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Circulatingtumordna (Ctdna) in Prostate Cancer: Current Insights and New Perspectives

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1. Abstract

Prostate Cancer (PC) is the common tumor in men, which represents one of leading cause of cancer death throughout the world. Most patients were diagnosed too late for curative treatment. So, it is necessary to develop a minimal invasive method to identify novel biomarkers. Currently, plasma DNA has attracted increasing attention as a potential tumor marker. However, to date its origin remains still unknown. Recent findings showed that a fraction of the plasma DNA is derived from the tumor itself, and genetic and epigenetic alterations are regularly detected in PC patients. Many studies have evidenced an association among the plasma DNA analysis, Gleason score, tumor stage, lymph node status, clinical progression, development of metastasis, and clinical outcome, concluding that plasma DNA levels could serve as a viable tool for diagnostic and prognostic information in prostate tumor. In addition, several genetic and epigenetic changes, identified by recent genotyping and sequencing technologies, such as copy number variation, point mutation, loss of heterozygosity, microsatellite instability, and gene methylation were correlated with treatment response and resistance mechanisms. Here, we reviewed the evidence of plasma DNA in PC and consider current and possible future applications in patient management.

2. Keywords

Plasma DNA; Prostate cancer; Biomarker; PCR; Sequencing

3. Introduction

Prostate Cancer (PC) is one of the three most common cancer type for the estimated new cancer cases and deaths, respectively, among men in worldwide [1]. PC is highly heterogeneous in terms of the clinical behavior and molecular pathogenesis. There is a plethora of clinical situations between indolent and aggressive tumors and within the same setting, particularly in the Castration-Resistant Prostate Cancer (CRPC). In addition, tumors with the same histopathologic grade are often biologically heterogeneous with different outcome. The remarkable variation in PC clinical behaviour reflects the broad landscape of molecular alterations among various prostate tumors and within the same tumor at different stages of disease progression [2-4].

On the basis of wide heterogeneity of prostate tumor, more precise biomarkers are needed to help accurately the identification of indolent or aggressive PC, a better knowledge of the genetic mechanisms of tumor progression and the optimization of PC management.

Over the years, the use of serum Prostate-Specific Antigen (PSA), the only routine test approved for the PC screening, detection and treatment response has become a highly debated question for the lack of specificity leading often to the over detection and overtreatment of prostate tumors [5-8]. Consequently, the identification of novel biomarkers is one of the most important issue for PC management and further studies for their validation and introduction into clinical practice are warranted the identification of novel biomarkers is one of the most important issue for PC management and further studies for their validation and introduction into clinical practice are warranted [9,10].

With advances in genotyping and sequencing technologies, the development of non-invasive methods to detect and monitor tumors continues to be a major challenge in oncology. New molecu-

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lar biomarkers are based on a combination of multiple genomic or proteomic biomarkers.

This review will focus on the role of circulating tumor DNA (ctDNA) as recent discovery in the "liquid biopsy" field of many solid tumors, including PC [11-14]. In recent years, measurement of ctDNA might become as efficient complementary tools to guide future therapeutic directions and improve the outcome of PC patients.

4. Biology of Circulating Cell Free DNA

Circulating free DNA (cfDNA) has been detected in the plasma of cancer patients as well as in that of healthy controls. However, circulating tumor DNA (ctDNA) is approximately three-four times the cfDNA amount of healthy controls, even if it often represents only a small fraction (less than 3.0%) of total circulating DNA [13,15,16]. Different potential mechanisms may be involved in the cfDNA release into the circulation and could include both cancer and healthy cells. Certainly, low levels of cfDNA in healthy individuals are due to the activity of infiltrating phagocytes that, however, under specific conditions (e.g., tumor, inflammation, etc.), might not clear apoptotic and necrotic debris properly [17].

The apoptosis and necrosis of tumor cells represent the most probable mechanisms for the ctDNA release into the bloodstream [13]. In addition, living cancer cells could actively release DNA into the circulation because of possible oncogenic property to affect the transformation of susceptible cells leading to metastasis formation [17,18]. ctDNA might also be released by Circulating Tumor Cells (CTCs) shed by tumor [13,16]. However, a single human cell contains 6 pg of DNA with an average of 17 ng of DNA per ml of plasma in advanced tumors. Therefore, there is a discrepancy between the number of CTCs and the amount of ctDNA. If CTCs were the main source of ctDNA, there would be more than 2,000 cells per ml of plasma; indeed, there are, on average, less than 10 CTCs per 7.5 ml blood [19-21]. In conclusion, at correlation between ctDNA and CTCs might exist but they represent separate entities and, in some cases, ctDNA could be identified also in the absence of detectable CTCs [11,22,23].

Other host elements that might contribute to the source of ctDNA are plasma nucleases (e.g., DNase 1) that could have a decreased DNase activity in the plasma of cancer patients [24,25]. Moreover, tumor microenvironment could have a role for ctDNA release because of the induction of apoptosis by pro-apoptotic cytokines by inflammatory cells or cancer cells. In fact, some studies showed higher level of ctDNA not only in PC, but also in patients affected by Benign Prostatic Hyperplasia (BPH) compared with healthy controls [26,27].

These data supported the theory that a large proportion of cfDNA was derived from noncancerous cells because of the induction of apoptosis by proapoptotic cytokines released from prostate cancer cells.

In addition, the host factors for ctDNA clearance [13], other factors influencing ctDNA amount are related to tumor (size, stage and, probably, type of tumor). In general, major burden tumor and metastatic disease are characterized by increased ctDNA levels [14,28].

The analyses of DNA fragmentation showed that apoptotic or necrotic pattern seems to be different in distinct types of cancer. In general, fragmentation of cell-free DNA is higher following apoptosis than following necrosis or phagocytosis [28]. In fact, ctDNA fragments longer than 10,000 bp originate usually from necrotic cells, whereas DNA fragments shorter than 1000 bp, particularly of 180 bp or multiples of this size, are observed in apoptotic cells [12,28]. A detailed analysis of the size of the ctDNA fragments could allow discerning the source of circulating nucleic acid in n various cancer entities. Specifically, necrotic pattern seems to be predominant in patients with breast and gynaecological [29,30], colon [31], testicular [32], and head and neck [29] cancer patients because necrotic breakdown leads to greater DNA integrity. On the other hand, the presence of mainly short DNA fragment in circulation of patients with bladder cancer [33] and PC [34] argues for apoptotic pattern of ctDNA release.

The combined analyses of ctDNA with CTCs or Disseminated Tumor Cells (DTCs) in bone marrow suggest that ctDNA may be derived not only from the primary tumor but also from micrometastatic cells. Consequently, some studies showed that the presence of ctDNA may be correlated to the detection of DTCs in bone marrow of PC patients [13,35,36].

5. Methods for ctDNA Analysis

Recent advances in the sensitivity and accuracy of DNA analysis are leading to genotype ctDNA for genomic aberrations, including somatic single nucleotide polymorphisms, chromosomal rearrangements and epigenetic alterations [37-41].

Preanalytical factors involved in sample collection (i.e., the preferred use of plasma compared to serum, the addition of anticoagulant EDTA in collection tube, proper blood centrifugation after blood collection, etc.) and highly sensitive techniques are mandatory for the ctDNA analysis because of the small fraction of ctDNA present within normal cfDNA [42].

Currently, many discoveries in genomics technologies are providing novel challenges for the analysis of ctDNA. In general, it is

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possible to subdivide the approaches for ctDNA analysis into three groups: a) Polymerase Chain Reaction (PCR) based methods, b) targeted deep sequencing, and c) whole sequencing [43]. The PCR based approaches are usually used for the evaluation of a low number of loci, even digital PCR is considered as a sensitive analysis tool for the identification of mutations at low allele fraction [44]. Methods involving the use of digital PCR include droplet-based systems [45,46], microfluidic devices [22,47], and the use of Beads, Emulsions, Amplification and Magnetics (BEAMing) [48,49]. An example of the utility of PCR based approaches for ctDNA analysis is showed by a work evaluating the prognostic utility of circulating plasma testing in 85 patients with different advanced solid tumors. Specifically, the presence of NRAS, PIK3CA and AKT1 was demonstrated in 3 of 11 (27.3%) circulating DNA specimens of CRPC patients and PIK3CA and AKT1 were found in the corresponding formalin-fixed paraffin-embedded tumor tissue pointing up that circulating plasma DNA in advanced cancer patients is largely derived from tumor [21].

Next Generation Sequencing (NSG) methods are characterized by more complete detection of mutations across larger genomic regions. They revolutionized the field of genomics, allowing rapid and cost-effective generation of genome-scale sequence data with excellent resolution and accuracy.

Targeted deep sequencing using PCR-based (e.g. TAm-Seq [22,50,51], Safe-Seq [52], Ion AmpliSeq™ [4,53] or capture-based (e.g. CAPP-seq [54]) approaches have been used to sequence specified genomic regions in plasma DNA. The utility of targeted deep sequencing in PC is showed in a recent study describing the clonal architectural heterogeneity at different stages of disease progression.This work evaluated genomic aberrations by sequencing serial plasma and tumor samples from 16 ERG-positive PC patients treated with abiraterone, achieving high coverage with lower input DNA (approximately 6 ng) and allowing applicability across a larger range of patients, including those with lower tumor burden. In addition, combining targeted deep sequencing and read based clonality computations provided timely non-invasive biomarkers for CRPC clinical management [4].

Whole-genome analysis of plasma DNA permits the complete characterization of mutation profiles, without focusing on predefined mutations [51]. Among these methods, there is personalized analysis of rearrangement ends, which allows to identify specific somatic rearrangements in human tumors and, subsequently, to design of PCR-based assays to detect these alterations in plasma DNA, using these alterations for development of tumor biomarkers [39,40]. Some studies have used whole genome sequencing to detect directly somatic chromosomal alterations and copy number aberrations in ctDNA genome-wide [38,40]. The first whole-genome sequencing analysis from plasma DNA of 13 patients with prostate cancer revealed multiple copy number aberrations and chromosomal aberrations [55].

With continued developments in the sensitivity of genomic approaches, NGS techniques will be increasingly able in ctDNA analysis for future management of many tumors, including PC at different disease stages, advancing precision medicine for PC through genomics clinical applications.

6. Application of Circulating DNA in Prostate Cancer

6.1. Early detection: The 2012 recommendations of the United States Preventive Services Task Force [8] and the National Comprehensive Cancer Network (NCCN) Guidelines for Prostate Cancer Early Detection Panel [56] show a decreased mortality in populations screened with PSA, stressing benefits from PC screening and early detection. However, many improvements are being made to enhance powerful tests that maximize early diagnosis of aggressive, but often curable disease, whereas diminishing the identification and treatment of indolent disease. cfDNA could be useful to discriminate between patients with PC and no malignant prostate disease. Many studies have explorated the utility of cfDNA as a marker of tumor dynamics in addition to conventional PSA or imaging techniques. However, the results have been often conflicting according to different cfDNA isolation and detection methods and various clinical setting.

Quantitative and qualitative alterations between patients with diagnosis of PC and nonmalignant lesions, including Prostatic Intraepithelial Neoplasia (PIN) and Benign Prostate Hyperplasia (BPH), have evaluated the role of cfDNA in screening and early detection. Many studies have demonstrated that higher cfDNA levels in PC patients are able to identify patients with malignant disease; however, contrasting results have emerged from other studies making the use of cfDNA a topic of much debate in oncology [25-27,36,57-62] **(Table 1)**.

The first study [27], published in 2004, did not demonstrate significant differences between patients with clinically localized PC and BPH; on the contrary, it showed a significant increase of plasma DNA levels in patients with metastatic PC compared to controls. Another study failed to detect significant differences between patients with PC and benign prostate diseases [26]. Seventy-eight PC patients had a significantly higher level of DNA compared with the control group, but they had significantly lower level of DNA than 74 patients with benign diseases ($P = 0.02$).Consequently, this study suggested that elevated cfDNA levels could not be used as a new non-invasive approach for PC early detection. These data have been confirmed in a subsequent study [25] showing a major cfDNA concentration in BPH patients than PC patients and, a lower cfDNA in healthy donors compared to PC patients ($P < 0.01$), most likely due to the reduced DNase activity in the blood plasma of PC patients

Table 1. Role of circulating free DNA levels for early detection of prostate cancer.

UTILITY OF QUANTITATIVE ALTERATIONS									
YES					NO				
Study	**PC (number setting)**	**Control (number, setting)**	**Source, Method**	**Results**	**Study**	**PC (number, setting)**	**Control (number, setting)**	**Method**	**Results**
Allen et al. [57]	27 (12 high grade PIN, 15 PC)	10 (BPH)	Plasma, PCR	S: 85% Sp: 73% AUC:n.r. P:0.04 (PIN vs HD); P:0.01 (PC vs HD)	Jung et al. [14]	91 (32 pNoMo, 30 pN1M0 29 M1)	93 (30 female controls, 29 HD, 34 BPH)	Plasma, Flurome-tric assay	S:n.s. Sp:n.s. AUC:n.s. P: 0.140*
Papadopoulou et al. [58]	12	13	Plasma, PCR	S: 58% Sp: 92% AUC: 0.708 P: n.r.	Boddy et al. [26]	78	99 (74 benign, 15 low-risk benign, 10 HD)	Plasma, PCR	S: n.s. Sp: n.s. AUC: n.s. P (benign vs. PC): 0.0001; P (PBH vs. PC): 0.02
Chun et al. [59]	142 (local-ized PC)	19 (BPH)	Plasma, Spectropho-tometry	S: n.r. Sp: n.r. AUC: n.r. P: 0.032	Cherepanova et al. [25]	5	52 (22 BPH, 30 HD)	Flurome-tric assay	S: n.r. Sp: n.r. AUC: n.r P: n.s. **
Altimari et al. [60]	64	45 (HD)	Plasma, PCR	S: 80% Sp: 82% AUC: 0.881 P <0.001					
Ellinger et al. [33,34]	173	53 (42 BPH, 11 HD)	Serum, PCR	S: 88% Sp: 64% AUC: 0.824 P <0.001					
Schwarzenbach et al. [36]	69 (PC M1)	12 (PC M0)	Plasma, PCR	S: n.r. Sp: n.r. AUC: n.r P: 0.03					
Wroclawsky et al. [37]	133	33	Plasma, Spectropho-tometry	S: 66.2% Sp: 87.9% AUC: 0.824 P<0.05					
Feng et al. [61]	96	112 (BPH)	Plasma, PCR	S: 73.2% Sp: 72.7% AUC: 0.864 P<0.001					

The failure to distinguish BPH and localized PC may have different causes, including the use of a less sensitive fluorometric assay, or the presence of other diseases within the prostate gland, such as prostatitis, which may be elevating the cell-free DNA level perhaps to a greater degree than that of PC.

Recent studies employed a real-time PCR and demonstrated significant higher DNA levels in PC then in BPH patients and healthy donors with good values of sensitivity and specificity (more than 58% and 64%, respectively) [34,36,57,58,60,61]. Among PCR-based studies, the largest one [34] included 216 patients, whose 173 with PC and 53 controls (11 healthy individuals and 42 with BPH) and had by a sensitivity of 88% and a specificity of 64%. In this study, the analysis of PTGS2 DNA fragment, that biochemically characterizes apoptosis, showed DNA fragment levels in serum of patients with PC significantly increased in comparison to BPH patients and healthy individuals (both P<0.0001). Another important study [60], quantifying by real-time PCR assessment

cfDNA in plasma samples from 64 patients with localized PC and 45 healthy males, showed a better discrimination between PC and healthy subjects with a sensitivity of 80% and a specificity of 82%. It concluded that ctDNA quantification could be a candidate biomarker for early diagnosis correlating with pathologic tumor stage.

A prospective study [59] also detected increased DNA levels in 161 PC patients using spectrophotometry. The median plasma concentration of cfDNA was 267 ng/mL in men with BPH and 709 ng/mL in men with PC. Uni-and multivariate analyses (after controlling for age, total PSA, free/total PSA, prostate volume) suggested that cfDNA was highly accurate and informative predictor (P=0.032 and predictive accuracy 0.643) for the presence of PC on needle biopsy. A recent study [62] of 133 patients affected by PC and 33 controls used also spectrophotometry that confirmed cfDNA as a potential tool for PC diagnosis with sensitivity of 66.2% and specificity of 87.9% and the additional role of cfDNA during follow-up of PC patients.

For the discrimination between PC and benign conditions, the identification of genetic and epigenetic alterations in cfDNA may be also an interesting tool for molecular screening of PC patients. The presence of allelic imbalance, including loss of heterozygosity and microsatellite DNA, has been investigated on circulating DNA of PC patients in PCR-based studies, characterized by a sensitivity ranging from 34% to 57% and specificity from 70% to 100% [35,36,63-65]. Diagnostic information derives also from cell-free DNA hypermethylation, especially the hypermethylation of GSTP1 (Glutathione S-Transferase 1 Pi gene), a detoxifying enzyme present in about 30% of PC patients. Many studies us in gmethylation-specific PCR tests, characterized by a high specificity nearly 100% for the presence of prostatic neoplasia, but by a variable sensitivity ranging from 11% to 100%, suggested that measurement of GSTP1 promoter methylation in plasma, serum, or other samples may complement PSA screening and early detection for PC diagnosis [58,60,64,66-75].

Finally, some studies [34,76] showed also the utility of the DNA Integrity Assay (DIA) as a plasma-based screening tool for the PC detection.In 2006, Hanley et al. [76]evaluatedblood samples patients with biopsy-proven PC prior to prostatectomy (n = 123). He studied three control groups including young men with no history of cancer (group 1, n = 20); cancer-free post-prostatectomy patients (group 2, n = 25), and patients with a negative prostate biopsy (group 3, n = 22). A baseline cutoff was used for individual DNA fragment lengths to fix a DIA score for each patient sample. PC patients (86 of 123; 69.9%) had a strongly positive DIA score. The DIA results from control groups 1, 2, and 3 showed specificities of 90%, 92%, and 68.2%, respectively. So this study concluded that DIA could detect approximately 70% of PC patients with a specificity of 68.2% to 92%, a range similar to that currently accepted for PSA (60-70%).

6.2. Prognostic role: Recent evidences highlighted the clinical importance of increased levels of cfDNA and presence of genetic and epigenetic alterations as an adverse prognostic marker in PC patients. In 2004, Jung et al. [27] published the first study that emphasized cfDNA concentration as a survival predictor in PC patients. It showed survival curves of 91 PC patients (stage pN0M0, n=32; stage pN1M0, n=30; stage M1, n=29) according to the plasmatic concentrations of DNA, total PSA, and osteoprotegerin as a marker for bone metastases. The association between plasma DNA and the survival was similarly strong as with PSA but only in patients with distant metastases.

Lately, a retrospective study of 59 taxane-based chemotherapy treated PC men [77] showed the role of cfDNA level as a prognostic marker according to PSA response and survival. Patients with a PSA decline of more than 80 % had a lower cfDNA concentration compared to patients with the least PSA decline of less than 30%. In addition, cfDNA level was associated with a significantly longer survival rate (31 months in patients with cfDNA levels of less than 55ng/μl compared to 17 months in patients with higher concentrations) (p=0.03). Another recent work [78] aimed to clinically qualify baseline and on-treatment cfDNA levels as biomarkers of patient outcome treated with taxane chemotherapy. The authors analyzed blood samples prospectively collected from 571 mCRPC patients participating in two phase III clinical trials, FIRSTANA (NCT01308567) and PROSELICA (NCT01308580). They identified that baseline log10 cfDNA concentration correlated with shorter radiographic PFS (HR 1.54; 95% CI 1.15–2.08; P=0.004), and shorter OS on taxane therapy (HR 1.53; 95% CI1.18–1.97; P=0.001), and cfDNA concentration before starting docetaxel or cabazitaxel was an independent prognostic variable on multivariable analyses for PFS and OS in both first- and second-line chemotherapy settings. Patients with a PSA response experienced a decline in log10 cfDNA levels during the first four cycles of treatment.

Survival analysis from a phase I exploratory cohort of 75 men with CRPC and a phase II independent validation cohort of 51 CRPC men [74] demonstrated that detection of plasma methylated GSTP1 in CRPC patients was associated with a shorter OS (HR 4.2, 95% CI 2.1-8.2; P<0.0001). The 2-year survival for men with no detectable plasma mGSTP1 was 71% compared with 23% for men with detectable plasma methylated GSTP1 levels.

The prognostic value of cfDNA and circulating DNA fragments of apoptotic origin (i.e., PTGS2 DNA fragment that biochemically characterizes apoptosis) has also been showed in PC men as predictor of PSA recurrence after radical prostatectomy [34,79] and of biochemical recurrence free survival during follow-up in plasma

samples of 133 PC patients collected prospectively every 3 months for 2 years (P = 0.048) [62].

In addition, cell-free DNA hypermethylation of GSTP1, Ras association [RalGDS/AF-6] domain family member 1 (RASSF1A) and retinoic acid receptor β, variant 2 (RARB2) was also correlated with the Gleason score, tumor stage, extent of metastasis [64,69,74].

Currently, the identification of genetic aberrations in cfDNA has emerged as a promising prognostic biomarker. Recent studies [4,80-86] demonstrated a significant correlation between aberrations (copy number variations and point somatic mutations) of Androgen Receptor (AR) detected in blood and treatment outcome in CRPC patients treated with second generation hormonal drugs, abiraterone or enzalutamide. One of the most recent biomarkers studies [83] was aimed to clinically qualify AR status measurement in plasma DNA from 265 CRPC patients (191 in the primary cohort and 94 in the secondary cohort), using an optimized multiplex droplet digital PCR (ddPCR) assay in pre- and post-chemotherapy CRPC.AR gain was observed in 10 (14%) chemotherapy-naïve and 33 (34%) post-docetaxel patients and was associated with a worse OS (HR 3.98; 95% CI, 1.74-9.10; P<0.001 and HR 3.81; 95% CI, 2.28-6.37; P<0.001 respectively), PFS (HR, 2.18; 95% CI, 1.08-4.39; P = .03, and HR, 1.95; 95% CI 1.23-3.11; P=0.01, respectively) and rate of PSA decline ≥50% (Odds ratio (OR) 4.7; 95% CI, 1.17-19.17; P=0.035 and OR 5.0; 95% CI 1.70-14.91; P=0.003 respectively). AR mutations (2105T>A (p.L702H) and 2632A>G (p.T878A)) were observed in 8 (11%) post-docetaxel but no chemotherapy-naïve abiraterone-treated patients and were also associated with worse OS (HR 3.26; 95% CI, 1.47-not reached; P=0.004). These data were confirmed in the secondary cohort of the study and plasma AR resulted an independent predictor of outcome on multivariate analyses in both cohorts.

6.3. Predictive role and treatment resistance: Treatment resistance may develop mainly for an incessant evolving spectrum of genetic and epigenetic alterations within the tumor under the selective pressure of therapy. The analysis of ctDNAcould integrate invasive biopsy approaches to evaluate noninvasively from plasma DNAgenomic alterations related to acquired drug resistance in advanced cancers [51]. Beyond the prognostic role, recent studies [4,79,83] have demonstrated the association between clinical, biochemical and radiographic response and genomic aberrations, especially involving AR, in ctDNA of CRPC patients treated with novel hormonal therapies, as abiraterone and enzalutamide [4,80-86]. Carreira et al. [4] sequenced serial plasma and tumor samples from 16 ERG-positive lethal PC patients and identified genomic lesions, including common tumor deletions, AR copy number gain, functionally active AR mutations. This study demonstrated a temporal association between clinical progression and emergence of AR mutations activated by glucocorticoids in about 20% of patients progressing on abiraterone and prednisolone or dexamethasone. A subsequently study [80] of 53 consecutive CRPC patients treated with abiraterone after chemotherapy showed that AR or CYP17A1 amplification in cfDNA led to early progression disease that occurred within 4 months of the start of abiraterone treatment. So, the authors concluded that CNVs of AR and CYP17A1 genes were predictive of early resistance to therapy. Azad et al. [84] confirmed the utility of cfDNA to identify therapeutic resistance to novel hormonal drugs. They used array Comparative Genomic Hybridization (aCGH) for chromosome copy number analysis and NGS of exon 8 of the AR in cfDNA samples from 62 metastatic CRPC patients stopping abiraterone (n=29), enzalutamide (n=19) or other agents (n=14) due to disease progression. The results from aCGH showed that AR amplification was significantly more frequent in patients progressing on enzalutamide than on abiraterone or other agents (53% vs. 17% vs. 21%, P=0.02). Missense AR exon 8 mutations were detected in 11/62 patients (18%), including enzalutamide- and abiraterone-resistant patients.

In addition, a recent prospective study [74] showed the associations between detectable plasma mGSTP1 prior to chemotherapy and no response to chemotherapy (docetaxel or mitoxantrone) and so epigenetic alteration could be also a potential surrogate therapeutic efficacy marker for chemotherapy.

6.4. Role in follow-up: In this era of survival prolonging drugs in the treatment of PC patients, the identification of noninvasive biomarkers for monitoring patients during follow-up is essential. Wroclawsky et al. [62] showed a significant shorter biochemical recurrence free survival for patients with at least one value of cfDNA greater than140 ng/mL during a mean follow-up of 13.5 months (P=0.048).

The utility of cfDNA in follow-up of PC patients has emerged also from the relation between the measurement of cfDNA and radiographic imaging. Kwee et al. [87] explored cfDNA content in relation to fluorine-18 Fluorocholine (FCH) positron emission tomography/computed tomography (PET/CT) in 8 CRPC patients receiving docetaxel-based chemotherapy. Serial cfDNA samples were assessed by microfluidic electrophoresis, quantified by real-time PCR, and compared with results from FCH PET/CT scans, used for whole-body measurement of tumor activity results. Promoter methylation of two PC-associated genes, GSTP1 and RARB2, was evaluated by methylation-specific PCR. Plasma cfDNA concentrations increased significantly after one and three treatment cycles, respectively (P=0.001).

GSTP1 and/or RARB2 promoter methylation was identified in all pretreatment samples. The appearance of large (200 bp-10.4 kb) cfDNA fragments and loss of methylation at GSTP1 and/or RARB were observed after treatment. Tumor activity on PET/CT correlated significantly with cfDNA levels and PET/CT tumor response had significantly lower before therapy cfDNA levels than those who did not (P=0.03). These preliminary data exploratory examinated the translational significance of cfDNA as therapeutic response marker, but larger studied are warranted.

7. Comparison between Circulating DNA in Plasma and in Other Body Fluids

Like blood, urine and semen represent a source of cfDNA which can be gained in a more noninvasive manner and could therefore be useful as an easy substrate for biomarker measurement in PC patients. In 2013, the pilot study [88] investigating the potential role of urinary cfDNA in early PC diagnosis. The authors analyzed urinary cfDNA fragments longer than 250 bp in three regions frequently amplified in solid tumors, including PC: c-Myc (8q24.21, HER2 (17q12.1), and BCAS1 (20q13.2) and concluded that urine DNA integrity could distinguishing between PC patients and healthy individuals with an accuracy of about 80%, similar to that observed previously for bladder cancer [89,90]. However, this finding of longer cfDNA fragments in urine of PC patients was apparently in contrast to the presence of short DNA fragments in blood, as shown in previous work [34]. Conversely, the same profile of GSTP1 gene promoter methylation has been demonstrated in the pool of circulating and extracellular DNA from the blood and urine of PC patients. This profile differs from those characteristic of healthy donors and BPH patients, confirming that the quantification of cfDNA in different body fluids from blood, such as urine and ejaculates, could represent a diagnostic tool characterized by the ability to detect PC and lower the rate of unnecessary biopsies [66,91].

7.1. Comparison between cfDNA and other circulating biomarkers: Over cfDNA, other cell-free nucleic acids, including mRNA and microRNA (miRNA) are released and circulate in the blood of PC patients, probably from the same apoptotic or necrotic cells originating cfDNA. Changes in the levels of other circulating nucleic acids have been associated also with tumor burden and malignant progression. RNA released into the blood stream is more stable in spite of the increased amounts of circulating RNases. Consequently, RNA may be protected from degradation by its packaging into exosomes, such as microparticles, microvesiclesor multivesicles, which are shed from cellular surfaces into the circulation. For the detection and identification of RNA microarray technologies or reverse transcription quantitative real-time PCR are used [13] **(Figure1)**.

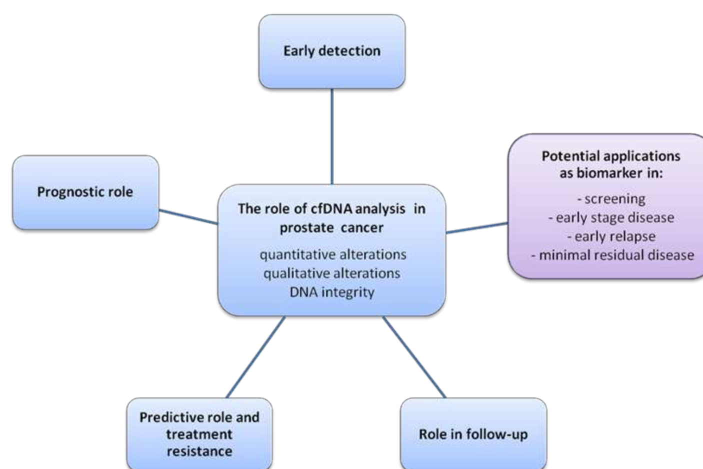

Figure 1. Current and future potential applications of circulating DNA in prostate cancer patients.

Prostate-derived exosomes (also called prostatosome) are endosome-derived vesicles with a diameter between 50 and 150 nm that contain cytoplasmic content (proteins and RNAs) encapsulated by a cholesterol rich phospholipid membrane. It is possible to detect exosomes in blood, urine and semen [92]. Some studies suggested the diagnostic and prognostic role of prostatosomes, revealing a higher number of exosomes in blood of PC patient compared to men with no disease and correlating exosome number with Gleason score [93,94]. In addition, recent studies revealed that the detection of a splice variant of AR (AR-V7) in plasma-derived exosomal RNA strongly predicted resistance to hormonal therapy in metastatic CRPC patients, making exosomes a potential source of clinically relevant biomarkers [94].

Among circulating biomarkers of PC, one area of expanding investigation is CTCs, rare cells that are shed from primary and metastatic tumor deposits into the peripheral circulation [95]. Enumeration of CTCs before and after therapy has shown that CTC burden correlates with outcome in CRPC patients [96-98]. Moreover, studies have demonstrated the potential of molecular analysis of CTCs in monitoring and predicting response to therapy in patients [99].

Both CTCs and ctDNA offer snapshots of genomic alterations in primary tumors and metastases at various stages during the course of disease. However, there are yet some limits for the use of CTC and ctDNA as liquid biopsies. Because CTCs are very rare cells, capturing them depends on rather sophisticated equipment, while plasma DNA is easily obtained, even tumor DNA fragments are diluted with various amounts of DNA from normal cells, which may hinder analysis [100]. Despite these limitations, combining both ctDNA and CTC analyses as a blood biomarker panel results in a higher diagnostic accuracy compared to the use of a single

marker. Concerning the heterogeneity of the disease, the future prospect will have to be focused on the combination of different circulating biomarkers to improve PC management.

Recently, it has emerged that detection of AR-V7 in CTCs may represent one such treatments election marker in men with metastatic CRPC associated with a lack of benefit of abiraterone and enzalutamide [101], but no with taxane chemotherapy [102,103].

8. Conclusions and Future Perspectives

Despite some limitations of using cfDNA related mainly to the sensitivity of measuring ctDNA using plasma versus serum [34] and the different detection methods [76], and lower concentration of ctDNA in early disease, plasma DNA is a clinically relevant marker offering new chances for management of PC patients adding a new helpful tool for diagnosis, staging and prognosis **(Figure 2)**.

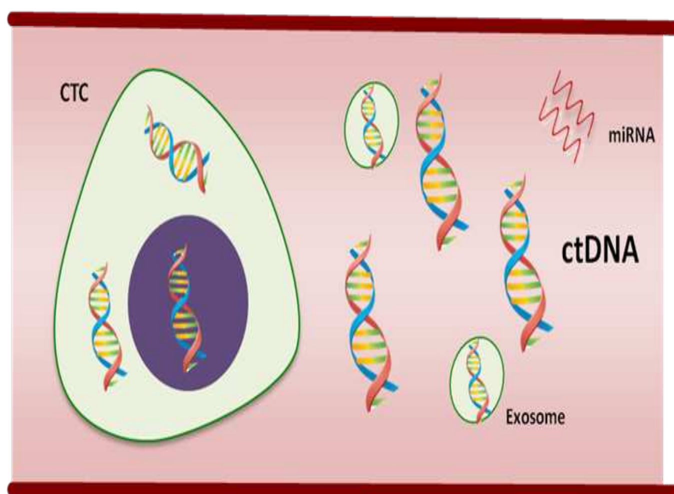

Figure 2. Circulating tumor DNA and other circulating biomarkers in prostate cancer.

All mCRPC have tumor lesions to detect in plasma matching perfectly with tumor tissue.

Future studies are also aimed at the improvement of cancer screening or as diagnostic biomarkers in early stage disease. In fact, there are ongoing some clinical trials carried out on early stages of PC. One study has main objective to evaluate genetic testing in predicting biomarkers of recurrence in PC patients undergoing surgery (NCT00977457) and two phase 3 trials performing plasma DNA analysis in PC patients after radiotherapy (NCT01411332 and NCT01411345). Moreover, next studies could also include patients with localized PC patients receiving neoadjuvant chemotherapy predicting pathological residual disease that usually correlates with poor prognosis, as already shown in other tumors. In the complex scenario of prostate tumor, the detection of ctDNA may become readily available for routine clinical decision making. Nowadays, circulating biomarkers are correlated with new imaging techniques, that do not only evaluate tumor extent and distribution, but it can also detect biologic characteristics of all lesions rather than those of a single biopsy or blood draw. A recent work [104] made a direct comparison between circulating AR CNV and 18F-Fluorocholine (FCH) uptake on PET/CT in patients with metastatic CRPC. AR CNV was determined by digital droplet PCR and Taqman on pre-treatment plasma from 80 patients with metastatic CRPC progressing after docetaxel treated with abiraterone (n=47) or enzalutamide (n=33). For all patients, an FCH PET/CT scans was performed. Plasma AR gain was significantly correlated with tumor metabolic activity. In addition, multivariate analysis revealed that AR CNV and FCH PET/CT values were associated with both shorter PFS and OS. These evidences suggested that choline uptake is higher in AR gained cancers, introducing the possibility of identifying this molecularly distinct group using non-invasive imaging, also obtaining further biologic tumor information performing new tracers, such as Prostate-Specific Membrane Antigen (PSMA).

In conclusion, the utility of plasma DNA derives from preclinical and clinical observations and from the integration of circulating and imaging markers that require, however, larger prospective trials with a clinically meaningful impact in PC patients.

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