatic copy number alteration, mCRPC patients (n = 65) | In ctDNA, AR mutations and copy number alteration were found in most cases. AR amplification and RB1 loss were associated with worse PFS. SCNA was therefore a biomarker for disease progression |
| Hendriks et al (2017)        | Plasma, Methylation-specific qPCR and copy number of GSTP1 and APC genes, CRPC patients (n = 47), controls (n = 30)                           | In CRPC patients the cfDNA quantity was significantly higher than age-matched controls. At baseline, GSTP1 was hypermethylated in patients. Both the copy number of methylated GSTP1 and APC were higher in patients than healthy controls. The increase of cfDNA levels, either each one of the methylated gene copies individually or together (GSTP1 + APC) or together with PSA (GSTP1 + APV + PSA), all correlated with decreased OS |
| Wyatt et al (2017)           | Plasma, targeted sequencing, Somatic copy number alteration, mCRPC (n = 45)                                                                   | The study proved a correspondence between SCNA in ctDNA and matched tissues. Such SCNA genes included AR, BRCA2, ATM, PTEN, PIK3CA, PIK3CB, PIK3R1, TP53, and RB1 |
| Rathkopf et al (2017)        | Plasma, dPCR of 11 relevant AR-liquid binding domain mutations. Non-metastatic CRPC (n = 51), AAP-naïve mCRPC (n = 25), post-AAP (n = 21) | In ctDNA, the AR-LBD mutations were found to be low at baseline (7.5%) and progression (7.3%). The AR-LBD mutations did not correlate with the AR score resistance to apalutamide |
| Goodall et al (2017)         | Plasma, Quant-IT, whole exome sequencing and targeted sequencing. Targeted genes, mCRPC patients (n = 40)                                      | At multivariate analysis, the ctDNA concentration was an independent prognostic biomarker: ≥ 50% reduction in ctDNA levels related to longer rPFS and OS. The ctDNA germline and somatic alterations in BRCA2 and PALB2 repair genes were found in ctDNA. All mutations found in the tissue were also detectable in ctDNA |
| Conteduca et al (2017)       | Plasma, dPCR. Somatic copy number gain of AR, mCRPC patients (n = 80)                                                                          | In ctDNA, the AR copy number gain was associated with a worse outcome in patients treated with abiraterone and enzalutamide. Independently from the type of antiandrogen treatment, there was a meaningful correlation among AR gain and TLA/MTV compared to AR non-gained cases (P = 0.001 and P = 0.004, respectively). AR copy number and TLA were associated with a shorter PFS and OS |
| Annala et al (2017)          | Plasma, somatic mutations of BRCA2 gene by qPCR, mCRPC germline-mutated patients (n = 11)                                                     | In 10 out of 11 germline mutated patients, biallelic gene loss of BRCA2 was found in ctDNA. This information help to guide clinicians to the best therapeutic choice |
| Conteduca et al (2017)       | Plasma, dPCR. Copy number gain of AR, CRPC patients (n = 265)                                                                                  | In ctDNA, the AR copy number gain before starting enzalutamide or abiraterone was associated with a decrease in both PFS and OS |
| Goldstein et al (2017)       | Plasma, NGS AR sequencing and validation by dPCR, somatic alterations in AR, mCRPC patients (n = 11)                                           | In ctDNA, the AR r (TC > CTC) F877L hotspot was prone to false positive mutations during NGS. Low-abundance mutations need to be verified by highly sensitive PCR, such as dPCR, but amplification conditions must be carefully optimized |
| Adalsteinsson et al (2017)   | Plasma WES, metastatic PCA PCa patients (n = 520)                                                                                             | There is a concordance between clonal somatic mutations (88%), copy number alterations (80%), mutational signatures and neantigens between tumor biopsies and ctDNA form 41 patients with ≥ 10% ctDNA |
| Wyatt et al (2016)           | Plasma, AR copy number qPCR and AR deep targeted sequencing, mCRPC patients (n = 65)                                                          | In ctDNA, the AR mutation and copy number alterations were found in 48% of baseline patients and in 60% patients at disease progression. The AR copy number gain (two or more AR mutations) and RB1 loss were associated with worse PFS |
| Salvi et al (2016)           | Plasma, qPCR. Copy number gain of AR, CRPC patients (n = 59)                                                                                | In ctDNA, the AR copy number gain was found in 36% of patients. AR copy number gain significantly associated with alkaline |
phosphatase and lactate dehydrogenase. At multivariate analysis, PSA decreasing ≥ 50% and AR copy number gain were significantly associated with worse OS and PFS

Fawzy et al[40], 2016

Plasma, qPCR of ALU 247bp and ALU115bp, cell-free DNA Integrity, cfDI metastatic PCa (n = 28), non-metastatic PCa (n = 22), BPH (n = 25), healthy controls (n = 30)

The cfDI levels, measured as ratio ALU247bp/ALU115bp, were significantly higher in metastatic PCa patients vs non-metastatic PCa patients, BPH patients and healthy controls

Azad et al[41], 2015

Plasma, AR qPCR copy number and deep sequencing of AR-LBD, mCRPC (n = 62)

In cfDNA, the AR copy number gain was associated with enzalutamide resistance; also abiraterone resistance was associated to AR mutations but to a lower extent

Deligezer et al[42], 2010

Plasma, qPCR for Sat-2 gene, PCa-localized (n = 22), locally advanced (n = 11), mCRPC (n = 28)

The average quantity of cfDNA measured as ratio ALU247bp/ALU115bp was significantly higher in metastatic PCa patients vs non-metastatic PCa patients, BPH patients and healthy controls

Schwarzenbach et al[43], 2009

Plasma, somatic LOH for D6S1631, D8S286 and D9S171 genes by qPCR, PCa patients (n = 69), metastatic PCa patients (n = 28)

In cfDNA, the somatic LOH significantly correlated with the diagnosis of subgroups made of localized and metastasized prostate cancers. cfDNA LOH significantly associated also with the tumor stage

Bastian et al[44], 2007

Serum, qPCR for GSTP1, MDRI and EBNRB genes, PCa patients (n = 192)

The levels of cfDNA was found to increase from PCa without recurrence to PCa with recurrence and then to metastatic PCa for all GSTP1, MDRI or EBNRB genes

**MMRd:** Mismatch repair deficiency; **AAP:** Acetate and prednisone treatment; **AR:** Androgen receptor; **ARV7:** Androgen receptor variant 7; **BPH:** Benign prostatic hyperplasia; **cfDI:** Cell-free DNA integrity; **cfDNA:** Cell-free circulating DNA; **CRPC:** Castrate-resistant prostate cancer; **CTC:** Circulating tumor cells; **ctDNA:** Circulating tumor DNA; **dPCR:** Digital PCR; **GOF:** Gain of function; **NGS:** Next generation sequencing; **PFS:** Progression-free survival; **LP-WGS:** Low pass whole-genome sequencing; **LOH:** Loss of heterogeneity; **OS:** Overall survival; **PCa:** Prostate cancer; **mCRPC:** Metastatic castrate-resistant prostate cancer; **mPCa:** Metastatic prostate cancer; **PSA:** Prostate specific antigen; **qPCR:** Quantitative PCR; **rPFS:** Radiographic progression-free survival; **SCNA:** Somatic copy number alteration; **scfDNA:** Seminal plasma cfDNA; **TGS:** Deep targeted sequencing; **TFx:** Tumor fractions; **TMB:** Tumor mutation burden; **ULP-WGS:** Ultra low pass whole-genome sequencing; **WES:** Whole exome sequencing; **LBD:** Ligand binding domain.

**Survival and chemoresistance.** Interestingly, TP53 mutations conferring gain of function were related to disease progression and drug resistance after abiraterone or enzalutamide treatments[13].

Different studies offer a comparison between ctDNA and the corresponding metastatic tissue. For example, Wyatt et al[30] sequenced 72 clinically relevant genes in 45 cfDNA samples and corresponding tissues obtained during biopsy. The comparison of data concerning alterations in ctDNA with those of the tissues has been demonstrated that for the majority of patients an assay of ctDNA could be enough to identify all the alterations in a metastatic tissue. The authors suggested that with appropriate validation methods, it could be possible to develop DNA-based biomarkers useful in identifying ctDNA for the management of patients with mCRPC. An important advantage of ctDNA is the ability to integrate somatic information from metastatic sites to discover mutation heterogeneity of the tumor, which should be taken into consideration to monitor the tumor stage and its progression. This information could add knowledge to the pathological analysis of the prostate tissue biopsy, which by itself may be not completely representative of the heterogeneous behavior of tumors[30].

**ctRNA in prostate cancer patients**

The necessity of a multi-parameter approach has been highlighted in different studies. Indeed, important additional knowledge could arise from a simultaneous approach.
investigation of molecules using liquid biopsy (i.e. cfDNA) and DNA extracted from circulating tumor cells and cfRNA, with the purpose of building a complete molecular profile to integrate with the clinical data.

RNA evaluation in liquid biopsy represents the next frontier in integrated molecular medicine. Table 2 summarizes the main clinical investigations on ctRNA. Most studies involved prostate cancer patients at diagnosis. Pioneer exploration of RNA molecules in liquid biopsy has involved the analysis of prostate specific membrane antigen mRNA, but only a limited number of newly diagnosed patients were positive, whereas the bone morphogenetic protein-6 mRNA, whose upregulation is strongly associated with bone metastasis, was found to be a biomarker for the metastatic disease.

Circulating mRNAs are generally degraded by RNases. However, circulating mRNAs are capable of forming complexes with transporters, which are proteins and/or lipids. When they form such complexes, they turn into a relatively stable structure in the blood circulation, thus becoming potentially useful biomarkers. Currently, androgen receptor variants are generally responsible for AR activity, survival and progression of prostate cancer. The AR-V7 is the only one of the androgen receptor variants observed as a protein, and it is therefore properly defined. Different studies in the literature have shown that AR-V7 is the main AR variant. The AR-V7 product is a truncated AR protein, which lacks the C-terminal ligand-binding domain but retains the transactivating N-terminal domain. Consequently, this protein is not capable of binding to ligands but remains a constitutively active transcription factor promoting the activation of target genes promoting cancer progression. Antonarakis et al. evinced that AR-V7 mRNA is expressed at high levels in circulating tumor cells and is associated with abiraterone and enzalutamide resistance in mCRPC patients. Indeed, Joncas et al. recently found blood levels of AR-V7 mRNA, which was shown to be correlated with response and resistance to abiraterone in mCRPC patients, demonstrating its potential as predictive biomarker. Particularly worthy of note, upregulation of the programmed death-ligand PD-L1 mRNA causes cancer cells to able to evade the host immune system.

In most patients, altered levels of miRNAs have been found, such as miR-21, miR-221, miR-1290 and miR-375. Such differential expression compared to healthy controls have been associated with different prognostic outcomes in mCRPC patients. For example, a potential diagnostic and prognostic role played by miR-141 has been suggested. miR-141 levels have been found to be progressively increased from hypertrophy of the prostate to prostate cancer and to the metastatic disease. Interestingly, the quantification of miR-141 in the liquid biopsy by droplet digital PCR has been described. However, a recent study showed that miR-18a has the highest potency to discriminate between healthy individuals and cancer patients, whereas miR-221 discriminated between patients with localized disease from those with metastasis. miR-141 did not show the same potency.

FUTURE PERSPECTIVES

Liquid biopsy represents an attractive field of research for many types of cancer, including prostate adenocarcinoma. In particular, liquid biopsy has been proven to provide support in therapeutic planning for patients with mCRPC, allowing detection of molecular changes in cell-free nucleic acids (i.e. DNA and RNA) that are associated with tumor progression and response or resistance to different drugs. The low invasiveness is particularly relevant because of the possibility of repeating the analyses frequently over time, allowing longitudinal monitoring of patients. In the near future, liquid biopsy will lead to a deeper understanding of the metastatic evolution of prostate cancer with the possibility of developing new targeted therapies in the perspective of an even more personalized oncology.

The analysis of ctDNA appears to be the most promising tool to monitor cancer diseases. In fact, ctDNA is the only target recommended by the Food and Drug Administration and the European Medicines Agency for cancer diagnosis and to monitor the efficacy of treatments. For prostate cancer, ctDNA is a very interesting biomarker for the anticipation of progression-free survival and overall survival in response to therapies and for improving the clinical management of patients avoiding overtreatments. The high concordance between ctDNA genomic alterations and those found in tumor tissue biopsies strongly supports the potential of liquid biopsy to integrate clinical data and improve patient management. The next generation
### Table 2 Diagnostic and prognostic outcomes methods of studies investigating circulating tumor ribonucleic acid in prostate cancer by liquid biopsy

| Ref. | Methods and patients | Prognostic or predictive outcomes |
|------|----------------------|---------------------------------|
| Joncas et al.\(^a\), 2019 | Plasma dPCR, AR-7 mRNA, PCa patients (n = 35) | AR-V7 mRNA expression was associated with shorter time to progression (median, 16.0 vs 28.0 mo; P = 0.0499) |
| Mohammad et al.\(^a\), 2019 | Serum qPCR, miR-20A, miR-26A, PCa patients (n = 40), healthy controls (n = 40) | In PCA samples miR-20A was significantly upregulated compared to healthy controls. On the other hand, there was no significant difference in the levels of pre- and post-operation miR-26A compared to controls |
| Ishiba et al.\(^b\), 2018 | Plasma dPCR, FDL-1 mRNA, PCa patients (n = 88) | PO-L1 mRNA was detected and quantified in ctRNA of cancer patients. Interestingly, there was a comparison between expression of PD-L1 protein in tumor tissues and PD-L1 gene expression in plasma of cancer patients |
| Wang et al.\(^b\), 2018 | Plasma qPCR, SAP30L-AS1 and SChLAP1 IncRNAs, PCa patients (n = 34), BPH patients (n = 46), Healthy controls (n = 30) | SAP30L-AS1 IncRNAs levels were upregulated in BPH and SChLAP1 IncRNAs levels were significantly higher in PCa than in BPH and healthy controls. The area under the ROC curve indicated that SAP30L-AS1 and SChLAP1 IncRNA had an adequate diagnostic value different from PCa and controls |
| Zedan et al.\(^c\), 2018 | Plasma qPCR, miR-93, miR-221, miR-125b, miR-139, PCa patients (n = 149) | Significantly lower levels of miRNA-93 and miRNA-221 in the follow-up of patients vs baseline z = −2.738, P < 0.006, and z = −4.486, P < 0.001, respectively. Similarly, miRNA-125b was significantly lower in the observational cohort (z = −2.656, P = 0.008). There was a correlation between both miRNA-125b and miRNA-221 with risk assessment r = 0.23, P = 0.015 and r = 0.203, P = 0.016, respectively. However, miRNA-93 was significantly correlated with prostatectomy Gleason score (r = 0.276, P = 0.0576) |
| Farran et al.\(^d\), 2018 | Plasma qPCR, miRNA signature, PCa patients (n = 114) | Aggressiveness of PCa could be segregated based on circulating miRNA signature consisting of an interaction between a combination of two miRNAs (miR-17/miR-192) and an independent miRNA (miR-181a) |
| Liu et al.\(^e\), 2018 | Plasma qPCR, miR-223, miR-24, miR-375, PCa patients (n = 329) | Patients could be significantly reclassified using a 3-miR (miRNA-223, miRNA-24 and miRNA-375) score (training OR 2.72, 95%CI 1.50e 4.94 and validation OR 3.70, 95%CI 1.29e 10.6) |
| Adalsteinsson et al.\(^f\), 2017 | Plasma WES, Metastatic PCa patients (n = 520) | There is a concordance between clonal somatic mutations (88%), copy number alterations (80%), mutational signatures and neoantigens between tumor biopsies and ctDNA form 41 patients with ≥10% ctDNA |
| Albitar et al.\(^g\), 2018 | Urine and plasma qPCR, mRNAs panel, PCa patients (n = 306) | The urine/plasma biomarker test, evaluating the mRNA levels of PCa-specific gene such as PDLIM5, HSPD1, PSA, IMPDH2, PC3, TMAPSS2, ERG, UAP1, PTEN, AR, the housekeeping B2M and GAPDH genes, accurately predicted high-grade cancer with sensitivity at 92%-97%, while core-biopsy sensitivity was 78% |
| Endzelniš et al.\(^h\), 2017 | Plasma qPCR, miR-375, miR-200-3p, miR-21-5p, mRNA Let-7a-5p, PCa patients (n = 50), BPH patients (n = 22) | miR-375 could be used to differentiate between PCa and BPH patients when analyzed in whole plasma, while miR-200-3p and miR-21-5p performed better in EVs. Let-7a-5p could be used to differentiate PCa patients, with Gleason score ≥ 8 vs ≤ 6 |
| McDonald et al.\(^i\), 2017 | Plasma qPCR, miRNA panel, PCa patients (n = 134) | miR-381, miR-34a, miR-523, miR-365, miR-122, miR-375, miR-125b, miR-34b, miR-450b-5p, and miR-639 were the most statistically significant miRNA after adjusting for age (P values ≤ 0.05) |
| Alhasan et al.\(^j\), 2016 | Plasma Scano-miR, miRNA panel, very high risk, PCa patients (n = 9), Low risk, PCa patients (n = 9), and healthy controls (n = 10) | miR-200c, miR-605, miR-135a, miR-433, and miR-106a were identified as useful for differentiating indolent and aggressive forms of PCa |
| Yan et al.\(^k\), 2015 | Urinary qPCR, TSPAN13 and S100A9 mRNAs, PCa patients (n = 129), BPH patients (n = 105) | qPCR was used to measure circulating urinary nucleic acid levels and tissue mRNA expression. The TSPAN13 and S100A9 mRNA ratio was selected to determine the diagnostic value of urinary nucleic acids in PCa (P = 0.037). It was significantly higher in PCA than in BPH in the mRNA and nucleic acid cohort analysis (P < 0.001 and P = 0.013, respectively). ROC analysis showed that the area under the ROC curve was 0.998 and 0.676 in tissue mRNA cohort and urinary nucleic acid cohort, respectively. This ratio could have a strong potential as a diagnostic PCa marker |
| Antonarakis et al.\(^l\), 2014 | Serum qPCR, AR-V7 mRNA, PCa enzalutamide-treated patients (n = 31), AR-V7 mRNA detectable (positive) patients receiving enzalutamide had lower PSA response rates compared to AR-V7 mRNA not detectable (negative) patients (0% vs 53%, P = 0.004) and shorter PSA PFS (median, 1.4 mo vs 6.0 mo; P < 0.001), clinical or radiological PFS (median, 2.1 mo vs 6.1 mo; P < 0.001), and OS (median, 5.5 mo vs not reached;
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| Study | Method | Patients | Outcome |
|-------|--------|----------|---------|
| Korzeniewski et al. (2014) | Urine qPCR, miR-483-5p, PCa patients (n = 71), healthy controls (n = 18) | miR-483-5p was expressed at higher levels in PCa than in control |
| Deligezer et al. (2010) | Plasma qPCR, cBMP6 mRNA, Local PCa patients (n = 22), local advanced PCa patients (n = 11) or mCRPC patients (n = 28) | The levels of cBMP6 mRNA in patients with metastatic disease were higher than those in patients with localized disease (P = 0.001) or in patients with local advanced disease (P = 0.05) |
| Papadopoulou et al. (2006) | PBMC and plasma qPCR, PSMA mRNA, newly diagnosed PCa patients (n = 12), under therapy PCa patients (n = 4) | Among the newly diagnosed patients 4/12 (33.3%) had positive mRNA for PSMA in plasma, whereas only 2/12 (16.7%) had positive PSMA mRNA in PBMC. Among under therapy PCa patients, three (15.8%) were positive for PSMA mRNA in plasma, while only one (5.3%) was positive in PBMC. Furthermore, > 60% of PCa had elevated levels of cfDNA |

AR: Androgen receptor; AR-V7: Androgen-receptor splice variant 7; BPH: Benign prostatic hyperplasia; cfDNA: Cell free DNA; dPCR: Digital polymerase chain reaction; lncRNAs: Exosomal circulating long non-coding RNAs; mCRPC: Metastatic castrate-resistant prostate cancer; PSA: Prostate specific antigen; WES: Whole exome sequencing; PFS: Progression-free survival; OS: Overall survival; PBMC: Peripheral blood mononuclear cells; PCa: Prostate cancer; qPCR: Quantitative polymerase chain reaction; lncRNA: long non-coding RNA; PSMA: Prostate specific membrane antigen; BMP-6: Bone morphogenetic protein-6.

Liquid biopsy data offers robust evidence to consider cell-free nucleic acid analysis useful to improve the clinical management of mCRPC patients. In this new approach the use of PSA as a biomarker must be considered. PSA is the biomarker approved for men by the Food and Drug Administration in 1986. From then on, it has been widely used to predict incidence and recurrence of prostate cancer, despite its poor specificity. However, in mCRPC the PSA seems to be more specific as a biomarker than in the onset of prostate cancer; its increase is related to cancer progression[73]. The significance of PSA measurements in mCRPC is still interesting for the scientific community. For example, Aggarwal et al.[74] recently demonstrated that low PSA secretion levels can stratify mCRPC patients with treatment-emergent small-cell neuroendocrine prostate cancer. In fact, low PSA secretors showed high treatment-emergent small-cell neuroendocrine prostate cancer, RB1 and TP53 loss and low AR transcription. In sequencing of cfDNA has demonstrated the potential for the follow-up of the mutational changes of the tumor by being able to identify all its heterogeneity and to anticipate drug resistance. Moreover, many affordable high-throughput technologies, e.g. digital PCR, are now available to precisely detect the copy number variations of selected target genes (e.g. AR, TP53, BRCA2, PIK3CA) that are relevant for the progression of the disease and in response to therapies.
addition, overall survival and progression-free survival were shorter in the low PSA secretor group[34]. In a retrospective study, Buttigliero et al[35] showed that early PSA drop was related to a better overall survival and progression-free survival in mCRPC patients treated with abiraterone or enzalutamide (docetaxel-naïve or post-docetaxel setting). Finally, a mathematical model of PSA dynamics has been proposed to predict individual response to intermittent androgen deprivation therapy[36].

CONCLUSION

In our opinion, PSA can play an important role as a biomarker for the management of mCRPC patients. However, PSA measurements could maintain some limitations due to the high individual variability. Liquid biopsy on circulating cell-free nucleic acid offers the same low invasiveness but important molecular details on each specific tumor heterogeneity evolution. In conclusion, liquid biopsy on circulating cell-free nucleic acid along with PSA measurements and other clinical data can assure the best treatment decision-making for mCRPC patients.

ACKNOWLEDGEMENTS

The authors thank dr. Giorgia Ficco for the English language editing of the manuscript.

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