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Circulating biomarkers in prostate cancer

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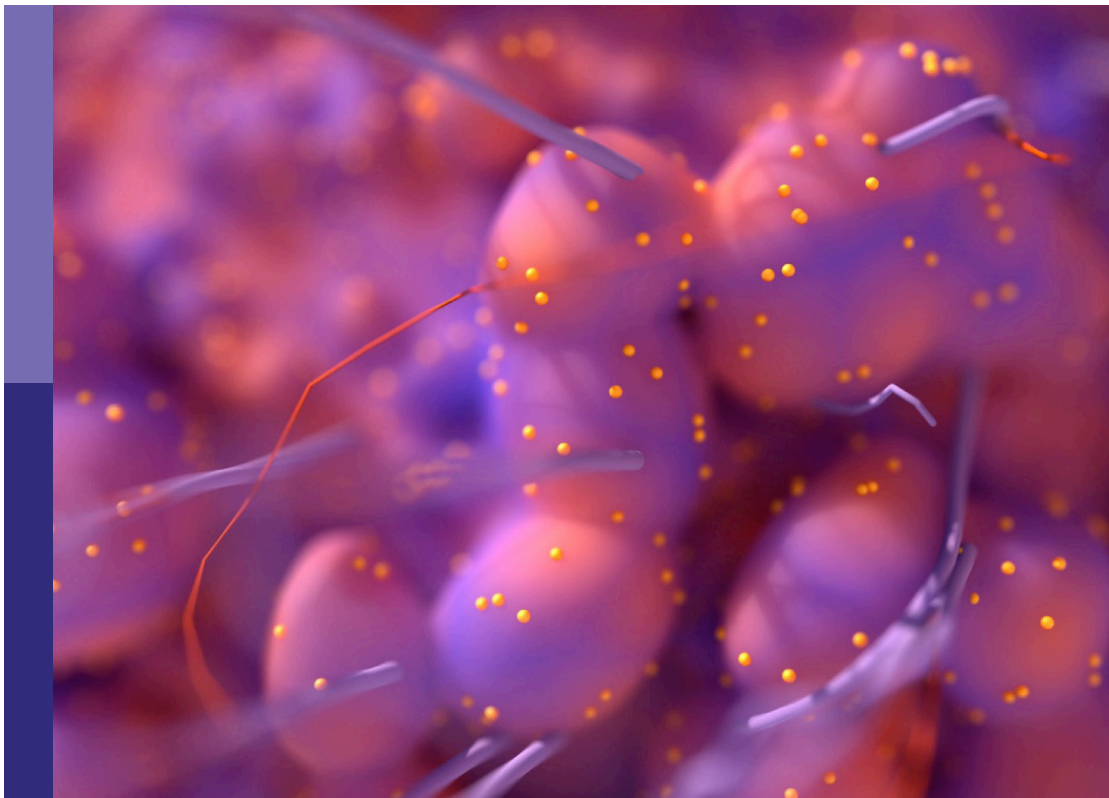

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Circulating biomarkers in prostate cancer

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Editorial: Circulating biomarkers in prostate cancer

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Editorial on the Research Topic
Circulating biomarkers in prostate cancer

Prostate cancer is the fifth leading cause of cancer death worldwide and biomarkers with improved specificity and sensitivity are in urgent need for precise prognosis and diagnose. Circulating biomarkers, compared to biomarkers detected in solid tissues, consist of a diverse array of components found in the blood or urine, serving as diagnostic and prognostic indicators and aiding in the selection of effective drugs. These components include substances routinely measured in clinical peripheral blood analyses, such as blood cell constituents, electrolytes, and proteins for example albumin, alkaline phosphatase (ALP), and prostate cancer specific antigen (PSA). Although commonly utilized across various cancer types, these biomarkers are nonspecific and often serve solely as prognostic markers, lacking the clinical utilizations in personalized medicine. Recent advancements in liquid biopsy technology, allowing for the analysis of cancer-derived cells and molecules from peripheral blood, offer a less invasive and cost-effective alternative to traditional tissue biopsies. This Research Topic titled "*circulating biomarkers in prostate cancer*" comprises a collection of 2 systematic reviews, 3 (mini)reviews and 4 original articles, focusing on both conventional circulating biomarkers and biomarkers, defined by liquid biopsy, such as circulating tumour cells (CTC), cell free tumour DNA (ctDNA), extracellular vesicles(exosomes).

Traditional circulating biomarkers

The geriatric nutritional risk index (GNRI), a conventional non-specific biomarker combining body weight and serum albumin levels, evaluates the nutritional status in cancer patients and proves valuable in predicting the prognosis in multiple cancer types (1, 2). Wu and Ye, through a meta-analysis, demonstrated the significance of pretreatment serum albumin as a circulating biomarker influencing the prognosis of urological cancer patients, and lower pretreatment GNRI predicts worse overall survival. Notably, PSA and its derivatives remain key traditional circulating biomarkers for early prostate cancer diagnosis, however PSA specificity is low. Ren et al. developed a predictive model for cancer positivity rates, incorporating various circulating biomarkers, including (f/T) PSA,

blood inflammatory indicators hemoglobin to platelet ratio (HPR), neutrophils (NEUT), alongside clinical background (age) and multiparametric prostate MRI imaging PI-RADS (Prostate Imaging–Reporting and Data System) score. Augmenting image diagnosis with circulating biomarkers enhances diagnostic efficiency and accuracy.

Biomarkers detected by liquid biopsy

Liquid biopsy demonstrates superiority in monitoring disease longitudinally and deciphering tumour evolutions. CTCs, ctDNA, exosomes (specially exosomal RNA), are commonly defined as liquid biopsy, offering insights into the genome and epigenome dynamics during prostate cancer progression. Lo et al. reviewed the genome-wide studies in prostate cancer focusing on cell free methylome (Figure 1) (3).

Molecular expression profiles and genetic abnormalities in CTCs, along with CTC count variations before and after treatment, provide powerful indicators of therapeutic efficacy (4). Furthermore, combining CTC measurements with clinical biomarkers, such as blood ALP levels and specific molecular expressions, enhances prognostic accuracy. In this Research Topic, Davies et al. captured pre-docetaxel treatment CTC from both metastatic castration resistant prostate cancer (mCRPC) and castration-sensitive PC (mHSPC), characterized CTC subtypes (epithelial, mesenchymal and EMTing) and gene expression analysis revealed the expression of docetaxel resistant gene ADAMTS1 and EMT transcription factors ZEB1 and SNAI1. Combination of total CTC number with PSA and ALP predict lack of partial response in mCRPC.

Homology recombination repair (HRR), the frequently altered DNA damage repair (DDR) mechanism in prostate cancer, involves genes like BRCA2. Poly ADP-ribose polymerase (PARP) inhibitors, such as olaparib and talazoparib, show antitumor activity in mCRPC with BRCA gene alterations (5, 6). Genomic testing for HRR gene mutations, especially using ctDNA, is crucial for guiding PARP inhibitor treatment. Liquid biopsy, particularly ctDNA testing, overcomes the limitations of tissue testing and allows longitudinal monitoring for emerging alterations and resistance mutations during disease progression. Catalano et al. reviewed current therapeutic indications in prostate cancer patients with DDR deficiency and provided the recommendations for germline and somatic -genomic testing in advanced PC and advantages of liquid biopsy in clinical utilities.

Dincman et al. emphasized the detection capabilities of ctDNA for abnormalities in various prostate cancer-related genes and highlighted the association of gene amplifications with disease features. Liquid biopsy, particularly plasma copy number analysis, aids in clinical decision-making for selecting appropriate therapeutic interventions during mCRPC treatment.

Long non-coding RNAs (lncRNAs), acting as regulatory transcripts of AR signaling and other important signaling pathways, play essential roles in prostate cancer development and treatment resistance. Urinary PCA3 (Prostate cancer antigen 3, a type of lncRNA) has been recognized as a biomarker for PC detection by U.S. Food and Drug Administration (FDA). In this Research Topic, Taheri et al. systematically reviewed the oncogenic roles of lncRNAs in prostate cancer pathogenesis and diagnosis and further emphasized the potentials and needs of early detection of prostate cancer with non-invasive methods, specially from blood.

The androgen receptor splice variant-7 (AR-V7) status, evaluated by both tissue and liquid biopsies, is highlighted as a potential biomarker for predicting drug resistance to enzalutamide and abiraterone, but not taxane chemotherapy in mCRPC (7–9). Zhao et al. performed a cumulative analysis on the relationship between AR-V7 status and the risk of CRPC, which only focused on the AR-V7 status in tissue biopsy.

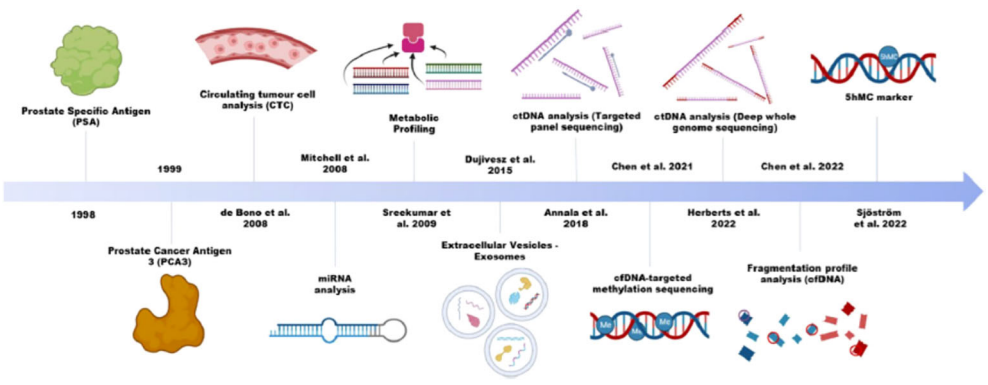

FIGURE 1
Application of various liquid biopsy technologies in prostate cancer.

Mo et al. explored Gene Expression Omnibus (GEO) database to discover potential prostate cancer associated biomarkers and identified 6 possible diagnostic markers, among which AOX1 could potential sever as a prognostic marker, further studies on its clinical value as a circulating biomarker are warranted.

Summary

Ongoing research utilizing liquid biopsy for prostate cancer, characterized by minimal invasiveness and periodic analysis, is anticipated. Advancements in CTC and ctDNA detection technologies, coupled with whole genome sequencing, will likely continue, providing new insights into prostate cancer biology. These developments will contribute to the discovery of more effective treatments and targeted molecules for prostate cancer.

Author contributions

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Development and validation of a predictive model for diagnosing prostate cancer after transperineal prostate biopsy

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Objective: This study aimed to develop and validate a nomogram to predict the probability of prostate cancer (PCa) after transperineal prostate biopsy by combining patient clinical information and biomarkers.

Methods: First, we retrospectively collected the clinicopathologic data from 475 patients who underwent prostate biopsy at our hospital between January 2019 to August 2021. Univariate and multivariate logistic regression analyses were used to select risk factors. Then, we established the nomogram prediction model based on the risk factors. The model performance was assessed by receiver operating characteristic (ROC) curves, calibration plots and the Hosmer–Lemeshow test. Decision curve analysis (DCA) was used to evaluate the net benefit of the model at different threshold probabilities. The model was validated in an independent cohort of 197 patients between September 2021 and June 2022.

Results: The univariate and multivariate logistic regression analyses based on the development cohort indicated that the model should include the following factors: age (OR = 1.056, p = 0.001), NEUT (OR = 0.787, p = 0.008), HPR (OR = 0.139, p < 0.001), free/total (f/T) PSA (OR = 0.013, p = 0.015), and PI-RADS (OR = 3.356, p < 0.001). The calibration curve revealed great agreement. The internal nomogram validation showed that the C-index was 0.851 (95% CI 0.809-0.894). Additionally, the AUC was 0.851 (95% CI 0.809-0.894), and the Hosmer–Lemeshow test result presented p = 0.143 > 0.05. Finally, according to decision curve analysis, the model was clinically beneficial.

Conclusion: Herein, we provided a nomogram combining patients' clinical data with biomarkers to help diagnose prostate cancers.

KEYWORDS

biopsy, biomarkers, nomogram, diagnosis, prostate cancer

Introduction

Prostate cancer (PCa) has the second highest incidence of all malignant tumors in men worldwide and ranks first in the incidence of male tumors in more than half of countries. Additionally, the mortality rate of PCa is the fifth highest among male cancers (1).PCa is a highly heterogeneous tumor. Cases with higher Gleason score (≥7), defined as clinically significant PCa (csPCa), usually show a high aggressiveness and a tendency for rapid progression. In contrast, the PCa patients with lower Gleason score progressed slowly (2, 3).Prostate-specific antigen (PSA) is the most used early detection marker for prostate cancer. However, the PSA specificity is weak (20-40%), and other conditions, such as benign prostatic hyperplasia (BPH), can affect PSA levels (4). Prostate biopsy is now the standard for prostate cancer diagnosis (5). Meanwhile, biopsy is an invasive operation, and systematic biopsy often shows false negative results. Besides, increasing the puncture points can lead to complications such as bleeding, urinary retention and infection (6).

Therefore, PCa screening based on PSA level as the sole indication for prostate biopsy lacks specificity and may lead to unnecessary biopsy. As such, clinical practice urgently needs new methods for early, noninvasive screening of prostate cancer. In recent years, several blood biomarkers (2, 3, 7–9), urine biomarkers (2, 3, 7, 10, 11) and multiparametric magnetic resonance imaging (mpMRI) (11, 12) have been developed to predict PCa. Several of PSA derivatives, especially free PSA/TPSA (f/TPSA), and PSA density (PSAD) have been demonstrated as promising biomarkers (8, 9). Urine metabolomics in the early detection, risk phase, and treatment prognosis of prostate cancer studies have been reported (2, 3, 7, 10, 11). mpMRI has also been validated as a reliable radiological technique for prostate cancer diagnosis, targeted biopsy, tumor staging, and monitoring (11, 12).

Current studies have shown that tumorigenesis and development are tightly linked with inflammation, and neutrophils are associated with the occurrence and development of human cancers, including lung (13) and breast (14) cancers. Additionally, the clinical significance of the hemoglobin to platelet ratio (HPR) has been demonstrated in colon cancer (15) and renal cell carcinoma (16). Furthermore, the clinical significance of neutrophil to lymphocyte ratio (NLR), platelet to lymphocyte ratio(PLR),monocyte-to-lymphocyte ratio (MLR) and systemic immune-inflammation index (SII) has also been confirmed (17, 18).

Abbreviations: AJCC, American Joint Committee on Cancer; AUC, Area under ROC curve; f/T, fPSA to TPSA ratio; HPR, Hemoglobin to platelet ratio; MLR, monocyte-to-lymphocyte ratio; NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; PSA, Prostate-specific antigen; PV, Prostate volume; PSAD, PSA density; PI- RADS, Prostate Imaging-Reporting and Data System; ROC, receiver operating characteristic; SII, systemic immune-inflammation index.

Therefore, in the present study, we developed and validated a PCa prediction model combining inflammatory biomarkers with the clinical data of patients. Based on our current results, the model can be used to assist in the screening of PCa.

Materials and methods

Patients' data

First, 752 patients who underwent transperineal prostate biopsy in our institution from January 2019 to June 2022 were enrolled, and 672 patients were finally included. The flowchart of patients enrolled is shown in Figure 1. Then, patients were separated into two groups according to the date of their prostate biopsy: development cohort: 475 patients who underwent prostate biopsy at our center between January 2019 to August 2021; validation cohort: 197 consecutive patients who received the same operation from September 2021 and June 2022.The pathological results of each patient were determined by the same pathologist based on the 8th American Joint Committee on Cancer (AJCC).

The inclusion criteria were as follows: man with abnormal PSA levels

(PSA>4ng/ml) or with suspicious lesions on imaging(PI-RADS score≥3) were recruited. The population included first-time biopsy and those with previous negative biopsy. All patients underwent MRI before biopsy, and prostate biopsy performed *via* ultrasound guidance. The interval between MRI and biopsy was less than 15 days.

Variables

Demographic and laboratory test results were retrospectively retrieved from our medical system. The HPR was calculated as the hemoglobin to platelet ratio. NLR was defined as neutrophil count divided by lymphocyte count, PLR was defined as platelet count divided by lymphocyte count, SII= (neutrophil × platelet)/lymphocyte. MLR was calculated as monocyte-to-lymphocyte count. The prostate volume (PV) was estimated with the MRI-based modified ellipsoid formula: 0.523*(max width × max length × max height). The PSA density (PSAD) was calculated by dividing the TPSA level by the PV. f/T (calculated as the fPSA to TPSA ratio). The PI-RADS score (1–5) was evaluated by specialists based on the T2WI, DWI, and DCE, according to the Prostate Imaging-Reporting and Data System version 2 (PI-RADS v. 2).

Biopsy methods

Patients who underwent prostate biopsy should present PSA > 10 ng/ml or PSA 4-10 ng/ml combined with suspicious lesions on imaging(PI-RADS score≥3) or normal PSA level combined with

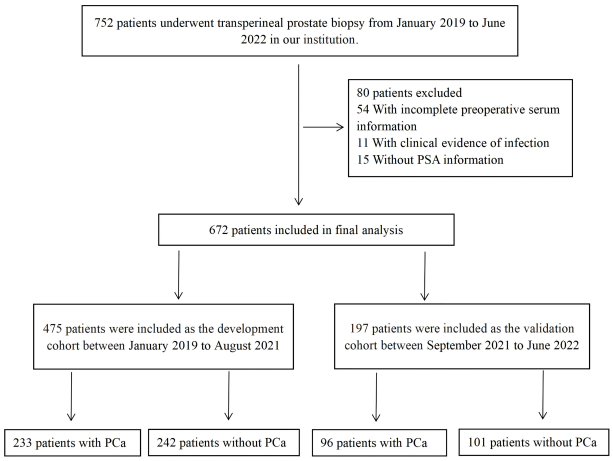

FIGURE 1
The flowchart of patients enrolled in this study. PSA, prostate specific antigen; PCa, prostate cancer.

imaging suspicious lesions(PI-RADS score≥3). Some patients underwent a second biopsy because their repeat PSA suggested suspicious prostate cancer. All operations were carried out by the same specialist doctor at our institute. Under local anesthetic, a systematic 13-core or 3 (Targeted) + 12 (systematic)-core prostate biopsy was performed *via* ultrasound guidance.

Statistical analysis

Data were analyzed using SPSS 23.0 statistical software (IBM SPSS INC., Chicago, USA) and R software (v. 4.1.3 - Institute of Statistics and Mathematics, Vienna, Austria).The reported statistical significance levels were two-sided, with p<0.05.Continuous variables with normal distribution were presented as means ± standard deviations (SD) and were analyzed by independent sample *t*-tests. Non-normal continuous variables were presented as medians (interquartile ranges) and were analyzed by the Mann–Whitney U-test. Categorical variables were presented as numbers (percentages). The correlations between categorical variables were analyzed using Pearson's or Continuity Corrected χ^2-test.

Univariate logistic regression analysis was used to investigate independent risk factors(P<0.05) associated with PCa in the development cohort. Then, a multivariate logistic regression analysis was performed for all significant risk variables, using backward stepwise regression to select prostate cancer risk predictors(P<0.05).A nomogram prediction model was established based on the risk factors selected by multivariate analysis.

The discrimination performance of the model was assessed by the area under the receiver operator characteristic curve (AUC). Calibration of the nomogram was evaluated using calibration plots (bootstrap method, 1000 repetitions) and Hosmer-Lemeshow test (P>0.05 indicates good agreement). The DCA curve was used to evaluate the net benefit ratio of the model at different probability thresholds. The performance of the nomogram was tested in an independent validation cohort divided by time.

Results

Baseline characteristics

A total of 475 patients were enrolled in the development cohort, and 197 were included in the validation cohort. The demographic and clinicopathological data of patients were presented in Table 1. A total of 233 (49.05%) patients in the development cohort and 96 (48.73%) in the validation cohort had PCa. Compared to the non-PCa group, the PCa group had a lower preoperative NEUT(neutrophils), HGB(hemoglobin), higher PLT(platelet), and lower HPR (Supplementary Figure 1).

In the development cohort, PCa patients were older (71.19 ± 8.66 years) than the control group (66.62 ± 8.60 years). The average TPSA, fPSA, and PSAD of PCa patients were higher compared to non-PCa patients. The f/T distribution was as follows: 0.14 (0.09-0.20) for PCa patients and 0.19 (0.13-0.25) non-PCa patients. Moreover, the distribution of coronary heart disease, ASA score, and PI-RADS scores significantly differed between the two groups. In the validation cohort, the differences between the groups of variables were consistent with the development cohort (Table 1).

TABLE 1 Characteristics and blood biomarkers of patients in this study.

Variable	Overall(n=672)			Development Cohort(n=475)			Validation Cohort(n=197)		
	PCa group n=329	non PCa group n=343	P value	PCa group n=233	non PCa group n=242	P value	PCa group n=96	non PCa group n=101	P value
Age (years)	71.64±8.48	66.89±8.38	<0.001	71.19±8.66	66.62±8.60	<0.001	72.74±7.94	67.53±7.83	<0.001
Hypertension						0.678			0.044
No	149 (22.2)	165 (24.5)	0.465	116 (49.8)	116 (47.9)		33 (16.7)	49 (24.9)	
Yes	180 (26.8)	178 (26.5)		117 (50.2)	126 (52.1)		63 (32.0)	52 (26.4)	
Diabetes mellitus			0.173			0.095			0.938
No	281 (41.8)	305 (45.4)		197 (84.5)	217 (89.7)		84 (42.6)	88 (44.7)	
Yes	48 (7.1)	38 (5.7)		36 (15.5)	25 (10.3)		12 (6.1)	13 (6.6)	
Coronary heart disease			<0.001			0.002			0.006
No	263 (39.1)	312		188 (80.7)	219 (90.5)		75 (38.1)	93 (47.2)	
Yes	66 (9.8)	31		45 (19.3)	23 (9.5)		21 (10.6)	8 (4.1)	
BMI (kg/m2)	23.01±3.31	23.25±2.96	0.311	22.86±3.24	23.17±2.90	0.278	23.37±3.47	23.46±3.08	0.842
ASA score 1/2/3/4	2.00 (2.00,3.00)	1.00 (1.00,2.00)	<0.001	2.00 (2.00,3.00)	2.00 (1.00,3.00)	<0.001	2.00 (2.00,3.00)	2.00 (1.00,2.00)	<0.001
Hematuria			0.269			0.323			0.592
No	306 (45.5)	311 (46.3)		220 (94.4)	223 (92.1)		86 (43.7)	88 (44.7)	
Yes	23 (3.4)	32 (4.8)		13 (5.6)	19 (7.9)		10 (5.1)	13 (6.5)	
History of biospy			0.467			0.347			1.000
No	319 (47.5)	329 (48.9)		227 (97.4)	232 (95.9)		92 (46.7)	97 (49.2)	
Yes	10 (1.5)	14 (2.1)		6 (2.6)	10 (4.1)		4 (2.0)	4 (2.0)	
History of prostate surgery			0.784			0.711			1.000
No	316 (47.0)	328 (48.9)		224 (96.1)	231 (95.5)		92 (46.7)	97 (49.2)	
Yes	13 (1.9)	15 (2.2)		9 (3.9)	11 (4.5)		4 (2.0)	4 (2.0)	
Family history of PCa			0.956			0.966			1.000
No	325	340		230	240		95	100	
Yes	4	3		3	2		1	1	
NEUT (10^9/L)	3.87±1.36	4.42±1.81	<0.001	3.89±1.44	4.30±1.67	0.005	3.83±1.17	4.72±2.08	<0.001
LYM (109/L)	1.67±0.61	1.75±0.61	0.099	1.66±0.59	1.73±0.60	0.196	0.65±0.07	0.63±0.06	0.306
MO (10^9/L)	0.43±0.18	0.45±0.16	0.171	0.43±0.14	0.45±0.16	0.150	0.24±0.02	0.17±0.02	0.013
HGB (g/L)	130.90±19.65	137.46±15.35	<0.001	131.03±20.49	135.96±16.19	0.004	130.60±17.53	141.04±12.46	<0.001
PLT (10^9/L)	191.29±59.39	166.73±49.68	<0.001	190.01±59.31	166.70±54.67	<0.001	194.20±59.80	166.82±35.18	<0.001
NLR	2.61±1.66	3.04±3.40	0.036	2.64±1.84	2.83±1.74	0.256	1.15±0.12	5.64±0.56	0.077
PLR	127.17±56.33	107.12±59.25	<0.001	112.80±49.33	118.64±51.62	0.208	59.57±6.08	80.33±7.99	0.065

(Continued)

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TABLE 1 Continued

Variable	Overall(n=672)			Development Cohort(n=475)			Validation Cohort(n=197)		
	PCa group n=329	non PCa group n=343	P value	PCa group n=233	non PCa group n=242	P value	PCa group n=96	non PCa group n=101	P value
SII	503.58±398.30	496.66±514.78	0.846	509.94±437.82	462.97±286.18	0.169	281.56±28.74	836.42±83.23	0.322
MLR	0.28±0.15	0.29±0.22	0.532	0.28±0.11	0.29±0.14	0.478	0.21±0.02	0.34±0.03	0.784
HPR	0.74±0.23	0.91±0.32	<0.001	0.75±0.23	0.91±0.35	<0.001	0.73±0.22	0.89±0.22	<0.001
TPSA (ng/ml)	15.22 (10.07,35.1)	10.99 (7.71,16.6)	<0.001	15.34 (9.57,37.00)	11.3 (7.85,17.77)	<0.001	16.50 (10.97,43.68)	10.30 (7.03,15.25)	<0.001
fPSA (ng/ml)	2.25 (1.21,4.31)	1.90 (1.16,2.80)	0.001	2.63 (1.32,5.09)	2.11 (1.29,3.19)	0.022	1.90 (1.09,5.72)	1.61 (1.12,2.40)	0.023
f/T	0.13 (0.09,0.19)	0.18 (0.12,0.23)	<0.001	0.14 (0.09,0.20)	0.19 (0.13,0.25)	<0.001	0.11 (0.08,0.15)	0.16 (0.11,0.21)	<0.001
PV (ml)	41.44 (27.27,63.03)	54.76 (40.79,77.72)	<0.001	44.04 (29.13,68.10)	49.81 (37.09,70.98)	0.015	41.80 (26.95,59.87)	58.70 (45.24,82.21)	<0.001
PASD (ng/ml/ml)	0.43 (0.21,0.85)	0.19 (0.12,0.33)	<0.001	0.43 (0.20,0.85)	0.21 (0.14,0.38)	<0.001	0.46 (0.24,0.91)	0.15 (0.11,0.24)	<0.001
PI-RADS score 1/2/3/4/5	4.00 (3.00,5.00)	3.00 (2.00,3.00)	<0.001	4.00 (4.00,5.00)	3.00 (2.00,4.00)	<0.001	4.00 (3.00,4.00)	2.00 (2.00,3.00)	<0.001

PCa, prostate cancer; BMI, body mass index; ASA, American Society of Anesthesiologists; NEUT, neutrophils; LYM, Lymphocyte; MO, Monocyte; HGB, hemoglobin; PLT, platelet; NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; SII, systemic immune-inflammation index; MLR, monocyte-to-lymphocyte ratio;HPR, hemoglobin-platelet ratio; PSA, prostate-specific antigen; fPSA, free prostate-specific antigen; f/T, fPSA/ TPSA; PV, prostate volume; PSAD, prostate specific antigen density; PI-RADS, Prostate Imaging Reporting and Data System.

Logistic regression analysis of clinical features and biomarkers

Further, the logistic regression analysis was used to identify the independent risk factors to predict PCa in the development cohort. The univariate analysis revealed that 11 variables were significantly associated with PCa: age, coronary heart disease, ASA score, NEUT, HGB, PLT, PLR, HPR, TPSA, fPSA, f/T, PASD, and PI-RADS score ($p < 0.05$) (Table 2).

Next, the multivariate analysis showed that NEUT (OR = 0.787, 95% CI: 0.658-0.941, $p = 0.008$), HPR (OR = 0.139, 95% CI: 0.047-0.417, $p < 0.001$), and f/T (OR = 0.013, 95% CI: 0-0.426, $p = 0.015$) were independent protective factors for PCa. Besides, our current results indicated that age (older) was also an independent risk factor for PCa (OR = 1.056, 95% CI: 1.022-1.092, $p = 0.001$), as well as a higher PI-RADS score (OR = 3.356, 95% CI: 2.445-4.606, $p \leq 0.001$) (Table 3 and Supplementary Figure 2).

Nomogram for PCa prediction

Based on the multivariate analysis, the nomogram included age, NEUT, HPR, f/T, and PI-RADS scores (Table 3 and Figure 2). In the nomogram, each clinical feature corresponds to a particular point. The score corresponding to this point was found on the "Points" axis, and the individual scores were added together to calculate the total score. On the "Prob of prostate cancer" axis, the probability corresponding to the point on the "Total Points" axis comprehends the probability of a patient having PCa.

The internal validation of the nomogram showed that the C-index was 0.851 (95% CI: 0.809-0.894). The AUC was 0.851 (95% CI: 0.809-0.894) for the development cohort and 0.874 (95% CI: 0.820-0.928) for the validation cohorts (Figures 3A, B). The calibration curve presented great agreement (Figure 3C). Additionally, the Hosmer–Lemeshow test showed $\chi^2 = 2.15$ and $p = 0.143$. These results demonstrated that the nomogram model could predict PCa risk and was greatly consistent with the real risk. According to decision curve analysis, patients with a 10 to 90% threshold probability will benefit from adopting this prediction model for PCa after biopsy (Figure 3D).

Discussion

PSA and its derivatives are widely used in PCa detection, including free PSA/TPSA (f/TPSA), PSA density (PSAD) and precursor forms of PSA (3, 8, 9, 19, 20). The f/TPSA is one of the early diagnostic tools of prostate cancer (9). In patients with a PSA range between 2.5 – 10ng/ml, f/T PSA <10% is an important risk factor for prostate cancer (19). In a Chinese patient-based study, f/T PSA was better than PSA in patients with predicted PSA of 4.0 – 10.0ng/ml. For those age> 60 years, the PSA range was adjusted to 10-20ng/ml (20). A study of PSA density (PSAD) showed that PSA

TABLE 2 Univariable analysis of patients in the development and validation cohorts.

Variable	Development Cohort				Validation Cohort			
	β	OR	95%CI	P value	β	OR	95%CI	P value
Age	0.062	1.064	(1.041,1.089)	<0.001	0.086	1.090	(1.047,1.135)	<0.001
Hypertension	-0.074	0.929	(0.648,1.331)	0.622	0.587	1.799	(1.013,3.194)	0.045
Diabetes mellitus	0.461	1.586	(0.919,2.737)	0.097	-0.034	0.967	(0.418,2.239)	0.938
Coronary heart disease	0.824	2.279	(1.330,3.907)	0.003	1.18	3.255	(1.365,7.764)	0.008
BMI	-0.033	0.968	(0.912,1.027)	0.276	-0.009	0.991	(0.910,1.080)	0.841
ASA score	0.54	1.716	(1.352,2.178)	<0.001	0.848	2.334	(1.461,3.728)	<0.001
Hematuria	-0.366	0.694	(0.334,1.439)	0.326	-0.239	0.787	(0.328,1.891)	0.592
History of biopsy	-0.489	0.613	(0.219,1.715)	0.351	0.053	1.054	(0.256,4.340)	0.942
History of prostate surgery	-0.170	0.844	(0.343,2.075)	0.711	0.053	1.054	(0.256,4.340)	0.942
Family history of PCa	0.448	1.565	(0.259,9.453)	0.625	0.051	1.053	(0.065,17.069)	0.971
NEUT	-0.174	0.840	(0.743,0.950)	0.005	-0.367	0.693	(0.558,0.860)	0.001
LYM	-0.202	0.817	(0.601,1.110)	0.197	-0.232	0.793	(0.509,1.235)	0.305
MO	-0.882	0.414	(0.124,1.378)	0.151	-0.300	0.740	(0.189,2.899)	0.666
HGB	-0.015	0.985	(0.975,0.995)	0.004	-0.048	0.953	(0.932,0.974)	<0.001
PLT	0.007	1.008	(1.004,1.011)	<0.001	0.014	1.014	(1.007,1.021)	<0.001
NLR	-0.061	0.94	(0.844,1.048)	0.265	-0.135	0.874	(0.724,1.054)	0.158
PLR	-0.002	0.998	(0.994,1.001)	0.209	0.004	1.004	(0.999,1.009)	0.083
SII	0.0004	1.000	(1.000,1.001)	0.181	0.0003	1.000	(0.999,1.000)	0.346
MLR	-0.513	0.599	(0.144,2.482)	0.479	-0.142	0.868	(0.315,2.391)	0.784
HPR	-2.039	0.130	(0.064,0.266)	<0.001	-3.527	0.029	(0.007,0.131)	<0.001
TPSA	0.023	1.024	(1.012,1.035)	<0.001	0.053	1.054	(1.027,1.082)	<0.001
fPSA	0.057	1.058	(1.011,1.108)	0.015	0.248	1.281	(1.103,1.488)	0.001
f/T	-5.297	0.005	(0,0.065)	<0.001	-8.819	0.001	(0,0.024)	0.001
PV	0.0004	1.000	(0.995,1.004)	0.872	-0.022	0.979	(0.968,0.990)	0.979
PASD	0.895	2.447	(1.576,3.799)	<0.001	3.221	25.047	(6.388,98.213)	<0.001
PI-RADS score	1.243	3.467	(2.690,4.468)	<0.001	1.139	3.124	(2.156,4.524)	<0.001

PCa, prostate cancer; BMI, body mass index; ASA, American Society of Anesthesiologists; NEUT,neutrophils;
LYM,Lymphocyte;MO,Monocyte; HGB, hemoglobin; PLT, platelet;NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio;SII, systemic immune-inflammation index;MLR, monocyte-to-lymphocyte ratio;HPR, hemoglobin-platelet ratio;
PSA, prostate-specific antigen; fPSA, free prostate-specific antigen; f/T, fPSA/ TPSA; PV, prostate volume; PSAD, prostate specific antigen density; PI-RADS, Prostate Imaging Reporting and Data System. Red values means P value <0.005.

density was significantly better than PSA in distinguishing intraprostatic inflammation from clinically meaningful PCa (csPCa). And this study further showed that in patients with PSA> 4 ng/ml, the PSA density of diagnosed csPCa is >0.15 ng/ml ^{2} and > 0.10 ng/ml ^{2}, respectively (8). In addition, other blood markers have also been tested clinically, including the prostate health index (PHI) test and four-kallikrein score (4Kscore). The prostate Health Index (PHI) test including free and total PSA and the [–2]pro-PSA isoform (p2PSA).The four kallikrein (4K) score including free, intact and total PSA and kallikrein-like peptidase 2

TABLE 3 Multivariable analysis of patients in the development and validation cohorts.

Variable	Development Cohort				Validation Cohort			
	β	OR	95%CI	P value	β	OR	95%CI	P value
Age	0.055	1.056	(1.022,1.092)	0.001	0.095	1.100	(1.041,1.163)	0.001
NEUT	-0.240	0.787	(0.658,0.941)	0.008	-0.762	0.468	(0.305,0.716)	<0.001
HPR	-1.971	0.139	(0.047,0.417)	<0.001	-3.088	0.046	(0.005,0.405)	0.006
f/T	-4.328	0.013	(0,0.426)	0.015	-10.359	0.001	(0,0.046)	0.005
PI-RADS score	1.211	3.356	(2.445,4.606)	<0.001	1.224	3.401	(2.075,5.573)	<0.001

NEUT, neutrophils;HPR, hemoglobin-platelet ratio;f/T, fPSA/ TPSA; PI-RADS, Prostate Imaging Reporting and Data System. Red values means P value <0.005.

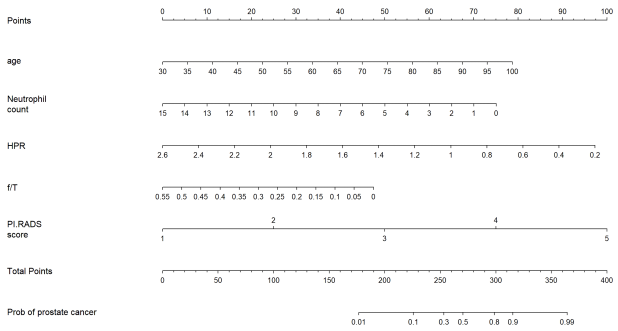

FIGURE 2
Nomogram for predicting the probability of prostate cancer in patients after transperineal prostate biopsy.

(hK2).They have great accuracy in the preliminary diagnosis and prediction of csPCa (2, 7). In this study, we included four PSA related indicators, include PSA, fPSA, free/total PSA ratio, and PSAD. Although they were significant association with prostate cancer (all $p<0.05$) according to the univariate analysis. However, only f/T PSA was significant in multivariate analysis (OR = 0.013, 95% CI: 0 - 0.426, p = 0.015). Meanwhile, previous studies have shown that the free/total PSA ratio has higher diagnostic accuracy

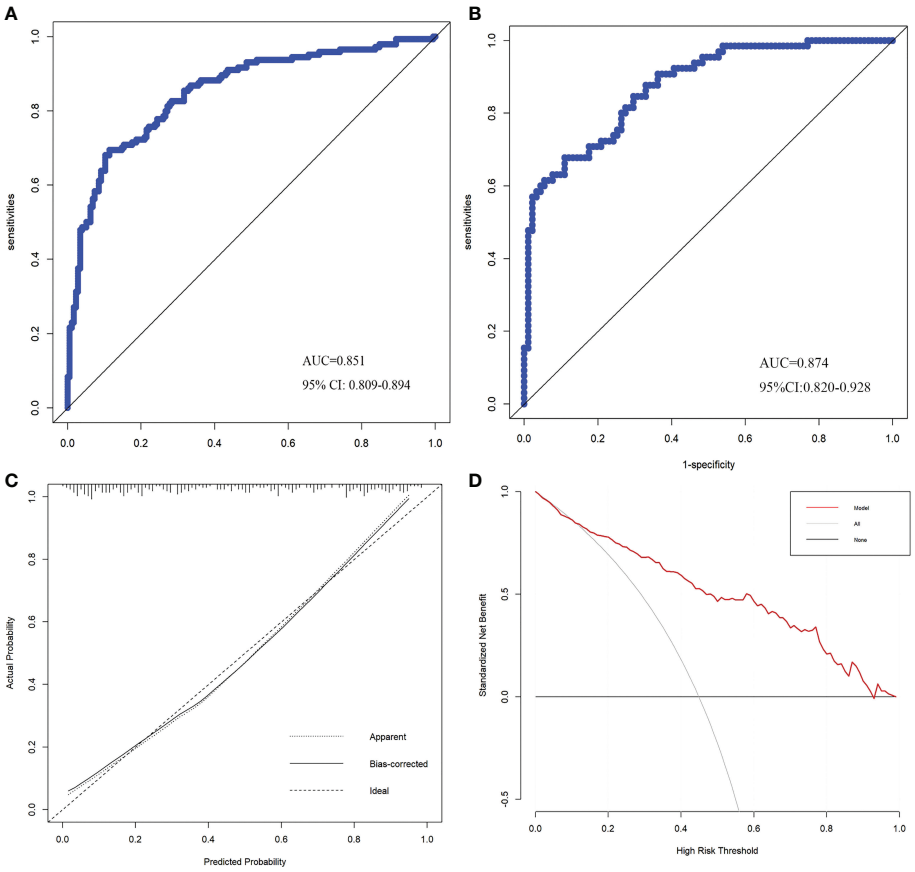

FIGURE 3
ROC curves of the development **(A)** and validation **(B)** cohorts. Calibration curve of the prediction model **(C)**. Decision Curve Analysis curve of the prediction model **(D)**.

than the TPSA (19–21). In our study, the clinical significance of f/T PSA exceeded that of other PSA related indicators and was negatively associated with PCa. Thus, the f/T PSA was included in the prediction model according to the multivariable analysis.

Compared with blood, urine has the advantages of convenient material extraction and large sample size. Prostate Cancer Antigen 3 (PCA3) is a prostate cancer marker in the urine. In clinical applications, it has shown satisfactory results in PC detection, staging, and prognosis. According to the study, the specificity of PCA3 in the diagnosis of prostate cancer was 56.3-89%, and the sensitivity was 46.9-82.3% (2). PCA3 positivity was associated with high-grade PCa in the pathology (11). Furthermore, the amino acids and carnitine derivatives were significantly increased in the urine of patients with PCa, but hardly in most of the urine of patients with BPH. This provides a direction for the subsequent exploration of PCa related biomarkers (3). Besides, another predictor of prostate cancer, the SelectMDx score (MDx Health). It was determined by combining different levels of HOXC6 and DLX1 with clinical risk factors (age, DRE, PSA, PSAD, family history, previous negative biopsy) (7, 10). According to the study of Busetto GM et al., the selectMDx score predicted PCa on biopsy with a sensitivity of 94.1% and 91.4% specificity, which was significantly better than PSA (17.1%). In predicting csPCa, the sensitivity and the PSA were 100% identical, and the specificity of 73.3% was significantly better than the PSA (13.3%) (7). Another study on the SelectMDx scores showed that a sensitivity and specificity of 86.5% and 73.8% predicted PCa at biopsy, and 87.1% and 63.7% predicted csPCa at biopsy, respectively. The negative predictive value (NPV) for PCa and csPCa was 91.6% and 95.2%, respectively (10).

MRI is a crucial imaging technique for PCa diagnosis. It can be used in clinical practice for prostate cancer detection, biopsy, and monitoring of disease progression. And every patient requiring a biopsy is recommended for an MRI (11). Using mpMRI before prostate biopsy reduces unnecessary biopsies by 25% and may improve detection of csPCa (22). However, 10% to 20% of csPCa is not detectable by mpMRI (11). Therefore, additional predictors are needed to complement the MRI results and refine the decision-making on biopsy. Moreover, Current models combining radiomics and genomic biomarkers improve prostate cancer prediction power and have better applications. In addition, artificial intelligence and machine learning in PCa is also crucial for the diagnosis plays an important role (11, 12). In 2012, the European Society of Urogenital Radiology (ESUR) developed the PI-RADS v. 1 based on mpMRI to standardize image interpretation, reporting, and diagnosis of PCa. In 2015, the American College of Radiology (ACR), ESUR, and the AdMeTech Foundation revised and improved PI-RADS v. 1 to develop PI-RADS v. 2 (23). According to a previous meta-analysis on the diagnostic value of the PI-RADS v. 2 score for PCa. In this study, the sensitivity and specificity of the PI-RADS v. 2 score in diagnosing PCa were 89.0 and 73.0%, respectively. Compared to the PI-RADS v. 1, the PI-RADS v. 2 score was more accurate in detecting PCa (24). However, both malignant and benign prostate tumors have many similar mpMRI features (25). Hence, different levels of PI-RADS score only indicate the probability of tumor occurrence (23, 26). Herein, the PI-RADS score (OR = 3.356, 95% CI: 2.445-4.606, $p < 0.001$) was included as a risk factor in the prediction model.

The incidence of prostate cancer increases with age. A study on the prevalence of prostate cancer reported that the prevalence of prostate cancer was 5% in patients < 30 years and 59% in those > 79 years (27). Consistent with this conclusion, in our study, age (OR = 1.056, 95% CI: 1.022-1.092, $p = 0.001$) in the development cohort was also a significant risk factor for PCa. The age and PI-RADS score were significantly associated with adverse pathology (AP) at radical prostatectomy (RP) (28). At RP, AP was considered as non–organ-confined disease, and/or lymph node invasion and/or pathological grade group ≥ 3.

The current study shows that inflammatory actions are essential at different phases of tumor growth (29).Therefore, the blood inflammatory indicators were included in the research.Neutrophils are associated with the prognosis of many separate cancers (30) and are involved in almost all phases of tumor progression (31). Neutrophils also participate in lung cancer development (13) and breast cancer metastasis (14). Tumour-associated neutrophils have two opposing mechanisms and are classified according to their function into the N1-phenotype (anti-tumor effects) and the N2-phenotype (promotes tumourigenesis) (32). The N1-phenotype is regulated by TGF-β and can be converted to N2. TGF-β can be provided by the tumor or the tumor microenvironment (31, 32). Moreover, previous studies have shown that PCa patients have lower neutrophil count (33, 34), consistent with our current findings. The distribution of neutrophils was 3.89 ± 1.44 in the PCa group vs. 4.30 ± 1.67 in the non-PCa group for the development cohort ($p = 0.005$) and 3.83 ± 1.17 in the PCa group vs. 4.72 ± 2.08 in the non-PCa group ($p < 0.001$) for the validation cohort.

The HPR is calculated from hemoglobin and platelets, and its diagnostic and prognostic utility for tumors has been demonstrated (15, 16). Our current multivariable analysis showed that low HPR was a risk factor for PCa(OR = 0.136, 95% CI: 0.047- 0.417, $p<0.001$). Lower HGB levels have also been related to the development of colorectal cancer in previous studies (35). Herein, we showed that the two groups had different hemoglobin distributions (development cohort: 131.03 ± 20.49 vs. 135.96 ± 16.19, $p = 0.004$; validation cohort: 130.60 ± 17.53 vs. 141.04 ± 12.46, $p < 0.001$). Additionally, tumor patients with low hemoglobin had a worse prognosis (36, 37). Low hemoglobin levels can cause tumor hypoxia in cancer patients (38). Hypoxia in tumors can also cause alterations in the genetic code that contribute to tumor progression and aggressiveness (38, 39). Furthermore, an increased platelet count is a predictive factor for various tumors (40). Cancer

patients with thrombocytosis have increased odds of adverse events. Besides, vascular embolism occurs in nearly 20% of cancer patients (41). Previous research has found that tumor cells cause thrombocytosis by boosting hepatic thrombopoietin (TPO) expression *via* IL-6 activation (42). Platelets can also accelerate tumor progression and invasion by producing cytokines, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β) (41, 42).

In this study, we included hematological indicators and clinical characteristics of patients to explore the risk factors of prostate cancer. Based on the results of the multivariate analysis for the development cohort, we included five risk factors associated with PCa development, including age (p = 0.001) and preoperative NEUT (p = 0.008), HPR (p < 0.001), f/T (p = 0.015), and PI-RADS score (p < 0.001). These factors included biological indicators and clinical information of patients. Therefore, we established a nomogram based on the relative risk of each factor to predict PCa probability, which was simple and convenient for clinical application. The internal validation of the nomogram showed that the C-index was 0.851, and the AUC was 0.851. The calibration curve presented great agreement. Herein, all indicators were collected from the patient before the biopsy. Thus, predicting patients' probability of cancer with non-invasive approaches can be used to avoid unnecessary punctures and reduce pain.

However, our current study also had some limitations. First, this was a retrospective study with small sample size and might be subject to selection bias and interference by other uncharted factors. Second, patients' data came from a single center, and the established predictive model was not externally validated. Therefore, the validity of this model needs to be tested in future studies. Despite these shortcomings, our findings demonstrated that combining patients' biomarkers and clinical information could contribute to diagnosing PCa. Finally, our study population only included Chinese people, and the results of this study may not apply to other ethnic groups.

Conclusion

In summary, we constructed a nomogram to predict PCa by integrating patients' biological markers and clinical features. This nomogram provided a handy and non-invasive prostate cancer screening method for male.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee board of the First Affiliated Hospital of Wannan Medical College. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

WR, YX: data collection, data analysis, and manuscript writing. CY, PY, SF: data collection. LC, JH, DZ: project development and manuscript revision.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1038177/full#supplementary-material

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Prognostic impact of geriatric nutritional risk index on patients with urological cancers: A meta-analysis

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Background: Despite previous research examining the predictive value of the geriatric nutritional risk index (GNRI) in individuals with urological cancers (UCs), results have been conflicting. This study aimed to comprehensively explore the potential link between GNRI and the prognosis of UCs using a meta-analysis.

Methods: The Cochrane Library, PubMed, Embase, and Web of Science databases were systematically and exhaustively searched. We estimated the prognostic importance of the GNRI in patients with UCs by calculating the pooled hazard ratios (HRs) and 95% confidence intervals (CIs) on survival outcomes. Publication bias was identified using Egger's test and Begg's funnel plot.

Results: Eight trials with 6,792 patients were included in our meta-analysis. Patients with UCs who had a lower GNRI before treatment had a higher risk of experiencing worse overall survival (HR = 2.62, 95% CI = 1.69–4.09, $p < 0.001$), recurrence-free survival/progression-free survival (HR = 1.77, 95% CI = 1.51–2.08, $p < 0.001$), and cancer-specific survival (HR = 2.32, 95% CI = 1.28–4.20, $p = 0.006$). Moreover, the subgroup analysis did not change the predictive significance of the GNRI in individuals with UCs. Neither Egger's nor Begg's test indicated substantial bias in this analysis.

Conclusion: As a result of our meta-analysis, we found that a low GNRI strongly predicts poor prognosis for patients with UCs. A lower pretreatment GNRI indicates poor survival outcomes in UCs.

KEYWORDS

GNRI, urological cancers, meta-analysis, survival, clinical use

1 Introduction

Urological cancers (UCs), including urothelial carcinoma (UC), renal cell carcinoma (RCC), and prostate cancer (PCa), are the primary causes of public health issues globally (1). UCs account for 380,480 new cases and 46,620 cancer-related deaths in men in the United States by 2022 (2). The incidence and mortality of UCs have been increasing in recent years, and UCs are more prevalent in Western countries than in Eastern regions (3, 4). Personalized medicine plays an important role in the treatment of UCs. The foundation of medical care includes androgen deprivation therapy (ADT) for PCa, tyrosine kinase inhibitors for RCC, and cytotoxic chemotherapy for UC (5). Patients undergoing urological oncology surgeries, such as radical prostatectomy, radical cystectomy, and radical nephroureterectomy, show a particular community at risk of poor prognosis (6). For example, for patients with bladder who underwent radical cystectomy (RC), the overall 3, 5 and 10-year survival after RC was 62%, 52% and 37%, respectively (6). However, finding new prognostic markers for patients with UCs is crucial for the design of therapeutic approaches.

Numerous studies have demonstrated a robust association between malnutrition and poor prognosis in patients with cancer. Nutritional evaluations, such as the prognostic nutritional index (7), controlling nutritional status score (8), and geriatric nutritional risk index (GNRI) (9), are commonly used to evaluate malnutrition in patients (7–9). In 2005, Bouillanne et al. (10) initially suggested the GNRI to evaluate the likelihood of death or disability in medically stable older adult individuals. The ideal weight, current weight, and serum albumin level (10) were used to determine GNRI. GNRI was calculated as GNRI = 14.89 * albumin (mg/dl) + 41.7 * (current/ideal body) weight. Nutritional status in patients with cancer may be evaluated using the GNRI because it is a straightforward method. Previous research has revealed the predictive usefulness of the GNRI in many different forms of cancer, including gastric cancer (11), hepatocellular carcinoma (9), pancreatic cancer (12), and oral squamous cell carcinoma (13). The prognostic factor of GNRI in patients with UC has been the subject of several studies with varying results (14–21). We collated relevant literature and conducted this study to evaluate the correlation between prognosis and GNRI in patients.

Abbreviations: GNRI, geriatric nutritional risk index; UCs, urological cancers; HR, hazard ratio; CI, confidence interval; RCC, renal cell carcinoma; PCa, prostate cancer; UC, urothelial carcinoma; ADT, androgen deprivation therapy; OS, overall survival; RFS, recurrence-free survival; PFS, progression-free survival; CSS, cancer-specific survival; TNM, tumor-node-metastasis; MVA, multivariate analysis; UVA, univariate analysis; NOS, Newcastle-Ottawa scale; FEM, fixed-effects model; REM, random-effects model; BMI, body mass index.

2 Materials and methods

2.1 Ethics statement

This meta-analysis did not require the use of an institutional review board or ethical committee. Additionally, the primary data were obtained from previously published research; therefore, there was no direct effect on the participants.

2.2 Study guideline

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines were used to compile the data for this meta-analysis (22).

2.3 Literature search

We systematically and extensively searched the Cochrane Library, Embase, PubMed, and Web of Science databases. Our exhaustive and targeted search methodology consisted of the following steps: (geriatric nutritional risk index OR GNRI) AND (bladder cancer OR renal cell cancer OR prostate cancer OR urothelial cancer OR urological cancer OR urinary cancer). A new search update was implemented on September 10, 2022. Articles written in languages other than English were also excluded. Furthermore, we also analyzed all the cited sources of the reviews and studies to find other papers that were relevant to our topic.

2.4 Inclusion and exclusion criteria

The inclusion criteria were as follows: (i) patients with upper tract urothelial cancer, bladder cancer, PCa, RCC, and UC were pathologically diagnosed; (ii) patients were divided into subgroups based on their GNRI; (iii) a GNRI cut-off value was determined; (iv) the GNRI was calculated as 14.89 × albumin (mg/dl) + 41.7 × (present/ideal body) weight (kg) before treatment; (v) hazard ratios (HRs) and 95% confidence intervals (CIs) were reported or adequate data were provided to compute them; and (vi) recurrence-free survival (RFS), cancer-specific survival (CSS), overall survival (OS), and progression-free survival (PFS) were reported. The following studies were excluded: animal studies, studies that did not provide enough data for analysis, studies that were duplicated and featured the same patients, reviews and conference abstracts, letters and case reports, and comments.

2.5 Data extraction and quality assessment

The literature review was conducted by two scholars working separately (QW and FY). All disagreements were

discussed and resolved verbally until agreement was reached. Data from relevant studies included the first author's name, year of publication, sample size, country, sex, time period, type of cancer, study design, study center (multicenter or single-center), treatment, tumor-node-metastasis (TNM) stage, duration of follow-up, GNRI cut-off value, type of survival analysis, survival outcomes, and HRs and 95% CIs. When both multivariate and univariate HRs and 95% CIs were used, the results of the multivariate analysis (MVA) were employed. In cases where only UVA was available, the HRs and 95% CIs were used instead. Each study included in the list was scored on the Newcastle-Ottawa scale (NOS) (23) to evaluate the research design quality. The final NOS score may range from 0 to 9, with points awarded for comparability (1–2), patient selection (0–4), and outcome (0–3). A high-quality study received a score of ≥ 6.

2.6 Statistical analysis

The predictive significance of the GNRI in patients with UCs was evaluated by calculating the 95% CI and HR for survival outcomes. The I^2 statistic and Cochrane Q statistic were used to assess statistical heterogeneity between studies. Owing to the low levels of heterogeneity, indicated by an I^2 value below 50% and a Q-test significance level above 0.10, a fixed-effects model (FEM) was used. Without this information, a random-effects model (REM) was utilized. To determine the origin of the observed variation, a subgroup analysis was performed, stratified by several clinicopathological characteristics. Publication bias was determined using Egger's test and Begg's funnel plot. The Stata version 12.0 was used for all statistical analysis (Stata Corporation, College Station, TX, USA) was used for all statistical analyses. Statistical significance was set at $p < 0.05$.

3 Results

3.1 Study selection

As shown in Figure 1, the initial literature search generated a total of 125 items. After filtering out 44 duplicates, the abstracts and titles of 81 papers were read. Thereafter, 66 papers were discarded, leaving only 15 for the full-text analysis. Seven studies were excluded for the following reasons: (1) they did not provide survival data (n = 3), (2) they did not perform a GNRI analysis (n = 2), (3) they did not determine a GNRI cut-off value (n = 1),

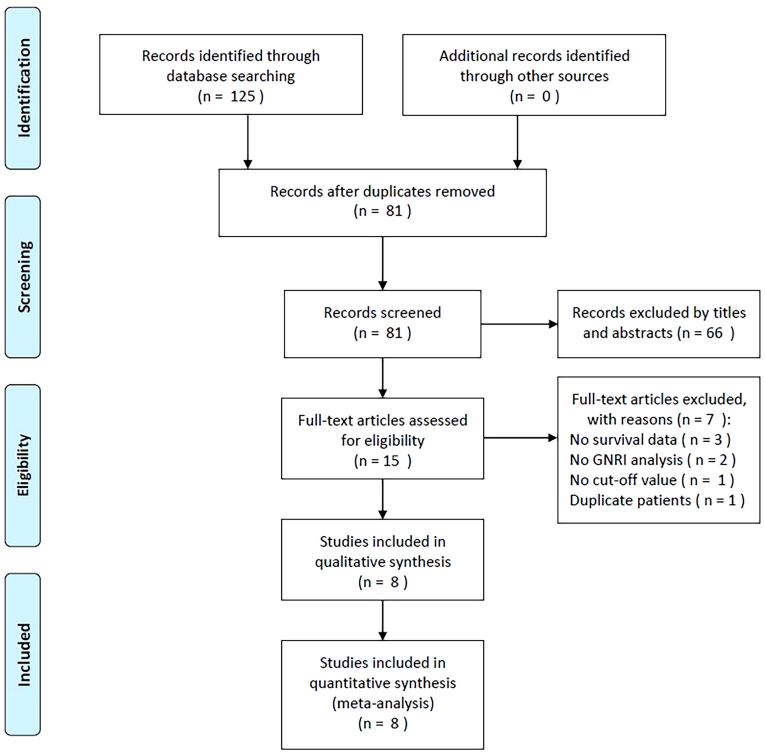

FIGURE 1
The flow diagram of this meta-analysis.

and (4) they included patients who had already been studied (n = 1). Eight studies with 6,792 patients (14–21) were included in the final meta-analysis (Figure 1).

3.2 Features of the included research

Table 1 shows the typical characteristics of the included studies. The articles considered were published in full-text format in the English language between 2015 and 2022 (14–21). Four studies were performed in Japan (15, 16, 19, 21), two in China (14, 20), and one each in Korea (17) and Taiwan (18). The sample sizes ranged from 68 to 4,591, with a median of 319.5. Four studies recruited patients with RCC (14, 15, 17, 20), two studies enrolled patients with PCa (16, 18), and two studies included patients with UC (19, 21). Seven studies were retrospective studies (15–21) and one was a prospective trial (14). Five studies recruited patients with TNM stage IV (14, 16, 18, 19, 21) and three studies enrolled patients with TNM stages I–III (15, 17, 20). Three studies included patients receiving surgery (15, 17, 20), two studies recruited patients undergoing chemotherapy (18, 19), and one study used ADT (16), immune checkpoint inhibitor (21), and targeted therapy (14). Seven studies adopted 92 as the cut-off value for the GNRI (14, 16–21) and one study adopted 98 (15). The significance of the GNRI as an OS prognostic factor was revealed in six studies (14, 16, 18–21), three studies presented the association between the GNRI and RFS (15, 17, 20), two studies reported the HR and 95%CI for PFS (18, 19), and three studies demonstrated a correlation between the GNRI and CSS (15–17). Six studies described the HRs and 95% CIs from the MVA (14, 17–21), and two studies reported data from the UVA (15, 16). Five studies were multicenter (14, 16, 17, 19, 21) and three were single-center (15, 18, 20). The NOS score of the considered studies varied from 7 to 9, with a median of 8, showing that the methodology of all considered studies was of a high standard.

3.3 GNRI and OS in UCs

The predictive importance of the GNRI for OS in patients with UCs was revealed in six investigations, including a total of 1,769 participants (14, 16, 18–21). In this case, substantial heterogeneity (I^2=75.6%, Ph=0.001) necessitated REM deployment. As shown in Table 2 and Figure 2, the combined results indicated that a low GNRI was significantly associated with poor OS in patients with UCs (HR = 2.62, 95% CI = 1.69–4.09, p < 0.001). The subgroup analysis revealed that regardless of study design, type of survival analysis, or sample size, a low GNRI was a clear indication of worse OS (Table 2). Patients with UC and PCa, but not RCC, had a low GNRI and poor OS (Table 2).

3.4 GNRI and RFS/PFS in UCs

We merged RFS and PFS into the RFS/PFS groups because they were both event-free survival endpoints. Five studies comprising 5,955 patients (15, 17–20) reported the relationship between RFS/PFS and GNRI. The pooled HR and 95% CI were as follows: p < 0.001, HR = 1.77, 95% CI = 1.51–2.08 in the FEM (Figure 3, Table 3), which suggested that patients with UCs with low GNRI had poor RFS/PFS. The prognostic significance of GNRI for RFS/PFS remained significant in various subgroups of sample size, cancer type, study center, TNM stage, and cut-off value, as shown in Table 3 from the subgroup analysis.

3.5 GNRI and CSS in UCs

Three studies, consisting of 5,362 patients (15–17) described the HRs and 95% CIs for CSS. REM was used, and the combined outcomes were as follows: HR = 2.32, 95% CI = 1.28–4.20, p = 0.006 (Figure 4). As shown in Table 4, subgroup analysis revealed that decreased GNRI was an important prognostic marker for poor CSS, regardless of the study center and cut-off value in patients with UCs.

3.6 Publication bias

This meta-analysis did not exhibit any significant publication bias according to Egger's test and Begg's test (Figure 5).

4 Discussion

Prior research has shown conflicting results regarding the prognostic efficacy of GNRI in patients with UCs. In the present meta-analysis, we included eight studies with a total of 6,792 patients and found that low GNRI predicted poor RFS/PFS, CSS, and OS in patients with UCs. In addition, the prognostic impact of the GNRI in these patients remained stable in diverse subgroups. The publication bias test identified non-significant publication bias and validated the accuracy of our findings. To our knowledge, this is the first meta-analysis to explore the association between pre-treatment survival outcomes and GNRI in UCs. Based on our meta-analysis, we know that a low GNRI is an easy and reliable prognostic indicator for patients with UCs in clinical practice.

The GNRI is a nutritional index based on body weight and albumin level. Therefore, the roles of these two components in cancer can provide insights into the processes underlying the association between GNRI and prognosis in UCs. Albumin levels are often used to assess patients' nutritional and

TABLE 1 Basic characteristics of included in this meta-analysis.

Author	Year	Country/ region	Sample size	Age (years) Median (range)	Cancer type	Gender (M/F)	Study duration	Study design	Study center	TNM stage	Treatment	Follow-up (month) Median (range)	Cut-off value of GNRI	Survival outcomes	Survival analysis type	NOS score
Gu, W.	2015	China	300	56.2(27-81)	RCC	203/97	2009-2013	Prospective	Multicenter	IV	Targeted therapy	30.8	92	OS	MVA	8
Miyake, H.	2017	Japan	432	≤70: 164 >70: 268	RCC	277/155	2005-2011	Retrospective	Single center	I-III	Surgical resection	1-100	98	RFS, CSS	UVA	7
Okamoto, T.	2019	Japan	339	72	PCa	339/0	2005-2017	Retrospective	Multicenter	IV	ADT	26(12-53)	92	OS, CSS	UVA	8
Kang, H. W.	2020	Korea	4,591	61	RCC	3,367/ 1,224	1988-2015	Retrospective	Multicenter	I-III	Surgical resection	37	92	RFS, CSS	MVA	9
Chang, L. W.	2021	Taiwan	170	74	PCa	170/0	2006-2012	Retrospective	Single center	IV	Chemotherapy	22.49 (11.35-41.32)	92	OS, PFS	MVA	8
Naiki, T.	2021	Japan	68	71(49-87)	Urothelial carcinoma	55/13	2016-2020	Retrospective	Multicenter	IV	Chemotherapy	12.9(1.7-50.2)	92	OS, PFS	MVA	8
Tang, Y.	2021	China	694	≤60: 449 >60: 245	RCC	442/252	2009-2014	Retrospective	Single center	I-III	Surgical resection	60.9	92	OS, RFS	MVA	7
Isobe, T.	2022	Japan	198	70(37-85)	Urothelial carcinoma	163/35	2009-2021	Retrospective	Multicenter	IV	ICI	1-60	92	OS	MVA	8

GNRI, Geriatric Nutrition Risk Index; RCC, renal cell carcinoma; PCa, prostate cancer; UC, urothelial carcinoma; OS, overall survival; CSS, cancer-specific survival; RFS, recurrence-free survival; PFS, progression-free survival; ADT, androgen-deprivation therapy; ICI, immune checkpoint inhibitor; MVA, multivariate analysis; UVA, univariate analysis; TNM, tumor-node-metastasis; NOS, Newcastle-Ottawa Scale; M, male; F, female.

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TABLE 2 Subgroup analysis of the prognostic value of GNRI for OS in patients with urologic cancers.

Factors	No. of studies	No. of patients	Effects model	HR (95%CI)	p	Heterogeneity I^2 (%)	Ph
Overall	6	1,769	REM	2.62 (1.69-4.09)	<0.001	75.6	0.001
Sample size							
≤300	4	736	FEM	3.54 (2.71-4.62)	<0.001	39.1	0.177
>300	2	1,033	FEM	1.51 (1.06-2.15)	0.022	22.3	0.257
Cancer type							
RCC	2	994	REM	1.97 (0.76-5.12)	0.165	87.0	0.006
PCa	2	509	REM	3.10 (1.06-9.04)	0.039	89.9	0.002
UC	2	266	FEM	2.80 (1.76-4.48)	<0.001	0	0.318
Study design							
Retrospective	5	1,469	REM	2.53 (1.45-4.41)	0.001	79.4	0.001
Prospective	1	300	–	3.16 (2.06-4.84)	<0.001	–	–
Study center							
Multicenter	4	905	FEM	2.55 (1.96-3.31)	<0.001	29.5	0.235
Single center	2	864	REM	2.54 (0.58-11.11)	0.216	93.8	<0.001
TNM stage							
I-III	1	694	–	1.19 (0.69-2.05)	0.529	–	–
IV	5	1,075	REM	3.06 (2.06-4.57)	<0.001	63.7	0.026
Survival analysis							
MVA	5	1,430	REM	2.86 (1.70-4.80)	<0.001	77.1	0.002
UVA	1	339	–	1.80 (1.13-2.87)	0.013	–	–

REM, random-effects model; FEM, fixed-effects model; RCC, renal cell carcinoma; PCa, prostate cancer; UC, urothelial carcinoma; MVA, multivariate analysis; UVA, univariate analysis.

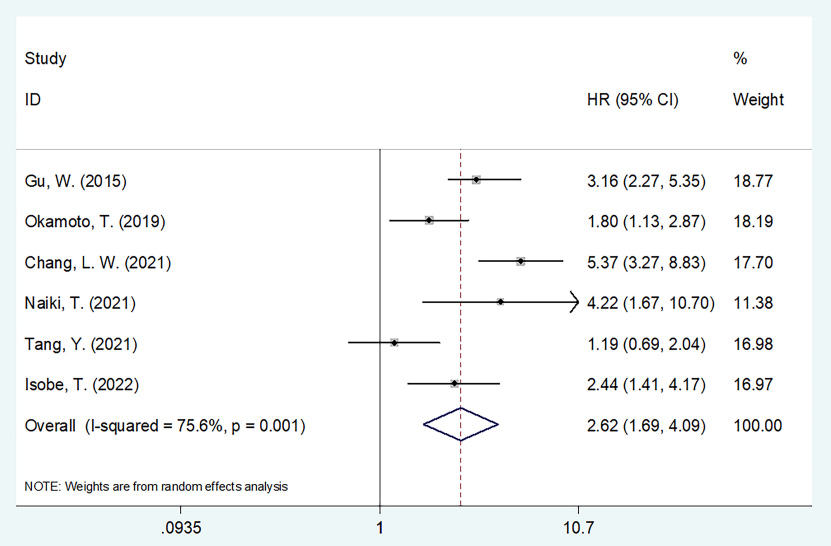

FIGURE 2
The forest plot of the association of pretreatment GNRI with overall survival (OS) of patients with UCs.

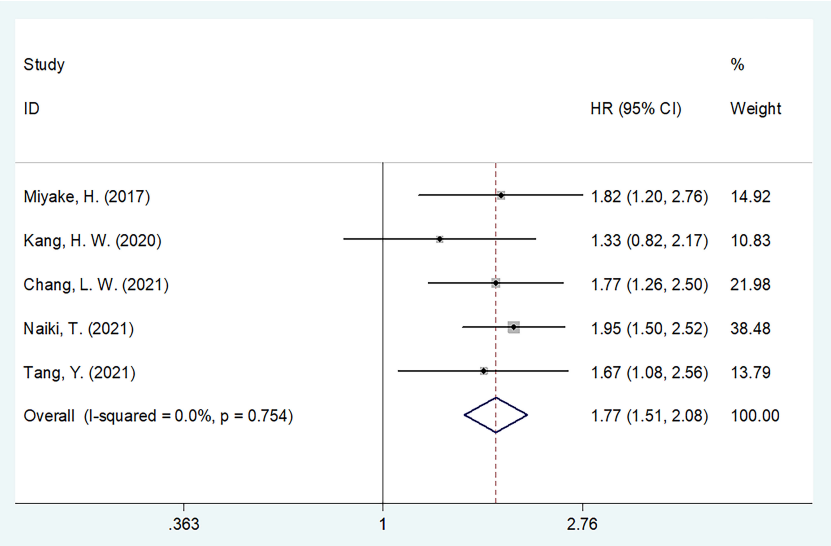

FIGURE 3
The forest plot of the association of pretreatment GNRI with recurrence-free survival/progression-free survival (RFS/PFS) of patients with UCs.

TABLE 3 Subgroup analysis of the prognostic value of GNRI for RFS/PFS in patients with urologic cancers.

Factors	No. of studies	No. of patients	Effects model	HR (95%CI)	p	Heterogeneity I^2 (%)	Ph
Overall	5	5,955	FEM	1.77 (1.51-2.08)	<0.001	0	0.754
Sample size							
≤300	2	238	FEM	1.88 (1.53-2.31)	<0.001	0	0.676
>300	3	5,717	FEM	1.62 (1.26-2.09)	<0.001	0	0.626
Cancer type							
RCC	3	5,717	FEM	1.62 (1.26-2.09)	<0.001	0	0.626
PCa	1	170	–	1.77 (1.26-2.50)	0.001	–	–
UTC	1	68	–	1.95 (1.50-2.52)	<0.001	–	–
Study center							
Multicenter	2	4,659	FEM	1.79 (1.42-2.25)	<0.001	44.6	0.179
Single center	3	1,296	FEM	1.76 (1.40-2.20)	<0.001	0	0.959
TNM stage							
I-III	3	5,717	FEM	1.62 (1.26-2.09)	<0.001	0	0.626
IV	2	238	FEM	1.88 (1.53-2.31)	<0.001	0	0.676
Cut-off value							
92	4	5,523	FEM	1.77 (1.48-2.10)	<0.001	0	0.597
98	1	432	–	1.82 (1.20-2.76)	0.005	–	–
Survival analysis							
MVA	4	5,523	FEM	1.77 (1.48-2.10)	<0.001	0	0.597
UVA	1	432	–	1.33 (0.82-2.17)	0.247	–	–

REM, random-effects model; FEM, fixed-effects model; RCC, renal cell carcinoma; PCa, prostate cancer; UC, urothelial carcinoma; MVA, multivariate analysis; UVA, univariate analysis.

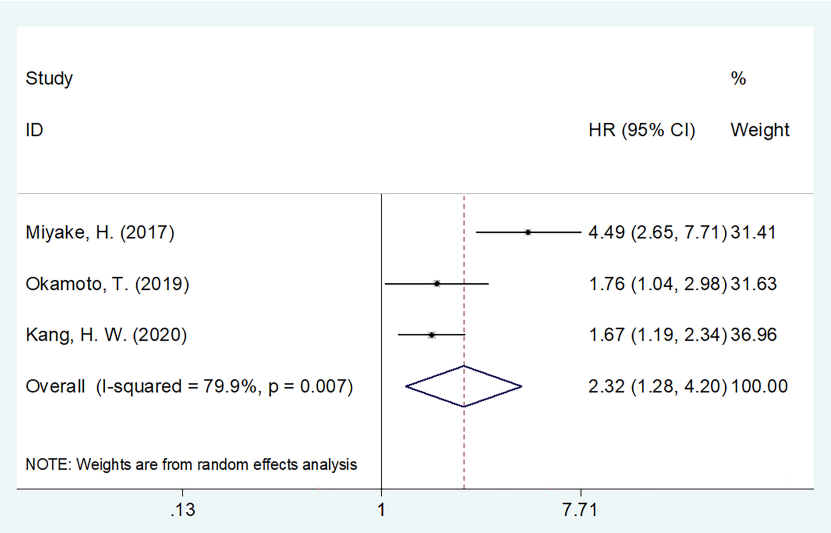

FIGURE 4
The forest plot of the association of pretreatment GNRI with cancer-specific survival (CSS) of patients with UCs.

inflammatory health when dealing with UCs. There was a correlation between low albumin levels and increased fetoprotein levels, portal vein thrombosis, larger maximal tumor diameters, increased tumor multifocality, and shorter overall survival time (24). Therefore, a lower serum albumin level directly indicates the malnutrition status of patients with cancer. Moreover, current evidence shows that malnutrition is a common issue among patients with cancer, with an incidence of

TABLE 4 Subgroup analysis of the prognostic value of GNRI for CSS in patients with urologic cancers.

Factors	No. of studies	No. of patients	Effects model	HR (95%CI)	p	Heterogeneity I^2 (%)	Ph
Overall	3	5,362	REM	2.32 (1.28-4.20)	0.006	79.9	0.007
Cancer type							
RCC	2	5,023	REM	2.68 (1.02-7.05)	0.046	89.4	0.002
PCa	1	339	–	1.76 (1.04-2.98)	0.035	–	–
Study center							
Multicenter	2	4,930	FEM	1.70 (1.28-2.26)	<0.001	0	0.870
Single center	1	432	–	4.49 (2.63-7.66)	<0.001	–	–
TNM stage							
I-III	2	5,023	REM	2.68 (1.02-7.05)	0.046	89.4	0.002
IV	1	339	–	1.76 (1.04-2.98)	0.035	–	–
Cut-off value							
98	1	432	–	4.49 (2.63-7.66)	<0.001	–	–
92	2	4,930	FEM	1.70 (1.28-2.26)	<0.001	0	0.870
Survival analysis							
MVA	1	4,591	–	1.67 (1.19-2.34)	0.003	–	–
UVA	2	771	REM	2.81 (1.12-7.03)	0.027	83.3	0.014

REM, random-effects model; FEM, fixed-effects model; RCC, renal cell carcinoma; PCa, prostate cancer; MVA, multivariate analysis; UVA, univariate analysis.

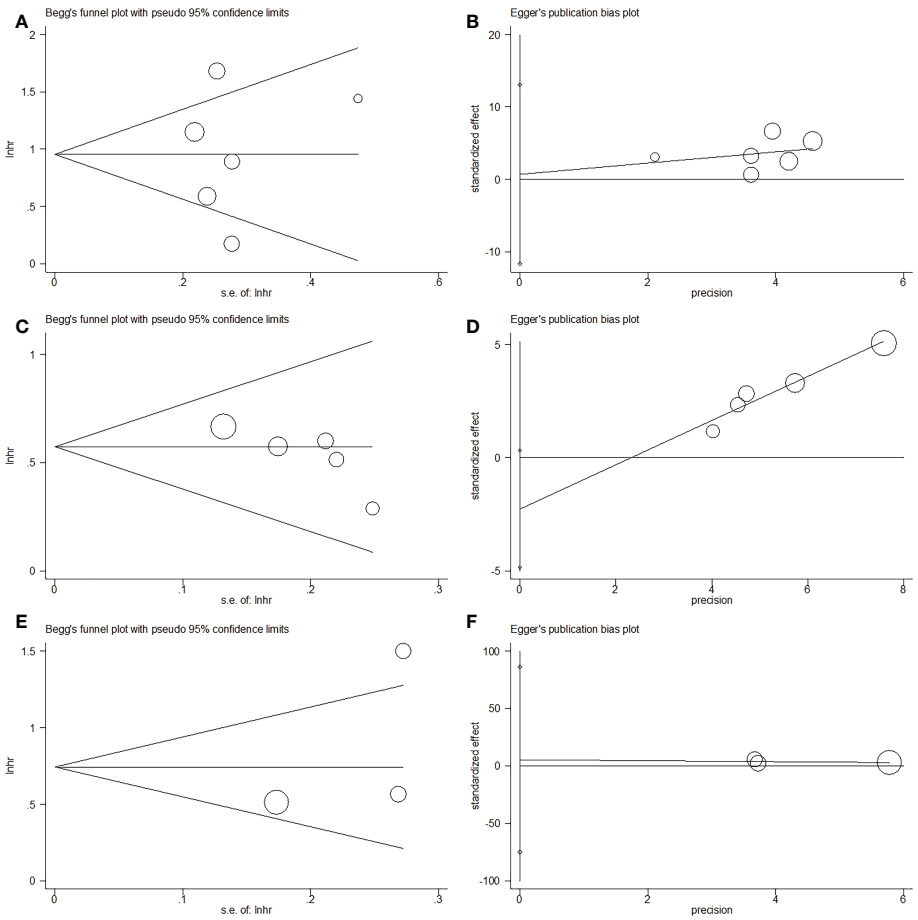

FIGURE 5
Publication bias by Begg's test and Egger's test in this meta-analysis. **(A)** Begg's test for OS, p=0.851; **(B)** Egger's test for OS, p=0.883; **(C)** Begg's test for RFS/PFS, p=0.086; **(D)** Egger's test for RFS/PFS, p=0.068; **(E)** Begg's test for CSS, p=0.296; **(F)** Egger's test for CSS, p=0.548.

39–71% (25, 26). Researchers have found that low albumin levels are a strong predictor of poor health outcomes in patients with advanced cancer (27). In contrast, weight is a proxy for the extent of a systemic ailment and reserves of protein and calories. To calculate the GNRI, we must first calculate the body mass index by comparing an individual's actual weight to their ideal weight. It is well established that low body mass index is associated with poor prognosis in patients with cancer (28).

Some recent studies have provided pivotal evidence for the clinical use of nutritional indices for the prognosis of patients with urological cancers (29, 30). A recent single-center retrospective study including 510 cases showed that the fibrinogen-to-albumin ratio (FAR) in patients with bladder cancer who had elevated preoperative FAR might be more likely to have advanced-stage cancer and malignancy (29). Another recent study proposed that the lymphocyte-to-monocyte ratio could be a promising prognostic indicator for tumor progression in patients with bladder cancer (30).

Several recent meta-analyses have documented the prognostic importance of GNRI (31–34). In a meta-analysis of 11 trials, Zhou et al. demonstrated that a low GNRI was associated with poor CSS and OS in patients with esophageal cancer (31). In a meta-analysis of 3,239 patients, Xu et al. found that a low GNRI score was associated with a higher risk of death and postoperative complications in Asian patients with colon cancer (35). The authors of a recent meta-analysis of 8 studies conducted by Wang et al. (36) found that low GNRI levels were associated with shorter RFS, CSS, and OS in patients with lung cancer. Consistent with earlier findings in other cancer types, our meta-analysis showed that a lower GNRI was an effective prognostic predictor of RFS/PFS, CSS, and OS in patients with UC.

This meta-analysis has some limitations. First, all included studies were conducted in East Asia. Therefore, it is important to confirm our meta-analysis results in locations other than Asia. Second, because many studies in this meta-analysis were retrospective, there is a possibility of intrinsic selection bias and heterogeneity. Third, there was no consistent GNRI cut-off value across studies that were considered; hence, an ideal cut-off value should be determined. It is important to conduct multinational large-scale prospective trials across nations to corroborate our findings.

In summary, our meta-analysis concluded that a low GNRI significantly predicts worse outcomes for patients with UC. A lower pretreatment GNRI indicates poor survival outcomes in UCs. The GNRI may be a potential parameter for evaluating prognosis and developing appropriate treatment approaches for patients with UC.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

QW and FY designed the study. QW and FY established the process of literature selection and screened the abstracts and articles. QW analyzed data and wrote the main manuscript. All authors reviewed and approved the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The potential of using circulating tumour cells and their gene expression to predict docetaxel response in metastatic prostate cancer

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Background: Docetaxel improves overall survival (OS) in castration-resistant prostate cancer (PCa) (CRPC) and metastatic hormone-sensitive PCa (mHSPC). However, not all patients respond due to inherent and/or acquired resistance. There remains an unmet clinical need for a robust predictive test to stratify patients for treatment. Liquid biopsy of circulating tumour cell (CTCs) is minimally invasive, can provide real-time information of the heterogeneous tumour and therefore may be a potentially ideal docetaxel response prediction biomarker.

Objective: In this study we investigate the potential of using CTCs and their gene expression to predict post-docetaxel tumour response, OS and progression free survival (PFS).

Methods: Peripheral blood was sampled from 18 mCRPC and 43 mHSPC patients, pre-docetaxel treatment, for CTC investigation. CTCs were isolated using the epitope independent Parsortix® system and gene expression was determined by multiplex RT-qPCR. We evaluated CTC measurements for post-

docetaxel outcome prediction using receiver operating characteristics and Kaplan Meier analysis.

Results: Detection of CTCs pre-docetaxel was associated with poor patient outcome post-docetaxel treatment. Combining total-CTC number with PSA and ALP predicted lack of partial response (PR) with an AUC of 0.90, p= 0.037 in mCRPC. A significantly shorter median OS was seen in mCRPC patients with positive CTC-score (12.80 vs. 37.33 months, HR= 5.08, p= 0.0005), ≥3 total-CTCs/7.5mL (12.80 vs. 37.33 months, HR= 3.84, p= 0.0053), ≥1 epithelial-CTCs/7.5mL (14.30 vs. 37.33 months, HR= 3.89, p= 0.0041) or epithelial to mesenchymal transitioning (EMTing)-CTCs/7.5mL (11.32 vs. 32.37 months, HR= 6.73, p= 0.0001). Significantly shorter PFS was observed in patients with ≥2 epithelial-CTCs/7.5mL (7.52 vs. 18.83 months, HR= 3.93, p= 0.0058). mHSPC patients with ≥5 CTCs/7.5mL had significantly shorter median OS (24.57 vs undefined months, HR= 4.14, p= 0.0097). In mHSPC patients, expression of *KLK2*, *KLK4*, *ADAMTS1*, *ZEB1* and *SNAI1* was significantly associated with shorter OS and/or PFS. Importantly, combining CTC measurements with clinical biomarkers increased sensitivity and specificity for prediction of patient outcome.

Conclusion: While it is clear that CTC numbers and gene expression were prognostic for PCa post-docetaxel treatment, and CTC subtype analysis may have additional value, their potential predictive value for docetaxel chemotherapy response needs to be further investigated in large patient cohorts.

KEYWORDS

prostate cancer, circulating tumour cells, docetaxel, response prediction, biomarker, liquid biopsy, prognosis

Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in Western males, accounting for 24% of all new cancers in 2018 (1). The effective first-line treatment for metastatic disease is androgen deprivation therapy (ADT), although after an initial response, progression to castration-resistant PCa (CRPC) occurs within 1-3 years (2). Adding docetaxel to ADT improves overall survival (OS) in metastatic (m)CRPC (3) and since 2014 as a result of the CHAARTED (4) and STAMPEDE (5) phase III trials, docetaxel has been used in combination with ADT as a first-line treatment for metastatic hormone-sensitive PCa (mHSPC) (3, 6). However, response to docetaxel is not universal due to inherent and/or acquired resistance. While numerous studies have investigated the underlying mechanisms and pharmacogenomic biomarkers of docetaxel resistance (7–11), there remains an unmet clinical need for new surrogate markers and a robust predictive test to stratify patients for treatment and develop personalised therapeutic approaches (12).

Tissue biomarkers representing cancer characteristics may help predict treatment outcome, but serial biopsies add morbidity and delay, and sampling of bone metastases is practically difficult. Furthermore, tissue biopsy fails to represent the entire cancer population due to intra-tumoural heterogeneity. In the case of therapy response prediction, markers that were detectable within the initial tissue biopsy sample are unlikely to truly represent the patient's disease due to continuous tumour evolution at the molecular level. This is particularly important when considering second line therapies and beyond. As an alternative to tissue biopsy, liquid biopsy refers to the analysis of tumour biomarkers such as circulating tumour cells (CTCs), circulating tumour DNA (ctDNA), microRNA (miRNA) and extracellular vesicles (EVs) in peripheral blood or other body fluids. Liquid biopsies are minimally invasive, easily repeatable and can provide real time information of the heterogeneous

tumour, providing a promising tool to overcome the limitations posed by tissue biopsy. Prostate-specific antigen (PSA) remains the standard serum biomarker for PCa diagnosis and progression, however with limited ability to predict therapeutic response (13).

CTCs are malignant cells that have gained an invasive phenotype, allowing them to shed from the tumour mass into the circulation where they travel to distant sites and form metastases (14). CTCs are unique amongst cancer biomarkers, as they provide a source of live tumour cells that carry molecular and biological information that may represent overall tumour burden and phenotypic characteristics present in both primary and metastatic sites. In addition to CTC enumeration, molecular profiling of enriched CTC populations or single CTCs provides a plethora of potentially clinically valuable markers of the metastatic process, disease status and predictors of patient individualised therapeutic response (15, 16). Therefore, CTC analysis may be a potentially ideal docetaxel response prediction biomarker.

Numerous studies have investigated various clinical applications of CTC enumeration and characterisation in PCa (17–22). Baseline CTCs have been shown to predict poor OS in patients with mCRPC (19, 23), which led to the FDA approval of CellSearch® detected CTCs for advanced PCa prognosis (24). The MAINSAIL phase III trial of mCRPC patients treated with docetaxel found a significant association between baseline ≥5 CTCs/7.5ml of peripheral blood and poor OS, but not PSA response or Response Evaluation Criteria in Solid Tumors (RECIST) (25–27). The recent PROPHECY prospective multicentre study in patients with mCRPC undergoing treatment with enzalutamide or abiraterone followed by taxane chemotherapy, focused on the detection of CellSearch® isolated CTCs expressing the androgen receptor splice variant, AR-V7. The study demonstrated that pre-treatment CTC AR-V7 status was independently associated with shorter progression free survival (PFS) and OS with abiraterone or enzalutamide, however men with AR-V7-positive disease still experienced clinical benefit from taxane chemotherapy (28). CTCs have also been investigated as predictive and prognostic biomarkers of clinical outcome, including mCRPC onset, in patients with mHSPC (17, 29–31), however to date there is limited information regarding their clinical utility in predicting docetaxel response in this patient cohort. Furthermore, the majority of studies to date have used epithelial epitope dependent isolation, missing a potentially important subpopulation of CTCs with epithelial negative phenotypes following epithelial-mesenchymal transition (EMT) during cancer cell invasion and metastatic spread. Previous research from ourselves and others has demonstrated that CTCs that are undergoing EMT, or those that have a fully mesenchymal phenotype have significant value as biomarkers of increased metastatic tumour burden (32), and disease progression (33, 34). Moreover, EMT is increasingly recognised as an important mechanism that drives inherent and acquired resistance to chemotherapies (35), including docetaxel (36). As such, exclusion of CTCs with epithelial negative phenotypes limits the detection of genes which might be developed into novel predictive biomarkers of docetaxel response, which may facilitate patient personalised treatment stratification.

We previously used the cell size and deformability based Parsortix® CTC isolation system, which was recently FDA approved, to detect CTCs with epithelial, mesenchymal and intermediate phenotypes, and demonstrated their biomarker potential in different clinical scenarios (32, 37, 38). In this study we used Parsortix® to capture pre-docetaxel treatment CTCs, evaluating CTC subtypes and their gene expression as biomarkers of docetaxel response in order to identify CTC markers with clinical value for the management of advanced PCa patients. Our strategy combined epitope independent CTC isolation for enumeration and molecular characterisation using multiplex RT-qPCR for a targeted panel of genes. We demonstrate the potential of analysing multiple CTC subtypes and their gene expression as predictive and prognostic biomarkers in both mCRPC and mHSPC patients.

Methods

Patients

Between January 2015 and January 2020, 18 mCRPC and 43 mHSPC patients were recruited with informed consented at St Bartholomew's Hospital, Barts Health NHS, London, UK. Clinical characteristics for individual patients are shown in Supplementary Table 1. Peripheral blood samples were collected into EDTA tubes ≤2 months before commencing 6 cycles of docetaxel. Patients with mHSPC had started initial hormone-therapy <3 months before blood collection. Patients received CT and bone scans before and after treatment. Serum PSA, ALP, and LDH were measured together with CTC sampling. Radiological response assessment was based on RECIST criteria (25): (1) complete response (CR): disappearance of all target lesions; (2) partial response (PR): at least 30% decrease in the sum of the longest diameter of target lesions, taking as reference the baseline since treatment started; (3) progressive disease (PD): at least 20% increase in the sum of the longest diameter of target lesions, taking as reference the baseline since treatment started; (4) stable disease (SD): neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD. Assessments of response by bone scan were classified as follows: (1) CR: disappearance of all bone metastasis; (2) PR: a decrease in number, extent or intensity of bone lesions was detected; (3) PD: appearance of new bone lesion(s) and/or apparent enlargement of the bone metastases; (4) SD: little or no change in the number, extent or intensity of bone metastases was observed. PSA progression was defined as two consecutive rises above PSA nadir at least two weeks apart. CTC measurements

were also investigated for their ability to prognose OS and PFS outcomes.

Cell lines

The docetaxel-resistant human PCa cell line PC3-D12 and the sensitive counterpart PC3-Ag, were gifted by A.J. O'Neill (10), University College Dublin. PC3-D12 cells were treated every 4 weeks with 12 nM docetaxel in order to maintain resistance. The cells were maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum and 2 nM L-glutamine (Invitrogen, Waltham, Massachusetts, Unites States).

CTC isolation, enumeration and characterisation

CTCs were isolated from 7.5 mL of whole blood using the Parsortix® (Angle Plc, Guildford, UK) isolation system and identified for CTC enumeration using four-colour immunofluorescence as previously described (32, 37). Briefly, 7 mL of blood was transferred to 50 mL LeucoSep tubes (Greiner Bio-One, Frickenhausen, Germany) with 15.3 mL of Ficoll-Paque Plus (GE Healthcare, Chicago, Illinois, Unites States) and centrifuged at 1000 g for 15 min with the break off at room temperature to recover the peripheral blood mononuclear cell (PBMC) fraction. The PBMC fraction along with the plasma above the fit of the LeucoSep tube was removed into a new 50 mL falcon tube and pelleted at 200 g for 8 min at room temperature. The pellet was then re-suspended in 4.5 mL of isolation buffer (PBS containing 1% BSA and 2 nM EDTA) and added back to the remaining 0.5 mL of whole blood and loaded onto the Parsortix® for CTC isolation. Once samples are loaded, cells are separated based on cell size and deformability according to a pre-set programme PX-S99F that uses 6.5 μm-gap cassette and 99 mbar pressure for isolation. Cells were then harvested using a pre-set programme and transferred onto glass slides for downstream analysis. All blood samples were processed within 4 hrs of collection. Slides were stained using mouse monoclonal PE-conjugated anti-CD45 (Miltenyi Biotec, Bergisch Gladbach, Germany), mouse monoclonal FITC-conjugated anti-Cytokeratin (Miltenyi Biotec, Bergisch Gladbach, Germany), Alexa Fluor 647-conjugated anti-Vimentin (Abcam, Cambridge, UK), and counterstained using SlowFade gold antifade mountant with DAPI (Life Technologies, Carlsbad, California, United States). CTCs were identified as Cytokeratin (CK)+/Vimentin (VIM)−/CD45- (epithelial-CTCs), CK+/VIM+/CD45- (EMTing-CTCs) and CK-/VIM+/CD45- (mesenchymal-CTCs). Patients with ≥1 epithelial-CTC and/or ≥1 EMTing-CTC and/or ≥4 mesenchymal-CTCs were defined as CTC-score 'positive' using our previously established definition based on the analysis of healthy control blood samples (32)

CTC RNA extraction and gene expression analysis

CTCs were isolated from a separate 7.5 mL of whole blood using the Parsortix® and collected into a 1.5 mL low-retention eppendorf. Total RNA was extracted using miRNeasy micro kit (Qiagen, Hilden, Germany) following manufacturer's instructions but eluted with a final volume of 11.5 μL. The total 11.5 μL of RNA extracted from CTCs was mixed with 0.5 μL of random primers and denatured at 65 °C for 5 min. After incubation for 5 min on ice, 4 μL of first strand buffer, 2 μL of 0.1 M DTT, 1 μL of 10 mM dNTPs (Roche, Basel, Switzerland), 0.5 μL of water and 0.5 μL of Superscript II (Thermo Fisher Scientific, Waltham, Massachusetts, Unites States) were added and cDNA synthesis was performed at 42 °C for 2 hrs, followed by enzyme inactivation by heating at 70 °C for 15 mins. Multiplex RT-qPCR was performed by Barts and the London Genome Centre using BioMark HD system (Fluidigm Corporation, South San Francisco, California, United States). 96.96 Dynamic Array Integrated fluidic circuit (IFC) was used to test expression levels of 32 assays in triplicates within one reaction plate. A list of TaqMan probes (Applied Biosystems, Massachusetts, Unites States) used are shown in Supplementary Table 2. The brief workflows were as follows: (1) pooling the TaqMan assays. Combine equal volumes of each 20X TaqMan Gene Expression assays in a 0.5 mL microcentrifuge tube, up to 100 μL in total. Dilute the pooled assays using DNA Suspension Buffer (10mM Tris, pH 8.0, 0.1 mM EDTA) so that each assay is at a final concentration of 0.2X. (2) Combine 2.5 μL of TaqMan® PreAmp MasterMix (Life Technologies, Carlsbad, California, Unites States), 1.25 μL of pooled assay mix and 1.25 μL of cDNA to make the final sample mixture in each aliquot. (3) Place reaction tubes in the thermal cycler and cycle as (95 °C for 10 minutes followed by 14 cycles of 95 °C for 15 secs and then 60 °C for 4 mins). Only the targets of interests are amplified and this results in small amount of cDNA being amplified equally without introducing bias. Following pre-amplification, the samples were diluted 1:5 (v/v) in DNA suspension buffer. Reactions were then assayed using Dynamic Arrays prepared as instructed by the manufacturer. PCR was performed with 40 cycles of reactions.

Gene panel selection

Two microarray expression profile data sets (GSE36135 (39), GSE33455 (11)) were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo), which are based on the GPL571 Affymetrix Human Genome U133A 2.0 Array [HG_U133A_2] and GPL570 Affymetrix Human Genome U133 Plus 2.0 Array [HG-U133_Plus_2], respectively. The original Series Matrix data files were analysed with GEO2R (using the GEOquery and limma R packages from Bioconductor (http://www.bioconductor.org/) to identify

differentially expressed genes (DEGs) in each of the paired docetaxel-resistant and docetaxel-sensitive cell lines in the datasets. A DEG was considered to be significant according to the following criteria: Fold-change (FC) >2 and false discovery rate (FDR) <0.05. Genes that were not upregulated in ≥2 DOC resistant cell lines were excluded to control for random variance in gene expression. Additionally, we identified reported PCa-specific and/or docetaxel-resistance related genes by literature search, and the two gene lists were combined to form a test gene panel. To select for genes suitable for CTC analysis, the genes were searched in The Genotype-Tissue Expression Portal V7 database for their expression in prostate and whole blood. Genes were selected based on their relative high expression in the prostate and low/no expression in whole blood. Candidate gene expression was subsequently validated in a panel of PCa cell lines and PBMC samples from five PCa biopsy negative males.

Statistical analysis

Mann-Whitney U test was applied to assess differences in clinical characteristics between patient groups. Data were shown as median (interquartile range [IQR]). Spearman's rank correlation was used to assess associations of CTC counts and gene expression with concurrent PSA, ALP, LDH levels and OS/PFS. A combined risk score (CRS) was computed as the linear predictor of the fitted bivariate logistic model with PSA, ALP, CTC-score, total-CTC number and KLK2 count as only predictors (as CRS = a * Y + b * X…, where the values of 'a' and 'b' are the estimated log odds ratios). Survival curves were generated using the Kaplan-Meier method and compared using the logrank test. Bonferroni correction method was applied to adjust p values (p_{adj}) for multiple testing. RT-qPCR $2^{-\Delta\Delta Ct}$ was used to compare mRNA expression levels in patient samples. Receiver operating characteristic (ROC) curve analysis was used to evaluate prediction values. Follow-up time started on the date of administration of the first docetaxel dose. Observations were censored on the date of last follow-up.

Statistical analyses were performed using GraphPad Prism 9. All tests were two-sided with p values of <0.05 considered statistically significant.

Results

Patient characteristics and association of pre-docetaxel CTC measurements with clinicopathological data

Clinical characteristics and CTC counts are summarised in Table 1. At least one CTC was detected in 12/18 (67%) of mCRPC patients, 50% of patients had a positive CTC-score (≥1 epithelial-CTC and/or ≥1 EMTing-CTC and/or ≥4 mesenchymal-CTCs) and the median total CTC count was 1.5 (interquartile range= 0-5.8). In mHSPC patients, ≥1 CTC was detected in 26/43 (60%), 51% of patients had a positive CTC-score and the median total CTC count was 1 (interquartile range = 0-4.3). Figure 1A shows an example of immunofluorescence staining for three CTC subtypes. Figure 1B shows individual CTC subtype counts in mHSPC and mCRPC patients. No significant differences were found between CTC subtype numbers.

We subsequently investigated the relationship between CTCs and clinicopathological data. Spearman's correlation was performed between CTCs and serum PSA and ALP, results are shown in Table 2. In mCRPC patients, serum PSA was significantly correlated with total-CTC (ρ= 0.51, p= 0.032), epithelial-CTC (ρ= 0.51, p= 0.030), EMTing-CTC numbers (ρ= 0.53, p= 0.024) and positive CTC-score (ρ= 0.68, p= 0.0021). Serum ALP was significantly correlated with total-CTC (ρ= 0.51, p= 0.046) and epithelial-CTC numbers (ρ= 0.62, p= 0.012). In mHSPC patients, serum ALP was significantly correlated with mesenchymal-CTC numbers (ρ= 0.34, p= 0.044), however no other significant correlations were observed.

Correlation of pre-docetaxel CTCs with RECIST response post docetaxel treatment

To assess if CTCs could predict radiological response to docetaxel following treatment cycles, we compared CTC numbers between partial response (PR), stable disease (SD) or progressive disease (PD) groups (Supplementary Table 1), and performed ROC analysis. Due to the limited sample size, we combined patients who had SD or PD at the end of docetaxel treatment into one group. While there were no significant differences in CTC numbers between patients with PR and SD/PD in this small cohort, trends were observed. In mCRPC patients with PR, limited mesenchymal-CTCs and no epithelial- and EMTing-CTCs were detected. Total-CTCs trended towards a significantly lower number in patients with a PR (p= 0.073) compared to those with SD/PD (Figure 2A) with an AUC of 0.80, p= 0.071 in predicting SD/PD (Figure 2C). In comparison, serum PSA had an AUC of 0.78, p= 0.089, and ALP had an AUC of 0.74, p= 0.20 (Figure 2C). In order to improve our ability to predict radiological response to docetaxel using blood-based biomarkers, we generated a combined risk score (CRS) combing the total number of CTCs, serum PSA and ALP levels for SD/PD prediction as CRS-TPA= 0.7414 * Total-CTC number + 0.02909 * PSA + 0.01423 * ALP, which resulted in an AUC of 0.90, p= 0.037, with a sensitivity of 84.62% and

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TABLE 1 Summary of clinical characteristics and CTC enumeration for metastatic PCa patients.

	n	mCRPC	n	mHSPC
Age at pre-docetaxel, y				
Mean ± SD	18	73 (66.5-75.8)	43	68 (63-73)
PSA at diagnosis, ng/mL				
Median (IQR)	14	21 (14.5-61.5)	39	54 (18.5-344.6)
Biopsy GS, n (%)				
7		8 (44)		6 (14)
>7		9 (50)		28 (65)
unknown		1 (6)		9 (21)
Pre-docetaxel PSA, ng/mL				
Median (IQR)	18	54.1 (12.9-111.8)	42	15.6 (3.6-50.1)
Pre-docetaxel ALP, U/L				
Median (IQR)	16	88 (74.8-387.5)	36	104 (70-328)
Pre-docetaxel CTC-score, n (%)				
Positive		9 (50)		22 (51)
Negative		9 (50)		21 (49)
Pre-docetaxel total CTC, n				
Median (IQR)	18	1.5 (0-5.8)	43	1 (0-4.3)
Pre-docetaxel Epithelial-CTC, n				
Median (IQR)	18	0 (0-2)	43	0 (0-1)
Pre-docetaxel EMTing-CTC, n				
Median (IQR)	18	0 (0-0)	43	0 (0-0)
Pre-docetaxel Mesenchymal-CTC, n				
Median (IQR)	18	1 (0-2.25)	43	0 (0-2)

mCRPC, metastatic castration-resistant prostate cancer; mHSPC, metastatic hormone-sensitive prostate cancer; IQR, interquartile range; PSA, prostate specific antigen; GS, Gleason score; SD, standard deviation.

specificity of 100% when the cut-off was set at >3.45 (Figure 2C). The CRS-TPA performed better than serum PSA and ALP alone, although the difference was not statistically significant (p= 0.66, and 0.23 respectively), likely due to the limited sample size.

In mHSPC, epithelial- and EMTing-CTC numbers did not differentiate patients based on radiological response to docetaxel. Conversely, high mesenchymal-CTC numbers trended towards (p= 0.079) higher chance of SD/PD (Figure 2B) with an AUC of 0.65, p= 0.099 (Figure 2D). Serum ALP levels were best able to predict SD/PD, with an AUC of 0.68, p= 0.077, compared to that of serum PSA which had an AUC of 0.52, p= 0.82 (Figure 2D). A CRS comprised of mesenchymal-CTC number and serum ALP levels as CRS-MA= 0.3599 * mesenchymal-CTC number + 0.002037 * ALP, increased the AUC to 0.69, however with only a trend towards significance (p= 0.064) (Figure 2D).

CTCs were significantly associated with PFS and OS in mCRPC and mHSPC patients

To assess the prognostic value of CTCs, we correlated CTC measurements with patient OS and PFS. Long term follow-up data was available for 18 mCRPC and 42 mHSPC patients. The median follow-up time for mCRPC patients was 22.7 months (range 8.0-53.1 months), during which time 13/18 (72%) patients progressed and/or died. Spearman's correlation (Table 3) showed that OS significantly inversely correlated with total- (ρ= -0.66, p= 0.0027), epithelial- (ρ= -0.62, p= 0.0057) and EMTing-CTC (ρ= -0.65, p= 0.0034) numbers and a positive CTC-score (ρ= -0.80, p< 0.0001) in the mCRPC cohort. Additionally, PFS was significantly inversely correlated

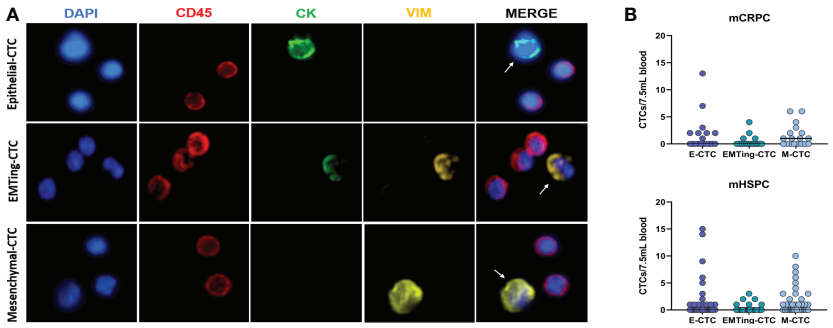

FIGURE 1
Detection of three subtypes of CTCs in PCa patient samples. **(A)** Three distinct CTC subtypes were identified by immunofluorescence in patient blood samples. Top: One CK+/VIM-/CD45- epithelial-CTC adjacent to two CD45+ leucocytes. Middle: One CK+/VIM+/CD45- EMTing-CTC adjacent to three CD45+ leucocytes. Bottom: One CK-/VIM+/CD45- mesenchymal-CTC adjacent to two CD45+ leucocytes. **(B)** Individual CTC numbers in each mCRPC and mHSPC patient sample, respectively. Median CTCs number per 7.5mL of blood is shown. Abbreviations: CK, Cytokeratin; VIM, Vimentin.

with epithelial-CTC numbers (ρ= -0.63, p= 0.0049) and a positive CTC-score (ρ= -0.65, p= 0.0033). We also evaluated the performance of serum biomarkers PSA and ALP. We found that serum PSA levels significantly inversely correlated with OS (ρ= -0.72, p= 0.0008) and PFS (ρ= -0.50, p= 0.035). With a view to improve the sensitivity and specificity of pre-docetaxel biomarkers for the prediction of OS, we generated a combined risk score using both PSA and CTC-score data. The AUC of a CRS comprised of PSA (AUC= 0.93) and CTC-score (AUC= 0.89) (CRS-PS= 0.08127 * PSA + 4.159 * CTC-score) to discriminate mCRPC patients with <24 months OS from those with ≥24 months OS reached 0.96, p= 0.0009, with a sensitivity

TABLE 2 Spearman's correlation between CTCs and serum PSA and ALP.

	PSA	ALP
	Spearman's ρ (p-value)	
mCRPC patients		
Total-CTCs	**0.51 (0.032)**	**0.51 (0.046)**
Epithelial-CTCs	**0.51 (0.030)**	**0.62 (0.012)**
EMTing-CTCs	**0.53 (0.024)**	0.38 (0.15)
Mesenchymal-CTCs	0.22 (0.37)	0.27 (0.31)
CTC-score	**0.68 (0.0021)**	**0.45 (0.091)**
PSA	–	–
ALP	0.35 (0.19)	–
mHSPC patients		
Total-CTCs	-0.017 (0.92)	0.27 (0.11)
Epithelial-CTCs	0.14 (0.37)	0.080 (0.65)
EMTing-CTCs	0.066 (0.68)	-0.032 (0.86)
Mesenchymal-CTCs	-0.047 (0.77)	**0.34 (0.044)**
CTC-score	-0.006 (0.97)	0.21 (0.23)
PSA	–	–
ALP	**0.35 (0.044)**	–

bold black numbers, significant results

of 88.9% and a specificity of 100% when the cut-off was set to <5.96 (Figure 3). This made an improvement on the AUC of PSA alone but without significance, p= 0.60.

We then performed Kaplan Meier survival analysis using total and subtype CTC numbers to predict OS and PFS with optimal CTC number cut-offs evaluated (Table 4). In mCRPC patients, each total-CTC number cut-off that was explored (<2 vs. ≥2 to <6 vs. ≥6 CTCs) was significantly associated with patients with short median OS. The detection of a positive CTC-score (12.80 vs. 37.33 months, HR= 5.08, p= 0.0005) (Figure 4A), ≥3 total-CTCs (12.80 vs. 37.33 months, HR= 3.84, p= 0.0053) (Figure 4B), ≥1 epithelial-CTC (14.30 vs. 37.33 months, HR= 3.89, p= 0.0041) (Figure 4C) and ≥1 EMTing-CTC (11.32 vs. 32.37 months, HR= 6.73, p= 0.0001) (Figure 4D) were most significantly associated with shorter median OS. Importantly, when the Bonferroni correction method was applied to adjust p values for multiple testing, a positive CTC-score (p_{adj}= 0.0055), ≥1 epithelial-CTC (p_{adj}= 0.045) and ≥1 EMTing-CTC (p_{adj}= 0.0011) remained significantly associated with shorter median OS. The detection of ≥2 epithelial-CTCs was most significantly associated with shorter median PFS (7.52 vs. 18.83 months, HR= 3.93, p= 0.0058) (Figure 4E).

The median follow-up time for mHSPC patients was 29.5 months (range 5.9-48.8 months), during which time 25/42 (60%) patients progressed and 11/42 (26%) died. Spearman's correlation did not show associations of CTC measurements with PFS and OS, however, ALP was significantly inversely correlated with OS (ρ= -0.45, p= 0.0068) and PFS (ρ= -0.62, p< 0.0001) (Table 3). Kaplan Meier analysis revealed that patients with ≥5 CTCs experienced the most significantly

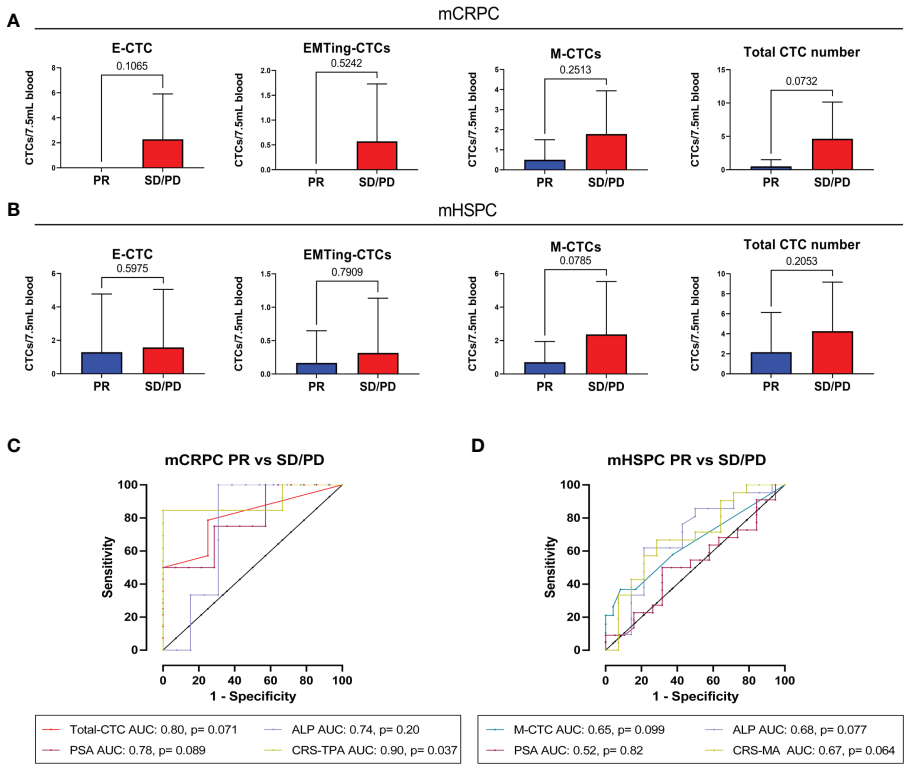

FIGURE 2
The relationship between pre-docetaxel CTCs and initial RECIST response. **(A)** Number of CTC subtypes and total-CTC numbers in mCRPC patients with PR or, SD and PD combined. Epithelial and EMTing-CTCs were not detected in patients with PR, although they did not significantly differentiate from patients with SD/PD, p=0.1065 and p=0.7907, respectively. Mesenchymal CTCs were detected in a small number of patients with PR but the majority were detected in patients with SD/PD (p=0.2513). Total CTC number trended towards a significant difference between patients with PR and SD/PD p=0.0732. **(B)** Number of CTC subtypes and total-CTC numbers in mHSPC patients with PR or, SD and PD combined. There was no significant difference in the numbers of epithelial-CTCs (p=0.5975), EMTing-CTCs (p=0.7909) and total-CTCs (p=0.2053) detected between patients with PR and SD/PD. However, mesenchymal-CTC numbers trended towards a significant difference between the patient outcome groups (p=0.0785). **(C)** For prediction of immediate tumour response in mCRPC patients, total-CTCs had an area under the curve (AUC) of 0.80, p= 0.071, PSA had an AUC of 0.78, p= 0.089 and ALP had an AUC of 0.74, p= 0.20. A combined risk score combining all three variables was calculated as CRS-TPA= 0.7414*Total-CTC number + 0.02909 *PSA + 0.01423*ALP, which increased the AUC to 0.90, p=0.037. **(D)** For prediction of immediate tumour response in mHSPC patients, mesenchymal-CTCs had that highest AUC of 0.65, p= 0.099, PSA had an AUC of 0.52, p= 0.82 and ALP an AUC of 0.68, p= 0.77. A combined risk score combining all mesenchymal CTC numbers and ALP was calculated as CRS-MA= 0.3599 * mesenchymal-CTC number + 0.002037 * ALP, resulted in an AUC of 0.69, p= 0.064.

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TABLE 3 Spearman's correlation of CTCs and serum biomarkers with OS and PFS.

	OS	PFS
	Spearman's ρ (p-value)	
mCRPC patients		
Total-CTCs	**-0.66 (0.0027)**	**-0.52 (0.075)**
Epithelial-CTCs	**-0.62 (0.0057)**	**-0.63 (0.0049)**
EMTing-CTCs	**-0.65 (0.0034)**	-0.14 (0.57)
Mesenchymal-CTCs	-0.40 (0.10)	0.34 (0.17)
CTC-score	**-0.80 (<0.0001)**	**-0.65 (0.0033)**
PSA	**-0.72 (0.0008)**	**-0.50 (0.035)**
ALP	-0.41 (0.13)	-0.16 (0.55)
mHSPC patients		
Total-CTCs	0.16 (0.31)	-0.087 (0.58)
Epithelial-CTCs	-0.084 (0.60)	-0.16 (0.30)
EMTing-CTCs	0.13 (0.41)	0.11 (0.43)
Mesenchymal-CTCs	0.20 (0.20)	-0.076 (0.63)
CTC-score	0.012 (0.94)	-0.18 (0.26)
PSA	-0.21 (0.19)	-0.16 (0.33)
ALP	**-0.45 (0.0068)**	**-0.62 (<0.0001)**

PFS, progression-free survival; OS, overall survival; bold black numbers, significant results

shorter median OS (24.57 vs undefined months, HR= 4.14, p= 0.0097) (Table 4, Figure 4F).

CTC gene expression predicted PFS and OS in mHSPC patients

Subsequently, we interrogated CTC mRNA expression to enhance the efficiency of CTCs as predictive biomarkers beyond CTC enumeration in the mHSPC cohort. Up-regulated differentially expressed genes in docetaxel-resistant cell lines from two microarray datasets were considered for docetaxel-resistant CTC detection. There were 162 genes commonly up-regulated in docetaxel-resistant cell lines and considered for further validation. Additionally, we identified 75 reported docetaxel-resistance related genes by literature search of relevant publications regarding the mechanisms of docetaxel resistance. These panels were combined to form a 237-candidate gene panel (Supplementary Table 3). As the enriched CTC fraction that is harvested from the Parsortix® is not pure, it was necessary to account for white blood cell contamination in the sample. Therefore, we performed two steps of gene expression analysis to exclude any of the 237 candidate genes that were expressed in leucocytes, 1. *in silico* analysis and 2. *in vitro* experiments using PCa cell lines and patient derived leucocyte samples. Firstly, the 237 genes were searched in The Genotype-Tissue Expression Portal V7 database for their expression in prostate and whole blood. Genes were selected based on their relatively high expression in prostate tissue and

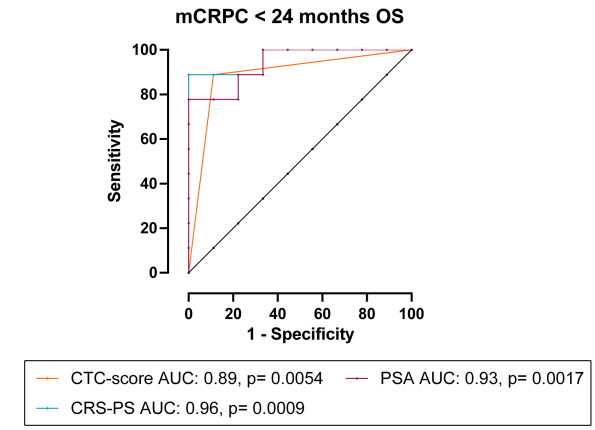

FIGURE 3
CRS of CTC-score and PSA for the prediction of <24 months OS in mCRPC patients. In mCRPC patients, combining CTC-score with PSA as CRS-PS= 0.08127 * PSA + 4.159 * CTC-score, increased the AUC to 0.96, p= 0.0009.

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TABLE 4 Kaplan Meier analysis of CTC enumeration cut-offs for OS and PFS in mCRPC and mHSPC.

CTC parameters	Patients per group (n)	OS			PFS		
		Median survival (months)	HR (95% CI)	p-value	Median survival (months)	HR (95% CI)	p-value
mCRPC patients							
CTC-score negative vs. CTC-score positive	9 vs. 9	37.33 vs. 12.80	5.08 (3.41 to 43.57)	**0.0005**	18.83 vs. 8.0	2.70 (1.18 to 15.03)	**0.042**
< 2 vs. ≥ 2 CTCs	9 vs. 9	34.80 vs. 12.80	3.13 (1.37 to 15.82)	**0.021**	16.43 vs. 8.0	1.54 (0.50 to 5.50)	0.43
< 3 vs. ≥ 3 CTCs	11 vs. 7	37.33 vs. 12.80	3.84 (2.08 to 34.72)	**0.0053**	16.43 vs. 8.0	2.03 (0.66 to 11.16)	0.20
< 4 vs. ≥ 4 CTCs	12 vs. 6	32.27 vs. 12.53	3.66 (1.80 to 39.08)	**0.010**	13.82 vs. 8.30	1.57 (0.41 to 7.95)	0.46
< 5 vs. ≥ 5 CTCs	13 vs. 5	32.27 vs. 12.27	3.39 (1.38 to 41.25)	**0.025**	11.20 vs. 9.80	1.25 (0.25 to 6.61)	0.77
< 6 vs. ≥ 6 CTCs	14 vs. 4	28.23 vs. 12.53	2.50 (0.68 to 22.90)	**0.014**	11.30 vs. 7.33	2.10 (0.38 to 21.12)	0.32
0 vs. ≥ 1 E-CTC	10 vs. 8	37.33 vs. 14.30	3.89 (2.17 to 28.45)	**0.0041**	18.83 vs. 7.70	3.57 (1.82 to 28.47)	**0.0088**
< 2 vs. ≥ 2 E-CTC	11 vs 7	37.33 vs. 12.80	3.72 (1.99 to 32.21)	**0.0065**	18.83 vs. 7.52	3.93 (2.20 to 51.88)	**0.0058**
0 vs. ≥ 1 EMTing-CTC	14 vs. 4	32.37 vs. 11.32	6.73 (11.47 to 1043)	**0.0001**	11.20 vs. 10.28	1.03 (0.23 to 4.72)	0.97
0 vs. ≥ 1 M-CTC	10 vs. 8	32.37 vs. 16.07	1.39 (0.60 to 5.29)	0.30	13.82 vs. 8.3	0.89 (0.30 to 2.61)	0.83
< 2 vs. ≥ 2 M-CTC	12 vs. 6	28.23 vs. 12.53	1.88 (0.56 to 8.89)	0.27	10.57 vs. Undefined	0.60 (0.18 to 2.31)	0.50
mHSPC patients							
CTC-score negative vs. CTC-score positive	20 vs. 22	Undefined vs. Undefined	2.53 (0.72 to 7.74)	0.15	36.40 vs. 12.52	1.90 (0.89 to 4.01)	0.10
< 2 vs. ≥ 2 CTCs	23 vs. 19	Undefined vs. 40.30	1.73 (0.53 to 5.60)	0.37	26.57 vs. 16.83	1.42 (0.67 to 3.02)	0.37
< 3 vs. ≥ 3 CTCs	27 vs. 15	Undefined vs. 40.30	2.74 (0.86 to 9.71)	0.090	21.80 vs. 13.33	1.58 (0.74 to 3.66)	0.23
< 4 vs. ≥ 4 CTCs	29 vs. 13	Undefined vs. 31.07	3.91 (1.31 to 16.89)	**0.018**	26.57 vs. 11.13	1.62 (0.74 to 4.06)	0.22
< 5 vs. ≥ 5 CTCs	32 vs. 10	Undefined vs. 24.57	4.14 (1.61 to 27.36)	**0.0097**	26.57 vs. 9.63	1.79 (0.77 to 5.27)	0.16
< 6 vs. ≥ 6 CTCs	34 vs. 8	Undefined vs. 24.57	3.98 (1.54 to 35.11)	**0.013**	26.57 vs. 9.63	2.18 (0.94 to 8.33)	0.067
0 vs. ≥ 1 E-CTC	28 vs. 14	Undefined vs. Undefined	2.86 (0.92 to 12.25)	0.068	29.37 vs. 11.42	2.07 (0.99 to 5.33)	0.098
< 2 vs. ≥ 2 E-CTC	35 vs. 7	Undefined vs. Undefined	2.07 (0.49 to 12.93)	0.27	21.80 vs. 11.13	1.54 (0.58 to 4.65)	0.39
0 vs. ≥ 1 EMTing-CTC	36 vs. 6	Undefined vs. Undefined	0.50 (0.12 to 2.81)	0.50	15.90 vs. 28.97	0.85 (0.31 to 2.34)	0.76
0 vs. ≥ 1 M-CTC	22 vs. 20	Undefined vs. 40.30	2.13 (0.65 to 7.0)	0.23	26.57 vs. 15.88	1.22 (0.57 to 2.61)	0.60

(Continued)

TABLE 4 Continued

CTC parameters	Patients per group (n)	OS			PFS		
		Median survival (months)	HR (95% CI)	p-value	Median survival (months)	HR (95% CI)	p-value
< 2 vs. ≥ 2 M-CTC	31 vs. 11	40.30 vs 31.07	1.86 (0.57 to 7.28)	0.29	26.57 vs. 13.33	1.56 (0.68 to 3.97)	0.27

PFS, progression-free survival; OS, overall survival; E-CTC, Epithelial CTC; M-CTC, Mesenchymal CTC; Undefined, The probability of survival exceeds 50% at the longest time point; bold black numbers, significant results; bold grey numbers, results with a trend towards significance.

low/no expression in whole blood. This resulted in a candidate panel of 39 genes. Secondly, using RT-qPCR in paired docetaxel-sensitive (PC3-AG) and docetaxel-resistant (PC3-D12) PCa cell lines, and five PBMC samples from biopsy-negative patients, we experimentally validated 23/39 genes for lack of expression in leucocytes. A further seven genes, including EMT and stem cell markers (*PTPRC* (*CD45*), *SNAI1*, *ZEB1*, *NANOG*, *POU5F1*, *PROM1* and *SOX2*) were included due to their potential prognostic value in clinical samples and lack of expression in leucocytes, along with housekeeping genes *GAPDH* and *MRFAP1*. Unfortunately, the mCRPC sample size available at this time point was limited and gene expression analysis was not performed. However, the relationship between candidate gene expression and OS/PFS was investigated in 33 mHSPC patient samples.

Survival analysis was performed by separating patients into the following groups: 1) Expression vs. no expression 2) 50% highest expression vs. 50% lowest expression. Kaplan Meier analysis revealed that *KLK2* expression was significantly associated with shorter median OS (27.17 vs. undefined months, HR= 3.87, p= 0.037) (Figure 5A) and PFS (8.13 vs. 26.57 months, HR= 5.15, p= 0.0002) (Figure 5B). *KLK4* expression was significantly associated with shorter median PFS (10.17 vs. 26.57, HR= 3.01, p= 0.034) (Figure 5C), while *KLK3* (PSA) expression had only a trend towards shorter median OS (30.13 vs. undefined months, HR= 3.18, p= 0.068)

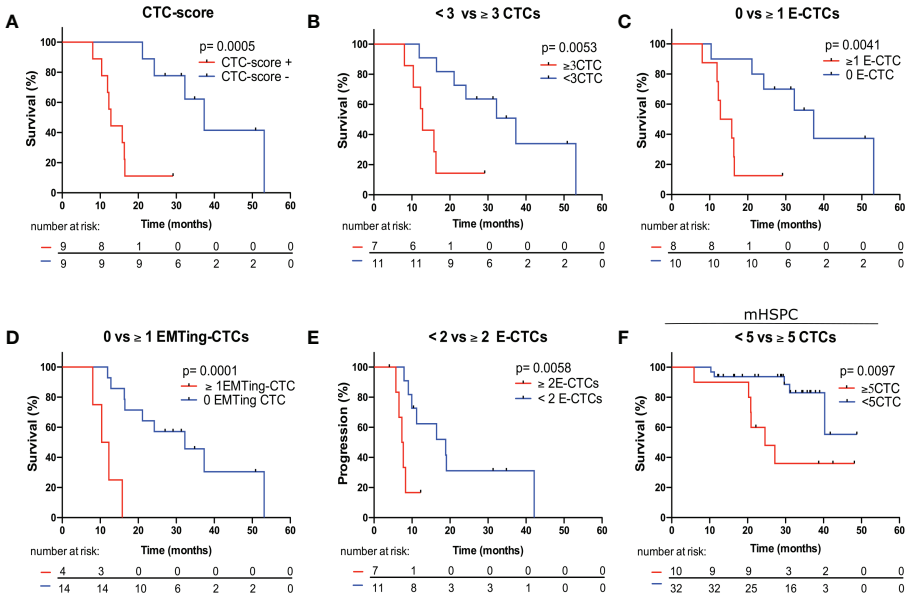

FIGURE 4
Kaplan Meier survival analysis of CTC measurements to predict OS and PFS in mCRPC and mHSPC patients. **(A)** mCRPC patients with a positive CTC-score experienced significantly shorter median OS compared to those with a negative CTC-score (12.80 vs. 37.33 months, HR= 5.08, p= 0.0005). **(B)** mCRPC patients with ≥3 total-CTCs experienced significantly shorter median OS compared to those with <3 total-CTCs (12.80 vs. 37.33 months, HR= 3.84, p= 0.0053). **(C)** mCRPC patients with ≥1 epithelial-CTCs experienced significantly shorter median OS compared to those with <1 epithelial-CTC (14.30 vs. 37.33 months, HR= 3.89, p= 0.0041). **(D)** mCRPC patients with ≥1 EMTing-CTCs experienced significantly shorter median OS compared to those with <1 EMTing-CTC (11.32 vs. 32.37 months, HR= 6.73, p= 0.0001). **(E)** mCRPC patients with ≥2 epithelial-CTCs experienced significantly shorter median PFS compared to those with <2 epithelial-CTC (7.52 vs. 18.83 months, HR= 3.93, p= 0.0058). **(F)** mHSPC patients with ≥5 CTCs experienced significantly shorter median OS compared to those with <5 CTCs (24.57 vs undefined months, HR= 4.14, p= 0.0097).

(Figure 5D) and did not separate patients based on PFS. Patients with 50% highest *SNAI1* and/or *ADAMTS1* expression experienced significantly shorter median OS (31.07 vs. undefined months, HR= 9.51, p= 0.0090 (Figure 5E); 31.07 vs undefined months, HR= 4.30, p= 0.047 (Figure 5F), respectively) and patients with the 50% highest *ZEB1* expression experienced significantly shorter median PFS (11.50 vs. 24.40 months, HR= 2.5, p= 0.036) (Figure 5G). Hazard ratios for each gene for OS and PFS are presented in Figure 5H.

We then investigated the clinical outcomes of patients who had CTCs expressing more than one high-risk gene. The median PFS for patients with *KLK2*+*ZEB1*^{hi} (8.13 months Logrank p= 0.0004) (Figure 6A) and/or *KLK2*+*SNAI1*^{hi} (5.9 months, Logrank p= 0.0019) (Figure 6B) and/or *KLK2*+*ADAMTS1*^{hi} (5.9 months, Logrank p= 0.0004) (Figure 6C) was significantly shorter than for patients with no *KLK2* expression and low expression of each gene. Patients expressing both genes also had high total CTC numbers detected in paired samples (*KLK2* +*ZEB1*^{hi}: ≥8 CTCs, *KLK2*+*SNAI1*^{hi}: ≥5 CTCs, *KLK2* +*ADAMTS1*^{hi}: 0, 5 and 9 CTCs).

Using receiver operating characteristic curve analysis, high expression of *ADAMTS1* was significantly predictive of shorter OS with an AUC of 0.73, p= 0.043 (Figure 7A). Neither serum PSA or ALP levels significantly differentiated patients based on OS, although ALP showed a trend towards significance (AUC= 0.55, p= 0.66; AUC= 0.73, p= 0.072, respectively) (Figure 7A). Combining *ADAMTS1*, ALP and ≥5 total CTCs to form a CRS as CRS-AA5o= 0.06386 * *ADAMTS1* + 0.001465 * ALP + 2.169 * ≥5 total CTCs, which increased the AUC to 0.83, p= 0.0070 (65% sensitivity and 87.5% specificity at a cut off of <0.48) vs AUC of 0.73 for ALP alone, (Figure 7A) however without a significant difference (p= 0.38).

We then evaluated the candidate genes for the prediction of PFS and found that high expression of *ZEB1, SNAI1* and *ADAMTS1* was significantly predictive of progression within 24 months (AUC= 0.77, p= 0.0092; AUC= 0.71, p= 0.039; AUC= 0.71, p= 0.043, respectively) (Figure 7B). Expression of *KLK2* had a trend of correlated with <24 months PFS, but the AUC was not significant (AUC= 0.64, p= 0.18) (Figure 7B). ALP but not serum PSA levels significantly discriminated patients with <24 months PFS from those with ≥24 months PFS (AUC= 0.86, p= 0.0015; AUC= 0.52, p= 0.86, respectively) (Figure 7B).

Discussion

Improvements in our understanding of the genetic landscape of PCa have advanced treatments for metastatic

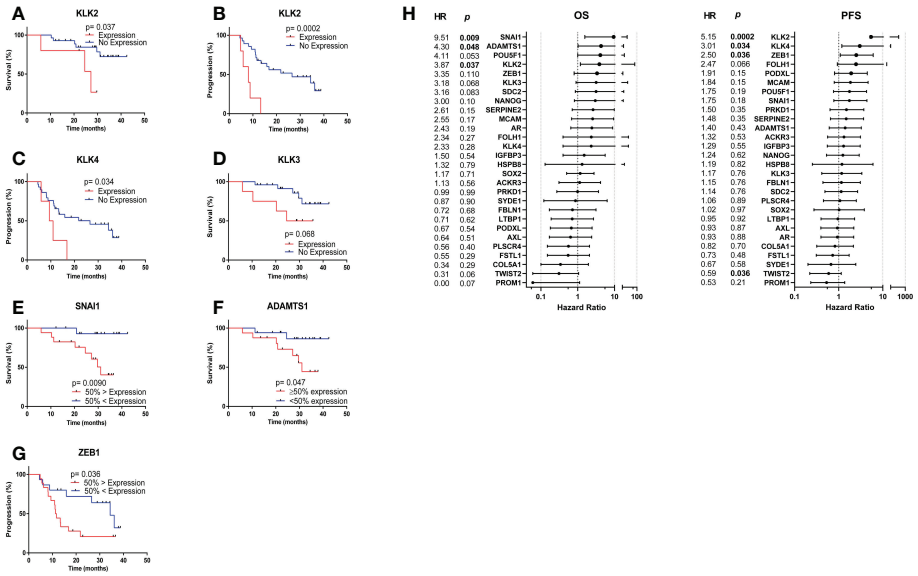

FIGURE 5
Kaplan Meier analysis based on CTC gene expression. **(A)** *KLK2* expression was significantly associated with shorter median OS (27.17 vs. undefined months, HR= 3.87, p= 0.037). **(B)** *KLK2* expression was significantly associated with shorter median PFS (8.13 vs. 26.57 months, HR= 5.15, p= 0.0002). **(C)** *KLK4* expression was significantly associated with shorter median PFS (10.17 vs. 26.57, HR= 3.01, p= 0.034). **(D)** *KLK3 (PSA)* expression had a trend towards significant association with shorter median OS (30.13 vs. undefined months, HR= 3.18, p= 0.068). **(E)** Patients with 50% highest *SNAI1* expression experienced significantly shorter median OS (31.07 vs. undefined months, HR= 9.51, p= 0.0090). **(F)** Patients with 50% highest *ADAMTS1* expression experienced significantly shorter median OS (31.07 vs undefined months, HR= 4.30, p= 0.047). **(G)** Patients with the 50% highest *ZEB1* expression experienced significantly shorter median PFS (11.50 vs. 24.40 months, HR= 2.5, p= 0.036). **(H)** Hazard ratios for each gene for OS and PFS. *Undefined months:* The probability of survival exceeds 50% at the longest time point.

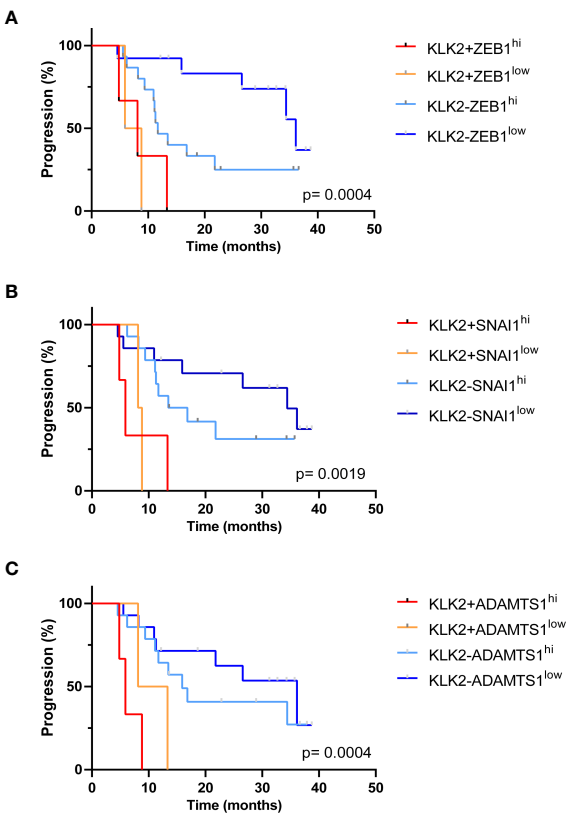

FIGURE 6
Kaplan Meier analysis of patients expressing multiple poor prognostic genes. **(A)** The median PFS for patients with *KLK2+ZEB1*^{hi} was 8.13 months, which was significantly shorter than *KLK2-ZEB1*^{low} group (36.13 months) with p= 0.0008. Overall Logrank p= 0.0004. **(B)** The median PFS for patients with *KLK2+SNAI1*^{hi} was 5.9 months. *KLK2+SNAI1*^{hi} vs. *KLK2-SNAI1*^{hi}: 5.9 vs. 15.15 months, p= 0.019; *KLK2+SNAI1*^{hi} vs. *KLK2-SNAI1*^{low}: 5.9 vs. 34.40 months, p= 0.0083. Logrank p= 0.0019. **(C)** The median PFS for patients with *KLK2+ADAMTS1*^{hi} was 5.9. *KLK2+ADAMTS1*^{hi} vs. *KLK2+ADAMTS1*^{low}: 5.9 vs. 10.73 months, p= 0.28; *KLK2+ADAMTS1*^{hi} vs. *KLK2-ADAMTS1*^{hi}: 5.9 vs 15.90 months, p= 0.0015; *KLK2+ADAMTS1*^{hi} vs. *KLK2-ADAMTS1*^{low}: 5.9 vs. 36.13 months, p= 0.0007. Logrank p= 0.0004.

disease. These treatments comprise androgen-receptor targeting therapies (abiratorine, enzalutminde) bone-targeting radiotherapies (Radium-223), immunotherapies, and cytotoxic chemotherapies (docetaxel, cabazataxel). Docetaxel is now a first-line therapy in both mCRPC and mHSPC, however its efficacy is limited by the vast inter- and intra-tumour heterogeneity of PCa, resulting in clonal populations with inherent and/or acquired resistance in a proportion of patients. Although multiple docetaxel resistance mechanisms have been revealed through extensive research, such as upregulation of drug efflux pumps (e.g. ABCB1) (40), alterations to β-tubulin and expression of tubulin isoforms (41), deregulation of apoptosis and survival signalling pathways (42), induction of EMT and cancer stem cells phenotypes (36, 43, 44), and deregulation of AR signalling (45), the development of clinically useful tools to predict response is currently still required (46–48). Thus, patients with resistance may undergo systemic chemotherapy with little survival benefit (49, 50). Our ability to elucidate biomarkers of resistance is limited by tissue biopsy, which samples only a small fraction of the entire heterogeneous tumour, is practically difficult in metastatic disease and as a repeated measure. However, liquid biopsy analysis of CTCs offers a minimally invasive, easily repeatable tool for cancer specific interrogation. Using the Parsortix® CTC isolation system, we investigated the potential of using CTCs, including subtype and CTC gene expression analysis, to predict docetaxel response and survival benefit in both mCRPC and mHSPC patients.

We found that individual CTC measurements alone showed some, but limited associations with RECIST response in mCRPC and mHSPC patients. However, when combined with serum biomarkers in mCRPC patients, initial progressive disease could be predicted with high accuracy. Furthermore, combining CTCs with serum biomarkers efficiently detected mCRPC patients at risk of shorter OS, supporting the potential use of CTCs to triage patients for docetaxel treatment. Importantly, we showed that ≥5 pre-docetaxel CTCs/7.5mL were also significantly associated with poor PFS in mHSPC patients treated with first-line chemo-hormonal therapy. Additionally, molecular analysis of CTC samples revealed that the expression of the candidate docetaxel-resistance gene *ADAMTS1*, and EMT transcription factors *ZEB1* and *SNAI1* along with the PCa specific kallikreins *KLK2* and *KLK4* significantly correlated with poor mHSPC patient outcome.

While the detection of CTCs is now a well-established marker of aggressive cancer with poor survival outcome, it is yet to be determined if CTCs have an association with initial docetaxel response. Immediate radiological or RECIST response criteria are commonly used to determine a treatment response upon the completion of a therapeutic regimen. In our study, although potentially due to the limited cohort sizes, individual CTC subtypes did not significantly discriminate between patients with a partial response and those with stable disease and/or progressive disease. This may suggest that in the pre-docetaxel setting the CTC subtype has limited relation to inherent docetaxel sensitivity and RECIST response. Nevertheless, we found that total CTC numbers showed potential for predicting initial radiological treatment response when used in combination with serum biomarkers (PSA and ALP) in mCRPC patients, with a good AUC of 0.90. Although we observed a higher number of mesenchymal CTC in mHSPC patients lacking a partial response to treatment, they did not add significant predictive value. Newly diagnosed mHSPC patients are treated with first-line ADT for a short period prior to starting and throughout docetaxel therapy. Unlike mCRPC patients, all mHSPC patients should be responsive to ADT at this time. Therefore, our results indicate that responsiveness to first-line

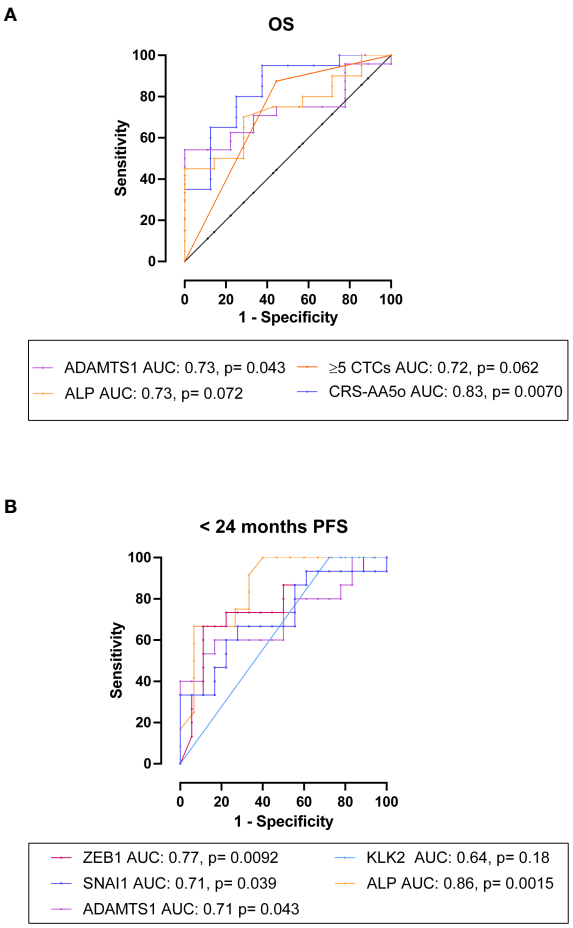

FIGURE 7
The association and predictive value of CTC gene expression with OS and PFS in mHSPC patients. **(A)** ROC analysis for OS: High expression of *ADAMTS1* had an AUC of 0.73, p= 0.043 for the prediction of OS. Neither serum PSA or ALP levels significantly differentiated patients based on OS, although ALP showed a trend towards significance (AUC= 0.55, p= 0.66; AUC= 0.73, p= 0.072, respectively). Combining *ADAMTS1*, ALP and ≥5 CTCs as CRS-AA5o= 0.06386**ADAMTS1* + 0.001465*ALP + 2.169*≥5CTCs, increased the AUC to 0.83, p= 0.0070. **(B)** ROC analysis for <24 months PFS: High expression of *ZEB1*, *SNAI1* and *ADAMTS1* was significantly predictive of <24 months PFS (AUC= 0.77, p= 0.0092; AUC= 0.71, p= 0.039; AUC= 0.71, p= 0.043, respectively). Additionally, ALP but not serum PSA levels showed significant AUCs (AUC= 0.86, p= 0.0015; AUC= 0.52, p= 0.86, respectively).

ADT might affect the value of CTCs for the prediction of near-term clinical response to docetaxel. This is also reflected in the lack of significant correlations between serum PSA level and CTC numbers before docetaxel treatment in mHSPC patients. In summary, CTCs might have a potential value in predicting docetaxel response, but data here is insufficient to make a conclusion. Further investigations in larger cohorts are required.

While pre-docetaxel CTC measurements showed limitations in the prediction of initial radiological response, we showed that CTCs were associated with OS and PFS subsequent to docetaxel treatment. The significant correlation of epithelial CTCs with shorter OS and/or PFS confirmed previous research findings in mCRPC patients to be treated with docetaxel (19, 23, 24, 26, 27, 51). However, by using the epitope independent Partsortix® isolation system, we were able to capture and analyse three different CTC subtypes. Interestingly, in the mCRPC patient cohort, the detection of ≥1 EMTing-CTCs (p= 0.0001) and a positive CTC-score (p= 0.0005) were the most significant predictors of shorter OS among the different CTC measurements. Our previous studies have demonstrated both to be valuable PCa biomarkers, and associated with increased metastatic burden (32, 38). Patients with a positive-CTC score and/or ≥1 EMTing-CTC present in 7.5mL of pre-docetaxel blood experienced an approximate three-fold reduction in median OS time compared to those with a negative CTC-score or no EMTing-CTCs. This demonstrates the value of analysing different subtypes of CTCs and the good prognostic value of the CTC score, in which mesenchymal CTCs were a component. A positive CTC-score combined with serum PSA (AUC= 0.96 for OS <24 months) may flag high-risk mCRPC patients with high sensitivity (88.89%) and specificity (100%) and facilitate timely therapeutic intervention post-docetaxel, such as Cabazitaxel administration, which has been shown to retain activity in patients after docetaxel treatment (52).

To date, reports on the use of CTCs as a prognostic biomarker in mHSPC patients treated with ADT plus docetaxel are limited. The prediction of significantly shorter OS by ≥5 total-CTCs in mHSPC patients may be useful for treatment stratification in this cohort. In this study, CTCs expressing cytokeratin alone did not significantly differentiate mHSPC patients with shorter OS from those with a good response and prolonged OS after docetaxel treatment. Again, these findings highlight the advantage of epitope independent CTC isolation, which allows for the capture and analysis of multiple CTC subtypes.

We showed that the RNA expression of certain genes in CTCs correlated with shorter OS and/or PFS in mHSPC patients. These poor prognostic genes included the EMT transcription factors *ZEB1* and *SNAI1*. EMT is responsible for tumour cell migration and metastasis, and is associated with drug resistance in multiple solid tumour types (53). In docetaxel resistance, upregulation of EMT genes has been shown to mediate resistance emergence in PCa cell line models (36, 54, 55) and increased expression in primary tumours prior to therapy has been correlated with radiological relapse (36). Several EMT genes have been assessed in CTCs, the most common being Vimentin, for detection of a mesenchymal subtypes which have been linked to higher metastatic burden, a more aggressive phenotype and disease progression in PCa (32, 56, 57). The shorter OS and/or PFS associated with the RNA expression of *ZEB1* and *SNAI1* by CTCs may indicate a more aggressive and potentially docetaxel resistant phenotype being present in the primary or metastatic tumour sites, which may promote disease progression in mHSPC patients.

Additionally, these findings highlight the importance of considering multiple different markers corresponding to the same cellular phenotype, as genes may be exhibiting different patterns of spatial–temporal expression under pathological conditions, yet could be controlled by different upstream signals.

Patients with mHSPC who had detectable *KLK2* RNA expression in CTCs were more likely to suffer shorter PFS compared to patients with *KLK3* (PSA) expression. The protein encoded by *KLK2*, hK2, has been utilised in the 4Kscore® Test to predict risk of aggressive PCa (58). Our findings suggest that *KLK2*/hK2 may also be used as biomarker to identify mHSPC patients who are likely to progress under chemo-hormonal treatment.

The metalloprotease, *ADAMTS1*, was upregulated in docetaxel-resistant cells in microarray analysis and was associated with both PFS and OS. Combining *ADAMTS1* expression with ALP levels and ≥5 CTCs predicted shorter median OS with high sensitivity and specificity. For PFS, ALP was highly predictive, with an AUC of 0.86, leaving little margin for improvement, so combined biomarker analysis was not performed.

The variety of CTC derived genes that were identified as biomarkers of poor prognosis indicates the heterogeneity of CTCs between patients. CTC heterogeneity may be the result of a spatio-temporally different microenvironment surrounding the tumour lesions, in the circulation, as well as differences in therapy response (14, 59). In the case of the mHSPC patient cohort, differing levels of response to initial ADT may alter tumour biology, influence CTC gene expression and fitness, and subsequent response to docetaxel therapy, such as induction of EMT machinery (60–62). To explore this, further analysis of CTC gene expression changes over multiple time points during therapy in warranted.

The limitations of this study include: 1. Small patient cohort, particularly for the mCRPC patients, although we observed several significant corrrelations. The small sample size was due to the several effective therapies been devloped in recently years for PCa, leading to competing treatment options. 2. The candidate CTC genes were selected based on the microarray dataset gene expression profile from 2D-cultured docetaxel-resistance cell line models, as currently, datasets from better models for docetaxel resistant versus sensitive samples are not available. It is well-known that 2D-induced resistance creates artificial resistance mechanisms. Therefore, validation of these candidate genes in clinically relevant samples is critical. 3. As the harvested CTC samples that we used for gene expression analysis were not pure CTCs (with predominantly leucocyte contamination), we had to exclude a large number of candidate docetaxel resistance genes which were expressed in leucocyte. This led to only 23 selected from the initial 237 candidate genes, thus potentially missing genes with good docetaxel therapeutic response prediction value. Further pure CTC selection (although a challenging task) or single cell RNA sequencing may be explored in the future to address this issue.

In summay, our study demonstrated that in mCRPC, elevated numbers of CTCs were inidcators of poor initial response when combined with serum biomarkers, and CTC measurements could be used to predict short OS and/or PFS in mCRPC and mHSPC patients. Addtionally, we showed that measuring RNA expression of candidate docetaxel-resistance and PCa related genes from CTC samples increased our ability to predict patient outcome in the mHSPC patient cohort. Importantly, we found that combining CTC data with clinical serum biomarkers has the potential to predict poor docetaxel treatment response, although this should be confirmed in a large series of samples.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by UK Research Ethics Committee: London - City & East Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: CD, PR, AG, GSh, JS, and Y-JL. Validation: CD and LX. Formal analysis: CD and Y-JL. Investigation: CD, TG, EB, ES, LX, and XM. Data Curation: CD, EB, ES, XM, and GSc. Writing – Original Draft: CD. Visualization: CD. Writing – Review and Editing: TG, ES, XM, PR, AG, TO, JS, and Y-JL. Methodology: XM, Y-JL, LX. Resources: GSh, PR, KT, CA, AW, MG, SC, TP, AG, SK, GSc, DB, and JS. Supervision: JS and Y-JL. Project administration: Y-JL. Funding: Y-JL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1060864/full#supplementary-material

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DNA repair deficiency as circulating biomarker in prostate cancer

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Deleterious aberrations in DNA repair genes are actionable in approximately 25% of metastatic castration-resistant prostate cancers (mCRPC) patients. Homology recombination repair (HRR) is the DNA damage repair (DDR) mechanism most frequently altered in prostate cancer; of note *BRCA2* is the most frequently altered DDR gene in this tumor. Poly ADP-ribose polymerase inhibitors showed antitumor activity with a improvement in overall survival in mCRPC carrying somatic and/or germline alterations of HHR. Germline mutations are tested on peripheral blood samples using DNA extracted from peripheral blood leukocytes, while the somatic alterations are assessed by extracting DNA from a tumor tissue sample. However, each of these genetic tests have some limitations: the somatic tests are related to the sample availability and tumor heterogeneity, while the germline testing are mainly related to the inability to detect somatic HRR mutations. Therefore, the liquid biopsy, a non-invasive and easily repeatable test compared to tissue test, could identified somatic mutation detected on the circulating tumor DNA (ctDNA) extracted from a plasma. This approach should better represent the heterogeneity of the tumor compared to the primary biopsy and maybe helpful in monitoring the onset of potential mutations involved in treatment resistance. Furthermore, ctDNA may inform about timing and potential cooperation of multiple driver genes aberration guiding the treatment options in patients with mCRPC. However, the clinical use of ctDNA test in prostate cancer compared to blood and tissue testing are currently very limited. In this review, we summarize the current therapeutic indications in prostate cancer patients with DDR deficiency, the recommendation for germline and somatic-genomic testing in advanced PC and the advantages of the use liquid biopsy in clinical routine for mCRPC.

KEYWORDS

DNA damage repair, prostate cancer, liquid biopsy, circulating biomarker, circulating tumor DNA

1 Introduction

Deleterious aberrations in DNA repair genes are found in a considerable rate of patients with advanced prostate cancer (PC) (1–3). With the advent of target therapy such as ribose polymerase poly-ADP inhibitors (PARPis) and immune checkpoint inhibitors (ICIs), genomic testing has become part of the clinical practice in metastatic castration resistant prostate cancer (mCRPC) patients with DNA damage repair (DDR) (4). Homology recombination repair (HRR) is the DDR mechanism most frequently altered in PC and mutation of the *BRCA2* gene is the most frequently detected among the DDR genes (5). Oppositely to *BRCA1* involvement, the germline *BRCA2* mutations have been associated with a 2 to 6 fold increase in the risk for PC (6). *BRCA2* mutant patients seems to have a more aggressive phenotype, and a significant reduction in survival times compared to the non-mutated patients (7–9). Others germline mutations such as ataxia mutated telangiectasia (*ATM*), checkpoint kinase 2 (*CHEK2*), and the partner and locator of *BRCA2* (*PALB2*) seems to correlate, albeit to a lesser extent, with an increase of the risk of PC (3, 10, 11). Currently, the peripheral blood samples are preferentially used to detect germline mutations; while somatic alterations are assessed by extracting DNA from the tumor tissue sample, whose detection could be affected by the sample availability and by tumor heterogeneity.

Liquid biopsy is a new approach increasingly used in clinical setting allowing the rapidly and/or simultaneously detection/capture of cell-free DNA or circulating tumor cells (CTCs) or DNA (ctDNA), and extracellular vesicles. The liquid biopsy is a less invasive molecular profiling resource able to obtain intratumoral heterogeneity and to track dynamic changes and resistance mechanism occurring during therapies (12). The ctDNA has become a viable option to perform genomic testing in PC patients, receiving Food and Drug Administration (FDA) approval in the last years (13). However, although the advantages are now known, several limitations to the use of the ctDNA test are still present.

Here, we review the role that liquid biopsy currently plays in PC, the reliability of the ctDNA test in detecting DDR mutations and the evidence in favor of its routinely introduction in clinical practice.

2 DNA damage repair deficiency and mutations in prostate cancer

The DNA repair process is a fundamental mechanism for identifying and correcting the DNA damage induced by environmental factors or normal cellular metabolic processes. DNA damage induces a complex cascade of signals involving various checkpoints capable of interrupting the cell cycle to guarantee the repair of the lesion or, if not possible, to induce senescence and apoptosis (14).

This process is critical for cell survival, as it promotes genomic stability and reduces the risk of inheriting damage during cell division. DNA repair pathways include single-stranded break (SSB) defect repair mechanisms (base excision repair, nucleotide excision repair and mismatch repair (MMR) and repair mechanisms for damage to the double stranded (DSB) (homologous recombination (HR) and non-homologous end joining) as dispatched in Figure 1. Other mechanisms such as direct chemical inversion and crosslink repair between strands, although less common, may be coincided in the removal of damage (15). However, the presence of cells with alterations in these pathways are related to fallible repair mechanisms with consequent accumulation of cellular mutations and tumor transformation.

MMR is a proteins system including MLH1, MSH2, MSH6, and PMS2, recognizing and repairing erroneous insertion, deletion, and mis-incorporation of bases caused by DNA polymerase during the DNA replication (16). Its alteration, represented phenotypically as microsatellite instability (MSI), has been firstly identified in tumors from patients with Lynch Syndrome and subsequently in different types of cancers becoming an overall biomarker of response to treatments (17).

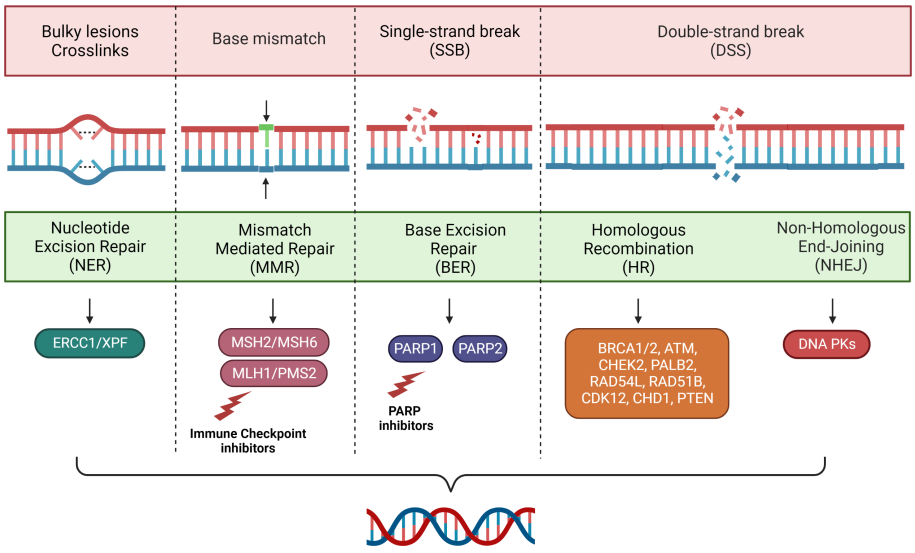

FIGURE 1
Main mechanisms of DNA repair.

In the Memorial Sloan Kettering Cancer Center (MSKCC) cohort, the MSI and defective MMR (dMMR) have been identified in approximately 5% of PC patients (18). Another prospective case series reported MSI-H/dMMR in 3.1% of PC patients, while an unselected cohort study of 3,607 patients with personal history of PC reported the presence of mutations germline in MLH1, MSH2, MSH6 or PMS2 in 1.7% of cases (19, 20). On 60 rapid tumor autopsies from metastatic PC patients, 12% resulted dMMR/MSI-H (21), while in another study on 150 mCRPC patient's tumor biopsy detected an MSI-H frequency of 3% (22). This high variable frequency of dMMR/MSI-H ranged from 1 to 12% in patients with mCRPC may be in part explained by the diversity of assays used to detect tumors with dMMR (23, 24).

HR is a complex DNA double helix repair system, allowing one stretch of the DNA double helix to serve as a template to restore lost or damaged information in the other stretch. The germinal alterations in various genes belonging to HR, mainly BRCA1 and BRCA2, have been associated with the development of familial tumors, primarily involving breast and ovarian cancer, and subsequently prostate and pancreas cancer (25). Failure in the HR repair system can compromise the elimination of genome mutations, favoring the accumulation of DNA damage events and oncogenesis (26).

Actionable molecular alterations and aberrations in HR and MMR pathways occur in a considerable fraction of localized prostate cancers and, even more frequently in metastatic disease (22).

Regarding the inactivation of HR-associated genes (i.e., BRCA1/2, ATM, CHEK2, PALB2, RAD54L, RAD51B, CHD, CDK12 or PTEN), a number authors have reported the frequencies of somatic and germline mutations at several disease stages of PC (27). Somatic mutations were recorded in 19% of localized PCs and 23% of mCRPC cancers, with the highest incidence in the BRCA2 and ATM genes (22, 23). BRCA2 somatic mutations are associated with germline mutations in 42% of patients with mCRPC (22) and in 60% of localized PC (23). Recent data indicate that 11.8% of patients with metastatic prostate cancer (mPC) have germline mutations in 1 of 16 DNA repair genes: (BRCA1, BRCA2, ATM, CHEK2, PALB2, RAD51D, ATR, and NBN, PMS2, MSH2, MSH6, GEN1, RAD51C, MRE11A, BRIP1, or FAM175A) (28). In the Cancer Genome Atlas (TCGA) cohort, patients with high-risk localized PC had a rate of germline DNA repair mutations of 6% versus 2% in those at low/intermediate risk (23, 28). Another study reported a rate of pathogenic germline mutations in MUTYH, ATM, BRCA1, BRCA2 and BRIP1 of 7.2% in patients with high-risk, very high risk or metastatic PC (29). Other authors have reported varying incidence rates of DDR mutations ranging from 11 to 28%. Robinson et al. found a rate of 22.7% of germline DDR mutations or somatic mutations in BRCA1, BRCA2, ATM, FANCA, CDK12, RAD51B and RAD51C in patients with mCRPC (22), while a rate of less than 11.8% of germline mutations in at least one DDR gene has been reported by Pritchard et al. in the same context of patients (28). In all stages of PC, Abida et al. found germline or somatic alterations in BRCA1/2, TMJ and CHEK2 in 27% of patients (30). More recently in the PROfound study, 28% of mCRPC patients had alterations in 15 genes with direct or indirect roles in HR (31).

3 Recommendations for germline and somatic genomic testing in advanced prostate cancer

A family history of prostate cancer, as well as the hereditary breast and ovarian cancer syndrome (HBOC) due to germline mutations in HR genes and Lynch Syndrome increases the risk of PC (32–34). However, approximately 30% of patients with mPC carrying the germline DDR had no family history of cancer. An increased risk of PC has been found in Ashkenazi Jews in whom more than 2% carry germline mutations in *BRCA1* or *BRCA2* and in PC with intraductal histology that appear to have greater genomic instability compared to those with adenocarcinoma histology (35–38). Moreover, a correlation between clinical pathological features (Gleason score ≥8, lymph node and distant metastases to the diagnosis) and germline *BRCA2* mutations has been observed, although the mutation cannot be excluded in the other patients (39). Below reported the main recommendations on carrying out the genetic and/or somatic test (40–42).

3.1 Germline testing

National Comprehensive Cancer Network (NCCN) panel recommends germline genetic testing, with or without pretest genetic counseling, for patients with PC and any of the following: a positive family history (multiple family members diagnosed with castration sensitive PC at the age <60 years, a family member died from PC, family history of high-risk germline mutations or of multiple cancers on the same side of the family); high-risk, very-high-risk, regional, or mPC regardless of family history; Ashkenazi Jewish ancestry and intraductal histology. Germline testing should include proteins of MMR and the HR genes (i.e., *BRCA2, BRCA1, ATM, PALB2*, and *CHEK2)*. A cancer predisposition next-generation sequencing (NGS) panel testing, at a minimum including who consider other genes in addition (i.e., *HOXB13)* to the above, and guided by clinical context can be considered (40, 43, 44).

3.2 Somatic tumor testing

Alternatively somatic tumor test follows these recommendations: tumor testing for somatic HR gene mutations (i.e., *BRCA1/2, ATM, PALB2, RAD51D*, *FANCA*, and *CHEK2*) and MSI/dMMR can be considered in patients with regional or mPC; multigene molecular testing can be considered for patients with low- and favorable-intermediate risk PC and life expectancy ≥10 years; the Decipher molecular assay can be considered as part of counseling for risk stratification in patients with prostatic specific antigen (PSA) resistance/recurrence after radical prostatectomy. If HR mutations, especially *BRCA1/2, ATM, CHEK2*, or *PALB2* are found, patient should be referred for genetic counseling to assess for the possibility of hereditary tumors such as HBOC. MSI testing should be performed using an NGS assay validated for prostate cancer and if positive, the patient should be referred for genetic counseling to assess for the possibility of Lynch Syndrome (40, 45–47).

Overall, the current recommendations are summarized below:

- Germline testing for DDR genes associated with cancer predisposition syndromes (especially *BRCA2*) is recommended for patients with a family history of cancer and should be considered in all patients withmPC;

- Somatic testing for HR and MSI genes should be considered in all patients with mCRPC;

- Patients with pathogenic mutations detected on somatic testing should be referred for germline testing and genetic counselling;

- In patients with localized prostate cancer, tissue molecular tests can be considered in the presence of suspected clinicopathological factors to aid decision making.

Notably European Association of Urology (EAU) reported genetic testing on circulating tumor DNA (ctDNA) as an alternative to tissue testing although still less common (42).

4 Damage of DNA damage repair as therapeutic target in prostate cancer

4.1 PARP inhibitors

PARP system is a nucleolar proteins complex involved in DNA repair, genomic stability and programmed cell death (48). Its main role consists in detecting and initiating an immediate cellular response to SSB damage (48). PARP inhibitors have developed as a possible therapeutic strategy in patients with DDR.

The anticancer effect of these drugs is attributed to the catalytic inhibition of PARP that interfere with efficient DNA damage repair inducing tumor cells death (49). While in normal cell PARP inhibition is tolerated, in tumors cells with concomitant HR alteration, the effect of PARPi are notable (50):the defective enzymatic function of PARP results in the accumulation of SSB that promote the accumulation of damage in the potentially lethal DSB, preferentially repaired by HR (51). The concomitant loss of PARP function in cancer cells with altered HR proteins involved in HR deficient repair with the accumulation of DSBs and subsequent cell death (51).

Based on this synergistic effect numerous PARP inhibitors (i.e., olaparib, rucaparib, niraparib, talazoparib and veliparib) have been tested firstly in patients with germline mutations in *BRCA1* or *BRCA2* (52, 53). Afterwards, sensitivity to PARPi has been proved in tumor with loss of other tumor suppressor DNA repair proteins (*e.g., ATR, ATM, RAD51, CHEK1/2, and PALB2*), suggesting the validity of this therapeutic strategy also in patients intrinsically deficient in HR without *BRCA1/2* mutations (54–57). The outcome benefits observed with PARPi in DDR mutated breast and ovarian cancers, led to the evaluation of PARPi efficacy in PC.

TOPARP-A was the first phase II study to test in 2014 olaparib in patients with mCRPC regardless of DDR mutations. Fourteen of the 16 patients with aberrations of the DNA repair genes (*BRCA2, ATM, BRCA1 or CHEK2 and HDAC2*) achieved a response to treatment measured by a composite methodology including the decline of CTCs (58). Based on these findings, olaparib received the FDA's breakthrough PC therapy designation (59).

A subsequent phase II study, TOPARP-B, further examined the anticancer effects of olaparib in mCRPC patients with DDR mutations who had progressed to an earlier line of therapy (60). Patients positive for pathogen mutation or homozygous deletion in a DDR gene tested with NGS on primary tumor biopsies received olaparib 300 or 400 mg twice daily. Subgroup analysis showed that patients with the *BRCA1/2* mutation predicted greater responses and a longer median radiographic progression-free survival (PFS), with an overall response rate (ORR) of 83.3%. In patients with *ATM* and *PALB2* mutations, rate of radiographic objective responses was 8.3% and 33,3%, respectively; while PSA declines of at least 50% were detected in 5.2 and 66.6% of patients with alteration of *ATM* and *PALB2*, respectively, suggestinga susceptibility of *PALB2*to PARP inhibition. Limitations in obtaining accurate and timely somatic genetic testing in this trial allowed to enlist only 13.7% (98/711) of the screened patients. Data derived from these trials showed the antitumor effects of olaparib both when used to treat mCRPC patients with certain DDR genetic aberrations and in some patients with non-BRCA mutations (60).

The phase III study, PROfound, tested the efficacy of olaparib versus androgen receptor signaling inhibitors (ARSi) (abiraterone or enzalutamide) in patients with mCRPC and mutations in 15 HR-associated genes (*BRCA1, BRCA2, ATM, BRIP1, BARD1, CDK12, CHEK1, CHEK2, FANCL, PALB2, PPP2R2A, RAD51B, RAD51C, RAD51D* and *RAD54L*) (31). The primary endpoint examined was imaging-based PFS. The patients were divided into two cohorts: the cohort A included patients with alterations in *BRCA1/2* or *ATM*: the cohort B included patients with alterations in any of the other 12 genes. All patients received 300 mg olaparib twice daily versus second ARSi in a 2:1 ratio. In the overall population (cohorts A and B), significantly longer PFS was recorded in patients treated with olaparib compared to control arm (5.8 vs 3.5 months; hazard ratio [HR], 0.49; 95% confidence interval [CI], 0.38-0.63; p<0.001). An even longer PFS was recorded in cohort A in the olaparib group compared to control (7.4 vs 3.6 months; HR, 0.34; 95% CI, 0, 25-0.47; p <0.001) as well as a better OS (18.5 vs 15.1 months; HR, 0.64; 95% CI, 0.43 to 0.97; p=0.02). Notably, failure to sequence DNA occurred in approximately 31% of the tumor samples. Based on these results, FDA recently approved olaparib for patients with mCRPC progressed to enzalutamide and/or abiraterone who have deleterious germline alterations in *BRCA1/2* or somatic deleterious alteration in *BRCA1/2, ATM, BARD, BRIP, CDK12, CHEK1, CHEK2, FANCL, PALB2, RAD51B, RAD51C, RAD51D*, and *RAD54L* (61).

Another PARP inhibitor, rucaparib, has been evaluated for the treatment of patients with mCRPC who have germline or somatic mutations in the DDR genes (62). Phase II study, TRITON2 enlisted patients with any mutation in the HR genes, showing initial efficacy and safety results that allowed for the designation of rucaparib as a breakthrough therapy by the FDA. Preliminary data from this study showed promising results: 43.9% of patients with *BRCA* achieved a confirmed radiographic response, and lasting responses (62). Partial radiographic responses have been observed in 10.5% of patients with non-*BRCA* DDR genes and patients with *ATM* mutations. Two patients with *CHEK2* aberrations had a confirmed partial response and a confirmed PSA response. No objective response was observed in patients with *CDK12* mutations. In the 13 patients whit other mutations including *FANCA, PALB2, BRIP1*, or *RAD51B*, ORR was 38.5% with one complete response and four partial responses. The FDA recently approved rucaparib for the treatment of mCRPC

patients with germline or somatic *BRCA1/2* mutations (63). Is ongoing a phase III study, TRITON3, comparing rucaparib with standard of care treatments, enrolling only patients with mCRPC and mutations in *BRCA1/2* and *ATM* (NCT02975934).

The GALAHAD study evaluated the activity of niraparib in patients with mCRPC and DDR gene alterations received three or more systemic therapies for mCRPC (64). In this phase II trial niraparib was tolerable and showed anti-tumour activity in heavily pretreated patients, particularly in those with *BRCA* alterations with an ORR of 34.2%. Niraparib combined with abiraterone acetate/prednisone versus abiraterone acetate/prednisone for patients with mCSPC and deleterious germline or somatic HRR gene mutated is under evaluation in the phase III trial, AMPLITUDE (NCT04497844).

Finally, talazoparib has been tested in TALAPRO-1 phase II study enrolling patients with measurable soft tissue disease, progressive mCRPC, and DDR mutated (*ATM, ATR, BRCA1/2, CHEK2, FANCA, MLH1, MRE11A, NBN, PALB2, RAD51C*), treated with one or more taxane-based chemotherapy regimens and ARSi for mCRPC to receive oral talazoparib 1 mg/day until radiographic progression, unacceptable toxicity, consent withdrawal, or death (65). Talazoparib monotherapy has encouraging antitumor activity in docetaxel-pretreated mCRPC patients with *BRCA1/2* alterations and was generally well tolerated. The efficacy and safety of talazoparib and enzalutamide combination in mCRPC patients with or without DDR mutations is currently under evaluation in the phase III trial TALAPRO-2 (NCT03395197). The phase III study, TALAPRO-3, is comparing talazoparib plus enzalutamide versus placebo plus enzalutamide in patients with mCSPC and DDR alterations (NCT04821622).

As the use of PARPi is limited by primary resistance mechanisms and the onset of secondary resistance in sensitive patients'numerous efforts have been aimed at developing combined treatment approaches (66)

PROpel is a phase III trial randomizing patients with mCRPC regardless of HRR status to receive olaparib or placebo and abiraterone plus prednisone or prednisolone (67). The primary endpoint was investigator-assessed radiographical (rPFS), OS was one of the multiple secondary endpoints. Treatment with olaparib plus abiraterone significantly prolonged rPFS in patients with mCRPC regardless HRR status compared to control (24.8 vs 16.6 months; HR, 0.66, 95% CI, 0.54-0.81; p <0.0001). The safety and tolerability profile of combination was consistent with the known safety profiles of the individual drugs.

MAGNITUDE is a randomized, double-blind phase III study enrolling mCRPC patients (≤4 months of prior abiraterone acetate/prednisone for mCRPC was allowed) with or without HRR biomarker positive (*ATM*, *BRCA1/2, BRIP1, CDK12, CHEK2, FANCA, HDAC2, PALB2*) to receive niraparib 200 mg once daily plus abiraterone acetate/prednisone or placebo plus abiraterone acetate/prednisone (68). The primary endpoint was rPFS. assessed by blinded independent central review in the *BRCA1/2* group then in all patients with positive HRR biomarkers. The preplanned futility analysis in HRR mutations negative patients showed no benefit of adding niraparib to abiraterone acetate/prednisone in the prespecified composite endpoint (PSA progression or rPFS: HR 1.09, 95% CI 0.75-1.59). Niraparib plus abiraterone acetate/prednisone significantly improved the primary clinical outcome in HRR biomarker positive patients, with a manageable safety profile and health-related quality of life. Therefore, while PROpel trial showed a global benefit of PARPi and ARSi without the need for HRR stratification in untreated mCRPC patient, the MAGNITUDE study results support the combination strategy only for patients with alterations in HRR genes.

4.2 Immune checkpoint inhibitors

Prostate cancer belong to those tumors whose microenvironment is defined as immune-excluded, as it is characterized by a low mutational load, a reduced expression of neoantigens, hyperactivity of myeloid-derived suppressor cells and T-regulator cells, loss of major histocompatibility complex class I expression and abnormal IFN-1 signaling (69). However, like other solid tumors, it has been shown that dMMR or MSI-H prostate cancer may respond better to the immune checkpoint blockade (70).

Based on the results of a phase II trial, KEYNOTE 158, pembrolizumab received the first tissue agnostic approval for an antineoplastic therapy granted by the Food and Drug Administration (71). Patients enrolled in this study presented several types of cancer, including PC in two cases, with MMRd and had received at least one prior therapy. Objective radiographic responses have been reached in 46 (53%) of patients, with 18 (21%) achieving a complete response. Two subsequent placebo-controlled, phase III trials testing CTLA-4 inhibitor, ipilimumab, did not find improvement in OS in mCRPC patients, while pembrolizumab as single-agent showed a low response rate of 3%-5% post chemotherapy (72–74). A phase II trial combined ipilimumab with nivolumab showed an overall response of 26% in mCRPC chemotherapy naïve, although with an significant rate of grade ≥3 adverse events (75). Follow-up studies have largely confirmed pembrolizumab's efficacy in men with MMRd prostate cancer. In a study conducted at MSKCC, 3.1% of enrolled mCRPC patients were characterized by MSI-H PC and 11 of these received ICI-based therapy (19). About half of the patients achieved a PSA decline of at least 50% from baseline (PSA50 response), and four patients achieved a radiological response. A small sample size-based study by Schweizer et al. showed that 4 out of 10 ductal PC patients were dMMR, and 3 of them were also characterized by MSI-H. Notably, one of these dMMR/MSI-H patients with ductal PC achieved a significant reduction in PSA levels during treatment with pembrolizumab (76). Several other series of studies enrolling patients with PC with MMRd reported a PSA50 response, ranging from 50 to 65% when treated with ICI monotherapy, with long term responses observed (77).

5 DNA damage repair deficiency testing type

5.1 Tissue and whole blood testing

Tests for identifying mutations in DNA repair genes can be detected on two main tissues: blood or tumor tissue. The main difference between these two strategies is based on the type of mutation that can be detected. Through blood analysis we can detect genomic rearrangements significant for patients and their family, without indications on somatic mutations. Both alterations

can be identified through tissue testing (78). The blood test has numerous advantages including the easy availability of the sample, the minimum invasiveness of the procedure and the repeatability of the test. Tissue testing can be performed on both surgical or biopsy specimens of the prostate and on metastatic sites, although with some limitations. Firstly, the multifocal nature of PC, which may result in the core biopsy analyzed not representing the metastatic disease clone (79). The execution of the biopsy on the metastatic site is an invasive procedure and not free from complications for the patient, in addition the frequent bone involvement as a metastatic site in patients with PC considerably reduces the probability of success of the test (78). Secondly, is related to quantity and quality of sample. In fact, the small size of prostate primary tumor biopsies and progressive degradation of DNA in paraffine after years, are two conditions to be carefully considered for the choice of the test. Contrary to the high quality of blood sample, quality in the tissue is low and variable in relation to factors including carrying out different molecular tests, presence of necrosis, high infiltration of inflammatory cells, degradation related to aging, poor formalin fixation and cauterized tissue (80, 81). This making up to 20-30% of core biopsies unsuitable for NGS testing using commercial platforms (31).

Regarding MSI the gold standard for determining MSI/dMMR status is immunohistochemistry (IHC) and polymerase chain reaction (PCR) testing performed on tumor tissue samples. IHC is highly sensitive and specific in Lynch Sindrome-associated tumors by exploring the expression of the four major MMR proteins or just the MSH6/PMS2 doublet (82, 83). The MSI-PCR method based on PCR amplification of microsatellite regions followed by capillary electrophoresis is a reliable alternative to IHC. PCR helps also to recover cases that can escape IHC due to preanalytical problems, indeterminate results, as well as false negatives (non-truncating missense mutations in MMR genes associated with intact antigenicity) (84). Recently, they have emerged new molecular approaches (histopathology-based approach, PCR-based test, NGS-based approaches computational tools for MSI diagnosis) on tumor tissue samples that could improve sensitivity and specificity compared to conventional tests, representing a valid future option.

To date, circulating free DNA (cfDNA) can also be used to accurately determine MMRd and MSI status with the advantage of being easily obtainable compared to a metastatic biopsy; however, there are technical limitations of ctDNA-based sequencing approaches such as the low tumor burden which can results in indeterminate results.

5.2 Liquid biopsy and ctDNA

In the last year liquid biopsy has emerged as a promising surrogate for tumor biopsy, capable of overcoming spatial and temporal heterogeneity by allowing longitudinal monitoring of the disease through iterative sampling (85–87). It is an emerging field in the management of patients with cancer and its relevance as a potential diagnostic, prognostic, monitoring, and therapeutic tool makes it an attractive strategy in the management of these patients (87–89). However, liquid biopsy still has some limitations, although seem to be within the reach of technological development soon. This strategy has shown to be able to reliably represent the tumor microenvironment and its modification. Different biomarkers such as CTCs, extracellular vesicles, ctDNA, circulating tumor RNA can be analyzed through liquid biopsy on the blood or on the other human fluids (*i.e.*, urine, sperm, etc.) for diagnostic, prognostic, and predictive purposes (88).

The detection of cell-free DNA (cfDNA) as a part of liquid biopsy in PC has been widely explored, despite its diagnostic value for PC remains controversial. cfDNA is the total amount of circulating DNA found in blood plasma representing the total DNA released by normal and cancer cells. Its concentration may be increased in stroke, trauma, myocardial infarction, and autoimmune diseases (90–92), and even more in patients with advanced cancer (93, 94). Circulating tumor DNA is plasmatic DNA derived specifically from the primary or secondary site of the tumor, or from circulating tumor cells. It can represent 0.01% up to 90% of total free DNA, with an inherent patient variability due to various factors such as location, size, vascularity, tumor stage and response to therapy. The ctDNA level is higher in metastatic cancer than in localized disease and appears to correlate with disease progression (95–98). The release of free DNA from circulating cells can occur passively, during apoptosis or necrosis, or through active secretion. The DNA fragments released during apoptosis differ from those poured into the circulation in case of necrosis in the shorter length (99). A smaller amount of ctDNA is released through active secretion from extracellular vesicles, such as exosomes and prostasomes (100).

Liquid biopsy and specifically ctDNA testing can ensure monitoring of tumor evolution during therapy bypassing the intratumoral heterogeneity that limits tissue testing, especially if performed on the primary site. This allows to ctDNA to also detect resistance mutations. Both somatic and germline mutations can be detected through the ctDNA test, considering the pros and cons of the test (Table 1). Among the advantages of this method, there are: readiness in obtaining samples, repeatability during therapy or disease progression and rapidity, 1-2 weeks for the ctDNA test compared to 2-4 weeks for the examinations of the blood (101–103). The main disadvantages are related to amount of DNA and ctDNA levels (104). The amount of DNA in ctDNA is usually a very small fraction of cell-free DNA, especially in the early stages of the disease (105). The level of ctDNA is critical for performing the test, indeed it may vary during treatment and appears to closely correlate with tumor response (106). Some authors have shown how ctDNA determination changes at various stages of treatment. CtDNA was detected in 74% of patients before anti androgen therapy (ADT) initiation versus 59% of patients who received ADT prior to collection, with significantly higher ctDNA fraction in treatment-naïve patients (1.0% vs 11%; $p = 0.02$). The reduction in the ctDNA fraction was more pronounced after one week of ADT (107, 108).

Another critical point lies in the interpretation of the test result. Indeed, a negative result does not exclude the presence of a mutation in the patient's tumor, while a positive result for gene alterations does not distinguish between germinal and somatic origin. In the former case the patient should receive a confirmatory tissue test, in the latter case they should be referred to an appropriate confirmatory test if a germline mutation is suspected.

Some authors have evaluated the false positive rate linked to specific ctDNA tests in healthy controls, recording a rate of 0.82% in unique short variants. These false positive results may derive from

TABLE 1 Pros and cons of tissue, blood, and ctDNA-based HHR gene tests.

	Tissue	Blood	ctDNA
Mutation detected	Somatic and germline	Germline	Somatic and germline
Sample quantity	Medium	High	Low
Sample quality	Low	High	Variable
Time to response	2-8 weeks	2-4 weeks	1-2 weeks
Advantages	Archivial tissue for tumor histology	Easy to obtain samples Feasiblility in all cases Minimally invasive Easly repeatable	Easly to onbtain samples Better representative o ftumor heterogeneity and metastatic sites Minimally invasive Easly repeatable
Limitations	Tumor heterogeneity Invasive procedute to obtain samples High percentage of tests failed	Does not detect HRRm of somatic origin Does not capture the potential changing genetic profile of disease progression	Low concentration of ctDNA Type of sentitive tests False positive Adeguate amount of ctDNA particularly in early stages.
Genetic counselling	After germline test confirmation	Required	After germline test confirmation

ctDNA, circulating tumor DNA; HRR, homologous recombination repair.

somatic non-tumor changes in genes derived from clonal hematopoiesis indeterminate potential (CHIP), including *ASXL1, ATM, CBL, CHEK2, DNMT3A, JAK2, KMT2D, MLL2, MPL, MYD88, SF3B1, TET2, TP53 and U2AF1* (109–113). Despite tumor biopsy represents the reference tissue for the determination of MSI, clinically it has several limitations mainly related to the complexity of the procedure and to the spatial heterogeneity of the disease (114). Furthermore, in some rare cases, sporadic tumors may have a late onset of MMR defects that tissue biopsy cannot detect, leading to a misclassification of the MSI. It is now known the clinical potentiality of liquid biopsy in establishing tumour molecular diagnostics albeits data regarding its utility in determining MSI status which are still unclear, especially in the prostate cancer.

6 Circulating tumor DNA in prostate cancer

In PC, tissue testing is currently the test of choice for the analysis of tumor genomic profiles, although several critical issues have emerged in the main studies. In fact, in 30% of PC cases in which the tissue test was performed before enrollment, it failed due to problems in the pathological review, and during and after DNA extraction (31, 115–117). Therefore, the possibility of evaluating molecular alterations using ctDNA has made its way among pathologists and clinicians (118). NGS of ctDNA from plasma provides a minimally invasive method to identify genomic profile and resistance mechanisms in patients with mCRPC (119). However, the fraction of ctDNA in mCRPC patients and the clinical validity of the genomic alterations detected in plasma remain still unclear.

Several authors have studied the level of agreement between the plasma and tissue testing (Table 2). Firstly, Wyatt et al. in their study reported a high concordance rate between the ctDNA test and the metastatic tissue test. A ctDNA rate greater than 2% of the cfDNA was present in 75.6% of the samples (123). In these patients, all somatic mutations identified in metastatic tissue biopsies were simultaneously present in the ctDNA. The concordance results stratified by variant, showed a high positive agreement for substitutions (92%) and indels (95%) and a much lower agreement for rearrangements and copy number loss. Negative concordance was of 100%. In several patients, ctDNA sequencing revealed robust changes do not present in solid biopsy including clinically relevant alterations in the AR, WNT and PI3K pathways (123). Similarly, Vandekerkhove et al., reported a rate of 80% of concordance for mutation detection in diagnostic prostate tissue and ctDNA (107).

De Bono et al. reported a very high agreement between tissue and ctDNA testing for the detection of deleterious alterations in *BRCA1* or *BRCA2* with a positive percentage agreement of 88% and a negative percentage agreement of 95%. Some degree of discrepancy has been attributed to biological differences and sampling times between tumor tissues and plasma samples (31). Likely, Mateo et al. reported a similar prevalence between NGS over 470 primary tumors and metastatic site biopsy findings in patients who later developed mCRPC (60).

In their large study of ctDNA in 3334 mCRPC patients Tukakinsky et al. showed a high agreement between the alterations identified by liquid biopsy and those detected by tissue biopsy (119). The 94% of patient plasma samples had detectable ctDNA. In 79.5% of all patients, liquid biopsy identified at least one genomic alteration (*Tp53, RA, BRCA2/1, PI3K/AKT/mTOR, WNT/β-catenin pathway genes, RAS/RAF/MEK, MSI-H*). Regarding BRCA mutations, 67 (8%) *BRCA1/2* alterations were detected in both tissue and liquid biopsy, 5 (0.6%) exclusively in tissue biopsy (in 4 samples the ctDNA fraction was less than 1%) and 20 (2.4%) exclusively in liquid biopsy. The 20 cases detected only with liquid biopsy, may represent secondary alterations to the collection of the tissue sample. The concordance between BRCA mutation identified by blood test and ctDNA analysis was 100%.

Warner et al. demonstrated that the frequency of harmful *BRCA2, ATM* and *CDK12* changes detectable in plasma ctDNA was like those observed in the population with metastatic tissue biopsy in a large cohort of mCRPC, supporting minimally invasive liquid biopsy as approaches to identify responders to PARP inhibitors (120).

TABLE 2 Summary of the studies evaluating concordance between tissue and cDNA testing.

Study (ref)	Patients	Number of samples	Types of tests	Method	Results
Wyatt et al., 2017 (118)	mCRPC	45	Metastatic tissue and ctDNA	Targeted sequencing across 72 clinically relevant genes	All the somatic mutation identified in matched metastatic tissue biopsies were concurrently present in ctDNA; concordance of 88.9% for individual gene CAN.
Vandekerkhove et al., 2019 (119)	mCSPC	53	Diagnostic prostate tissue and ctDNA	Targeted sequencing strategy capturing the exon of 73 driver genes	80% of concordance for mutation detection in the matched samples. Combined ctDNA and tissue analysis identified potential driver alterations in 94% of patients; ctDNA or prostate biopsy alone failed in the 36% of cases.
Tukakinski et al., 2021 (120)	mCRPC	3334 (1674 screening samples from TRITON2/3 trial)	Tissue biopsy and ctDNA	Plasma assay with 62 (FoundationACT) or 70 genes (FoundationOne Liquid)	93% of concordance between BRCA 1/2 mutations detected in tissue biopsy and those identified by ctDNA 100% of concordance for germline variants. In 20 patients, BRCA 1/2 gene alterations were identified using ctDNA but not tissue testing.
Schweizer et al., 2021 (121)	mCRPC	51	Primary prostate tissue, metastatic tissue and ctDNA	Plasma assay with 324 (FoundationOne CDx) or 70 genes (FoundationOne Liquid)	Of the 53 paired samples, at least partial concordance in DDR genes was identified in 43 cases (84%) Concordance was numerically higher between ctDNA primary pairs compared with metastatic primary pairs; however, this difference was not statistic ally significant (92% vs 79%. 2 monoallelic DDR gene alterations only found in primary tissue.
Warner et al., 2021 (122)	mCRPC	1615	Archival primary tissue and ctDNA	Plasma assay with 22 genes (Illumina MiSeq or HiSeq 1500/2500 machine)	DDR gene status was concordant (94%) between archival primary tissue taken at cancer diagnosis and serial ctDNA-positive samples collected in the mCRPC setting.

mCSPC, metastatic castration sensitive PC; mCRPC, metastatic castration resistant prostate cancer; ctDNA, circulating tumor DNA; DDR, damage DNA repair.

Finally, Schweizer at al. in their study, showed as primary prostate tissue accurately reflected the mutational status of activatable DDR genes in metastatic tissue. After excluding probable CHIP events, the ctDNA profile accurately detected DDR mutations including alterations suggesting potential related mechanisms of resistance (122). Only one patient developed a *BRCA2* alteration later, while two cases *BRCA1/2* mutation positive in the primary sample, got lost in downstream samples. This may be reconducted to the intraprostatic genomic heterogeneity. However, it is also plausible a selective therapeutic pressure in the first case and an eradication of clones sensitive to DNA-damaging therapies in the second.

The confirmation of the high prevalence of HRR-associated gene mutations in advanced PC has led to some controversy regarding the use of archival primary prostate tumor biopsies for genomic profiling once patients have developed mCRPC (30, 121, 124).

Recently, Hussain et al. tried to outline the correct use of tissue for mutational analyzes by formulating the following recommendations (125): -in presence of more samples with similar tumor content, the choice must fall on the younger sample; -if the samples available exceed 5 years from collection, it is necessary to use those with the highest tumor content and high yield of DNA such as lymph nodes; -for the samples just collected, the recommendation is to optimize the fixation and storage of formalin and avoid descaling.

Regarding MSI, a good overall agreement was observed between conventional tissue-based tests and newly developed ctDNA-based approaches (126–128). This suggested that ctDNA-based MSI diagnosis could be performed as part of clinical practice to identify patients who might benefit from immune checkpoint inhibitors when tissue samples are unavailable or scarce (126, 127, 129). In Nakamura et al., changes in basal MSI levels during ICI treatment has been correlated well with those of other ctDNA markers and reliably reflected tumor response to treatment (128). Longitudinal analysis of ctDNA also allowed to detect the acquisition of somatic MSI that can appear during cancer evolution in patients initially diagnosed with MSS tumors (130). At the present state of knowledge, there are few cases in which the MSI phenotype is acquired during the disease (19, 130). This phenomenon could be partly explained by the fact that most cancers are screened for MSI only at the time of diagnosis, underestimating cases. Further studies are needed to evaluate the true impact of such an acquired MSI phenotype in clinical practice.

In addition to a predictive value, a prognostic value may be recognized to ctDNA. Already in localized disease, *BRCA1/2* germline changes have been associated with poor outcome, including disease progression among patients under active surveillance (131) or a higher risk of recurrence and death among patients undergoing salvage therapy (132). In a retrospective study of mCRPC profiled patients with a 70-gene NGS cfDNA panel an alteration was recorded in over 94% of cases, and a greater number of ctDNA alterations were associated with a shorter time to treatment failure with chemotherapy (HR, 1.05, p=0.026) or androgen inhibitors. In the study conducted by the detection of a ctDNA fraction greater than 30% was strongly associated with a poor response to enzalutamide or abiraterone therapy even after adjustment for other clinical prognostic factors (133). In this study, a ≥50% reduction in cfDNA concentration after eight weeks of therapy was independently associated with longer OS suggesting free DNA concentration as predictive factor to PARPi response.

7 Conclusion and future perspectives

Given the significant percentage of mCRPC patients with DNA repair genes mutations and the therapeutic possibilities currently available, the most important guidelines recommend the performance of genomic testing in all these patients. The test can be performed on

various samples mainly whole blood and tissue, with relative advantages and limitations. In recent years, ctDNA has become a viable option for performing genomic testing receiving FDA approval in 2020 (13). The ctDNA can overcome the limitations of tissue testing, which can fail in up to a third of cases. Additionally, ctDNA testing can be performed longitudinally by detecting new alterations and resistance mutations that may emerge during disease progression.

Several authors demonstrated high agreement between tissue testing and ctDNA testing suggesting that the analysis on ctDNA is sufficient to identify all DNA alterations and be used as a guide for patient management with mCRPC. Ideally, the combined use of the two techniques could ensure the study of the molecular subtype, paving the way for the implementation of precision therapy, but still far from possible clinical practice.

To consider the ctDNA test results as reliable as possible, it should be performed in certified institutions using the standard NGS procedure. New sequencing technologies such as PacBioScience and Oxford Nanopore allow for the acquisition of additional information, such as large intermediate chromosomal aberrations that appear to correlate with a worse prognosis of PC (134, 135). These new technologies, still burdened by a high sequencing error rate and high costs, will enable the generation of more complete and easy-to-read data.

The large proportion of patients with a rich genomic signal from ctDNA and the sensitive and specific detection of *BRCA1/2* alterations position liquid biopsy as a compelling clinical complement for comprehensive tissue genomic profiling for mCRPC patients. However, despite the findings, there are still several barriers limiting the clinical implementation of genomic sequencing, including cost, access, and feasibility based on often limited tissue availability or quality. Furthermore, only a fraction of patients with PC and genomic aberrations respond durably to targeted therapy.

The integration of analyzes that combine genomics with transcriptome, epigenome and tumor microenvironment study could help identify patients who have more likely to benefit from targeted therapies. In the future, these integrated systems, combined with clinical information, will ensure a further push towards precision oncology.

Author contributions

GR had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: GR, DG. Acquisition of data: GR, MC, MG, BR, LP. Analysis and interpretation of data: GR, Drafting of the manuscript: GR, MC Critical revision of the manuscript for important intellectual content: DG Statistical analysis: None Obtaining funding: None. Administrative, technical, or material support: None. Supervision: DG, GN. Financial disclosures: None. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The positive relationship between androgen receptor splice variant-7 expression and the risk of castration-resistant prostate cancer: A cumulative analysis

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Background: At present, androgen deprivation therapy (ADT) is still the standard regimen for patients with metastatic and locally advanced prostate cancer (PCa). The level of androgen receptor splice variant-7 (AR-V7) in men with castration-resistant prostate cancer (CRPC) has been reported to be elevated compared with that in patients diagnosed with hormone-sensitive prostate cancer (HSPC).

Aim: Herein, we performed a systematic review and cumulative analysis to evaluate whether the expression of AR-V7 was significantly higher in patients with CRPC than in HSPC patients.

Methods: The commonly used databases were searched to identify the potential studies reporting the level of AR-V7 in CRPC and HSPC patients. The association between CRPC and the positive expression of AR-V7 was pooled by using the relative risk (RR) with the corresponding 95% confidence intervals (CIs) under a random-effects model. For detecting the potential bias and the heterogeneity of the included studies, sensitivity analysis and subgroup analysis were performed. Publication bias was assessed Egger's and Begg's tests. This study was registered on PROSPERO (ID: CRD42022297014).

Results: This cumulative analysis included 672 participants from seven clinical trials. The study group contained 354 CRPC patients, while the other group contained 318 HSPC patients. Pooled results from the seven eligible studies showed that the expression of positive AR-V7 was significantly higher in men with CRPC compared to those with HSPC (RR = 7.55, 95% CI: 4.61–12.35, $p < 0.001$). In the sensitivity analysis, the combined RRs did not change substantially, ranging from 6.85 (95% CI: 4.16–11.27, $p < 0.001$) to 9.84 (95% CI: 5.13–18.87, $p < 0.001$). In the subgroup analysis, a stronger association was detected in RNA *in situ* hybridization (RISH) measurement in American patients, and those studies were published before 2011 (all $p < 0.001$). There was no significant publication bias identified in our study.

Conclusion: Evidence from the seven eligible studies demonstrated that patients with CRPC had a significantly elevated positive expression of AR-V7. More investigations are still warranted to clarify the association between CRPC and AR-V7 testing.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42022297014.

KEYWORDS

androgen receptor variant-7, castration-resistant prostate cancer, systematic review, cumulative analysis, expression

Introduction

According to the data from Cancer Statistics 2022, prostate cancer (PCa) is the most frequently diagnosed male cancer in Western countries (1, 2). Moreover, PCa is one of the leading causes of cancer mortality in developed countries (1, 2). The growth and differentiation of normal prostate cells depend on androgens for the activation of androgen receptors (ARs) (3). Also, androgens play an essential role during all phases of the growth of PCa cells (4). AR signaling is the foundation for the proliferation and survival of PCa cells. A human AR gene can be found on chromosome Xq11-12. It is composed of eight exons encoding a 110-kDa protein. Structurally, the human AR protein is composed of an N-terminal transactivation domain, a hinge region, a central DNA-binding domain, and a C-terminal ligand-binding domain (5). The binding of androgen to the AR ligand-binding domain (LBD) allows the ligand-bound receptor to enter the nucleus and regulate androgen-responsive genes in the nucleus (6). At present, androgen deprivation therapy (ADT) is still the mainstay therapy for metastatic and advanced PCa. To a great extent, men with advanced PCa may initially respond to ADT, termed hormone-sensitive prostate cancer (HSPC). Unfortunately, the majority of patients may experience progression to castration-resistant prostate cancer (CRPC) within a median time frame of 24 to 36 months, although the levels of androgens continue to be low (7).

The current evidence suggests that CRPC may be not independent of the effect of androgen, but AR signaling continues to be essential (8). The researchers found that CRPC could express not only AR but also the androgen-responsive genes. The AR signaling pathway is still functional when the androgen is depleted (9). The AR axis still plays a role in CRPC and is accountable for the progression of the disease (10), and a new generation of AR-directed agents have recently emerged, i.e., androgen biosynthesis inhibitor "abiraterone" (11) and the AR antagonist "enzalutamide" (12). The mechanisms of ablation-resistant AR-mediated signaling pathways have not yet been fully elucidated. The mechanisms by which AR are activated in CRPC have been hypothesized to be different, including mutation and augmentation of AR gene (13, 14). AR may turn out to be more susceptible to stimulation by androgens or other ligands due to intratumoral synthesis of androgens, as well as epigenetic and genetic alterations (15–17). Moreover, the androgen receptor splice variants (AR-Vs) are frequently expressed in CRPC (18, 19). To date, more than 20 AR-Vs were identified, and AR-V7 (also named AR3) is one of the most clinically significant variants (20).

AR-V7 has been identified as one of the leading splice variants in both localized and advanced PCa (21). AR-V7 is deficient with the ligand-binding domain (androgen-binding site) but retains the transactivating N-terminal domain. Since AR-V7 serves as an important transcription factor, it constitutively activates and promotes the activation of its target genes (22). Several clinical trials have reported that the AR-V7 level was significantly greater in CRPC when compared with HSPC (23–25). According to these studies, a trend toward a higher level of AR-V7 was observed in patients with CRPC.

Since a number of studies have identified the potential relationship between CRPC and AR-V7, it is clinically meaningful to summarize the evidence on this issue by conducting a meta-analysis. In this study, we performed a cumulative study to summarize and analyze the evidence on the association between CRPC and the AR-V7 expression.

Methods

The current cumulative analysis was conducted according to the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (26), which was registered on the PROSPERO (ID: CRD42022297014).

Search strategy

The Medline, Cochrane Library, and Embase were searched for systematic literature reviews. The time frame for searching the eligible studies was up to April 2022. Searches were conducted by using the subject headings and keywords. The following search terms were used: ((((((“Prostatic Neoplasms”[Mesh]) OR (Prostate Cancer)) OR (Prostatic Cancer)) AND ((((AR-V7) OR AR3) OR receptor splicing variant 7) OR androgen receptor 3))) AND castration-resistant prostate cancer). Moreover, we further reviewed the reference lists of the relevant articles to detect more eligible studies. Participants and the language of the search were restricted to American patients and English, respectively.

Quantification of AR-V7

Clinical trials in which AR-V7 was investigated by any of the existing instruments were considered to be eligible. These included immunohistochemical (IHC) staining analysis, RNA *in situ* hybridization (ISH), PCR, and protein analysis.

Study selection

Study eligibility was fully determined by the PICOS criteria, namely, the patient population, intervention or exposure, comparison, and outcome (PICOS) study design. The inclusion criteria included the following: 1) participants: men with prostate cancer PCa. 2) Interventions: CRPC. 3) Comparisons: HSPC. 4) Outcomes: the positive expression of AR. 5) Study design: any study designs. Furthermore, additional studies included in this review were supposed to provide the relative risk (RR) estimates with the corresponding 95% confidence intervals (CIs). A list of exclusion criteria was provided, as follows: 1) no control data; 2) updated or duplicated data; 3) review articles; 4) meeting abstract, commentaries, editorials, congress reports, letters, or case reports; and 5) animal or *in vitro* experiments.

Quality assessment and data extraction

According to the predetermined selection criteria, two authors independently extracted the data. Data were obtained from the included studies, as follows: names of the first author, study design, publication year, study areas, the sample sizes of the study group and the control group, methods of AR-V7 detection, and the effect measures (HR, RR, or OR) with their 95% CI. The quality of cross-sectional studies was assessed by using the quality methodology checklist (27). The Newcastle–Ottawa Scale was applied to evaluate the study quality of the case–control/cohort studies (28).

Statistical analyses

The strength of the association between CRPC and AR expression was assessed by the pooled RRs and 95% CIs. Results with p-values <0.05 were defined as statistically significant. Heterogeneity across studies was determined by using the I^2 statistic and Cochran's Q statistic (29). Fixed-effect models were used when there was no significant heterogeneity (I^2 < 50%, p > 0.10). Otherwise, a random-effects model was applied. Moreover, an analysis of sensitivity was conducted by omitting one study at one time to assess how it affected the overall risk estimate. The origins of heterogeneity were further examined by subgroup analyses. Publication biases were determined by using Begg's and Egger's tests (30, 31). p > 0.05 indicated no publication bias, while p < 0.05 was judged to be a statistical publication bias. All the analyses presented within the present study were conducted the Stata 12.0 software (Stata Corp LP, College Station, TX, USA).

Results

Results from the literature search

A diagram of the study selection process is shown in Figure 1. A total number of 747 articles were identified in the initial search, of which 285 duplicates were eliminated. Of the remaining articles, after the titles and abstracts were read, 315 articles were excluded. A total of

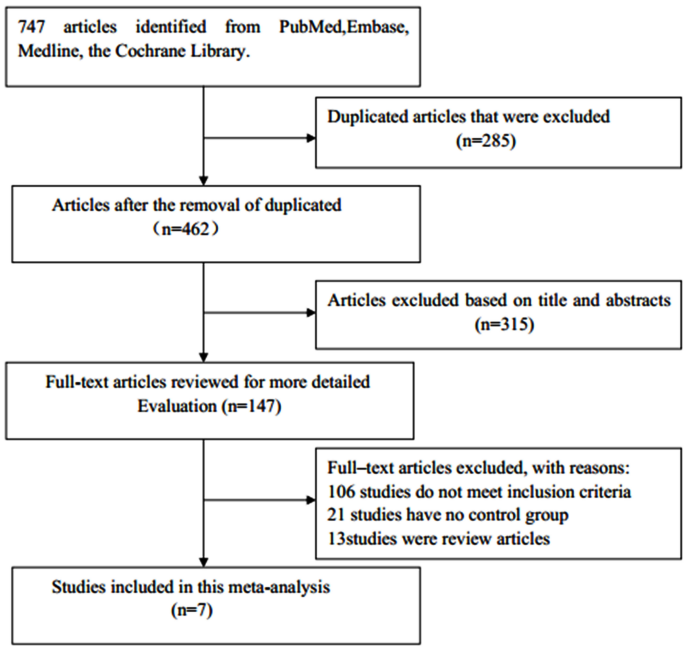

FIGURE 1
Flowchart of study selection.

147 potentially relevant studies remained for further review. Among them, 106 studies were eliminated for not meeting the inclusion criteria, 21 for having no control group, and 13 for being reviews. Finally, seven observational studies were included in this meta-analysis (23–25, 32–35).

Study characteristics

The characteristics of eligible publications are listed in Table 1. The study design of all included studies was cross-sectional. All of the eligible publications were published from 2009 to 2017. Among the seven included studies, a total of 672 participants were enrolled, 354 of whom were CRPC patients, while the remaining 318 participants were HSPC patients (the sample sizes ranged from 9 to 162). The assessment of AR-V7 expression was inconsistent among studies.

Study quality

Supplementary Table 1 summarizes the quality assessment results for the cross-sectional studies.

Synthesis of results

No significant statistical heterogeneity was detected among all the eligible studies (I^2 = 20.6%, p = 0.272); thus, the fixed-effects model was conducted to pool the data. As displayed in Figure 2, the combined results revealed that patients with CRPC had a significantly higher expression of AR-V7 than the individuals with HSPC (RR = 7.55, 95% CI: 4.61–12.35, p < 0.001), which showed that CRPC was strongly correlated with an increased positive expression of AR-V7.

Sensitivity analysis

A sensitivity analysis of this meta-analysis was conducted to evaluate its reliability with regard to the association between CRPC and positive expression of AR-V7. Each of the individual studies was excluded in turn to recalculate the synthesized RR. The overall pooled return on investment did not change substantially, with a range from 6.85 (95% CI: 4.16–11.27, p < 0.001) to 9.84 (95% CI: 5.13–18.87, p < 0.001) after eliminating any one of the included studies (Table 2 and Figure 3). These results suggested that no single study dominated the combined RR, which strengthened the evidence of this study.

Subgroup analyses

To further obtain the potential relationship between CRPC and the positive expression of AR-V7, subgroup analyses were performed according to the methods of AR-V7 detection, the geographical region, and the publication year (Table 3). In the subgroup analysis according to the methods of AR-V7 detection, a stronger association was detected in the RNA *in situ* hybridization (RISH) group (RR = 39.81, 95% CI: 4.90–323.51, p < 0.001) compared with the IHC group (RR = 6.72, 95% CI: 3.83–11.81, p < 0.001) and PCR and protein analysis group (RR = 7.41, 95% CI: 2.33–23.56, p < 0.001). Moreover, in the subgroup analysis according to the geographical region, a statistically significant association between CRPC and positive expression of AR-V7 could be observed in the UK, the USA, and China, with RR (95% CIs) of 9.29 (3.06–28.20, p < 0.001), 10.09 (4.21–24.18, p < 0.001), and 5.30 (2.50–11.24, p < 0.001), respectively. However, a similar association was not identified in Sweden (RR = 17.94, 95% CI: 0.78–411.52, p > 0.05). Finally, when further stratified by publication years 2009–2011 and 2012–2017, the RR of CRPC associated with positive AR-V7 expression was 8.00 (95% CI: 3.35–19.13, p < 0.001) and 7.34 (95% CI: 4.04–13.33, p < 0.001), respectively.

Publication bias

In accordance with Begg's rank correlation analysis and Egger's linear regression analysis, no publication bias was observed in the studies reviewed (Begg's, $p > |z| = 0.133$; Egger, $p > |t| = 0.069$) (Figures 4, 5).

TABLE 1 Characteristics of the included studies in the meta-analysis.

Study	Year	Country	Study design	AR-V7 detection	Hormone-sensitive prostate cancer		Castration-resistant prostate cancer		RR (95% CI)
					Patients	Age	Patients	Age	
Welti et al.	2016	UK	Cross-sectional	IHC	7/33	NA	25/35	67.5 (64.2–75.3)	9.29 (3.06–28.20)
Zhang et al.	2011	USA	Cross-sectional	IHC	2/50	NA	39/162	NA	7.61 (1.77–32.75)
Saylor et al.	2016	USA	Cross-sectional	RISH	1/30	NA	12/12	NA	491.67 (18.72–12913.73)
Qu et al.	2015	China	Cross-sectional	IHC	22/104	70 (43–84)	27/46	65 (50–79)	5.30 (2.50–11.24)
Hornberg et al.	2011	Sweden	Cross-sectional	IHC	8/10	79 (60–85)	30/30	70 (51–86)	17.94 (0.78–410.42)
Hu et al.	2009	USA	Cross-sectional	PCR and protein analysis	34/82	LNA	21/25	NA	7.41 (2.33–23.55)
Zhu et al.	2017	USA and UK	Cross-sectional	RISH	0/9	NA	15/44	NA	6.89 (0.45–105.79)

IHC, immunohistochemistry; RISH, RNA in situ hybridization; NA not available; RR, relative risk.

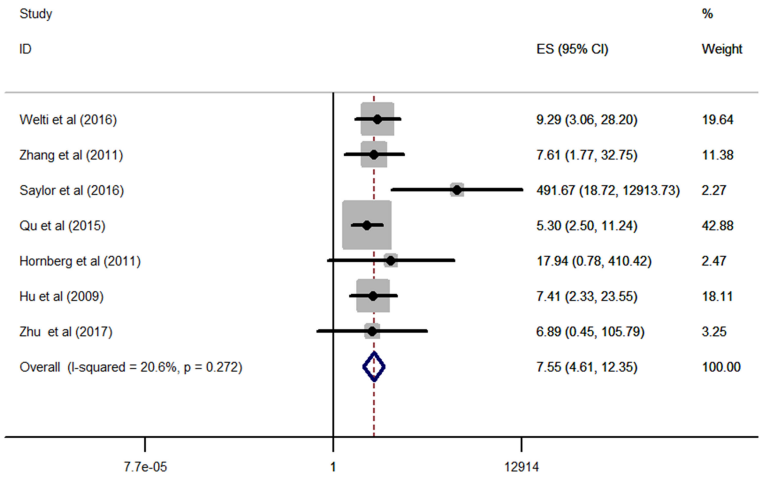

FIGURE 2
Forest plots of meta-analysis of the included studies on the association between CRPC and positive expression of AR-V7. CRPC, castration-resistant prostate cancer.

Discussion

Recently, numerous studies (36, 37) had speculated the relationship between CRPC and the positive expression of AR-V7. In this study, we summarized the evidence of the association between CRPC and positive AR-V7 expression. Based on data from the seven included studies, the present study suggested that CRPC patients have a significantly increased positive expression of AR-V7 than the individuals with HSPC. The results derived from this study were consistent with several previous clinical studies (23–25), which demonstrated that the expression of AR-V7 protein was more frequently identified in men with CRPC compared to those with HSPC. Furthermore, we also examined the impacts of potential confounders on our findings by performing the subgroup analyses. A stronger association was detected in RISH measurement in American patients, and those studies were published before 2011 (all $p < 0.001$). Based on the sensitivity analyses, the positive association between CRPC and AR-V7 remained significant in nearly all of the included studies.

A majority of tumors eventually progress to CRPC after a period of an initial response to the ADT regimen (38). The underlying mechanism for the development and progression of CRPC is the expression of AR-Vs, particularly AR-V7, which is a popular area of related research. In ligand-free forms of AR-V7, ligand-binding domains are absent, but transcriptional element binding domains are preserved, facilitating intracellular AR signaling even in the absence of androgens or antiandrogens (39). It is capable of translocating into nuclei and binding AR-responsive elements without ligand interaction and regulating gene transcription (32).

Accumulating evidence has demonstrated that there is a correlation between the level of AR-V7 and resistance to both abiraterone and enzalutamide, as well as poor survival in CRPC (40). In a previous study developed by Seitz et al. (41), 85 CRPC patients were under abiraterone (n = 56) or enzalutamide (n = 29) treatments. High AR-V7 expression levels were associated with shorter prostate-specific antigen (PSA)–progression-free survival (PSA-PFS) (median, 2.4 *vs.* 3.7 months; $p < 0.001$), shorter clinical progression-free survival (median, 2.7 *vs.* 5.5 months; $p < 0.001$), and shorter overall survival (median, 4.0 *vs.* 13.9 months; $p < 0.001$). Moreover, Todenhöfer et al. (42) found that AR-V7 transcripts in peripheral blood were significantly associated with a shorter median PSA-PFS (0.7 *vs.* 4.0 months, $p < 0.001$) and median overall survival (5.5 *vs.* 22.1

TABLE 2 Sensitivity analysis after each study was excluded by turns.

Study omitted	RR (95% CI) for remainders	Heterogeneity	
		I^2 (%)	p
Welti et al., 2016	7.17 (4.14, 12.42)	32.3	0.19
Zhang et al., 2011	7.54 (4, 47, 12.72)	33.8	0.18
Saylor et al., 2016	6.85 (4.16, 11.27)	0	0.95
Qu et al., 2015	9.84 (5.13, 18.87)	17.6	0.30
Hornberg et al., 2011	7.38 (4.49, 12.15)	31.1	0.20
Hu et al., 2009	7.58 (4.40, 13.05)	33.8	0.18
Zhu et al., 2017	7.57 (4.59, 12.49)	33.8	0.18

RR, relative risk.

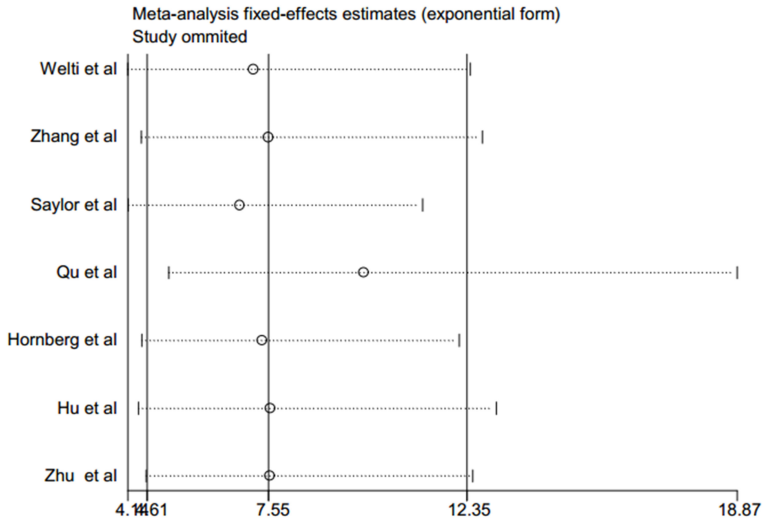

FIGURE 3
Sensitivity analysis after each study was excluded by turns.

months, $p < 0.001$) in CRPC patients treated with abiraterone. This association was confirmed in another clinical study that demonstrated that patients with positive expression of AR-V7 had a poor prognosis than those with negative AR-V7 expression in CRPC treatment with either enzalutamide or abiraterone (43).

Preclinical studies have shown that expression of AR-V7 protein might be a treatment-specific biomarker in CRPC (44). AR-V7-positive patients would benefit from taxanes more than those managed with androgen receptor signaling inhibitors (ARSIs). Antonarakis et al. (45) reported that PSA responses were higher in AR-V7-positive CRPC patients. Moreover, PSA-PFS and clinical and/or radiographic PFS were significantly longer in taxane-treated than those under enzalutamide or abiraterone treatments. However, the outcomes turned out to be non-significant different when the therapy type was changed to AR-V7-negative CRPC. Similar to their results, Scher et al. (46) observed that circulating tumor cell expression of AR-V7 protein in CRPC patients was correlated with superior survival on taxane therapy over ARSI-directed therapy. This evidence suggested the presence of AR-V7 was associated with a better clinical outcome for patients treated with taxanes when compared to those under enzalutamide or abiraterone therapies. Based on the above studies, detecting the AR-V7 expression might be useful to serve as a therapeutic biomarker in patients with CRPC.

In this study, the AR-V7 expression in CRPC patients was found to be significantly ascending as compared with that of patients with HSPC. Moreover, on the basis of the findings in previous studies, the AR-V7 expression might be correlated to resistance to abiraterone and enzalutamide treatment but not taxane chemotherapy. Thus, CRPC patients with positive expression of AR-V7 are recommended to be treated with taxane rather than ARS inhibitors. Accordingly, the AR-V7 status should be evaluated when managing patients with CRPC in clinical practice.

TABLE 3 Subgroup analysis of the association between CRPC and positive expression of AR-V7.

Study or subgroup		No. of studies	Heterogeneity		RR (95% CI)	*p*
			I^2 (%)	*p*		
AR-V7 detection	IHC	4	0	0.78	6.72 (3.83, 11.81)	<0.001
	RISH	2	74.1	0.05	39.81 (4.90, 323.51)	<0.001
PCR and protein analysis	1	–	–	7.41 (2.33, 23.56)	–	
Country	UK	1	–	–	9.29 (3.06, 28.20)	–
	USA	3	65.8	0.054	10.09 (4.21, 24.18)	<0.001
	China	1	–	–	5.30 (2.50, 11.24)	–
Sweden	1	–	–	17.94 (0.78, 411.52)	–	
Year	≤2011	3	0	0.87	8.00 (3.35, 19.13)	<0.001
	>2011	4	28.6	0.064	7.34 (4.04, 13.33)	<0.001

IHC, immunohistochemistry; RISH, RNA in situ hybridization; RR, relative risk.

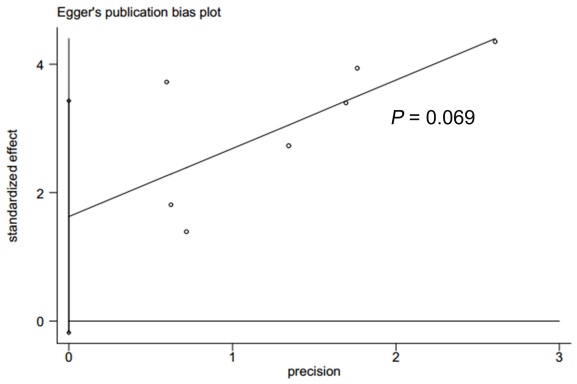

FIGURE 4
Eggers test to detect publication bias.

It was speculated that the possible mechanism of progression from HSPC (also known as castration-sensitive prostate cancer (CSPC)) to CRPC is the existence of AR splice variants. AR-V1 was the most common AR-V in hormone-naïve PCa, while AR-V7 was the most common during ADT and in the CRPC stage (47). However, several other variants also have been found to play roles in this action. AR-Vs can be categorized into the following four groups depending on their nuclear localization ability: ligand stimulated in a similar manner to canonical full-length AR (AR-FL) (i.e., AR-23), constitutively active (i.e., AR-V3, 4, 7, and 12), conditionally active (i.e., AR45, AR-V1, and 9), and inactive (i.e., AR-V13, 14, and AR8) (48, 49). For example, in addition to AR-V7, AR-V3, AR-V7, and AR-V9 were also found to be co-expressed in the metastases of CRPC (50). Interestingly, the mRNA of expression of these variants (including AR-V7) was also detected in benign prostatic hyperplasia (BPH) and hormone-naïve primary tumors with lower abundance and frequency (51). De Laere et al. assessed 30 circulating tumor cell (CTC) samples from 26 metastatic CRPC (mCRPC) patients, and 15/26 (57.7%) patients were AR-V-positive with AR-V7 being the most frequently detected variant (12/15, 80%), followed by AR-V3 (11/15, 73%), AR45 (10/15, 67%), AR-V9 (6/15, 40%), AR-V1 (5/15, 30%), AR-V2 (3/15, 20%), and AR-V5 (3/15, 20%) (52). However, a subsequent study (53) developed by To et al. showed that

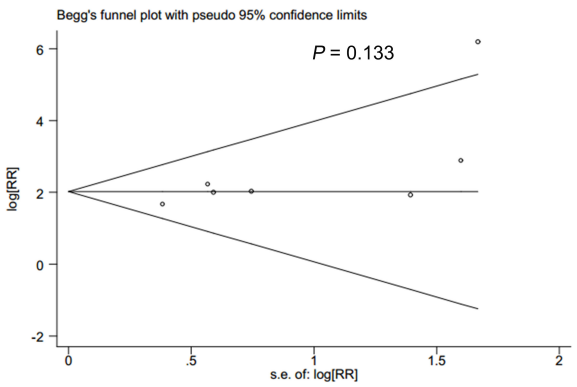

FIGURE 5
Beggs test to detect publication bias.

AR-V7 or AR-V9 expression does not predict outcomes in mCRPC patients receiving abiraterone or enzalutamide by conducting a whole blood assay. Nevertheless, based on outcomes from the majority of the previous relevant studies and the meta-analysis of our, AR-V7 was found to be associated with the development and progression of CRPC. At present, most investigators are focusing on AR-V7, but the clinical importance of the detection of other AR-Vs in CRPC is largely unknown, and it deserves further research. According to the above evidence, the specific role of AR-V7 in CRPC is still controversial among different studies. In addition to AR-V7, the expression of several other variants should also be evaluated in both blood and tissues when making a decision on CRPC patients.

In addition to distinct demographic characteristics (i.e., race, sample size, and age), different disease states, and the diverse treatments of CRPC or CSPC, it should be known that various detection methods and measurements for assessing the AR-V7 might also play a role in the outcomes among different studies. For example, Li and colleagues (54) showed that 21% (64/310) of the patients with metastatic CSPC had positive AR-V7 immunohistochemical staining on diagnostic biopsies using clone EPR15656 from Abcam. However, a more recent study (4) reported by Sowalsky et al. demonstrated that AR-V7 mRNA and protein abundance was low in CSPC prior to treatment using robustly validated assays applying both IHC and RNA sequencing-based approaches. The teams previously showed that AR-V7 protein was rarely expressed (<1%) in primary PC but is frequently detected (75% of cases) following androgen deprivation therapy (55). The authors, as have others, also demonstrated that AR-V7 mRNA was commonly identified in benign prostate and primary prostate cancer tissues (4, 51). At present, the majority of the studies were conducted in the metastatic CRPC setting. Our meta-analysis has confirmed the positive relationship between a high level of AR-V7 and the risk of CRPC. However, the clinical importance of AR-V7 detection in HSPC is rarely reported. In HSPC/CSPC research, Li et al. and Sowalsky et al. presented conflicting results about the expression of AR-V7 in CSPC. The following factors may cause this inconsistency: 1) the participants: Li et al. investigated Chinese metastatic HSPC (mHSPC) patients receiving ADT, while Sowalsky et al. investigated US and UK HSPC patients with high-risk localized prostate cancer treated with ADT plus enzalutamide prior to surgery (that is, non-metastatic). 2) The antibody: the AR-V7 antibody used in their studies was different (Li used clone EPR15656 from Abcam; Sowalsky used clone RM7 from RevMAb and Abcam clone EPR15656). 3) The assessment methods for IHC: diverse dilution rate (data were not available) and the cutoff were used for defining "positive or negative" samples. Thus, in the future, evidence for the potential link between AR-V7 and CSPC development may be enhanced if the detection methods are under the same condition. In the CRPC setting, the positive association between AR-V7 and CRPC seems to be a little controversial.

The relationship between AR-V7 and CRPC has been extensively investigated, but whether AR-V7 itself or the ratio of full-length (FL) AR and AR-V7 plays a key role in CRPC remains to be resolved. It is common for patients with CRPC to co-express the full-length receptor and spliced variant AR-V7 (56). After hormone-sensitive PCa progression into CRPC, the expression of full-length AR and AR-V7 increases. AR splice variants exert activating effects in DNA repair

genes similar to full-length androgen receptors. However, even in the absence of AR-FL, AR splice variants can provide the necessary transcriptional support for DNA repair genes. AR-Vs have mRNA sequences that are structurally different from the canonical full-length AR. The ARV-7 preferentially leads to the expression of cell cycle regulatory genes, while the full-length AR represses that program and favors instead genes related to metabolism, differentiation, and macromolecular synthesis. Blocking AR-FL using antiandrogens has been shown to retain AR-V activity (47). It was reported that the antibody used for the detection of AR-V7 has high specificity with no cross-reaction to full-length AR (57). In the study of Li et al., mHSPC patients were AR-FL-positive, and 64 (21%) were AR-V7-positive (54).

However, it was reported that AR-V7 could activate target genes irrespective of AR-FL, leading to the development and growth of prostate cancer under low androgen levels (58). AR-V7 are already located at a high fraction in the nuclei of primary PCA cells, while AR-FL remains cytoplasmatic in the absence of activating ligands (59). AR-FL and many AR-Vs may share common chromatin binding sites, genomic binding sites, and transcriptional programs specific to AR-V7. Although AR-V7 co-exists with AR-FL, genomic functions mediated by AR-V7 do not require the presence of AR-FL. AR-V7 expression was found to be lower than AR-FL in CRPC clinical samples (33). AR-V7 is negatively regulated by androgen signaling mediated by AR-FL (60). Detection of AR-V7 indicates the resistance of CRPC to AR signaling inhibitors (43). It was reported that dihydrotestosterone treatment results in a greater decrease in the AR-V7 expression than AR-FL, indicating a mechanism that preferentially reduces the expression of AR-V7 (58). It was also reported that AR-V7 and the AR-V7/AR-FL ratio increase as the disease progresses from CSPC to CRPC (18). Moreover, high nuclear AR-V7 expression and high nuclear AR-V7/AR-FL ratio were associated with a shorter biochemical recurrence-free survival of PCa (59). Among the CRPC patients before their treatment with abiraterone or enzalutamide, positive AR-V7 detection, but not higher AR-FL was significantly associated with shorter PSA-PFS (35). Based on this evidence, both AR-V7 itself and the AR-V7/AR-FL ratio play important roles in CRPC development, but AR-V7 plays a central functional role in this action. However, studies on the relationship between the AR−V7/AR−FL ratio and CRPC are insufficient; whether AR-V7/AR-FL forms heterodimers under low androgen conditions needs further investigation.

Our study is a high-quality cumulative analysis that was conducted following the PRISMA statement. However, some non-negligible limitations should be acknowledged when interpreting the outcomes derived from this study. First, heterogeneity was unavoidable among different studies, even though the potential causes of heterogeneity were identified by sensitivity analysis and subgroup analyses. The effects of small sample studies might be problematic in these meta-analyses, which resulted in the exaggeration of the summary estimates. Second, the various methods of assessment for AR-V7 among studies might also be one of the limitations of this study. Different kinds of assessments for AR-V7 may have a huge impact on the pooled analysis. Therefore, diverse measurements for detecting the AR-V7 expression might be another source of heterogeneity.

Conclusion

Our study demonstrated that the positive expression of AR-V7 was significantly higher in patients with CRPC than that in those with HSPC. To improve clinical prognostic, the level of AR-V7 should be evaluated when clinicians determined the preferred treatments for CRPC. However, the present evidence was based on retrospective clinical trials with limited included studies. Therefore, further high-quality, large sample size and multicenter cohort studies are still warranted to validate our findings. At present, the predictive effects of the AR-V7 expression in CSPC/HSPC are controversial among studies, and this needs to be addressed in the near future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

ShaZ: project development and manuscript writing. JL and MS: data collection. XL: data analysis. LZ and ShiZ: manuscript editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1053111/full#supplementary-material

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Importance of long non-coding RNAs in the pathogenesis, diagnosis, and treatment of prostate cancer

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Long non-coding RNAs (lncRNAs) are regulatory transcripts with essential roles in the pathogenesis of almost all types of cancers, including prostate cancer. They can act as either oncogenic lncRNAs or tumor suppressor ones in prostate cancer. Small nucleolar RNA host genes are among the mostly assessed oncogenic lncRNAs in this cancer. PCA3 is an example of oncogenic lncRNAs that has been approved as a diagnostic marker in prostate cancer. A number of well-known oncogenic lncRNAs in other cancers such as DANCR, MALAT1, CCAT1, PVT1, TUG1 and NEAT1 have also been shown to act as oncogenes in prostate cancer. On the other hand, LINC00893, LINC01679, MIR22HG, RP1-59D14.5, MAGI2-AS3, NXTAR, FGF14-AS2 and ADAMTS9-AS1 are among lncRNAs that act as tumor suppressors in prostate cancer. LncRNAs can contribute to the pathogenesis of prostate cancer *via* modulation of androgen receptor (AR) signaling, ubiquitin–proteasome degradation process of AR or other important signaling pathways. The current review summarizes the role of lncRNAs in the evolution of prostate cancer with an especial focus on their importance in design of novel biomarker panels and therapeutic targets.

KEYWORDS

lncRNA, prostate cancer, biomarker, expression, diagnostic

Introduction

Prostate cancer is the most commonly diagnosed cancer among males being responsible for 27% of all diagnosed cases (1). It also accounts for the greatest number of deaths from cancer among men after lung cancer (1). A number of risk factors have been identified for prostate cancer among them are age, ethnicity, genetics, family history,

obesity, and smoking (2, 3). Prostate cancer is developed *via* a multistep process, starting from prostatic intraepithelial neoplasia and being evolved to localized, advanced prostate cancer with local invasion and metastatic prostate cancer, respectively (4). The aggressiveness of prostate cancer is best described by the Gleason grading system (5). The hormone responsiveness is an important feature in this cancer resulting in tumor regression following castration (6). Therefore, androgen deprivation therapy has been suggested as the regular therapeutic regimen for prostate cancer. However, resistance to this therapeutic modality can develop (4).

Identification of the underlying cause of initiation and progression of prostate cancer is an imperative step in development of novel therapies for this kind of malignancy. Moreover, it can facilitate design of novel biomarkers for early detection of cancers. Long non-coding RNAs (lncRNAs) are promising transcripts for both purposes (7–9). These transcripts have sizes more than 200 nucleotides and are responsible for a variety of regulatory mechanisms at different levels of gene expression regulation (10). Aberrations in the expression of lncRNAs might be representative of certain phases of cancer progression, and can be used to predict early progression of cancer or induction of cancer-related signaling pathways (11, 12). Therefore, these transcripts have attained much attention during recent years for their contribution in the pathogenesis of almost all kinds of cancers, including prostate cancer. The current review summarized the role of lncRNAs in the evolution of prostate cancer with an especial focus on their importance in design of novel biomarker panels and therapeutic targets. We used PubMed and Google Scholar databases with the key words "lncRNA" or "long non-coding RNA" and "prostate cancer". Then, we screened the obtained articles and included the relevant ones in the manuscript. Finally, we tabulated the data obtained from these articles for the purpose of better classification of the data.

Up-regulated lncRNAs in prostate cancer

Using quantitative real time PCR method, several lncRNAs have been shown to be over-expressed in prostate cancer tissues compared with adjacent non-cancerous tissues or benign prostate hyperplasia (BPH) samples, representing an oncogenic role for these transcripts in the progression of prostate cancer (Table 1). Small nucleolar RNA host genes (SNHGs) are among the mostly

TABLE 1 Summary of function of up-regulated lncRNAs in prostate cancer (Official HUGO Gene Nomenclature symbols are used).

lncRNA	Samples	Cell lines	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
UBE2R2-AS1	74 PTNTs	RWPE-1, DU145, and PC-3	PCNA, CDK4, Cyclin D1, Bcl-2, N-cadherin, Vimentin, E-cadherin	–	Poor prognosis of PC patients	Might serve as a biomarker for diagnosis and a promising target in case of PC therapy	(13)
CASC11	66 PTNTs	PC-3, DU145, 22Rv1, LNCaP, and RWPE-1	YBX1	p53 pathway	–	CASC11 enhances the proliferation and migratory capacity of PC cells.	(14)
CASC11	29 tumor and 5 benign prostate samples	PNT1a, PC3, DU145, and LNCaP	miR-145	PI3K/AKT/ mTOR and CASC11/miR-145/IGF1R axis	–	Its high expression suppresses miR-145, and activates PI3K/ AKT/mTOR pathway.	(15)
SNHG17	52 PTNTs	RWPE-1, RV-1, PC-3, DU145, and LNCaP	miR-23a	SNHG17/miR-23a/OTUB1 Axis	Advanced tumor stage	SNHG17 may enhance the progression of PC.	(14)
SNHG17	58 PTNTs	LNCaP, C4-2, and HPrEC	TCF1, TCF4, LEF1, c-myc, cyclin D1 and axin2	Wnt/β-catenin pathway	Poor outcomes	SNHG17 promotes the proliferation and viability, but suppresses apoptosis.	(16)
SNHG17	36 PTNTs	RWPE-1, DU145, LNCaP, VCaP, and PC-3	SNORA71B, miR-339-5p, and STAT5A	SNHG17/miR-339-5p/ STAT5A/ SNORA71B axis	Low PFS	SNHG17/miR-339-5p/STAT5A modulates SNORA71B expression.	(17)
SNHG17	46 patients with CRPC and 149 patients with HSPC	LNCaP, C4-2, PC-3, and DU145	miR-144 and CD51	miR-144/CD51 Axis	–	Expression of SNHG17 was elevated in CRPC tissues and cells.	(18)

(Continued)

TABLE 1 Continued

lncRNA	Samples	Cell lines	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
SNHG16	80 PTNTs	DU-145 PCa cells	miR-373-3p	TGF-β-R2/ SMAD signaling	–	SNHG16 facilitates the proliferation and migration by modulating the miR-373-3p/ TGF-β-R2/SMAD axis.	(19)
SNHG16	52 cancer tissues and 36 normal prostate samples	22Rv1 and HPrEC	GLUT1	–	–	SNHG16 silencing suppresses the growth of PCa cells through downregulating GLUT1.	(20)
SNHG14	60 PTNTs	WPMY1, LNCaP, 22RV1, PC-3, and DU145	miR-5590-3p, YY1, Cyclin D1, Bcl-2, N-cadherin, Bax, Caspase-3, and E-cadherin	miR-5590-3p/ YY1 axis	Advanced stage and poor diagnosis	SNHG14 enhances the proliferation and invasion of PCa cells through miR-5590-3p/ YY1.	(21)
SNHG12	85 PTNTs	WPMY-1, LNCAP, DU145, and PC-3	apoptosis-related and invasion-related proteins	PI3K/AKT signaling pathway	–	SNHG12 Silencing suppresses PCa cells proliferation.	(22)
SNHG12	Blood samples from 56 PCa patients and 45 patients with BPH	22RV1, Du145, LNCaP, MDaPCa2b, and RWPE1	CCNE1 and miR-195	PI3K/AKT/ mTOR pathway and miR-195/ CCNE1 axis	Poor prognosis	SNHG12 silencing suppresses viability and induces apoptosis and autophagy of PCa cells.	(23)
SNHG11	120 PCa patients and 45 cases of BPH patients	22RV1	–	–	Shorter OS time and biochemical recurrence-free survival	SNHG11 silencing prevents the proliferation, invasion, and migration.	(24)
SNHG11	30 PTNTs	RWPE-1, LNCaP, C4-2, PC3, and DU145	miR-184	miR-184/IGF-1R signaling axis	–	SNHG11 promotes progression of PC by increasing the expression of IGF-1R.	(25)
SNHG10	gene expression profiles of PC patients from TCGA database	VCaP, LNCaP, 22RV1, PC3, DU145, and RWPE-1	–	Immune infiltration and oxidative phosphorylation	Advanced clinical parameters	SNHG10 affects proliferation, migration, and invasion.	(26)
SNHG9	52 PTNTs	–	–	maintenance of cell metabolism and protein synthesis	Poor prognosis	SNHG9 may serves as a possible prognostic biomarker in patients with PCa.	(27)
SNHG8	53 PTNTs	RWPE1, LNCaP, PC3, DU145, VCap, and 22RV1	miR-384 and HOXB7	–	–	SNHG8 enhances the proliferation, migration and invasion of PCa cells by sponging miR-384.	(28)
SNHG7	30 PTNTs	PC-3 and DU-145 cells	c-Myc	SRSF1/c-Myc axis	–	SNHG7 knocking down inhibits the proliferation and glycolysis in PCa cells.	(29)
SNHG7	127 PTNTs	–	–	–	Metastasis, pelvic lymph node metastasis, and TNM stage	SNHG7 may serve as a possible prognostic marker and target for the treatment of PCa.	(30)
SNHG6	63 PTNTs	PC-3 and DU145	miR-186	SNHG6/miR-186 axis	–	SNHG6 was upregulated in drug-resistant PCa tissues and cells.	(31)

(Continued)

TABLE 1 Continued

lncRNA	Samples	Cell lines	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
SNHG3	30 PTNTs	RWPE-1, PC-3, DU145, VCaP and LNCaP	miR-1827	Wnt/AKT/ mTOR pathway	Poor prognosis	SNHG3 may be a prognostic marker for PCa.	(32)
SNHG3	40 PTNTs	WPMY-1, PC-3, Du 145, LNCaP, and 22RV1	miR-152-3p	SNHG3/miR-152-3p/ SLC7A11 axis	–	Promotes proliferation, invasion, and migration of PCa cells *via* sponging miR-152-3p.	(33)
SNHG3	26 PTNTs	REPW-1, DU145, VCaP, LNCaP, C4-2B, 22RV1,and PC3	miR-214-3p	SNHG3/miR-214-3p/TGF-β axis	Advanced clinicopathological features and poor prognosis	SNHG3 silencing suppresses bone metastasis in PCa cell.	(32)
SNHG3	PTNTs	LNCaP and PC-3	miR-487a-3p and TRIM25	EMT	–	SNHG3 sponges with miR-487a-3p, and affects migration, invasion, and EMT of PCa cells.	(34)
SNHG3	–	RWPE-1, PC3, DU145, 22RV1, and LNCaP	miR-577 and SMURF1	SNHG3/miR-577/SMURF1 axis	–	SNHG3 affects the proliferation, migration, EMT process and apoptosis.	(35)
SNHG1	Formalin fixed paraffin —embedded PCa specimens and BPH or ANTs (n=14)	RWPE-1, LNCaP, 22Rv1, PC-3, DU145	E-cadherin, vimentin	EMT pathway	Tumor metastasis	SNHG1 is a possible target for treatment of PCa.	(36)
SNHG1	20 PTNTs	LNCaP, PC-3, DU-145, and RWPE-1	EZH2	Wnt/β-catenin and PI3K/AKT/ mTOR signaling pathway	–	SNHG1 affects PCa cells proliferation, apoptosis, migration, invasion, and autophagy by targeting EZH2.	(37)
SNHG1	134 PTNTs	PC3 and DU145	–	–	Aggressive malignant behavior	SNHG1 may serves as a possible marker and target for treatment of PCa.	(38)
SNHG1	142 PTNTs	DU-145, LNCaP, 22Rv1, PC-3, and RWPE-1	miR-195-5p, E-cadherin, N-cadherin, and Vimentin	EMT	–	SNHG1 affects PCa cells proliferation, invasion and EMT *via* sponging miR-195-5p.	(39)
SNHG1	Normal tissues (n=318) and PCa tissues(n=92)	22Rv1 and LNCaP	miR-377-3p and AKT2	SNHG1/miR-377-3p/AKT2 axis	Poor overall survival rate	SNHG1 sponges with miR-377-3p in PCa cells.	(40)
lncHUPC1	70 PTNTs	RWPE-1, LNCaP, 22RV1, DU145, and PC3	FOXA1, SDCCAG3, and miR-133b	lncHUPC1/ miR-133b/ SDCCAG3 axis	Advanced TNM stages	lncHUPC1 acts as an oncogene and increases the metastasis and growth of PCa cells.	(41)
MNX1-AS1	40 PTNTs	LNCaP, PC-3, C4-2B, Du-145 and RWPE1	miR-2113	miR-2113/ MDM2 axis	Worse overall survival rates	MNX1-AS1 enhances the proliferation, migration and invasion of PCa cells through miR-2113/MDM2 axis.	(42)
CERS6-AS1	PTNTs	DU145 and RWPE-1	miR-16-5p	miR-16-5p/ HMGA2 axis	–	Its knockdown can prevent the proliferation and migration of DU145 cells.	(43)
DANCR	30 PTNTs	HPrEC, RWPE-1, PC3, DU145, LN96, and OPCT-1	miR-33b-5p	Glucose Metabolism	–	DANCR affects the proliferation, migration, and taxol resistance of PCa cells.	(44)

(Continued)

TABLE 1 Continued

lncRNA	Samples	Cell lines	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
DANCR	53 PCa patients and 47 healthy persons	DU145, 22Rv1, RC-92a, PC-3M, and RWPE-1	miR-214-5p	TGF-β signaling pathway	Poor prognosis	Elevated expression of DANCR can facilitate PC progression.	(45)
DANCR	40 paired PCa tissues and ANTs	5 PCa cell lines and 1 epithelial cell line	miR-185-5p	FAK/PI3K/ AKT/GSK3β/ Snail pathway	–	DANCR exerts its oncogenic effects *via* miR-185-5p/LASP1 axis in prostate cancer.	(46)
MALAT1	98 paraffin-embedded clinical specimens (3 normal samples and 95 cancer tissues)	C-3, C4-2, and RWPE-1	MYBL2	MALAT1/ MYBL2/mTOR Axis	–	Its knockdown inhibits the expression of p-mTOR.	(47)
MALAT1	52 PTNTs	RWPE-1, PC-3, and DU145	miR-140 and BIRC6	miR-140/BIRC6 axis	Poor OS	MALAT1 silencing suppresses PC progression.	(48)
MALAT1	–	DU145, PC3, and LNCaP	miR-423-5p	–	Decreased survival	MALAT-1 expression affects progression and survival of PCa patients.	(49)
MALAT1	gene expression profiles of PC patients from TCGA database	LNCaP and CWR22Rv1	miR-145	miR-145-5p-SMAD3/ TGFBR2 axis		Long ncRNA MALAT1 enhances the proliferation, migration, and invasion by acting as a ceRNA for miR-145.	(50)
MALAT1	602 urine samples from patients with PCa and BPH	–	–	–	–	MALAT-1 and PCA3 may serve as noninvasive exosomal markers for detection of PCa.	(51)
PCA3							
PCGEM1	26 PTNTs	LNCAP, 22RV1, MDA-PCA-2B, and RWPE1	miR-129-5p	PCGEM1/miR-129-5p/CDT1 axis	–	PCGEM1 promotes the progression of PCa through sponging miR-129-5p.	(52)
PCGEM1	50 PTNTs	PC-3, LNPCa, Du-145, C4-2B, and RWPE1	miR-506-3p	miR-506-3p/ PCGEM1/ TRIAP1 axis	Distant metastasis	Facilitates the proliferation, invasion, and migration through sponging miR-506.	(52)
NEAT1	RNA sequencing data from TCGA and GEO databases	PC3	LDHA	–	–	NEAT1 regulates LDHA expression	(13)
NEAT1	130 PTNTs	–	–	–	Distant metastasis, TNM stage, and lymph nodes metastasis	It has been reported that NEAT1 plays a role in the prognosis of PCa patients.	(53)
NEAT1	50 PTNTs	RWPE-1, PC3, P4E6, LNCaP, and DU145	miR-766-5p	miR-766-5p/ E2F3 axis	–	NEAT1 promotes progression of PCa.	(54)
NEAT1	plasma of 15 PCa patients and 15 HCs and 8 FFPE tissues of PCa and ANTs	–	–	–	–	NEAT1 acts as an oncogene in PCa development.	(55)
NEAT1–1	FFPE or fresh-frozen hormone-naïve primary prostate cancer and bone metastatic tissues (n=60)	PDXs related primary cells	CYCLINL1 and CDK19	CYCLINL1/ CDK19/ NEAT1-1 axis	Poor prognosis	NEAT1 induces bone metastasis of PCa *via* N6-methyladenosine.	(56)
LINC00624	PCa tissues	–	TEX10	LINC00624/ TEX10/NF-κB axis	Poor prognosis	LINC00624 plays an oncogenic role in PCa progression.	(57)

(Continued)

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TABLE 1 Continued

lncRNA	Samples	Cell lines	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
TP73-AS1	–	DU-145 and PC-3 cells	TP73	TP73/TP73-AS1 axis		Knockdown of TP73-AS1 suppresses the proliferation of PCa cells by TP73 regulation.	(58)
LINC01207	–	PC-3, LNCaP, Du-145, C4-2B, and RWPE1	miR-1182	miR-1182/ AKT3 axis	Poor prognosis	LINC01207 could directly binds with miR-1182.	(59)
PCAT14	499 PCa samples and 52 adjacent normal tissue samples	–	–	immune pathways	–	PCAT14 is a potential diagnosis marker in case of PCa.	(60)
DLEU2	Prostate tumor tissues from TCGA database	PC-3 and DU145	miR-582-5p	miR-582-5p/ SGK1 axis	Poor prognosis	High expression of DLEU2 promotes the proliferation invasion, and migration of PCa cells.	(61)
BCAR4	90 PTNTs	PC346, LNCap, MDAPC1 2a/b, C4-2, PC3, BPH1, and DU145	miR-15 and miR-146	GLI2 signaling	–	Beclin-1 expression is regulated by BCAR4 *via* miR-146 and miR-15 in PC cells.	(62)
EIF3J-AS1	36 PTNTs	PC-3, LNCaP, DU-145, and RWPE-1	MAFG	–	–	EIF3J-AS1 induces progression of PCa through interaction with MAFG.	(63)
ZEB2-AS1	PTNTs and BPH tissues	–	–	apoptosis	–	No significant association was reported between the relative expression of this lncRNA and the tumor grade.	(64)
HOXD-AS1	36 and 9 cases paraffin embedded PCa and BPH tissues	LNCaP, PC-3, LNCaP-Bic, and LNCaP-AI	miR-361-5p	miR-361-5p/ FOXM1 axis	High volume disease	Exosomal lncRNA HOXD-AS1 enhances distant metastasis.	(65)
HOXA11-AS	25 PTNTs	RWPE-1, PC-3, Du-145, and LNCaP	miR-24-3p	HOXA11-AS/ miR-24-3p/ JPT1 axis	–	HOXA11-AS1 functions as ceRNA for microRNA-24-3p, and regulates Jupiter microtubule associated homolog 1.	(66)
HOXA-AS2	68 PTNTs	RWPE, LNCaP, DU145 and PC3	miR-509-3p and PBX3	miR-509-3p/ PBX3 axis	Advanced stages	Its knockdown inhibits the proliferation and migration.	(67)
LncAY927529	exosomes derived from PCa patient serum	BPH-1, RWPE-1, VCaP, LNCaP, DU145, and PC3	CXCL14	–	–	Exosomal lncRNA lncAY927529 induces proliferation and invasion of PCa cells.	(66)
HCG18	–	PC cells	miR-370-3p	miR-370-3p/ DDX3X Axis	–	HCG18 promotes cell proliferation, invasion, and migration of PCa.	(68)
LINC00115	24 PTNTs	PC-3, DU145, LNCap, 22RV2, and RWPE	miR-212-5p	miR-212-5p/ FZD5/Wnt/β-catenin axis	Poor prognosis	LINC00115 acts as a ceRNA for miR-212-5p, and regulates FZD5 level.	(69)
FOXD1-AS1	–	RWPE-1, LNCap, PC3, and DU145	miR-3167	miR-3167/ YWHAZ axis	–	FOXD1-AS1 induces malignant phenotype of PCa cells through	(70)

(Continued)

TABLE 1 Continued

lncRNA	Samples	Cell lines	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
						regulating the miR-3167/ YWHAZ axis.	
AC245100.4	PCa tissues	PCa cells	–	STAT3/NR4A3 axis	–	Its silencing suppresses the tumorigenesis of PCa cells by regulating STAT3/NR4A3 axis.	(62)
LNC992	Gene expression microarray data from the GEO database and cancer tissues from PCa patients	PCa cells	EIF4A3		–	LNC992 enhances the growth and metastasis of PCa cells by regulating SOX4 expression.	(71)
PCBP1-AS1	4 BPH patients, 28 HSPC patients, and 12 CRPC patients	LNCaP and C4-2 cells	NTD domain of AR	ubiquitin–proteasome degradation process of AR	Poor prognosis	It has been reported that PCBP1-AS1 expression was significantly increased in CRPC.	(62)
CCAT1	10 PTNTs	RWPE-1, LnCaP, DU145, PC3, and 22RV1	miR-490-3p	miR-490-3p/ FRAT1 axis	–	CCAT1 enhances the proliferation, migration, and invasion of PCa cells.	(72)
CCAT1	30 PTNTs	RWPE-1, PC3, and DU145	miR-24-3p and FSCN1	CCAT1/miR-24-3p/FSCN1 axis	–	CCAT1 affects the sensitivity of PCa cells to PTX by regulating miR-24-3p and FSCN1.	(73)
LOC100996425	110 PTNTs	C4-2, PC-3, 22RV1, LNCap, DU-145, and WPMV-1	HNF4A	AMPK/mTOR signaling pathway	Lower overall survival rate	LOC100996425 serves as a promoter in PCa by modulating the AMPK/Mtor signaling pathway.	(72)
OGFRP1	Docetaxel-sensitive (n = 70) and docetaxel-resistant (n = 72) PCa tissues	PC3 and DU-145 and corresponding normal control PrEC prostate epithelial cells	miR-149-5p	OGFRP1/miR-149-5p/IL-6 axis	Poorer overall survival	It was reported that OGFRP1 was upregulated in docetaxel-resistant PC tissue samples in comparison to samples from docetaxel-sensitive patients.	(74)
AATBC	86 PTNTs	LNCaP, DU145, 22RV1, VCaP, PC3, and RWPE-1	miR-1245b-5p	miR-1245b-5p/ CASK Axis	–	AATBC promotes prostate cancer progression.	(74)
AGAP2-AS1	–	PCa cells	miR-628-5p	AGAP2-AS1/ miR-628-5p/ FOXP2 axis and WNT pathway	–	AGAP2-AS1 enhances PCa cell growth by modulating WNT pathway.	(75)
PCAT6	CRPC tissues (n=17) and NEPC tissues (n=9)	NE-like cells (PC3, DU145, and NCI-H660), LNCaP, C4-2	miR-326	PCAT6/miR-326/Hnrnpa2b1 signaling	–	It has been reported that PCAT6 was upregulated in NE-like cells (PC3, DU145, and NCI-H660) in comparison to androgen-sensitive LNCaP cells.	(74)
PCAT6	20 PTNTs	–	IGF2BP2	PCAT6/ IGF2BP2/ IGF1R axis	Poor prognosis	The mentioned lncRNA was upregulated in tumor tissues with bone metastasis, and may act as a potential prognostic marker and therapeutic target in case of PCa patients with bone metastasis.	(76)
CRNDE	25 PTNTs	RWPE-1, LNCaP, PC3,	miR-146a-5p	–	–	CRNDE knocking down suppresses PC cells proliferation.	(71)

(Continued)

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TABLE 1 Continued

lncRNA	Samples	Cell lines	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
		DUL145, and VCaP					
LncRNA NCK1-AS1	116 PTNTs	WPMY-1, PC-3, LNCaP, 22Rv1, and DU145	–	–	Poor prognosis	lncRNA NCK1-AS1 is upregulated in PCa. its silencing can suppress PCCs proliferation.	(76)
AFAP1-AS1	30 PTNTs	HprEC, PC3, and DU145	miR-195-5p	miR-195-5p/ FKBP1A axis	–	AFAP1-AS1 affects the sensitivity of PCa cells to paclitaxel.	(77)
AFAP1-AS1	–	C4-2 cells and NE-like cells (PC3, DU145, and NCI-H660)	miR-15b	miR-15b/IGF1R Axis	–	Its expression was upregulated in castration-resistant C4-2 cells and NE-like cells, in comparison to androgen-sensitive LNCaP cells.	(74)
LINC00467	22 PTNTs	CaP, LNCaP, 22RV1, PC3, DU145, HrPEC, and RWPE-1	miR-494-3p	M2 macrophage polarization, STAT3 pathway and miR-494-3p/STAT3 Axis	–	Downregulation of LINC00467 prevents migration and invasion of PCa cells.	(78)
LINC01194	62 PTNTs	RWPE-1, PC3, DU145, and LNCap	PAX5, miR-486-5p	LINC01194/ miR-486-5p/ GOLPH3 axis	–	LINC01194 serves as a tumor promotor, and enhances progression of PCa by regulating LINC01194/miR-486-5p/ GOLPH3 axis.	(79)
PlncRNA-1	34 PTNTs	DU145 and 22Rv1	–	PTEN/Akt pathway	–	PlncRNA-1 facilitates PCa cells proliferation, migration and invasion.	(80)
MIR4435-2HG	–	WPMY-1, VCaP, LNCaP, DU145, and PC-3	ST8SIA1	FAK/AKT/β-catenin signaling pathway	–	MIR4435-2HG affects the clone formation aptitude, proliferation, invasion, and migration of PC-3 cells.	(81)
PTV1	PVT1 RNA-Seq data from TCGA-PRAD database	–	–	–	Worse prognosis	PTV1 is a potential diagnosis and prognosis marker in PCa.	(74)
PTV1	–	DU 145, PC-3, and RWPE-1	miR-15b-5p, miR-27a-3p, miR-143-3p, miR-627-5p, and NOP2	PVT1-NOP2 axis	–	PVT1 induces metastasis in PCa.	(82)
PVT1	25 PTNTs	22RV1, DU145, RWPE-1, and 293T	miR-15a-5p and KIF23	PVT1/miR-15a-5p/KIF23 axis	–	PVT1 modulates KIF23 *via* miR-15a-5p.	(83)
LINC01116	–	RWPE-1, DU145, PC3, LNCAP, 22RV1, and VCaP	miR-744-5p	miR-744-5p/ UBE2L3 axis	–	LINC01116 enhances the proliferation, migration, invasion and EMT progress of PCa cells.	(84)
PAINT	tissue microarray samples from normal prostate and prostate adenocarcinoma from stages I, II, III and IV	PC-3, C4-2B, 22Rv1, LNCaP-104S, and MDA-PCa-2b	Slug, Vimentin, E-cadherin	epithelial mesenchymal transition (EMT) and apoptosis	Aggressive PCa	PAINT functions as an oncogene in PCa.	(85)
PTTG3P	CRPC tissues and tumor tissues of patients	androgen-independent	miR-146a-3p, PTTG1	–	–	PTTG3P is the ceRNA of miR-146a-3p to increase PTTG1	(86)

(Continued)

TABLE 1 Continued

lncRNA	Samples	Cell lines	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
	with hormone-naive PCa	PC cell lines and androgen-dependent PCa cell line LNCaP				expression in the progression to CRPC.	
NORAD	74 PTNTs	22Rv1, DU145, PC-3, RWPE-1, C4-2B, HS-5, and HEK293T	miR-541-3p	NORAD/miR-541-3p/PKM2 axis	–	NORAD functions as a ceRNA of miR-541-3p to enhance the expression of PKM2, leading to development of bone metastasis in PCa.	(87)
NORAD	45 PTNTs	RWPE-1, PC-3, LNCap, 22RV1, and DU-145	miR-30a-5p and RAB11A	miR-30a-5p/ RAB11A/WNT/ β-catenin pathway	–	NORAD facilitates the proliferation, invasion, EMT, and suppresses apoptosis of PCa cells.	(88)
NORAD	30 PTNTs	DU145, 22Rv1, LNCaP, and RWPE-1	miR-495-3p and TRIP13	miR-495-3p/ TRIP13 axis	–	NORAD sponges with miR-495-3p, and increases malignant features of PCa cells.	(89)
KCNQ1OT1	30 PTNTs	DU145 and LNCaP	miR-211-5p	miR-211-5p/ CHI3L1 Pathway	–	lncRNA KCNQ1OT1serves as a ceRNA of miR-211-5p, and upregulates CHI3L1 levels.	(90)
KCNQ1OT1	30 PTNTs	DU145 and PC-3	miR-15a	Ras/ERK signaling	–	KCNQ1OT1 induces immune evasion and malignant phenotypes of PC by sponging miR-15a.	(89)
BLACAT1	42 PTNTs	DU145, LNCap, PC-3, and RWPE-1	miR-29a-3p and DVL3	miR-29a-3p/ DVL3 Axis	–	BLACAT1 facilitates the proliferation, migration and invasion of PCa cells.	(91)
FAM83H-AS1	8 normal prostate tissues and 20 PCa tissues	PCa cells	miR-15a	AR signaling and miR-15a/ CCNE2 Axis	–	FAM83H-AS1 plays an oncogenic role in PCa, and affects cell proliferation and migration.	(92)
RAMS11	42 PTNTs	RWPE-2, LNCap, PC3 and DU145	CBX4	–	Poorer OS and DFS	RAMS11 enhances the growth and metastasis of PCa cells.	(86)
AC245100.4	–	RWPE1, DU145, PC3, and 293T	miR-145-5p and RBBP5	AC245100.4/ miR-145-5p/ RBBP5 axis	–	AC245100.4/miR-145-5p/RBBP5 ceRNA network promotes PCa cells development.	(90)
Linc00662	PTNTs	WPMY-1, PC-3, and DU145	–	–	Lymph node metastasis and distant metastasis	Linc00662 affects PCa cells proliferation, migration, invasion, and apoptosis.	(93)
HOTAIRM1	–	PC3 and RWPE-1	Bad, Bax, Bid, and Bcl-2	Wnt pathway	–	HOTAIRM1 suppresses the progression of PCa.	(90)
LEF1-AS1	AIPC samples from 45 patients	AIPC cell lines PC3, DU145, and RWPE	miR-328	Wnt/β-catenin pathway	–	LEF1-AS1 enhances the proliferation, migration, and invasion of AIPC cells through its angiogenic activity.	(94)
PCAL7	104 PTNTs	LNCaP and VCaP cells	HIP1	AR signaling	–	PCAL7 acts as an oncogene in PCa.	(95)
LINC00852	Data from TCGA database	PC-3, VCaP and androgen-stimulated LNCaP cell lines	epithelial-mesenchymal transition-related proteins	EMT	–	Its upregulation promotes PC3 cells proliferation and colony formation abilities.	(96)

(Continued)

TABLE 1 Continued

lncRNA	Samples	Cell lines	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
AGAP2-AS1	50 PCa tissues and 20 BPH tissues	VCaP, 22Rv1, CRL-1740, CRL-2422, PC3M, and WPMY-1	miR-195-5p and PDLIM5	–	–	AGAP2-AS1 affects the proliferation, migration, and invasion.	(97)
LINC01006	–	RWPE-1, DU145, PC3, LNCAP, and VCaP	miR-34a-5p and DAAM1	LINC01006/ miR-34a-5p/ DAAM1 axis	–	LINC01006 serves as a ceRNA for miR-34a-5p, and up-regulate DAAM1 levels.	(92)
MCM3AP-AS1	64 PTNTs	PC-3, DU145, 22RV1, LNCaP, and WPMY-1	miR-543-3p	miR-543-3p/ SLC39A10/ PTEN axis	–	MCM3AP-AS1 induces PCa cells proliferation and invasion.	(98)
DLX6-AS1	20 PTNTs	WPMY1, LNCap, DU145, PC-3, and VCap	miR-497-5p and SNCG	miR-497-5p/ SNCG pathway	–	DLX6-AS1 exerts oncogenic role in PCa.	(99)
LINC00173	124 PTNTs	RWPE-1, DU145, PC-3, and LNCap	miR-338-3p	LINC00173/ MiR-338-3p/ Rab25 Axis	Reduced patient survivals	LINC00173 inhibits PCa cells proliferation, migration and invasion, and enhances apoptosis.	(100)
NNT-AS1	–	LNCaP clone FGC, VCaP, LNCaP C4-2B, PC3, and RWPE-1	miR-496 and DDIT4	NNT-AS1/miR-496/DDIT4 regulatory axis	–	NNT-AS1 acts as the sponge of miR-496 in PCa, and upregulates DDIT4 expression.	(101)
UCA1	40 PTNTs	RWPE1, 22RV1, and DU145	miR-331-3p and EIF4G1	UCA1/miR-331-3p/EIF4G1 axis	–	Its knockdown increases PCa cells radiosensitivity.	(100)
UCA1	86 PTNTs	DU145, PC-3, LNCaP, 22Rv1, and RWPE-1	miR-143 and MYO6	UCA1/miR-143/MYO6 axis	–	UCA1 plays an oncogenic role in prostate cancer.	(102)
IDH1-AS1	20 PTNTs	PC3, DU145, LNCaP, 22RV1, and WPMY-1	–	IDH1-AS1-IDH1 axis	–	IDH1-AS1 is a potential target for treatment of PCa.	(103)
CCAT2	18 PTNTs	PCa, PC3, DU145, and RWPE-1	TCF7L2 and microRNA-217	Wnt/β-catenin signaling pathway	–	CCAT2 sponges with miR-217 to regulate TCF7L2 levels.	(98)
AC245100.4	42 PTNTs	RWPE-1, DU145, PC3, 22RV1, and LNCaP	HSP90	NFκB signaling pathway	–	AC245100.4 is located in cytoplasm of PCa cells.	(97)
LINC00992	60 PTNTs	RWPE-1, PC3, LNCaP, DU145, and C4–2	miR-3935 and GOLM1	–	–	LINC00992 promotes the proliferation and migration of PCa cells, and inhibits apoptosis.	(92)
LINC00675	9 primary PCa tissues and 8 CRPC tissues	LNCaP-SF and LNCaP-JP human PCa cells	GATA2	LINC00675/ MDM2/ GATA2/AR signaling axis	–	Expression of LINC00675 was elevated in CRPC patients.	(104)
LINC01207	62 PTNTs	PC-3, DU145, and RWPE-1	miR-1972 and LASP1	LINC01207/ miR-1972/ LASP1 axis	–	LINC01207 serves as a tumor promoter in PCa.	(105)

(Continued)

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TABLE 1 Continued

lncRNA	Samples	Cell lines	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
MCM3AP-AS1	30 PTNTs	PrSC cell, C4-2, PC-3, LNCaP, DU145, and 22Rv1	WNT5A and miR-876-5p	MCM3AP-AS1/ miR-876-5p/ WNT5A axis	Poor prognosis	MCM3AP-AS1 partakes in PCa progression.	(94)
LINC00920	125 prostate tumor and 10 normal tissue samples	RWPE-1, LNCaP, VCaP, DU145, and PC-3	ERG and 14-3-3ϵ protein	FOXO signaling pathway	–	LINC00920 facilitates the interaction between14-3-3ϵ protein and FOXO1.	(106)
lncAMPC	32 primary PCa tissues from patients undergoing radical prostatectomy and 157 urine samples from patients with positive prostate biopsy	PC-3 and RM-1 prostate cells	LIF and miR-637	lncAMPC/LIF/ LIFR axis	–	lncAMPC enhances PCa cells proliferation, viability, migration, and invasion abilities.	(94)
LINC00689	80 PTNTs	RWPE1, DU145, LNCaP, PC-3 and C42B	miR-496 and CTNNB1	Wnt pathway	Short OS time	LINC00689 involves in progression of prostate cancer by increasing CTNNB1 levels.	(107)
LINC00473	–	DU145, LNCaP, PC-3, and P69	miR-195-5p and SEPT2	JAK-STAT3 signaling pathway and miR-195-5p/ SEPT2 axis	–	LINC00473 partakes in PCa cell proliferation through JAK-STAT3 signaling pathway.	(108)
FAM66C	Prostate carcinoma dataset of the TCGA	DU145, LNCaP, PC-3, PC-3M-IE8, and WPMY-1	–	EGFR-ERK signaling, proteasome and lysosome pathways	Shorter OS	Its upregulation induces cell growth in PCa cells.	(109)
OGFRP1	57 PTNTs	PC-3, DU-145, C4-2, VCAP, RWPE-1, and 293T	miR-124-3p and SARM1	–	TNM stages III and IV and perineural invasion	OGFRP1 sponges with miR-124-3p, and induces PCa cells growth.	(110)
TUG1	39 PTNTs	RWPE-1, PC-3, and DU145	miR-496	miR-496/Wnt/ β-catenin pathway	–	TUG1 sponges with miR-496, thus suppressing expression of miR-496.	(111)
TUG1	50 PTNTs	WPMY-1, LNCaP, 22RV1,PC-3, and DU145	miR-139-5p and SMC1A	TUG1/miR-139-5p/SMC1A axis	Lower survival rate and poor prognosis	TUG1 partakes in prostate cancer radio-sensitivity.	(92)
TUG1	–	RWPE1, PC-3, and DU145	Nrf2, HO-1, FTH1, and NQO1	Nrf2 signaling axis	–	TUG1 exerts oncogenic role in PCa cells.	(111)
TUG1	30 PTNTs	PC-3, DU145, and RWPE-1	miR-128-3p and YES1	miR-128-3p/ YES1 axis	Poor prognosis	TUG1 may serves as a potential target for treatment of prostate cancer patients.	(112)
SOX2-OT	27 PTNTs	NPrEC. LNCaP, and DU145	HMGB3 and miR-452-5p	miR-452-5p/ HMGB3 Axis and Wnt/β-Catenin Pathway	lymph metastasis, and TNM stages	SOX2-OT sponges with miR-452-5p, and modulates HMGB3 levels, and regulates the Wnt/b-catenin signaling pathway.	(105)
LINC00665	41 PTNTs	LNCaP, PC-3, DU-145, 22RV1, and RWPE-1	miR-1224-5p and SND1	miR-1224-5p/ SND1 pathway	Poor prognosis	Its knockdown inhibits the migration and invasion of PCa cells.	(113)

(Continued)

TABLE 1 Continued

lncRNA	Samples	Cell lines	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
ZEB1-AS1	30 PTNTs	RWPE-1, DU145, and LNCaP	miR-342-3p and CUL4B	PI3K/AKT/ mTOR signal pathway and miR-342-3p/ CUL4B axis	–	ZEB1-AS1 silencing represses PCa cells proliferation, migration, and invasion.	(110)
UNC5B-AS1	50 PTNTs	PC-3, DU-145, 22RV1, Lncap and WPMY-1	caspase-9	–	Distant metastasis and advanced pathological stage	UNC5B-AS1 regulates the expression of Caspase-9 in PCa tissues and cell lines.	(114)
CRNDE	64 PTNTs	PC3 and 22RV1	miR-101	miR-101/Rap1A axis	Poor outcomes	Increased CRNDE levels induces the proliferation, migration, and invasion of Pca cells.	(110)
ZFAS1	30 PTNTs	RWPE-1, PC3, DU145, 22RV1, and LNCAP	miR-135a-5p	–	–	ZFAS1 silencing suppresses PCa cell proliferation, invasion, and metastasis through modulating miR-135a-5p.	(115)
PRRT3-AS1	GSE55945 and GSE46602 datasets	DU145, LNCaP, PC3, IA8, IF11, and RWPE-1	PPARγ	mTOR signalling pathway	–	Its silencing suppresses the mTOR signaling pathway.	(116)
LINC00673	48 PTNTs	PC3, LNCap, DU145, paclitaxel-resistant cell line (DU145/ pr), and RWPE-1	KLF4	–	TNM stage and LNM	LINC00673 modulates KLF4.	(117)
VPS9D1-AS1	PRAD tissues from TCGA database	RWPE-1, DU145, VCaP, PC-3, and LNCaP	miR-4739, ZEB1 and MEF2D	miR-4739/ MEF2D axis	–	VPS9D1-AS1 enhances the proliferation, migration, and invasion.	(116)
NCK1-AS1	Blood samples from 60 patients with PCa, 58 patients with BPH, and 60 healthy males	DU145, 22Rv1, and RWPE-1	TGF-β1	TGF-β pathway	–	Expression of NCK1-AS1 was elevated in plasma of PC patients in comparison to patients with BPH and healthy controls.	(118)
VIM-AS1	88 PCa and 31 normal prostate tissue samples	RWPE-1, LNCaP, DU145, 22RV1, and PC3	vimentin	EMT	Large tumor size, metastasis and advanced TNM stage	Expression of VIM-AS1 affects the migration and invasion of PCa cells.	(119)
MALAT1	10 pairs of PCa tissues and ANTs	DU145 and 22RV1	METTL3	PI3K/AKT signaling pathway	Tumor recurrence	Elevated level of MALAT1 results in tumor recurrence in PCa patients.	(120)
MAFG-AS1	495 PCa tissues and 50 ANTs	PC-3 and DU145	ribosome-related genes	ribosome and DNA replication pathways	Poor prognosis	MAFG-AS1 silencing suppresses the proliferation, migration, and invasion of PCa CELLS.	(121)
lncRNA AC008972.1	PCa tissues	PC3 and LNCaP	miR-143-3p	lncRNA AC008972.1/ miR-143-3p/ TAOK2 axis	Low OS	AC008972.1 plays an oncogenic role in the progression of PCa and may serve as a possible therapeutic target in case of PCa.	(122)

BPH, benign prostate hyperplasia; PCa, prostate cancer; PTNTs, paired tumor-non-tumor tissues; HSPC, hormone-sensitive prostate cancer; CRPC, castration-resistant prostate cancer.

assessed lncRNAs in this field. A number of well-known oncogenic lncRNAs in other cancers such as DANCR, MALAT1, CCAT1, PVT1, TUG1 and NEAT1 have also been shown to act as oncogenes in prostate cancer. For instance, DANCR has been found to contribute to the taxol resistance of in this type of cancer *via* modulation of miR-33b-5p/LDHA axis (44). Expression of this lncRNA has been up-regulated in serum samples of prostate cancer patients, parallel with down-regulation of miR-214-5p. Notably,

DANCR expression has been correlated with PSA level, Gleason score and T stage in these patients. DANCR expression not only can be used for prostate cancer diagnosis, but also can predict poor prognosis of this type of cancer with high diagnostic value. Mechanistically, up-regulation of DANCR or down-regulation of miR-214-5p could enhance proliferation and migration, preclude apoptosis, and induce activity of TGF-β signaling (45). DANCR can also target miR-185-5p to increase expression of LIM and SH3 protein 1 promoting prostate cancer through the FAK/PI3K/AKT/GSK3β/snail axis (46).

In addition, MALAT1 has been found to regulate glucose metabolism through modulation of MYBL2/mTOR axis (47). Moreover, *in vitro* and *in vivo* studies have shown the importance of MALAT1/miR-140/BIRC6 axis in the progression of prostate cancer (48). In fact, MALAT1 acts as a molecular sponge for miR-140 to enhance expression of the anti-apoptotic protein BIRC6 (48). In turn, expression and activity of MALAT1 have been shown to be regulated by miR-423-5p, a miRNA that impedes activity of MALAT1 in enhancement of proliferation, migration, and invasiveness of prostate cancer cells (49). Most importantly, up-regulation of miR-423-5p could enhance survival and decrease metastasis formation in a xenograft model of prostate cancer (49). In addition, MALAT1 has a possible diagnostic value in prostate cancer. Expression levels of PCA3 and MALAT1 in urinary exosomes have been shown to be superior to the currently used clinical parameters in detection of prostate cancer, particularly high-grade ones (51).

NEAT1 has also been shown to regulate aerobic glycolysis to affect tumor immunosurveillance by T cells in this type of cancer (13). It can also promote progression of prostate cancer through modulation of miR-766-5p/E2F3 axis (54).

CTBP1-AS is reported as the antisense-RNA transcript positively regulated by androgen and promotes castration-resistant prostate cancer tumor growth (123). This lncRNA is localized in the nucleus and its levels are mostly increased in prostate cancer. It enhances both hormone-dependent and castration-resistant tumor growth. From a mechanistical point of view, CTBP1-AS suppresses the expression of CTBP1 through recruitment of PSF and histone deacetylases. It also exerts androgen-dependent function through inhibition of tumor-suppressor genes and enhancement of cell cycle progression (123).

Epigenetic repression of AR corepressor is an important mechanism for AR activation. ARLNC1 is also regulated by androgen and upregulates AR mRNA stability by binding to the 3'-UTR. In line with this, ARLNC1 silencing leads to inhibition of AR expression and suppression of AR signaling as well as of growth of prostate cancer. In fact, ARLNC1 has a role in the preservation of a positive feedback loop that induces AR signaling in the course of prostate cancer progression (124). In addition to these lncRNAs, several CRPC-specific AR-regulated lncRNAs are important for overexpression of AR and its variant. These AR-regulated lncRNAs are over-expressed in CRPC tissues. An experiment in these cells has shown that knock-down of PRKAG2-AS1 and HOXC-AS1 leads to suppression of CRPC tumor growth in addition to inhibition of expression of AR and AR variant. Mechanistically, PRKAG2-AS1 modulates the subcellular localization of the splicing factor, U2AF2. This splicing factor is involved in the AR splicing system (125).

SChLAP1 is another up-regulated lncRNA in prostate cancer whose up-regulation is associated with poor patient outcomes, such as metastases and prostate cancer specific mortality. It has a critical role in invasiveness and metastasis. Functionally, SChLAP1 influences the localization and regulatory function of the SWI/SNF complex (126).

PCAT-1 is another up-regulated lncRNA in prostate cancer which enhances cell proliferation through cMyc. Mechanistically, PCAT-1–associated proliferation depends on stabilization of cMyc protein. Moreover, cMyc has an essential role in a number of PCAT-1–induced expression alterations (127).

HOTAIR as regarded as an AR-repressed lncRNA is upregulated after androgen deprivation therapy and in CRPC. Mechanistically, HOTAIR binds to the AR protein to inhibit its interactions with the E3 ubiquitin ligase MDM2, thus suppressing AR ubiquitination and its degradation. Therefore, HOTAIR induces androgen-independent AR activation and drives the AR-mediated transcriptional program in the absence of androgen (128). Another study has shown that NEAT1 induces oncogenic growth in prostate tissue through changing the epigenetic marks in the target genes promoters to induce their transcription (129). Moreover, PCGEM1 and PRNCR1 bind to AR and enhance selective looping of AR-bound enhancers to target gene promoters (130). Similarly, SOCS2-AS1 interacts with AR for co-factor interaction (131).

The importance of other up-regulated lncRNAs in prostate cancer is summarized in Figure 1 and Table 1.

Down-regulated lncRNAs in prostate cancer

A number of other lncRNAs have been found to act as tumor suppressors in prostate cancer (Table 2). For instance, LINC00893 can inhibit progression of this type of cancer *via* modulation of miR-3173-5p/SOCS3/JAK2/STAT3 axis (132). Similarly, the sponging effect of LINC01679 on miR-3150a-3p has a role in inhibition of progression of prostate cancer through affecting expression of SLC17A9 (133). MIR22HG is another tumor suppressor lncRNA that acts as a molecular sponge for miR-9-3p (134). The tumor suppressor role of RP1-59D14.5 in prostate cancer is mediated through activation of the Hippo signaling and enhancement of autophagy (135). Moreover, MAGI2-AS3 has been shown to inactivate STAT3 signaling and suppress proliferation of prostate cancer cells through acting as a miR-424-5p sponge (136). NXTAR is another tumor suppressor lncRNA that modulates expression of androgen receptor (AR) and resistance to enzalutamide (137). Totally, the number of identified tumor suppressor lncRNAs in prostate cancer is far below that of oncogenic lncRNAs (Figure 2). Table 2 summarizes the information about tumor suppressor lncRNAs in prostate cancer.

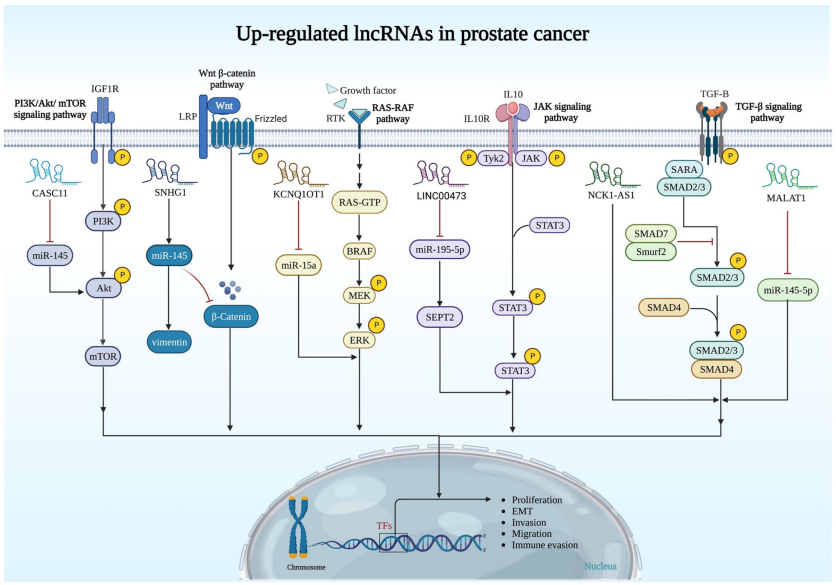

FIGURE 1
Upregulation of oncogenic lncRNAs and their relation with signaling pathways in prostate cancer. PI3K/AKT/mTOR, Wnt/β-catenin, RAS/RAF, JAK and TGF-β pathways are regulated by oncogenic lncRNAs in prostate cancer.

TABLE 2 Summary of function of down-regulated lncRNAs in prostate cancer (Official HUGO Gene Nomenclature symbols are used).

lncRNA	Samples	Cell line	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
LINC00893	66 PTNTs	PC-3, DU145, VCaP, LNCaP, and RWPE-1	miR-3173-5p	miR-3173-5p/SOCS3/JAK2/STAT3 axis	Poorer overall survival rate	LINC00893 is a tumor-suppressor in PCa.	(132)
LINC01679	55 PTNTs	RWPE-2, DU145, PC-3, LNCaP, C4-2B, and 22RV1	miR-3150a-3p	miR-3150a-3p/SLC17A9 axis	Poor survival	LINC01679 serves as a molecular sponge for miR-3150a-3p in prostate cancer.	(133)
MIR22HG	–	RWPE-2, 22Rv1, DU145, LNCaP, and PC3	miR-9-3p	MIR22HG/miR-9-3p axis	–	MIR22HG reduces cell proliferation and enhances apoptosis in DU145 cells.	(134)
RP1-59D14.5	–	LNCaP, PC3, DU145, and RWPE-1	miR-147a/ LATS1/2 axis	Hippo signaling pathway	–	RP1-59D14.5 acts as a ceRNA for miR-147a, and regulates large tumor suppressor kinase 1/2.	(135)
MAGI2-AS3	109 PTNTs	WPMY-1, PC-3 and DU145	miR-424-5p and COP1	STAT signaling	–	Elevated expression of MAGI2-AS3 suppresses PCa cell proliferation.	(136)
NXTAR	PTNTs	RWPE-1, 22Rv1, LNCaP, VCaP, PC3,	–	ACK1/AR signaling	–	NXTAR expression was lower in various AR-positive PCa cell lines in	(137)

(Continued)

TABLE 2 Continued

lncRNA	Samples	Cell line	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
		LAPC4, and C4-2B				comparison to normal prostate cells.	
FGF14-AS2	Gene expression profiles of PC patients from TCGA database	RWPE-1, DU145, PC-3, PC-3 M, and LNCaP	miR-96-5p	iR-96-5p/AJAP1 axis	–	lncRNA FGF14-AS2 affects proliferation and metastasis of PCa cells by regulating iR-96-5p/AJAP1 axis.	(138)
ADAMTS9-AS1	68 PTNTs	PC3, DU145 and Normal human prostate epithelial cells	miR-142-5p	miR-142-5p/CCND1 axis	TNM stage and perineural invasion	ADAMTS9-AS1 suppresses the progression of PCa by affecting the miR-142-5p/CCND1 axis.	(139)
MBNL1-AS1	Tissues of prostate adenocarcinoma (PARD) and normal tissues	LAPC4, LNCaP, DU145, C4-2B, and RWPE-1	miR-181a-5p	PTEN/PI3K/AKT/mTOR pathway	–	MBNL1-AS1 regulates PTEN by sequestering miR-181a-5p.	(140)
LINC00641	23 PTNTs	PC-3, C42B, LNCaP, and RWPE-1	VGLL4 and miR-365a-3p	miR-365a-3p/VGLL4 axis	Lower survival rate	LINC00641 is a tumor suppressor lncRNA in PCa, and modulates miR-365a-3p/VGLL4 axis.	(141)
PGM5-AS1	PCa-related microarray datasets (GSE3325 and GSE30994)	PC-3, LNCap, 22RV1, DU145, and RWPE-1	miR-587, GDF10	PGM5-AS1/miR-587/GDF10 axis	–	PGM5-AS1 acts as a ceRNA for miR-587, and upregulates GDF10 levels.	(142)
GAS5	51 PTNTs	DU145, LNCaP, and WPMY-1	miR-320a and RAB21	miR-320a/RAB21 axis	–	Its upregulation inhibits viability and migration of PCa cells.	(143)
GAS5	–	–	–	GAS5/miR-18a-5p/serine/threonine kinase 4	–	GAS5 functions as a tumor suppressor, and inhibits the metastasis and proliferation of paclitaxel-resistant PCa cells	(121)
LINC00261	83 PTNTs	LNCap, PC-3, DU145, 22Rv1, ARCaP, and RWPE-1	DKK3 and GATA6	LINC00261/GATA6/DKK3 axis	–	LINC00261 modulates DKK3.	(144)
EMX2OS	25 PTNTs	LNCaP, DU145, PC3, RWPE-1 and HEK293A	FUS and TCF12	cGMP-PKG pathway	–	EMX2OS suppresses tumor growth *in vivo*.	(145)
LINC00844	62 PTNTs	22Rv1, VCaP, LNCaP, Du145, PC-3, and RWPE-1	GSTP1 and EBF1	LINC00844/EBF1/GSTP1 axis	–	LINC00844 may serve as a potential target for PCa treatment.	(146)
Erbb4-IR	60 PTNTs	22Rv1 and DU145	miR-21	–	Poor survival	Erbb4-IR mediates the proliferation and apoptosis of PCa cells through miR-21.	(147)

(Continued)

TABLE 2 Continued

lncRNA	Samples	Cell line	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	Ref
MIR22HG	9 normal and 13 prostate tumor sample	LNCaP, WPMY-1, PC-3 and C4-2B	–	TNF, Cytokine-cytokine receptor interaction, MAPK, NF-κB, Jak-STAT, p53, NOD-like receptor signaling, Toll-like receptor, Cytosolic DNA-sensing, and PI3K-Akt	T stage	MIR22HG may acts as a potential biomarker in case of prostate cancer diagnosis.	(148)
FER1L4	78 PTNTs	PC-3, LNCaP, DU145, and RWPE-1	FBXW7 and miR-92a-3p	ER1L4/miR-92a-3p/FBXW7 axis and key signaling pathway	–	FER1L4 inhibits cell proliferation and promotes cell apoptosis by increasing expression of FBXW7 in PCa cells.	(145)
BLACAT1	25 PTNTs	PC3, DU145, and RWPE-1	DNMT1, HDAC1, EZH2, MDM2 and miR-361	–	–	Its silencing reduces the growth of PCa cells, and induces cell death.	(102)
LINC00908	55 PTNTs	VCaP, LNCaP, DU-145, PC-3, and RWPE-1	miR-483-5p and TSPYL5	LINC00908/miR-483-5p/TSPYL5 axis	–	LINC00908 sponges with miR-483-5p and suppresses PCa progression.	(149)
DGCR5	64 PTNTs	22Rv1 and DU145	TGF-β1	–	Poor survival	High expression of DGCR5 reduces PCa cells stemness.	(150)
MAGI2-AS3	PCa serum samples	LNCaP and PC3 cells	miR-142-3p	–	–	High level of MAGI2-AS3 inhibits proliferation, migration, and invasion of PCa cells.	(151)

PCa, prostate cancer; PTNTs, paired tumor-non-tumor tissues.

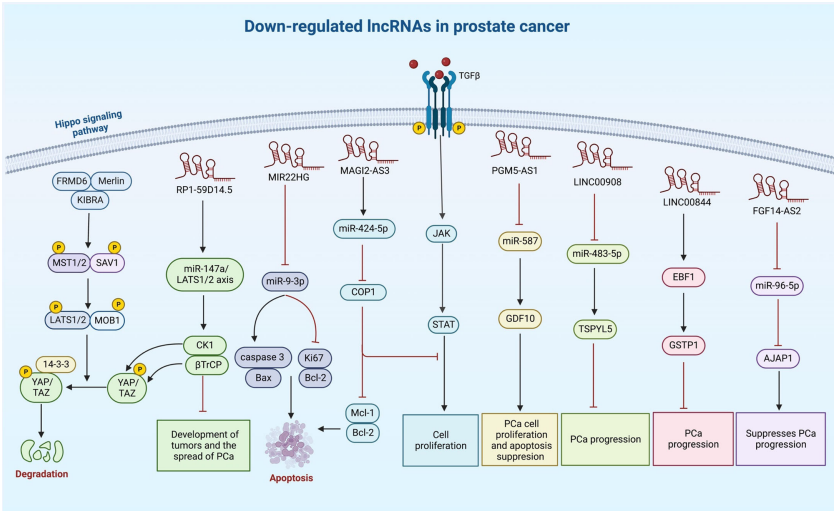

FIGURE 2
A synopsis of the known roles of lncRNA tumor suppressors in prostate cancer. Several lncRNAs can reduce cell proliferation and invasiveness of prostate cancer cells, particularly through sponging oncogenic miRNAs.

Contribution of lncRNAs variants in prostate cancer

Contribution of single nucleotide polymorphisms (SNPs) within *GAS5*, *POLR2E*, *MEG3*, *MALAT1* and *HOTAIR* in the risk of prostate cancer has been assessed in different ethnic groups (Table 3). Three SNPs within *GAS5* have been the subject of these investigations. First, rs145204276 (delCAAGG) is located within the promoter region of *GAS5*. Compared with subjects carrying ins/ins genotype, cases with ins/del or del/del genotype of this polymorphism have shown decreased risk of pathological lymph node metastasis (152). The rs17359906 in *GAS5* is another SNP whose A allele has been shown to be a risk allele for prostate cancer. Similarly, A allele of rs1951625 SNP within *GAS5* has been associated with higher risk of this cancer. Both rs17359906 G > A and rs1951625 G > A have been associated with high plasma level of PSA. Most importantly, the recurrence-free survival of patients with prostate cancer has been lowest in patients having AA genotype of rs17359906 and highest in those having GG genotype. Similar findings have been reported for the rs1951625 (153).

A systematic review and meta-analysis of 5 studies on the role of rs3787016 within *POLR2E* has revealed increased susceptibility to prostate cancer for carriers of T allele in all genotype models (154). The results of other studies on contribution of lncRNAs SNPs in prostate cancer are shown in Table 3.

Importance of lncRNAs as prognostic factors in prostate cancer

Several studies have indicated the importance of dysregulation of lncRNAs in the prediction of survival times of patients with prostate cancer (Table 4). Overall, up-regulation of oncogenic lncRNAs is predictive of lower survival time of patients in terms of overall survival or progression-free survival. For tumor suppressor lncRNAs, an opposite effect has been seen.

Discussion

Several lncRNAs have been shown to contribute to the pathogenesis of prostate cancer *via* modulation of AR signaling, ubiquitin–proteasome degradation process of AR or other important signaling pathways. Some of them such as PCA3 are highly specific for this kind of cancer, representing an appropriate biomarker for prostate cancer (151). Others might be over-/under-expressed in a variatey of cancers, being therapeutic targets for a wide range of human malignnacies. The observed differences in expression of some lncRNAs between castration-resistant prostate cancer and androgen deprivation therapy-responsive cases imply the importance of these transcripts in defining response of patients to this therapeutic modality and represent these transcripts as targets for management of resistance to this therapy.

TABLE 3 Contribution of lncRNAs SNPs in prostate cancer.

Gene	Polymorphism	Samples	Population	Association	Ref
GAS5	rs145204276	Blood samples from 579 PCa patients and 579 healthy controls	Taiwan	Compared with subjects carrying ins/ins genotype, cases with ins/del or del/del genotype of this polymorphism demonstrate decreased risk of pathological lymph node metastasis.	(152)
GAS5	rs17359906 G > A	Blood samples from 218 PCa patients and 220 healthy controls	Chinese Han	The mentioned SNP is correlated with increased plasma PSA levels.	(153)
	rs1951625 G > A			Subjects who carry the A allele of this polymorphism show a significantly higher risk of PCa compared to those who carry the G allele.	
POLR2E	rs3787016	5 eligible case-control studies including 5472 cases and 6145 controls	–	Genotypes carrying the T allele of the mentioned polymorphism show an increased risk for PCa.	(154)
MEG3	rs11627993 C>T	Blood samples from 65 prostate cancer patients and 200 healthy subjects	Chinese Han	No statistically significant results.	(155)
	rs7158663 A>G				
MALAT1	rs619586	Blood samples from 579 patients with prostate cancer	Taiwan	Cases with G allele of this polymorphism have an elevated risk of being in an advanced Gleason grade group.	(156)
	rs3200401			No statistically significant results.	
	rs1194338			Subjects who carry at least one polymorphic A allele of the mentioned SNP are positively associated with node-positive PCa.	
HOTAIR	rs12826786	Peripheral blood samples of 128 PCa patients, 143 BPH patients and 250 normal males	Iranian	Mentioned polymorphism is associated with PCa risk in co-dominant and recessive models.	(157)
	rs1899663			T allele of this SNP is associated with BPH risk.	
	rs4759314			No statistically significant results.	

TABLE 4 Importance of lncRNAs as prognostic factors in prostate cancer (PTNTs, paired tumor-non-tumor tissues; PCa, prostate cancer; OS, overall survival; PFS, progression-free survival).

lncRNA	Sample number	Kaplan-Meier analysis	Univariate cox regression	Multivariate cox regression	Ref
UBE2R2-AS1	74 PTNTs	Its high expression is associated with poorer survival rate.	–	Gleason score and expression of UBE2R2-AS1 are independent prognostic factors for OS of PC patients.	(13)
SNHG17	52 PTNTs	Its high expression is associated with poor BCR-free survival.	Over expression of SNHG17 is associated with poor OS in PC patients.	Its expression is an independent prognostic factor for OS in patients with PC.	(14)
LINC00893	66 PTNTs	Its low expression is correlated with poorer OS.	–	–	(132)
LINC01679	55 PTNTs	Its low expression is correlated with reduction in DFS.	–	–	(133)
SNHG3	30 PTNTs	Its high expression is associated with shorter OS time.	–	–	(32)
lncHUPC1	70 PTNTs	High lncHUPC1 expression is correlated with poor PFS.	–	–	(41)
MNX1-AS1	40 PTNTs	Its high expression is correlated with worse OS rates.	–	–	(42)
NEAT1	50 PTNTs	Its high expression is associated with lower survival rate.	–	–	(54)
SNHG3	50 PTNTs	Its upregulation is associated with shorter OS and BMFS.		Its high expression is an independent risk factor for death and progression in patients with PCa.	(32)
DLEU2	Prostate tumor tissues from TCGA database	Its high expression is correlated with lower survival rate.	Its upregulation is associated with a poor progression-free interval.	Its upregulation is independently associated with a poor progression-free interval.	(61)
HOXD-AS1	36 PCa and 9 BPH cases	Its high expression is associated with shorter PSA.	Serum exosomal HOXD-AS1 in conjunction with tumor stage is a prognostic factor for PRFS.	Serum exosomal HOXD-AS1 is an independent prognostic factor for PFS	(65)
SNHG10	gene expression profiles of PCa patients from TCGA database	Its high expression is associated with poor PFS of PC patients.	Elevated expression of SNHG10, T stage, N stage, Gleason score, primary therapy outcome, residual tumor, and PSA were associated with PFS in patients with PCa.	SNHG10 is an independent prognostic factor for PFS in PC patients	(26)
PCBP1-AS1	4 BPH patients, 28 HSPC patients, and 12 CRPC patients	Its high expression indicates a poor prognosis for PCa patients.	–	–	(62)
LOC100996425	110 PTNTs	Its elevated expression is associated with a lower OS rate of PCa patients.	–	–	(72)
OGFRP1	70 docetaxel-sensitive and 72 docetaxel-resistant PCa tissues	Its higher expression in docetaxel-resistant patients is associated with poorer OS relative to the docetaxel-sensitive patients.	–	–	(74)
DANCR	53 PTNTs	Its high expression is associated with lower OS in PCa patients.	Its expression might be prognostic indicators of PC patients.	DANCR is an independent prognostic indicator for PCa.	(45)
SNHG17	53 PTNTs	Its high expression is associated with poor OS time.	–	–	(16)
PVT1	RNA-Seq data from TCGA-PRAD database	Its high expression is associated with poor vital survival rates.	Its expression is associated with OS and relapse-free survival.	Its high expression is an independent prognostic factor	(74)

(Continued)

TABLE 4 Continued

lncRNA	Sample number	Kaplan-Meier analysis	Univariate cox regression	Multivariate cox regression	Ref
				for poor OS and poor relapse-free survival in PCa.	
NORAD	74 PTNTs	Its high expression is positively associated with OS of patients with PCa.	–	–	(87)
ADAMTS9-AS1	68 PTNTs	Its low expression is associated with TNM stage and perineural invasion.	–	–	(139)
RAMS11	42 PTNTs	Its upregulation is correlated with poorer OS and DFS.	–	–	(86)
SNHG9	495 PCa tissues and 52 adjacent prostate tissues	Its high expression is associated with poor prognosis.	Its expression level is associated with poorer PFS.	Its expression is independently associated with PFS in PCa patients.	(27)
LINC00641	23 PTNTs	Its low expression is associated with lower survival rate.	–	–	(141)

Although numerous prostate cancer-specific or prostate cancer-associated lncRNAs have been recognized, few lncRNAs have been verified in independent patient cohorts or approved for using in clinical settings. The most important milestone in the field of lncRNA research is probably approval of urinary PCA3 as a biomarker for detection of prostate cancer by the United States Food and Drug Administration (158). This lncRNA is a promising factor for urine test for prostate cancer and has a superior performance compared with PSA in urinary detection of this disorder. Further reseraches are needed to find other appropriate lncRNA biomarkers for this kind of cancer.

LncRNA profiles can also been used to identify prostae cancer patients that benefit from radiotherapy. For instance, UCA1 has beens shwon to mediate radiosensitivity in prostate cancer cell lines and therefore might be a marker to predict response to radiotherapy in these patients. This lncRNA affects radiosensitivity through influencing cell cycle progression (159).

The importance of lncRNAs in the mediation of cell proliferation, invasiveness and metastasis has potentiated them as therapeutic targets for prostate cancer. The results of animal studies have been promising particularly for some AR-regulated lncRNAs. However, clinical studies are missing in this field.

Notably, LncRNAs are also involved in drug resistance in prostate cancer cells, thus they are proper candidates for therapeutic targeting (160). For instance, HORAS5 up-regulation can trigger taxane resistance in CRPC cells through upregulation of BCL2A1. HORAS5 silencing can reduce resistance of prostate cancer cells to cabazitaxel and enhance the efficacy of chemotherapy (161).

PI3K/AKT/mTOR, Wnt/β-catenin, TGF-β, p53, FAK/PI3K/AKT/GSK3β/Snail, STAT3, FAK/AKT/β catenin, Ras/ERK, NF-κB and FOXO signaling pathways are among signaling pathways that are modulated by lncRNAs in the context of prostate cancer. Moreover, several lncRNAs have been shown to act as molecular sponges for miRNAs to regulated expression of miRNA targets. miR-145/IGF1R, miR-23a/OTUB1, miR-339-5p/STAT5A/SNORA71B, miR-144/CD51, miR-5590-3p/YY1, miR-195/CCNE1, miR-184/IGF, miR-152-3p/SLC7A11, miR-214-3p/TGF-β, miR-577/SMURF1, miR-377-3p/AKT2, miR-133b/SDCCAG3, miR-2113/MDM2, miR-16-5p/HMGA2, miR-140/BIRC6 axis, miR-145-5p-SMAD3/TGFBR2, miR-129-5p/CDT1 axis, miR-766-5p/E2F3, miR-1182/AKT3, miR-582-5p/SGK1, miR-361-5p/FOXM1, miR-24-3p/JPT1, miR-509-3p/PBX3, miR-370-3p/DDX3X, miR-212-5p/FZD5, miR-3167/YWHAZ, miR-490-3p/FRAT1, miR-24-3p/FSCN1, miR-149-5p/IL-6, miR-1245b-5p/CASK, miR-628-5p/FOXP2, miR-326/Hnrnpa2b1, miR-195-5p/FKBP1A, miR-15b/IGF1R, miR-494-3p/STAT3, miR-486-5p/GOLPH3, miR-15a-5p/KIF23 and miR-101/Rap1A are among putative miRNA/mRNA axes that are modulated by oncogenic lncRNAs in the context of prostate cancer.

Although expression profile of lncRNAs have been comprhensively assessed in tumoral tissues of patients with prostate cancer, less effort has been made for analysis of their expression in urine or serum samples. Based on the availability of these sources for non-invasive diagnostic procedures, future studies should focus on these biofluids to facilitate early detection of prostate cancer *via* non-invasive methods.

Taken together, lncRNAs have been found to contribute to the pathogenesis of prostate cancer through various mechanisms. These transcripts can be used as targets for therapeutic interventions in this kind of cancer.

Author contributions

MT and AB designed and supervised the study. SG-F wrote the draft and revised it. EB, BH, and AK collected the data and designed the figures and tables. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Identification and validation of immune-related hub genes based on machine learning in prostate cancer and AOX1 is an oxidative stress-related biomarker

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To investigate potential diagnostic and prognostic biomarkers associated with prostate cancer (PCa), we obtained gene expression data from six datasets in the Gene Expression Omnibus (GEO) database. The datasets included 127 PCa cases and 52 normal controls. We filtered for differentially expressed genes (DEGs) and identified candidate PCa biomarkers using a least absolute shrinkage and selector operation (LASSO) regression model and support vector machine recursive feature elimination (SVM-RFE) analyses. A difference analysis was conducted on these genes in the test group. The discriminating ability of the train group was determined using the area under the receiver operating characteristic curve (AUC) value, with hub genes defined as those having an AUC greater than 85%. The expression levels and diagnostic utility of the biomarkers in PCa were further confirmed in the GSE69223 and GSE71016 datasets. Finally, the invasion of cells per sample was assessed using the CIBERSORT algorithm and the ESTIMATE technique. The possible prostate cancer (PCa) diagnostic biomarkers AOX1, APOC1, ARMCX1, FLRT3, GSTM2, and HPN were identified and validated using the GSE69223 and GSE71016 datasets. Among these biomarkers, AOX1 was found to be associated with oxidative stress and could potentially serve as a prognostic biomarker. Experimental validations showed that AOX1 expression was low in PCa cell lines. Overexpression of AOX1 significantly reduced the proliferation and migration of PCa cells, suggesting that the anti-tumor effect of AOX1 may be attributed to its impact on oxidative stress. Our study employed a comprehensive approach to identify PCa biomarkers and investigate the role of cell infiltration in PCa.

KEYWORDS

biomarker, prostate cancer, AOX1, CIBERSORT, diagnostic

1 Introduction

Prostate cancer (PCa) is a significant global health concern, with 1.6 million cases and 366,000 deaths occurring worldwide each year. It ranks as the second most common cancer globally and the fifth leading cause of male mortality (1). Due to its biological characteristics, distant micrometastases, and localized residuals, PCa has an increasing likelihood of recurrence. However, there are certain curative therapies available, such as radical prostatectomy (RP). When utilized early during recurrence, salvage therapy can effectively decrease the risk of distant metastases, prolong lifespan, and potentially lead to a cure. Therefore, the early detection of PCa plays a vital role in improving prognosis and reducing patient mortality (2).

Historically, PCa has been diagnosed using a blood test for prostate-specific antigen (PSA), a digital rectal examination (DRE), and a prostate biopsy. However, PSA lacks specificity, which leads to the over-diagnosis and overtreatment of PCa. As a result, there is a growing clinical need for the identification of new biomarkers that can serve as prognostic, predictive, and therapeutic response indicators. These biomarkers can be utilized to implement a precision medicine strategy for the management of PCa (3). For instance, studies have demonstrated that the deletion of phosphatase and tensin homolog (PTEN) is associated with a poor prognosis in PCa patients (4). The loss of PTEN in biopsy samples has been shown to predict an increased risk of castration-resistant prostate cancer (CRPC), metastasis, and PCa-specific mortality (5, 6). To analyze the molecular processes and genomic effects of the co-deletion of BRCA2 and RB1 in PCa, previous research has shown that the deletion of BRCA2 leads to a castration-resistant phenotype in human PCa cell lines (LNCaP and lapc4) (7). This suggests that it is possible to investigate the molecular pathways involved in the progression of PCa and explore new diagnostic approaches for this disease. However, to date, no study has combined the least absolute shrinkage and selector operation (LASSO) regression model with support vector machine recursive feature elimination (SVM-RFE) to identify PCa biomarkers.

In recent years, immunology research has shown that immune cell infiltration plays a crucial role in the development and progression of PCa. For instance, Flammiger et al. conducted a study on prostate cancer specimens and used forkhead box P3 (FOXP3) immunohistochemistry to detect regulatory T cells (Tregs). They found that the increase in Tregs was associated with an advanced and worsening prognosis in prostate cancer tissues (8). Additionally, Eastham et al. compared normal patients with PCa patients and observed that the level of transforming growth factor-beta (TGF-β) was higher in PCa patients. This increase in TGF-β promoted both migration and invasion of PCa cells (9). Tissue microarray analysis confirmed decreased levels of FOXA1 protein and increased TGF-β signaling pathway in castration-resistant prostate cancer (CRPC) compared to primary tumors, which suppresses CRPC progression (10). Furthermore, some studies have demonstrated that tyrosine hydroxylase 2 (Th2) and central memory T cell (TCM) are associated with prostate cancer recurrence after radical prostatectomy (RP) and act as independent protective factors (11). However, so far, only a limited number of studies have utilized the CIBERSORT technique to investigate the infiltration of immune cells and potential biomarkers in prostate cancer.

We obtained six publicly available datasets on PCa from the Gene Expression Omnibus (GEO) database. To create a metadata cohort, we combined four of these datasets and used them as the training group. The remaining two datasets were merged into another metadata cohort, which served as the treatment group. Within the training group, we compared 127 PCa cases with 52 normal controls to identify differentially expressed genes (DEGs). Machine-learning techniques were then employed to screen and identify diagnostic biomarkers for PCa. These candidate genes were subsequently validated in the treatment group. Additionally, the CIBERSORT methods were used to examine the correlation between biomarkers and immune cells infiltrating PCa. This analysis aimed to enhance our understanding of the molecular immunological processes involved in PCa and establish a practical and conceptual framework for future research.

2 Methods and materials

2.1 Gene expression data acquisition and processing

We utilized the GEO database to gather information on PCa. Specifically, we downloaded raw data from the GSE8511, GSE14206, GSE46602, GSE55945, GSE69223, and GSE71016 datasets. These datasets were then divided into two groups: the training group (consisting of GSE8511, GSE14206, GSE46602, and GSE55945) with 127 PCa cases and 52 normal controls, and the test group (consisting of GSE69223 and GSE71016) with 63 PCa cases and 62 normal controls. To ensure consistency and eliminate any potential biases, we merged the datasets within each group and applied preprocessing techniques, including the use of the 'SVA' package's combat capabilities to remove any batch effects (12) (Table 1).

2.2 Identification of DEGs in PCa

The R package 'limma' from http://www.bioconductor.org/ was used to detect differentially expressed genes (DEGs) between 127 PCa patients and 52 normal controls in the training group (13). DEGs were identified based on a threshold of |log fold change (FC)| > 1 and an adjusted false discovery rate ($P < 0.05$). The comparison was made between 127 PCa cases and 52 normal controls in the training group. The volcano plot was generated using the R software package 'ggplot2' to visualize the DEGs. Additionally, a heat map of the DEGs was created using the R package 'heatmap'.

TABLE 1 Information for selected GEO datasets.

GEO accession	country	Platform	Samples		Category
			PCa	Normal	
GSE8511	USA	GPL1708	24	16	Train group
GSE14206	Italy	GPL887	53	14	Train group
GSE46602	Denmark	GPL570	36	14	Train group
GSE55945	USA	GPL570	13	8	Train group
GSE69223	Germany	GPL570	15	15	Test group
GSE71016	USA	GPL16699	48	47	Test group

2.3 Functional enrichment analysis

Gene module-related functions were identified through functional enrichment studies conducted using the R package 'cluster profile'. These studies utilized the gene ontology (GO), the disease ontology (DO) ontologies, and the Kyoto encyclopedia of genes and genomes (KEGG) (14). To perform gene set enrichment analysis (GSEA) on the training group, we examined signal pathway differences. GSEA analysis was conducted on the gene expression matrix using the 'cluster Profiler' and 'enrich plot' programs, with the reference gene set as 'c2.cp.kegg.v7.4.symbols.gmt' (15). KEGG GSEA analysis was separately performed on the PCa and normal cases of the training group. Significant saturation was defined as $P < 0.05$.

2.4 Screening characteristic related biomarkers *via* machine learning

Two machine learning methods were utilized to evaluate potential prognostic factors in prostate cancer (PCa). The LASSO technique, implemented with the R package 'glmnet', was employed to identify genes that were significantly associated with the differentiation between PCa and normal patients (16). Additionally, the support vector machine (SVM) was utilized as a surveillance machine learning technique to identify the optimal variables by eliminating feature vectors. To mitigate overfitting, a recursive feature elimination (RFE) approach was used to select the best genes. Therefore, SVM-RFE was employed to determine the gene set with the highest discriminatory power. To conduct classification analysis on the selected biomarkers for PCa diagnosis, we utilized the SVM-RFE classifier from the R package 'e1071' (17). Subsequently, a Venn diagram was employed to identify the overlapping genes obtained from both algorithms. These genes will be further validated in the test group.

2.5 Diagnostic value of the biomarkers in PCa

To compare the differences of these genes in the test group and assess the predictive value of established biomarkers, we utilized the R package 'ggpubr'. A significance level of $P < 0.05$ was considered statistically significant (18). Subsequently, we employed the R package 'proc' to generate an ROC curve in the training group consisting of 127 PCa cases and 52 normal controls (19). Hub genes were defined as those with a value greater than 85% (AUC). The diagnostic impact of PCa on normal samples was evaluated by calculating the AUC value, which was then verified in the test group comprising 63 PCa cases and 62 normal controls.

2.6 Assessment of immune cell infiltration

The CIBERSORT method was used to classify 22 different kinds of immune cell matrix. A reference set of 22 immune cell subtypes was utilized to assess the presumed abundance of immune cells, with 1,000 permutations (20). The invasion of the immune cell matrix was generated based on a significance level of $P < 0.05$. The program 'corrplot' was used to illustrate the association within 22 different kinds of immune cell infiltration and to create a correlation between heatmap and boxplot (21). Violin plots were created using the R package 'vioplot' to illustrate the variations in the infiltration of immune cells between PCa and normal samples (22).

2.7 Analysis of correlations between identified genes and immune cell infiltration

We conducted a Spearman's rank correlation analysis using R software to examine the correlation between the levels of expression of the identified biomarkers and the level of infiltrating immune cells (23). The resulting correlations were visualized using the charting approach provided by the 'ggpubr' package (24).

2.8 Cell culture and transfection

The PCa cell lines (LNCaP, PC3, and DU145) and the normal prostate cell line (RWPE-1) were cultured in RPMI-1640 medium. Oe-AOX1 and its negative control (Oe-ctrl) were synthesized by GenePharma (Shanghai, China). LNCaP and PC3 cells were evenly plated in 24-well plates. Once the two cell lines reached approximately 80-90% confluence, they were transfected following the provided instructions.

2.9 Western blot analysis

Cell proteins were separated by electrophoresis on a 12% SDS-PAGE gel and then transferred to PVDF membranes. The membranes were blocked with a 5% solution of silk milk at room temperature for 1 hour. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies against AOX1 (ab92519; 1:500; Abcam; USA) and β-actin (ab8226; 1:2,500; Abcam; USA), followed by incubation at room temperature for 1 hour with secondary antibodies (ab6721; 1:3,000; ab6728; 1:3,000; Abcam; USA). The membranes were then visualized using an enhanced ECL detection kit (Beyotime, China).

2.10 RT-qPCR analysis

RNA was extracted from PCa cells using TriZol (Beyotime, China). The extracted RNA was then reverse transcribed into complementary DNA. The quantified expressions were detected using SYBR Green qPCR Master Mix and the $2^{-\Delta\Delta Cq}$ method.

2.11 Cell proliferation assay

Ninety-six well plates were used to seed PCa cells (LNCaP and PC3). The plates were then incubated at 37°C and 5% CO_2. After incubation, a CCK-8 reagent test kit (Tiangen) was added at a volume of 10 μl per well. The PCa cells were further incubated at 37°C and 5% CO2 for 1 hour. Finally, the optical density (OD) value at 450nm was measured using a microplate reader for analysis.

2.12 Clone formation assay

The cultured cells in the logarithmic growth phase were diluted and seeded into dishes containing culture medium at the appropriate gradient density. The cells were then cultured at 37°C with 5% CO_2 for a period of 2 weeks. Afterward, the cells were washed twice with PBS and fixed with paraformaldehyde for 15 minutes. Subsequently, the colonies were stained with 0.1% crystal violet for 15 minutes and washed off with water. Finally, clones consisting of more than 10 cells were counted.

2.13 Measurement of malondialdehyde

Cells were lysed and centrifuged at 10,000g for 10 minutes. The MDA content of the cells was measured using the MDA Assay Kit (S0131, Beyotime, China). The samples were tested at 532 nm using a microplate reader and compared to the standard curve of MDA.

2.14 Measurement of Glutathione and ROS

The contents of GSH and ROS were detected using the corresponding kits, following the reference protocols provided by the manufacturer.

2.15 Statistical analysis

All statistical analyses were performed using Perl version 5.32.1 and R software version 4.1.2. $P < 0.05$ was used to determine statistical significance.

3 Results

3.1 Identification of DEGs in PCa

The differentially expressed genes (DEGs) from the GEO databases (GSE8511, GSE14206, GSE46602, and GSE55945) in 127 PCa cases and 52 normal controls were identified using the R package 'limma.' Out of the 37 DEGs, 17 were up-regulated and 20 were down-regulated. A log fold change (FC) > 0 indicates up-regulation in the training group, while a log FC < 0 indicates down-regulation. These findings are visually represented in the volcano plot (Figure 1A). The expression levels of the 37 DEGs are further illustrated in the heat map (Figure 1B).

3.2 Functional enrichment analysis

To evaluate the probable biological activities of the 37 DEGs, we conducted GO, KEGG, DO, and GSEA analyses using the R package 'cluster profile'. The GO results revealed that the majority of these genes were associated with basement membrane organization, cornification, and positive regulation of secretion by cells (Figure 2A). KEGG enrichment analysis identified genes involved in drug metabolism, specifically cytochrome P450, nicotinate and nicotinamide metabolism, and retinol metabolism (Figure 2B). The results from the DO analysis revealed that the diseases enriched by DEGs were primarily associated with chronic myeloproliferative diseases, epidermolysis bullosa, integumentary system disease, vesiculobullous skin disease, peripheral primitive neuroectodermal tumor, and prostate cancer (Figure 2C). In the PCa group, the GSEA results demonstrated that the enriched pathways mainly included bladder cancer, cell cycle, purine metabolism, ribosome, and toll-like receptor signaling pathway (Figure 2D). On the other hand, in the control group, the GSEA results showed that the enriched pathways mainly involved glutathione metabolism, focal adhesion, cytochrome P4 metabolism of xenobiotics, cytochrome P450 metabolism of drugs, and vascular smooth muscle contraction (Figure 2E).

3.3 Screening diagnostic feature biomarkers for PCa

To identify potential diagnostic biomarkers, we employed two distinct approaches. Firstly, we utilized the LASSO logistic regression approach to detect twenty-one genes as potential biomarkers for PCa from the robust DEGs (Figure 3A). Secondly, we employed the SVM-RFE technique to determine 28 genes from the DEGs (Figure 3B). Finally, we employed the Venn diagram to identify the overlapping gene markers obtained from both methods. As a result, we obtained

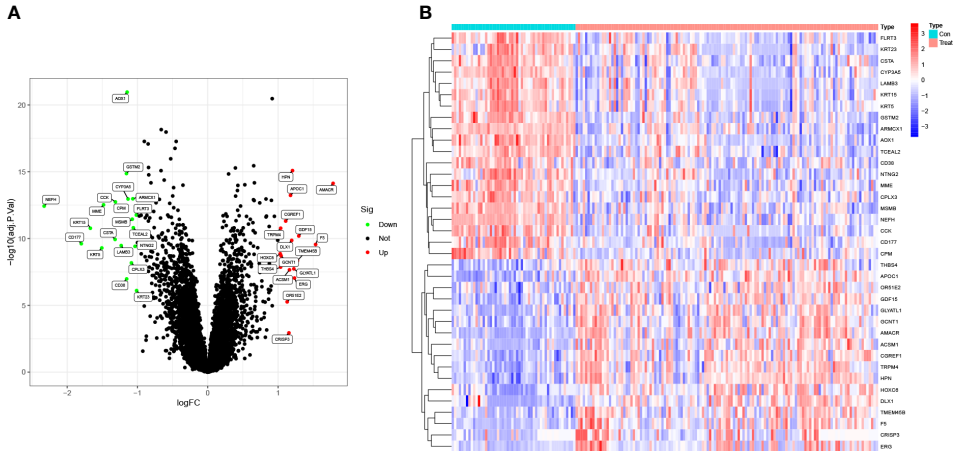

FIGURE 1
Identification of DEGs in PCa. **(A)** The volcano plot showed that 17 upregulated genes (red) and 20 downregulated genes (green) from 127 PCa cases and 52 normal controls in GEO datasets (*P*<0.05). Black dots mean meaningless (*P*>0.05). **(B)** The heat map showed the expression levels of 37 DEGs.

sixteen related biomarkers, namely AOX1, HPN, GSTM2, APOC1, ARMCX1, FLRT3, MSMB, KRT15, GDF15, DLX1, CD177, NTNG2, CPLX3, ACSM1, ERG, and CD38 (Figure 3C).

3.4 Identification and validation of diagnostic feature biomarkers for PCa

To determine the expression levels of six genes, ACSM1, APOC1, DLX1, GDF15, HPN, AOX1, ARMCX1, CD177, FLRT3, GSTM2, KRT15, and NTNG2, we utilized the GSE69223 dataset and GSE71016 dataset. Our findings revealed that the expression levels of ACSM1, APOC1, DLX1, GDF15, and HPN were significantly higher in PCa tissues compared to normal tissues (Figures 3D–H); all $P < 0.05$). On the other hand, the opposite outcome was seen for AOX1, ARMCX1, CD177, FLRT3, GSTM2, KRT15, 233 and NTNG2 (Figures 3I–O); all $P < 0.05$). Additionally, there was no significant change in the amounts of CD38, CPLX3, ERG, and MSMB between PCa tissues and the normal tissue (all $P > 0.05$). In the training group, we constructed ROC curves for these twelve genes, defining hub genes as those with an AUC greater than 85%. Then, we identified six PCa-related diagnostic genes, and the AUC of AOX1 was 0.921 (95% CI 0.878-0.956), APOC1 was 0.853 (95% CI 0.782-0.919), ARMCX1 was 0.883 (95% CI 0.834-0.928), FLRT3 was 0.854 (95%CI 0.796-0.904), GSTM2 was 0.877 (95%CI 0.823-0.923) and HPN was 0.871 (95%CI 0.817-0.921).Then, a

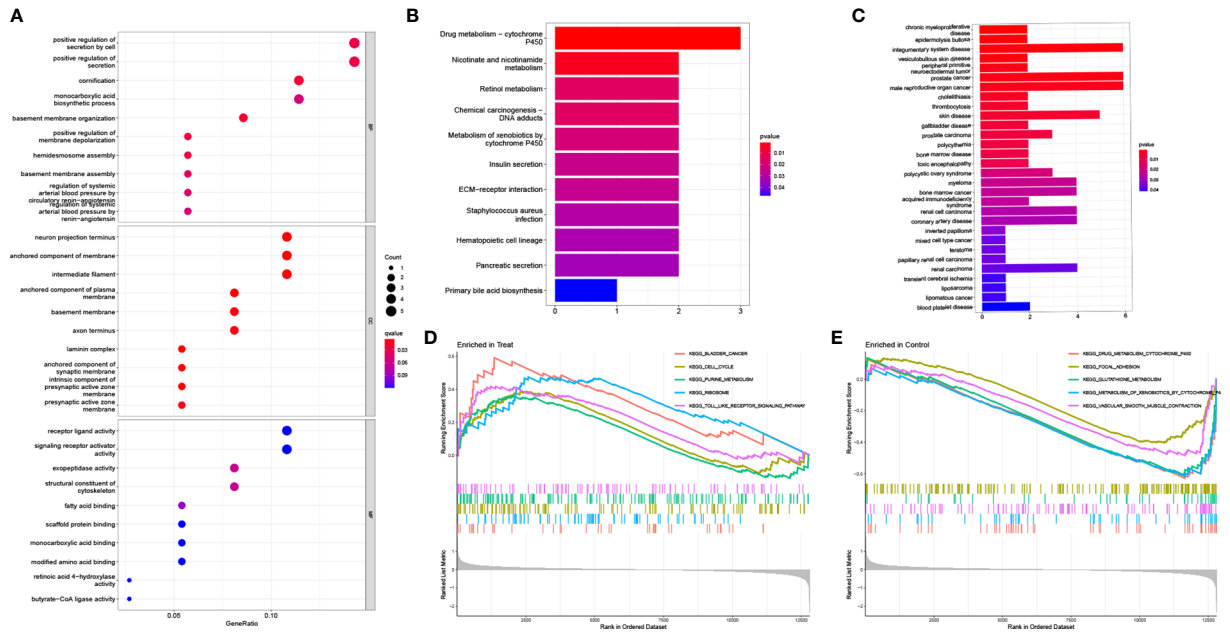

FIGURE 2
Functional enrichment analysis. **(A)** GO enrichment analysis, **(B)** KEGG enrichment analysis and **(C)** DO enrichment analysis of DEGs between PCa and control samples in train group. Enrichment analyses *via* gene set enrichment analysis in **(D)** PCa patients and **(E)** control samples of train group, respectively.

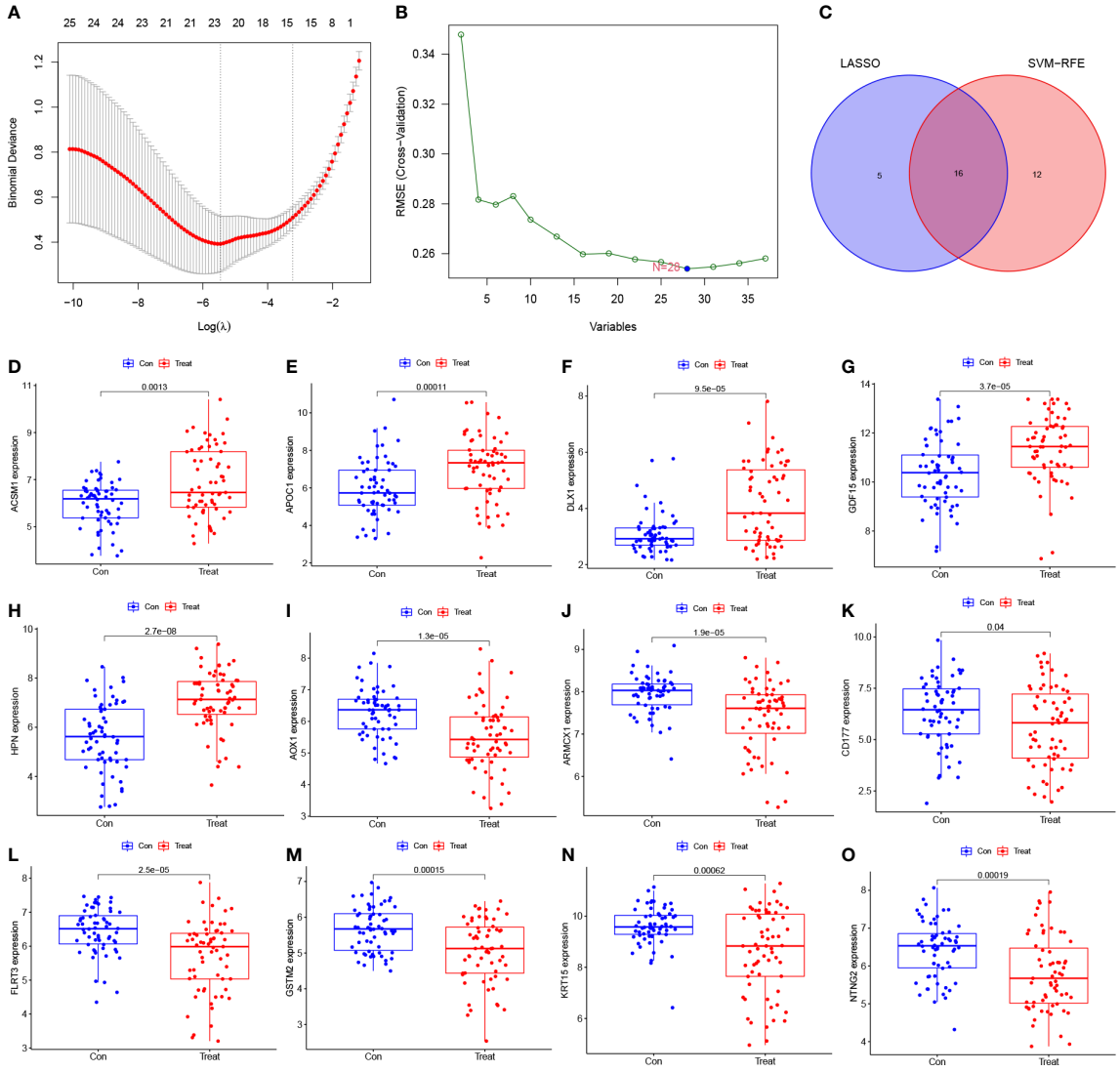

FIGURE 3
Screening diagnostic feature biomarkers for PCa. **(A)** Twenty-one genes were identified by LASSO regression. **(B)** A plot of biomarkers selection *via* SVM-RFE algorithm. **(C)** Venn diagram demonstrating sixteen diagnostic markers shared by the LASSO and SVM-RFE algorithms. **(D-O)** Validation of the expression levels of PCa-related diagnostic biomarkers in the test group (all $P < 0.05$), including **(D)** ACSM1; **(E)** APOC1; **(F)** DLX1; **(G)** GDF15; **(H)** HPN; **(I)** AOX1; **(J)** ARMCX1; **(K)** CD177; **(L)** FLRT3; **(M)** GSTM2; **(N)** KRT15; **(O)** NTNG2. LASSO, least absolute shrinkage and selection operator; SVM, support vector machine; RFE, recursive feature elimination.

robust discrimination was proved in the GSE69223 and GSE71016 datasets, and the AUC of AOX1 was 0.726 (95% CI 0.641-0.810), APOC1 was 0.701 (95% CI 0.607-0.792), ARMCX1 was 0.722 (95% CI 0.625-0.808), FLRT3 was 0.719 (95% CI 0.625-0.804), GSTM2 was 0.696 (95% CI 0.600-0.785) and HPN was 0.789 (95%CI 0.707-0.863) (Figures 4A–F).

3.5 Assessment of immune cell infiltration

Following that, we utilized the CIBERSORT technique to visualize the invasion of 22 distinct immune cell kinds in the training group (Figure 5A). Additionally, the CIBERSORT method demonstrated the invasion of 22 distinct immune cell types, and the heatmaps showed strong positive relationships between T cells CD4 memory resting and plasma cells (r=0.54) and strong inverse relationships between T cells CD4 memory resting and macrophages M1 (r= -0.51) (Figure 5B). We studied the component of immune cells in PCa and normal tissues. T cells CD8 in PCa was remarkably higher compared with the normal controls as indicated in the findings ($P = 0.032$), while mast cells resting was lower than the normal controls ($P = 0.005$; Figure 5C).

3.6 Correlation analysis between PCa-related biomarkers and immune infiltrating cells

The correlation analysis revealed that AOX1 was positively associated with mast cells resting (R = 0.4, $P = 0.036$) and negatively associated with macrophages M0 (R = -0.46, $P = 0.013$; Figure 6A). APOC1 was positively associated with macrophages M0

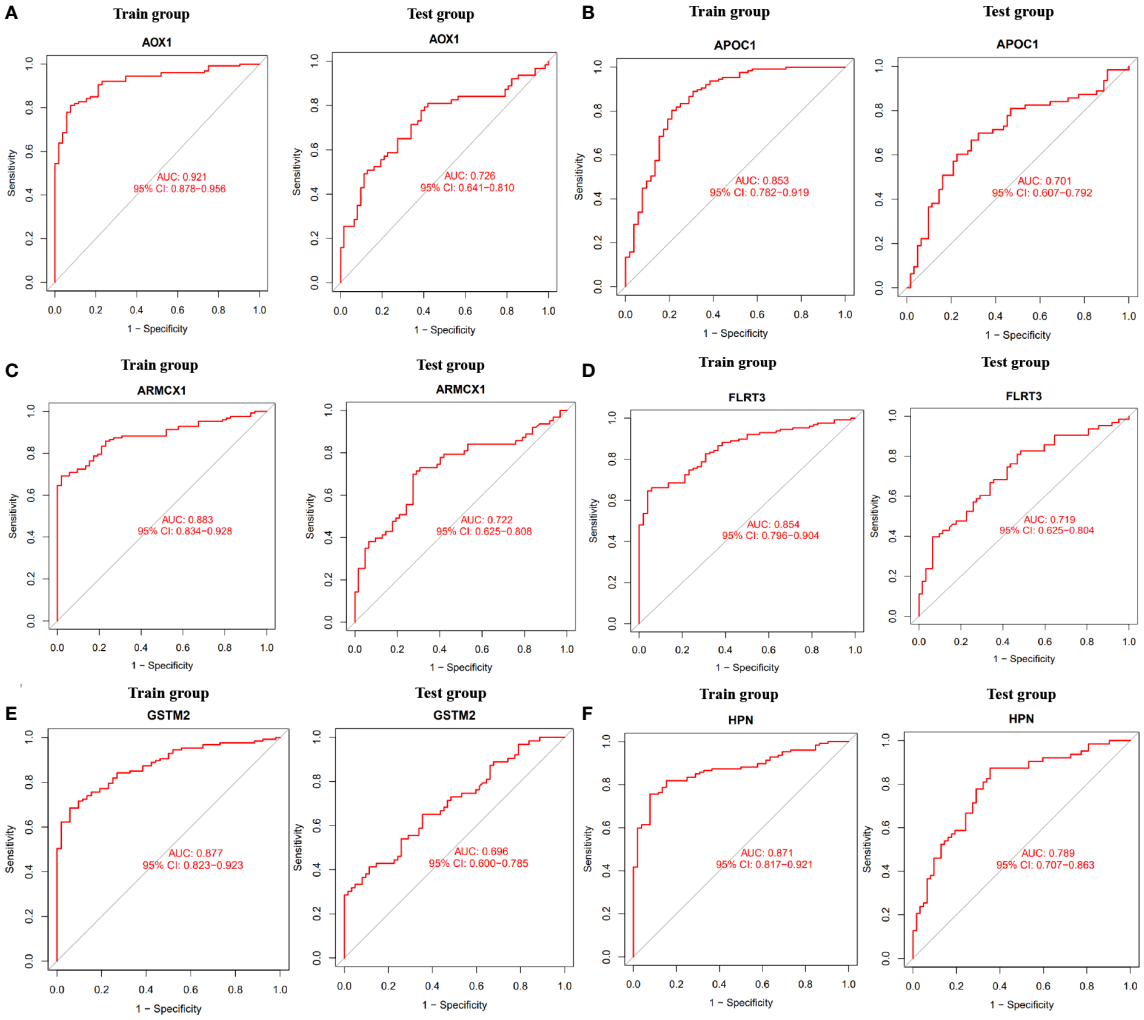

FIGURE 4
The ROC curve of the diagnostic effectiveness of the six diagnostic markers in the train group and test group. **(A)** AOX1; **(B)** APOC1; **(C)** ARMCX1; **(D)** FLRT3; **(E)** GSTM2; **(F)** HPN.

(R = 0.63, $P < 0.001$), neutrophils (R = 0.4, $P = 0.035$), and macrophages M2(R = 0.4, $P = 0.036$) and negatively associated with mast cells resting (R = -0.59, $P = 0.001$; Figure 6B). ARMCX1 was positively associated with NK cells activated (R = 0.38, $P = 0.045$) and mast cells resting (R = 0.38, $P = 0.049$; Figure 6C). FLRT3 was positively associated with T cells CD4 memory resting (R = 0.41, $P = 0.032$) and negatively associated with T cells gamma delta (R = -0.43, $P = 0.023$; Figure 6D). GSTM2 was positively associated with T cells follicular helper (R = 0.43, $P = 0.022$) and negatively associated with T cells CD4 naive (R = -0.41, $P = 0.029$; Figure 6E). HPN was positively associated with B cells memory (R = 0.47, $P = 0.011$) and macrophages M0 (R = 0.41, $P = 0.032$) and negatively associated with mast cells resting (R = - 0.41, $P = 0.031$; Figure 6F).

3.7 Experimental verification of AOX1 in PCa

Oxidative stress is closely related to cancer. To further identify whether these six PCa-related diagnostic genes are related to oxidative stress, we downloaded the gene sets of oxidative stress genes from the website GeneCards (https://www.genecards.org/). After taking the intersection, only AOX1 among the DEGs was classified as an oxidative stress gene (Figure 7A). The expression of AOX1 was verified. We obtained several PCa cell lines (LNCaP, PC3 and DU145) for experimental validation, with normal prostate cell line (RWPE-1) as the ctrol group. In Figures 7B, C, not only mRNA level but also protein level showed the same significant decrease ($P < 0.05$) trend of AOX1 in PCa cell lines. Subsequently, in order to detect the specific role of AOX1 in the progression of PCa, we applied the functional overexpression (oe-AOX1) into PCa cell lines (LNCaP and PC3). Figure 7D showed that the overexpression transfection was clearly successful in PCa cell lines. CCK-8 detection reveled that overexpression of AOX1 could significantly inhibit the proliferation activity of LNCaP and PC3 cells (Figures 7E, F). Similarly, the colony formation assays clearly revealed that the clone capacity of LNCaP and PC3 cells were inhibited by the overexpression of AOX1 (Figures 7G, H). Moreover, we detected the levels of MDA, ROS, and GSH in LNCaP and PC3 cells. The results showed a significant increase in

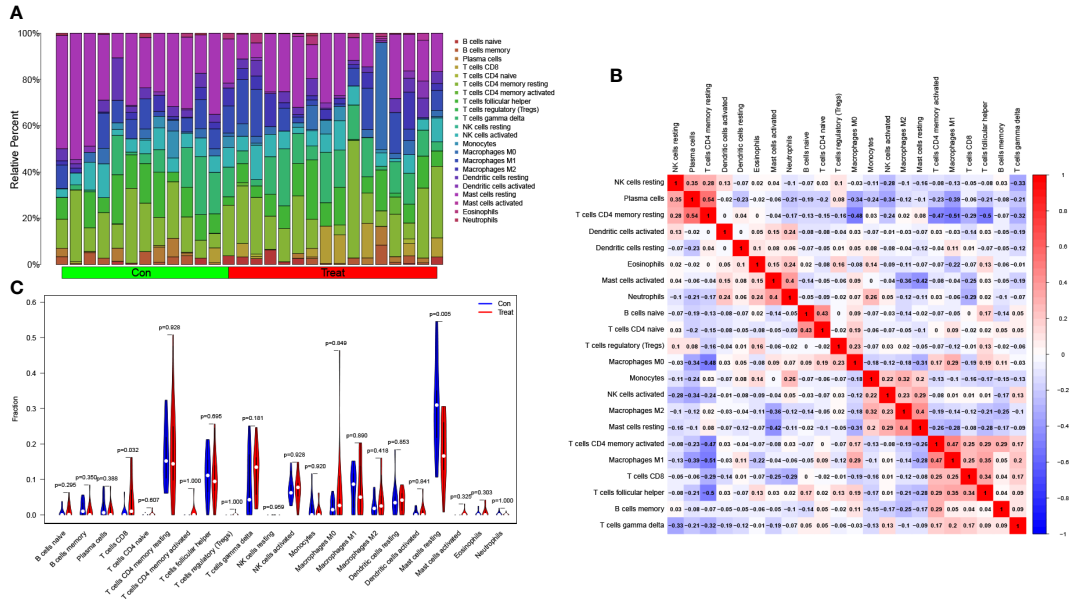

FIGURE 5
Assessment of immune cell infiltration between PCa and normal controls. **(A)** The box-plot diagram indicating the relative proportions of 22 different immune cell subtypes between PCa and normal controls; **(B)** The heat map shows the correlation among 22 different immune cell populations, with red and blue corresponding to positive and negative correlations, respectively. White indicates an absence of any correlation between the indicated immune cell populations; **(C)** The difference of immune infiltration between PCa (red) and normal (blue) controls (P<0.05 was regarded as statistically significant).

ROS and MDA levels, while an obviously decrease in GSH level (Figures 7I–K). To sum up, AOX1 acted as the role of cancer suppressor during the progression of PCa, which may be partly achieved by triggering oxidative stress.

4 Discussion

Nowadays, PCa continues to be one of the leading causes of cancer-related deaths in males. Considering the recent achievements of immunotherapy in various hematological and solid malignancies, there is a growing interest in investigating its potential in the treatment of PCa (25). An increasing number of researchers are acknowledging the connection between immune cell infiltration and various diseases, including cancer (26). As a result, immunotherapy is being considered as a potential approach to combat PCa. The CIBERSORT technique has been effectively utilized to determine the presence of immune cells within tumors and assess their impact on the prognosis of gastric cancer, colorectal cancer, breast cancer, and osteosarcoma (20, 27–29). The importance of immune cell infiltration in PCa has not yet been fully understood. The objective of this study was to investigate the significance of immune cell infiltration in PCa and identify potential diagnostic biomarkers.

To the best of our knowledge, this is the first retrospective study to use the combination of the LASSO and RVM-RFE algorithms, along with the CIBERSORT algorithm, to analyze immune cell invasion in PCa. We obtained six datasets from the GEO database, with two datasets merged for the test group and the remaining four datasets merged for the training group. In the training group, we identified a total of 37 differentially expressed genes (DEGs), with 17 genes being up-regulated and 20 genes being downregulated. The results of the gene set enrichment analysis (GSEA) in PCa cases revealed that the enriched pathways primarily involved bladder cancer, cell cycle, purine metabolism, ribosome, and toll-like receptor signaling pathway.

Using two algorithms, we selected sixteen genes as potential PCa-related biomarkers based on their robust differential expression. Subsequently, we analyzed the differences among these sixteen genes in the test group and found that twelve genes showed statistical significance (P < 0.05). Finally, we constructed ROC curves for these twelve genes and identified six final PCa-related diagnostic biomarkers (AOX1, APOC1, ARMCX1, FLRT3, GSTM2, and HPN). To evaluate the predictive efficacy of these six diagnostic biomarkers, we computed their ROC curves in the test group.

Additionally, we used CIBERSORT to assess immune cell infiltration in PCa and investigate its role in the disease. It has been observed that an increase in CD8 T cell infiltration and a decrease in mast cell infiltration during rest are associated with the occurrence and progression of PCa. Correlation analysis between biomarkers associated with PCa and immune invading cells revealed significant associations between AOX1, APOC1, ARMCX1, GSTM2, and HPN with resting mast cells. Furthermore, HPN, AOX1, and APOC1 showed significant correlations with macrophages M0. In a study by Florent et al., immunohistochemistry was performed on tumors from 51 patients with node-positive PCa. The presence of a large density of CD8 + T cells in tumors was discovered to be related to an increased risk of clinical progression in patients with node-positive PCa (30). Mast

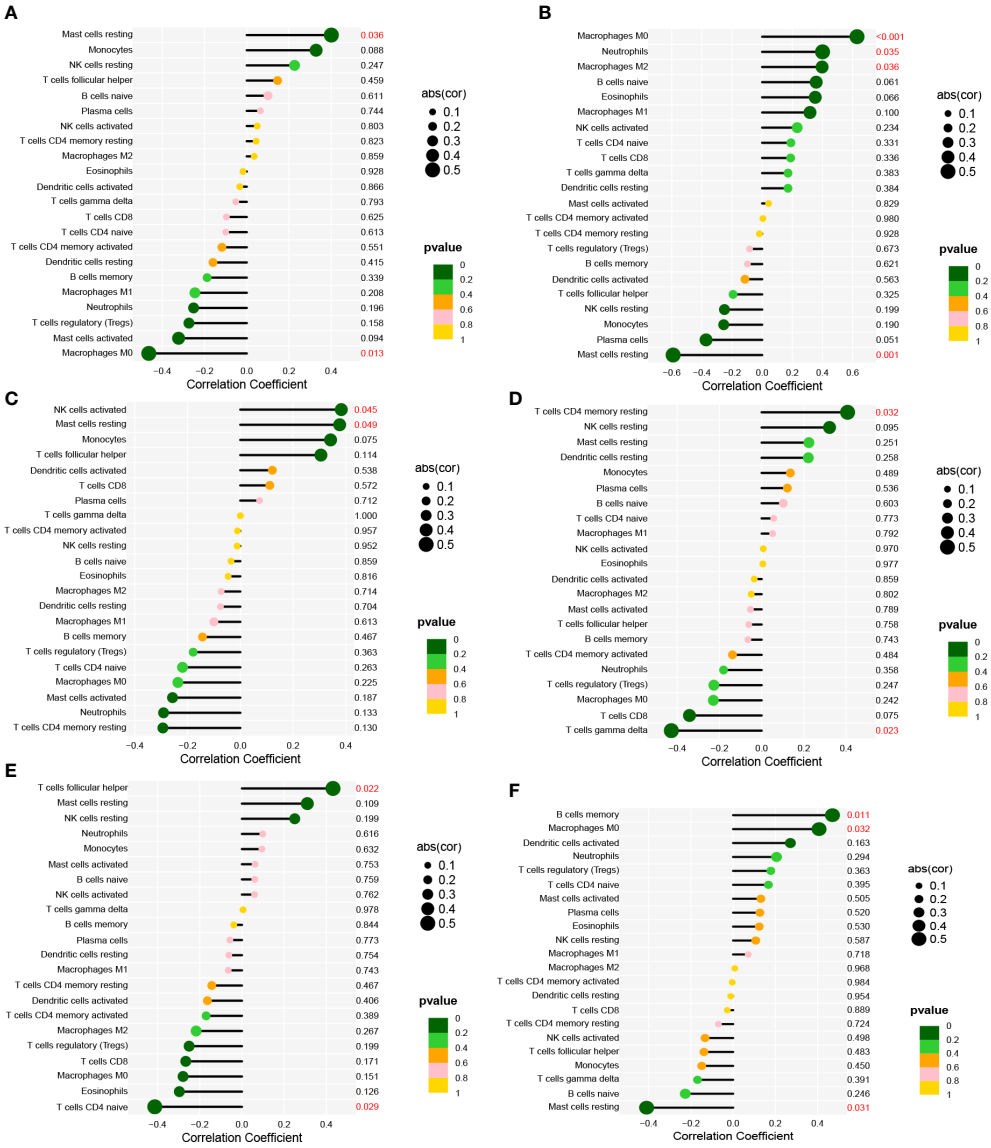

FIGURE 6
Correlation between **(A)** AOX1; **(B)** APOC1; **(C)** ARMCX1; **(D)** FLRT3; **(E)** GSTM2; **(F)** HPN and infiltrating immune cells in PCa.

cells are implicated in various disorders, such as hypersensitivity, inflammation, and fibrosis. It is worth noting that mast cells also play a crucial role in tumor progression. In this study, the CIBERSORT algorithm was employed to analyze 52 normal prostate tissues and 497 primary tumors of patients with prostate cancer (PCa) from TCGA. The results revealed a significant difference in the fraction of static mast cells between PCa and normal tissues. Moreover, an increased number of resting mast cells is associated with a poor prognosis. It is important to consider that radiotherapy and targeted molecular treatments may impact the infiltration of resting mast cells in the immune system (31). Somaiyeh et al. (year) conducted a study where they investigated the protective effect of M0 macrophages and THP-1 cells treated with toll-like receptor 4 (TLR4) agonists on etoposide-induced apoptosis in PCa cells. They cultured these cells with the supernatant of P human prostate cancer cell line (PC3) cells and analyzed the results using enzyme-linked immunosorbent assay with flow cytometry (ELISA) (32). Recent studies, including our own findings, suggest that various types of invasive immune cells play a significant role in PCa and should be the focus of future research.

Additionally, emerging evidence has shown a close relationship between oxidative stress and the development and progression of cancer (33, 34). In this study, we examined the gene AOX1 in relation to 6 hub genes and 664 oxidative stress-related genes. Xiong et al. have previously reported that AOX1 is downregulated and functions as a tumor suppressor gene in clear cell Renal Cell Carcinoma (ccRCC) and PCa (35, 36). In this study, we demonstrated that AOX1 expression was reduced in PCa cells. We then conducted functional experiments by transfecting oe-AOX1, which showed that the overexpression of AOX1 inhibited the proliferation and migration of PCa cells, consistent with previous findings. Notably,

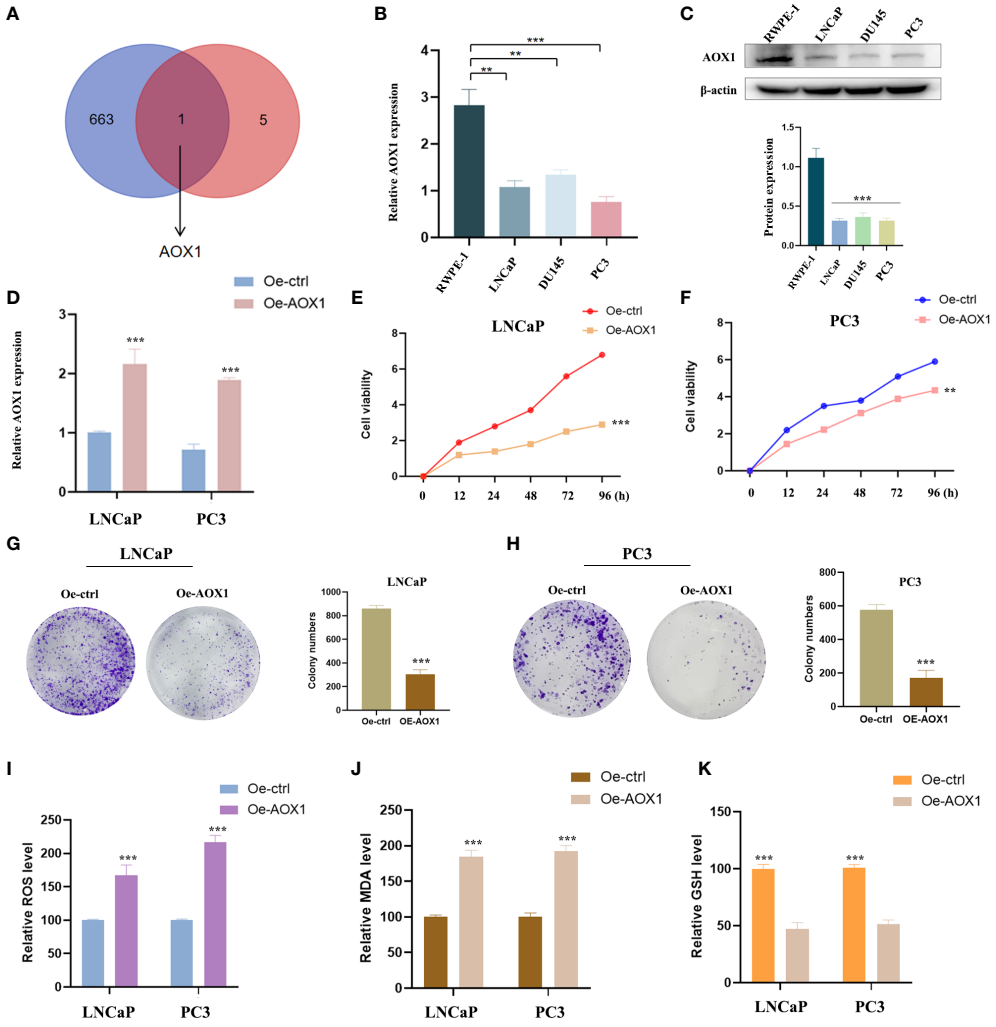

FIGURE 7
Experimental verification of AOX1 in PCa. **(A)** AOX1 was classified as an oxidative stress gene. **(B, C)** Both mRNA level and protein level showed AOX1 in PCa cell lines expressed higher than those in normal prostate cell line. **(D)** The overexpression transfection was clearly successful in PCa cell lines. **(E, F)** CCK-8 reveled that overexpression of AOX1 could significantly inhibit the proliferation activity of LNCaP and PC3 cell lines. **(G, H)** The clone capacity of LNCaP and PC3 cells were inhibited by the overexpression of AOX1. **(I-K)** Overexpression of AOX1 showed a significant increase in ROS and MDA levels, while an obvious decrease in GSH level. $^{**}P$ <0.01; $^{***}P$ <0.001.

AOX1 overexpression led to the accumulation of reactive oxygen species (ROS) and malondialdehyde (MDA), while also restoring the glutathione (GSH) content. Overall, our results suggest that the anti-cancer effect of AOX1 may be mediated through the activation of oxidative stress.

The investigation has several limitations that should be considered. Firstly, the sample size of the published datasets is small, which means that our findings need to be validated in larger datasets and clinical trials to determine whether AOX1, APOC1, armcx1, FLRT3, gstm2, and HPN can be used as biomarkers of PCa. Additionally, the CIBERSORT algorithm used in our study was based on limited retrospective gene data. While some earlier studies have found similar results to ours, the analysis of immune cell infiltration in PCa is currently limited, and our conclusion should be verified by a prospective study with a larger sample size. Moreover, We have conducted initial research on the anti-oncogenic role of AOX1 in the malignant progression of PCa. We have also identified a potential mechanism, which involves triggering oxidative stress *in vitro*. However, further rigorous testing is required for thorough verification.

5 Conclusions

In our study, we identified AOX1, APOC1, ARMCX1, FLRT3, GSTM2, and HPN as biomarkers associated with prostate cancer (PCa). Further research should focus on investigating the relationship between PCa and immune cell infiltration to enhance the effectiveness of immunomodulatory treatments for PCa patients. Moreover, we conducted experimental validation and

discovered that AOX1 functions as a tumor suppressor in PCa by inducing oxidative stress. This finding not only contributes to a better understanding of the pathogenesis of PCa but also opens up new possibilities for clinical treatment.

Data availability statement

The datasets supporting the conclusions of this article are available in the GEO database, [unique persistent identifier andhyperlink to datasets in http://www.ncbi.nlm.nih.gov/geo]. The datasets generated during and analyzed during the current studyare available from the corresponding author on reasonable request.

Ethics statement

The study was approved by the Ethics Committee of the First Affiliated Hospital of Jinan University, China.

Author contributions

XM, KY, DH and YL conceived and designed the experiments; XM, KY, DH and JL conducted the research; XM, DH, YL, KY and HL contributed materials and analysis tools; XM, DH, JL, KY and CH analyzed the results; XM, KY and DH wrote the paper. All authors reviewed the manuscript. XM, KY and DH contributed equally to this work. YL is the corresponding author.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Genome-wide studies in prostate cancer poised liquid biopsy as a molecular discovery tool

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Liquid biopsy is emerging as an intriguing tool in clinical disease detection and monitoring. Compared to a standard tissue biopsy, performing a liquid biopsy incurs minimal invasiveness, captures comprehensive disease representation, and can be more sensitive at an early stage. Recent genome-wide liquid biopsy studies in prostate cancer analyzing plasma samples have provided insights into the genome and epigenome dynamics during disease progression. In-depth genomic sequencing can offer a comprehensive understanding of cancer evolution, enabling more accurate clinical decision-making. Furthermore, exploring beyond the DNA sequence itself provides opportunities to investigate the regulatory mechanisms underlying various disease phenotypes. Here, we summarize these advances and offer prospects for their future application.

KEYWORDS

prostate cancer, liquid biopsy, deep genomic sequencing, genome-wide methylation analysis, disease progression

Main

Treatment options for metastatic lesions of prostate cancer (PCa) are limited, and resistance to androgen signalling inhibitors (ASI) is ultimately inevitable (1–3). Detecting aggressive disease while it is still manageable and understanding the underlying biology are clinical imperatives. The standard invasive tissue biopsy procedure for PCa diagnosis poses a risk to the patient (4), is limited in the early stages of disease (5), and is impractical for longitudinal disease monitoring. Liquid biopsies utilizing body fluids such as blood, urine and saliva and analyzing biomaterials in circulation show promise for revolutionizing tumour profiling and monitoring practices. It contributes to understanding the signals determining threshold tumour development (6), highlights metastatic markers, and provides complementary information for treatment response (7, 8). In addition to the

biomarker potential, it begins to serve as a method for evaluating mechanisms behind therapy resistance (9).

Characterization of metastatic PCa remains scarce, and previous studies are limited in scale and depth (9–11). Four recent studies (7, 12–14) analyzed the genome-wide genetic and epigenetic landscape using blood samples and brought deep biological insights associated with disease progression. In this mini-review, we summarize the key findings from these genome-wide studies and their implications for the potential applications of liquid biopsy. A concise overview of liquid biopsy research in prostate cancer was included to offer a more comprehensive context for our discussion.

Liquid biopsy analytes commonly used in the clinical practice

Circulating tumour cells (CTC), extracellular vesicles, and membrane free biomolecules, including various types of nucleic acids and proteins, constitute the most commonly used analytes for liquid biopsy. In prostate cancer, a single protein biomarker, the prostate cancer specific antigen (PSA) remains active in clinical practice, despite its tendency to overdiagnose (Figure 1) or overemphasize the severity of low grade and slow growing tumours (15). PSA is a highly sensitive marker, but it is limited for specific detection in patients. There is in fact limited evidence for the practicality of PSA in a primary care setting (16). Thus, many efforts were instead devoted to improving diagnostic accuracy, with the most studied being the urine-based test of long non-coding RNA (lncRNA) prostate cancer antigen 3 (PCA3). The test was approved by the US Food and Drug Administration, and unlike PSA, it shows moderate sensitivity and adequate specificity in differential diagnosis of PCa and non-PCa (17). However, it remains controversial in terms of the degree of additional clinical benefit it can provide (18–21). For RNAs to be robustly analyzed in liquid biopsies, they need to survive the RNase-rich extracellular environment. Certain RNA species, such as the microRNAs (miRNA), exhibit greater stability, can be abundant with high specificity in patient plasma and are increasingly explored (22, 23). The formation of RNA-protein, RNA-DNA and RNA-lipid complexes are potential mechanisms mediating this increased stability of endogenous RNA transcripts. Alternatively, encapsulation in extracellular vesicles (EV) can help stabilize the transcripts, as in the case of PCA3. In 2016, an exosomal RNA assay became commercially available to help detect aggressive disease while reducing unnecessary biopsies (24, 25). Meanwhile, research on other types of cargo in EVs, such as DNA, is on the rise (26).

CTC is another important liquid biopsy biomarker in clinical settings and the CellSearch system for CTC enumeration was FDA approved in 2010 (27). While multiple studies including clinical trials have validated the usefulness of CTC for prognostication and disease monitoring, the majority of current studies have focused on its applications in late-stage disease (28–30). An apparent limitation of their use in clinical and laboratory settings is their low detection rate at early stages of the disease (31). With the CellSearch system, the percentage of localized prostate cancer patients with detectable CTC in a 7.5ml blood sample ranges from 5% to 27%, and the median count can be as low as 1 (32–35). As potential clinically relevant predictors of future metastasis, many studies have taken efforts to improve overall detection. Using microfluidic devices, Stott et al. and (36) were able to achieve detection in approximately half of their localized patient cohorts, with medians of 95 and 4.5 cells per mL of peripheral blood, respectively (37). Additionally, combination of the CellSearch system with apheresis technology dramatically increases the volume of blood analyzed (mean 59.9 ml) and improves the recovery of CTCs (mean 12,546) in metastatic PCa, showing great promise in analyzing localized diseases.

Liquid biopsy in disease detection and monitoring in recent prostate cancer research

With minimal invasiveness, liquid biopsies are most well studied as biomarkers. Being able to capture a more holistic view of the disease is another attractive advantage of liquid biopsy. It is

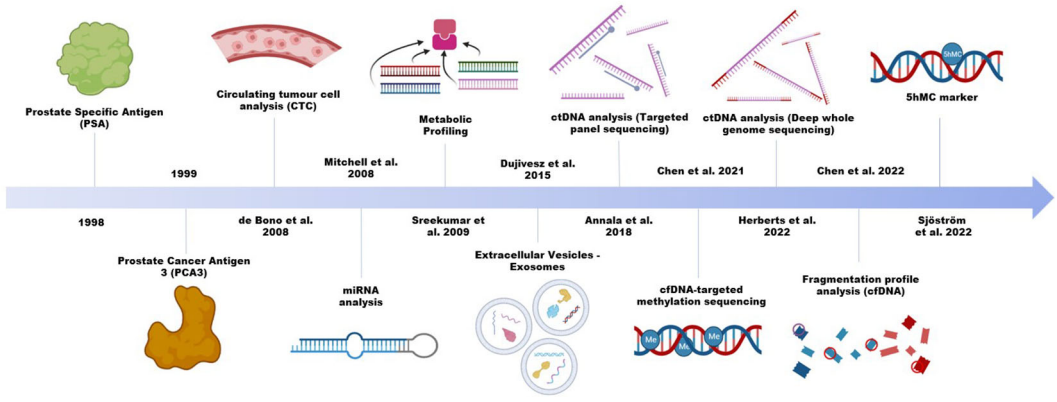

FIGURE 1
Application of various liquid biopsy technologies in prostate cancer.

particularly important for managing metastases, as they cannot be represented by individual lesions and are difficult to biopsy. In contrast to CTC, ctDNA can be obtained more readily from patient plasma without the need for rare cell type enrichment procedures and can be more sensitive. It allows the detection of prognostic and predictive genomic alterations in driver genes, including AR, TP53, and those in the DNA repair pathways (9, 38–40). Specifically, blood-based identification of DNA damage repair (DDR) defects can help uncover potential candidates for DDR-directed therapies and immunotherapy, which might be overlooked when relying on primary tissue samples (41, 42). Herberts et al. (12) further showed that single-matched tissue biopsy failed to identify the dominant clone detected in plasma, potentially misinforming clinical decision-making.

With limited detectable mutations and low concentration at early stage, research focus was redirected towards advanced metastatic castration-resistant prostate cancer (mCRPC) to aid in prognostication and provide guidance for targeted therapies (30, 43–47). A study by Stover and colleagues (48) applied a novel NGS panel for evaluating patient-derived models, allowing for somatic variant detection over time across several prostate cancer-associated genes. This was found to be useful not only for primary tumours, but also for CTCs and cfDNA (48). In clinical practice, classification of patients as plasma tumour DNA positive or negative was done using an orthogonal approach designed to utilize known information on heterozygous SNPs (47). Prior to treatment with abiraterone acetate, higher levels of gene alterations were found in mCRPC patients with a higher initial disease burden. Plasma changes over time established that a sample post-abiraterone acetate + glucocorticoid treatment could identify resistant clones more effectively than a pre-treatment liquid biopsy sample (47). On the other hand, epigenetic alterations, specifically DNA methylation, are thought to occur early in the progression of the disease and have a greater number of recurrent sites with detectable frequencies (49). These features make them attractive candidates for early cancer detection and have been extensively investigated (50, 51).

While liquid biopsy studies have traditionally made use of molecules like nucleic acids and proteins (52), emerging types of analytes, including lipids, glycans, and microbiomes, are being explored as potential biomarkers for prostate cancer (52–55). Studies on the blood microbiome have revealed distinctive signatures between major cancer types, indicating potential as a complementary diagnostic tool to ctDNA/ctRNA assays (56). The approach to screening is also evolving from single analytes to multi-gene panels, and now whole genome investigations are becoming more common. Genome-wide studies have advanced not only in size but also in depth, accuracy, and methodology. They now delve deeper into the underlying biology of diseases rather than solely focusing on biomarker discovery (7, 12–14).

For example, by capturing alterations not commonly present at the DNA level and beyond the tumours themselves, DNA methylation can offer additional layers of information (13, 14). This is particularly useful for early cancer diagnosis, for which the sensitivity is limited by the low amount of ctDNA and the even lower number of variable biomarkers available. Chen and colleagues (13) showed that fragmentation profiles inferred from the methylation sequencing data differ significantly between healthy control and localized samples, while Sjostrom et al. captured 5hmC alterations not detected in the DNA. The ability of DNA methylation to capture lineage-specific features can be further explored to facilitate the development of multi-cancer early detection tests (57). A study by Bjerre et al. found hypermethylation rates in ctDNA to be as high as 61.5% in *de novo* metastatic PCa patients. A shorter progression duration towards resistant PCa was also correlated with detection of ctDNA methylation (58). Practically, the detection process also appears to be minimally invasive, and has been found to be associated with higher rates of medical compliance and cost efficiencies (59). In terms of its supplemental monitoring capabilities, it can make up for what PSA assessments currently lack. ctDNA monitoring is currently in transition towards potential clinical implementation. ctDNA percent levels do not necessarily reflect the same tumour characteristics as current evaluation methods, which as previously mentioned, can provide more information alongside current popular markers (60). For AR-directed therapy regimens, the changes in monitored ctDNA levels may act as indicators for early cancer progression, thus warranting therapy alterations (61, 62). Overall, ctDNA methylation analysis is showing to be capable of being a valuable tool for both detection and cancer management.

Liquid biopsy as a tool for molecular discovery

Additional models and approaches have also been utilized to overcome challenges such as low ctDNA content and cancer diversity. The use of patient-derived xenograft (PDX) mouse plasma helped define nucleosome pattern analysis frameworks that can distinguish mCRPC phenotypes with up to 97% accuracy (7, 63). Two high-performance models were developed to approximate the proportion of neuroendocrine prostate cancer (NEPC) and androgen receptor-positive prostate cancer (ARPC), as well as predict their presence. An analysis framework implementing a GC correction procedure for cfDNA fragmentation patterns was also developed to achieve sensitive cancer subtype prediction (63).

Sarkar and colleagues employed PDX models with corresponding tissue samples to establish computational frameworks that can infer transcriptional activity by analyzing the nucleosome positioning pattern of ctDNA (7). They were able to link variations in nucleosome organization to changes in histone modifications, chromatin accessibility, and transcription factor activity that are specific to diverse tumour phenotypes (7). Using plasma ctDNA, the transcriptional activities of key phenotype regulators, including hepatocyte nuclear factor 4 gamma (HNF4G), AR, and achaete-scute homolog 1 (ASCL1), were detected, and the results showed high consistency compared to those obtained from tissue multi-omic profiling. Furthermore, direct estimation of phenotype proportion revealed that diverse molecular subtypes often coexist.

As well, the utilization of liquid biopsy has moved beyond its biomarker discovery ability. With deep whole-genome sequencing on the plasma samples from mCRPC patients, Herberts et al. (12) showed that different dominant clones exist for individual metastatic lesions. These differences could only be captured by liquid biopsies rather than tissue biopsies. They identified clinically relevant alterations that are difficult for bulk tissue sequencing to resolve, such as subclonal whole genome duplications, prevalent and diversified AR alterations, and convergence on AR augmentation after potent ASI treatment.

Chen et al. (13) and Sjöström et al. (14) used liquid biopsy to evaluate the DNA methylation landscapes. Through the use of immunoprecipitation in tandem with sequencing, Chen et al. were able to distinguish diverse forms of methylation and provide genome-wide cell-free profiles for 5mC, the most common form of DNA methylation (13). The cell-free methylomes revealed alterations apart from the tumour itself, coupled with global hypermethylation and hypomethylation at pericentromeric regions for mCRPCs compared to localized diseases. Using these data, the authors further inferred copy number alteration and fragmentation profiles, which showed notable distinctions among various disease stages.

For 5mC to reverse, it must first be oxidized to 5hmC, a mark for activated and poised transcription. Counting only a fraction of the total DNA methylation and unable to be distinguished from 5mC by the widely used bisulfite conversion-based methods, 5hmC was poorly understood in PCa until recently (14, 64). Sjostrom et al. used biotin labelling to specifically enrich 5hmC and provide a global landscape with paired liquid and tissue biopsies (14). The 5hmC dynamics throughout PCa progression identify cancer hallmarks and provide an additional layer of prediction by capturing non-canonical alterations. PCa-specific 5hmC patterns can track lineage plasticity and can be used to predict tumour burden in circulation (14).

Summary

The biomarker potential of liquid biopsies has been extensively explored. Although ctDNA has demonstrated success in disease monitoring and DNA methylation has shown promise in early cancer detection, there is currently no single method that is comprehensive enough to achieve sufficient clinical accuracy and stability in both scenarios. Application of ctDNA analysis is greatly restricted due to the limited number of tumor-specific mutations, especially for early cancer detection where the amount of shedded ctDNA is low. While measuring epigenetic alterations can provide more detectable features, it is impeded by technology and analytical limitations. Traditional chemical methods are more accurate, yet they are not as cost-effective and can result in loss of the already limited DNA materials available. Conversely, enrichment-based methods are susceptible to influence of sequence specificity and antibody effectiveness, leading to potential inaccuracies. Similarly, while RNA transcripts are more readily detectable, they are highly variable and present challenges in reproducibility. Certain types of RNA, such as miRNA and circRNA, have proven to be relatively stable and are gaining increasing attention in research.

Recent studies reiterated the necessity of using liquid biopsy to avoid potentially ill-informed clinical decisions and opened up new avenues towards developing more accurate multi-modality assays. Information beyond the ctDNA sequence itself, such as epigenetic alterations and fragmentation profiles, are reflective of gene regulatory patterns and can provide more detectable features, thus enhancing the potential for early cancer detection. Therefore, conducting multi-omics sequencing can improve sensitivities, while implementing a stringent analysis pipeline that uses multi-factor verification can reduce false positives and promote overall accuracy. Moreover, as evidence suggests the presence of unique features in liquid biopsy samples emerging, genome-wide strategies are now more commonly employed to facilitate unbiased biomarker discovery. Such comprehensive and in-depth analysis of liquid biopsies has also led to significant biological insights, establishing it as a powerful tool for molecular discovery. The continued research with liquid biopsy will no doubt yield stunning insights into disease biology and facilitate the development of more effective therapeutics.

Author contributions

HH and SC conceived and designed the research. NL, HH and SC wrote, revised and approved the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Genomic amplifications identified by circulating tumor DNA analysis guide prognosis in metastatic castration-resistant prostate cancer

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Purpose: Analysis of circulating tumor DNA (ctDNA) in patients with metastatic prostate cancer (mPC) provides an opportunity to identify and monitor genomic alterations during a patient's treatment course. We evaluated whether the presence of specific gene amplifications (GAs) and plasma copy number (PCN) alterations are associated with disease features.

Methods: This is a single-institution retrospective study of patients with mPC who underwent ctDNA profiling using Guardant360® (Guardant Health Inc.). This test identifies single nucleotide variants (SNVs) and GAs of select genes by next-generation sequencing. A total of 155 men with mPC were studied. Patients were stratified by GA status. The Kaplan-Meier method and multivariate cox regression models were used to estimate overall survival (OS) or failure-free survival (FFS) from either the date of GA detection or the initiation of systemic therapy. The chi-square test was used to evaluate associations between clinical factors and GAs.

Results: The presence of liver and/or lung metastases was associated with GAs of *BRAF, CDK6, PI3KCA*, and *FGFR1*. Survival analyses were completed on a subset of 83 patients with metastatic castration-resistant prostate cancer (mCRPC). Median OS was improved in patients with 1 GA compared to patients with ≥2 GAs, whether determined from the date of initial GA(s) detection (14.9 mo vs. 8.9 mo) or date of therapy initiation nearest to GA detection (16.7 mo vs. 9.0 mo). Patients without GAs had not reached median OS. Patients with androgen receptor (*AR*) GA only were also found to have better median OS compared to patients with *AR* GA plus at least one other additional GA (19.3 mo vs. 8.9 mo). Patients with *PIK3CA* GA had significantly lower median OS compared to patients with GAs that did not have a *PIK3CA* GA (5.9 mo vs. 16.0 mo). In patients with *AR* and/or *MYC* GA(s), median OS

improved in those with reduced *AR* or *MYC* PCN during therapy compared to those without such a reduction (25.1 mo vs. 15.9 mo).

Conclusions: The association of select GAs with survival provides an additional tool for assessing mCRPC prognosis and informing management. Serial monitoring of ctDNA GAs is also useful to guide prognosis and therapeutic response.

KEYWORDS

prostate cancer, metastasis, castration-resistant prostate cancer, androgen receptor, PIK3CA, aneuploidy, genomic amplification, circulating tumor DNA

Introduction

Prostate cancer (PC) remains the most diagnosed malignancy and the second leading cause of cancer-related death among men in the United States. Between 2014 and 2019, prostate cancer incidence has increased by 3% per year (1, 2). Although most men diagnosed with PC have local disease, recurrences frequently occur (3, 4). Furthermore, diagnosis of metastatic prostate cancer (mPC) has been modestly increasing in the context of changing screening practices and is a dynamic disease with a poor prognosis, particularly as it progresses to castration-resistant prostate cancer (CRPC) (5–8). Prostate cancer has the propensity to develop resistance across various treatment paradigms, which include androgen axis targeting agents and chemotherapy. Thus, uncovering the genomic phenotypes as the disease evolves is critical to understanding patient prognosis, treatment sequencing, and developing novel therapeutics. Circulating tumor DNA (ctDNA) provides an essential tool for understanding such genomic phenotypes, guiding therapeutic decisions, and characterizing tumor heterogeneity in a cost-effective manner. It poses minimal patient risk and little technical challenge compared to (repeated) biopsy of tumor tissue (8–10).

Recently, the use of ctDNA analysis has increased, enhancing our understanding of PC progression and helping to guide clinical management (9). An analysis of more than 500 patients with metastatic CRPC (mCRPC) identified frequent androgen receptor (*AR*) and *MYC* alterations associated with clinical outcomes, such as overall survival (OS) and failure-free survival (FFS) (11). Several *AR* mutations that are associated with resistance to anti-androgen axis therapies were identified by ctDNA analysis and can guide therapeutic decision-making (12). Although distinct from ctDNA, circulating cell-free DNA (cfDNA), which consists of ctDNA and hematopoietic cell-derived DNA, is elevated in patients with mPC and can also serve as a marker of therapeutic response. For example, decreased cfDNA levels were associated with improved outcomes in patients harboring mutations in genes involved in homologous recombination and demonstrating clinical response to poly(ADP)ribose polymerase (PARP) inhibitor (13). Similarly, cfDNA levels were reduced in patients receiving taxane therapy, and that reduction was associated with both radiological progression-free survival (rPFS) and OS (14). Finally, ctDNA levels were associated with rPFS in mCRPC patients in the A.MARTIN phase II study of abiraterone with or without pan-AKT inhibitor ipatasertib (15).

In patients with mPC, the genomic landscape remains fluid throughout the treatment course. ctDNA can be used to profile somatic mutations and genomic amplifications (GAs) with their corresponding variant allele frequency (VAF) plasma copy number (PCN) during any stage of the treatment course. GAs may be representative of either chromosomal duplication and/or gene-specific amplification, which is particularly interesting because aneuploidy is a relevant feature of aggressive prostate cancers that may be more likely to be lethal (16). Copy number alterations frequently occur in the form of loss of regions, with the loss of tumor suppressors *CHD1*, *RB1*, *TP53* and *PTEN* as the most common alterations (17). A recently published copy number alteration analysis from 300 patients in the androgen deprivation therapy control arm of the STAMPEDE trial showed that loss of segments of chromosome 5 containing *CHD1* and amplifications in segments of chromosome 8 containing *MYC* were associated with a higher burden of copy number alterations. Copy number alteration burden was associated with a statistically significant increase in metastasis at diagnosis, risk of progression, and death (18). This follows prior demonstration of the presence of *AR,* or *MYC,* or *BRAF* amplifications (as detected by ctDNA) are associated with worse OS (11). Furthermore, we previously demonstrated that elevated carcinoembryonic antigen (CEA) is associated with higher rates of liver metastases and increased copy number alterations of select genes (19).

The prognostic significance of GAs and PCN detected prior to or serially through the course of treatment in a cohort of patients remains to be fully characterized. The majority of ctDNA assays do not commonly report on actionable deletions, germline mutations, or less frequent GAs such as chromosomal rearrangements (20). This limitation led us to focus on whether GAs and PCN measured through ctDNA analysis alone can inform us on clinical outcomes and therefore continue to build from prior studies. To investigate the associations between GAs and PCN with specific clinical factors and treatment outcomes, we conducted a retrospective analysis of patients at our institution with mPC who harbor GAs.

Methods

Patient selection

Patients with mPC who were seen at the Medical University of South Carolina's Hollings Cancer Center (Charleston, SC) from March 2015 through March 2020 and underwent ctDNA evaluation using the Guardant360® platform (Guardant Health Inc., Redwood City, CA) were eligible for analysis (21). Eligible patients were de-identified, and demographic, clinical, and corresponding ctDNA data were collected. Patients with one or more GAs (i.e., increase in PCN) were selected for downstream analysis. Our cohort (Figure 1) included mPC patients with a ctDNA-detectable increase in PCN in at least one gene. Collected clinical data included Gleason score at diagnosis, prostate-specific antigen (PSA), hemoglobin (Hgb), metastatic disease present at diagnosis, sites of metastases, castrate-resistant status, Eastern Cooperative Oncology Group (ECOG) performance status, and systemic therapies received prior to collection of plasma for ctDNA analysis. Metastatic sites included bone only, lymph node only, liver/lung only, bone and lymph node, and liver/lung with other sites. Patients who had been diagnosed with mCRPC were divided into two groups. Patients in Group A (Figure 1) had evaluable survival and systemic therapy time points in addition to ctDNA analysis. Overall survival was defined as duration from ctDNA sample collection or treatment start date to study end date or death. Failure-free survival was defined as duration from treatment start date to date treatment was last administered or death. Patients in Group B had detectable *AR* or *MYC* amplification and two serial ctDNA analyses. The conduct of this study was approved by the institutional review board of the Medical University of South Carolina.

ctDNA profiling

Blood samples for subsequent ctDNA analysis were collected from patients at scheduled clinic visits as part of their routine care. Next-generation sequencing (NGS) of plasma ctDNA (i.e., liquid biopsy) was completed as previously described by Guardant Health (Guardant360®), a College of American Pathologists (CAP)-accredited and Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory (21). Briefly, cfDNA was extracted from whole blood collected in 10-mL Streck® tubes using the QIAmp Circulating Nucleic Acid Kit (Qiagen, Inc). Hybrid-capture sequencing libraries were prepared for up to 30ng cfDNA and labeled with non-random oligonucleotide barcodes (IDT, Inc.), followed by library preparation, hybrid capture enrichment (Agilent Technologies, Inc.), and sequencing at 15,000X read depth of the critical exons in the targeted panel by paired-end synthesis (NextSeq 500 and/or HiSeq 2500, Illumina, Inc.). Bioinformatics analysis and variant detection were performed as previously described (22). NGS data were interpreted by N-of-One, Inc. (Lexington, MA). Over the course of the study, the panel composition expanded from 68 to 70 to 73 to 74 genes. In the 68-gene panel, coverage of gene amplifications included 16 genes. The 74-gene panel includes coverage of 18 gene amplifications. Most

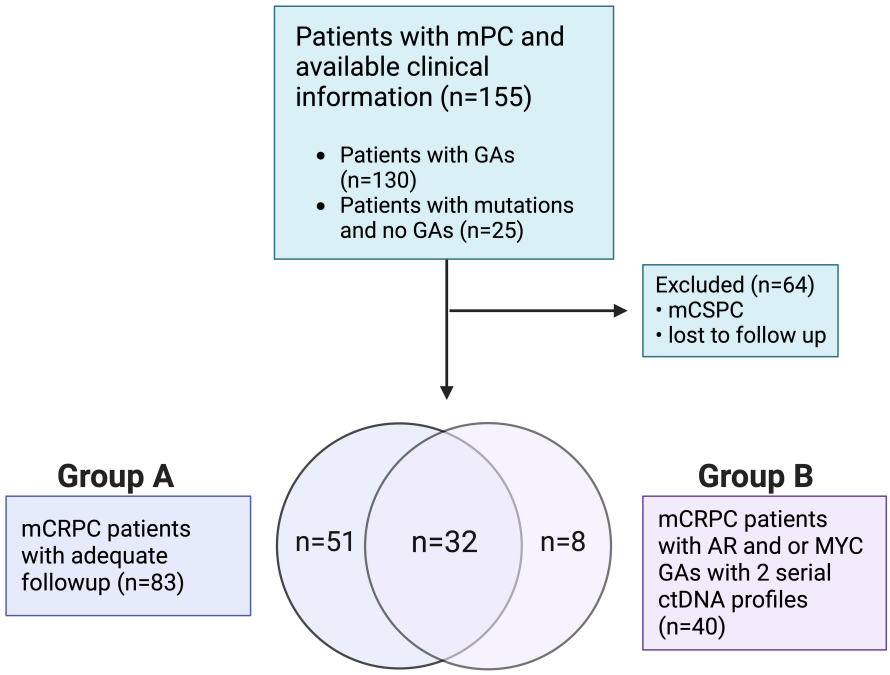

FIGURE 1
Retrospective study design flowchart.

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samples in this study were tested using the 74-gene panel. All gene amplifications analyzed for this study were sufficiently covered by all iterations of the Guardant ctDNA assay during the study period.

Statistical analyses

Descriptive statistics were used to summarize all baseline patient characteristics, frequency of GAs, and missense/frameshift mutation frequencies. Chi-square analyses and Odds Ratios were used to evaluate for associations between select clinical factors and GAs. The Kaplan-Meier method was used to estimate OS and FFS outcomes in patients with mCRPC from the time points indicated to either the end of the study (OS), final administration of treatment (FFS), or death (OS, FFS). Survival analyses for patients in Group A were stratified by the presence of one or more than one GA at the time of initial GA detection. Univariable Cox proportional hazards regression was used to identify associations of clinical variables and GAs with OS and FFS. These clinical variables included age, race, metastases at the time of diagnosis, PSA, Hgb, ECOG performance status, metastatic sites, therapy administered prior to ctDNA profiling, and select GAs. Systemic therapies evaluated included abiraterone, enzalutamide, docetaxel, cabazitaxel, carboplatin, sipuleucel-T, and radium-223 in which a p-value less than or equal to 0.2 was used as the initial variable selection criteria. Then multivariable Cox regression models were fit in which a forward variable selection approach was used to generate the final clinical factors which have significant impact on outcomes. Patients in Group B were categorized as either having a "Response" or "No Response". The term "Response" was defined as having a 10% reduction in *AR* PCN in the ctDNA analysis completed after the initial detection of *AR* amplification. If these variables remained the same or increased, patients were categorized as having "No Response". Statistical tests were 2-sided with significance defined as $p \leq 0.05$.

Results

Study design and patient characteristics

Among mPC patients seen at our institution, a total of 130 out of 155 tested patients had at least one GA (Figure 1). Baseline clinical variables of the study-eligible patient population are detailed in Table 1 and are reflective of the time of ctDNA analysis and initial GA detection. The median age was 71 years (range 46-91 years), and 36.8% of patients were African American. Almost three-quarters of patients (72.9%) had a Gleason score of 7-10, and the median PSA was 46.2 ng/ml (range 0.1-6000 ng/ml). Nearly half of the patients had metastasis at diagnosis (42.6%). Most patients had mCRPC (87.1%). Select systemic therapies included the androgen axis-targeting drugs abiraterone (47.1%) and enzalutamide (43.9%) and chemotherapy such as docetaxel (44.5%), cabazitaxel (22.6%), and carboplatin (14.2%). Of note, carboplatin was administered in

TABLE 1 Baseline characteristics of the mPC cohort.

CHARACTERISTIC	STATISTIC	RESULT
Age, y	Mean (SD)	70.5 (8.3)
	Median (range)	71 (46-91)
Race	No. (%)	
Caucasian	No. (%)	94 (60.6)
African-American	No. (%)	57 (36.8)
Other	No. (%)	4 (2.6)
GS at diagnosis	No. (%)	
5	No. (%)	1 (0.6)
6	No. (%)	10 (6.5)
7	No. (%)	32 (20.6)
8	No. (%)	20 (12.9)
9	No. (%)	46 (29.7)
10	No. (%)	15 (9.7)
Unknown	No. (%)	31 (20.0)
Metastasis at diagnosis	No. (%)	66 (42.6)
CRPC	No. (%)	135 (87.1)
PSA, ng/ml	Mean (SD)	338.3 (949.0)
	Median (range)	46.2 (0.1 -6000)
HGB, g/dl	Mean (SD)	11.3 (2.2)
	Median (range)	11.5 (6.4 - 16.0)
ECOG Performance Status	No. (%)	
0	No. (%)	46 (29.7)
1	No. (%)	79 (51.0)
2	No. (%)	18 (11.6)
3	No. (%)	10 (6.4)
Unknown	No. (%)	2 (1.3)
Metastatic Sites	No. (%)	
Bone	No. (%)	141 (91.0)
LN/Soft Tissue	No. (%)	104 (67.1)
Liver/Lung	No. (%)	34 (21.9)
Bone + LN/Soft Tissue	No. (%)	92 (59.4)
All Sites	No. (%)	21 (13.5)
Prior Systemic Therapy	No. (%)	
Abiraterone	No. (%)	73 (47.1)
Enzalutamide	No. (%)	68 (43.9)
Docetaxel	No. (%)	69 (44.5)
Cabazitaxel	No. (%)	35 (22.6)
Carboplatin	No. (%)	22 (14.2)

(Continued)

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TABLE 1 Continued

CHARACTERISTIC	STATISTIC	RESULT
Sipuleucel-T	No. (%)	24 (15.5)
Radium-223	No. (%)	16 (10.3)

combination with either docetaxel or cabazitaxel and not as a single agent. Sipuleucel-T and Radium-223 were also administered in 15.5% and 10.3% of patients, respectively.

Association between certain GAs and visceral metastases

At the time of initial detection of at least one GA, the most frequently occurring GA was in *AR* (Figure 2A; 59.2%), followed by *MYC* (29.2%), *BRAF* (27.7%), *CDK6* (22.3%), *PIK3CA* (21.5%), and *MET* (20.0%). Given the relative genomic positions of *BRAF* (7q34), *CDK6* (7q21.2), and *MET* (7q31.2) genes on chromosome 7, these GAs frequently co-occur. For instance, 69% of *CDK6* GAs co-occur with *BRAF* GAs, and 88.5% of *MET* GAs co-occur with *BRAF* and/or *CDK6* GAs. Of the GAs, *AR* had the largest range in plasma copy number (PCN) (1.2 – 35.4), but a median PCN of 2.03 (Figure 2B). Of all other identified GAs occurring in at least 10% of patients, the median PCN ranged from 2.37 (*RAF1*) to 2.87 (*FGFR1*) (Figure 2B).

We also evaluated the frequency of GAs co-occurring with select mutations. *TP53* was the most frequently mutated gene (Figure 2C). Interestingly, despite *AR* amplification being the most frequently occurring GA, patients harboring *TP53* mutations had a greater frequency of concurrent *MYC* and *BRAF* amplifications. Furthermore, patients with mPC with *APC* mutations rarely had the two most frequently occurring GAs, *AR* and *MYC*.

In patients with mPC, liver and/or lung metastases are associated with poorer prognosis and may be indicative of more aggressive mPC subtypes such as neuroendocrine prostate cancer (NEPC) (19, 23). We evaluated whether GAs that occurred in at least 10% of patients were associated with the incidence of liver/lung metastases present at the time of initial GA detection (Figure 2D). Liver/lung metastases were found to be significantly associated with amplification of *BRAF* (OR 2.51, 95%CI 1.09 – 5.81), *CDK6* (OR 3.23, 95%CI 1.36 – 7.93), *PIK3CA* (OR 4.37, 95%CI 1.79 – 10.67), and *FGFR1* (OR 3.13, 95%CI 1.1 – 8.95). *CDK6* is frequently co-amplified with *BRAF*, given the relative proximity of its genomic position. Thirty-three percent of patients with *CDK6* GA without co-occurring *BRAF* GA had liver/lung metastases.

Increased number of GAs associated with patient survival

From patients in this cohort, we then identified 83 patients with mCRPC and clinically evaluable complete treatment records (Group A, Figure 1). Patients were stratified by whether they had no GAs, one GA, or two or more GAs at the time of ctDNA analysis. There was no statistically significant difference in the mean number of therapies prior to ctDNA analysis amongst these three stratified groups. This analysis demonstrated that patients with two or more

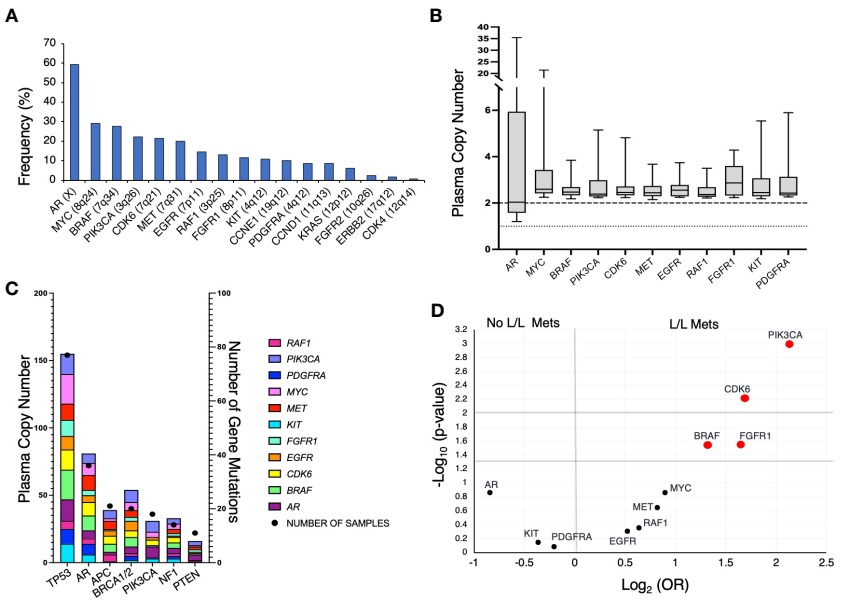

FIGURE 2
(A) Frequency of genomic amplifications (GAs) in our mPC cohort at time of initial identification of increased gene plasma copy number. **(B)** Distribution of PCN in GAs present in at least 10% of patients. Dotted line represent PCN of 1 to illustrate baseline for *AR* and dashed line at PCN of 2 to represent baseline for all described other genes. **(C)** Frequency of mutations and co-occurring GAs. X-axis represents the commonly occurring mutations in cohort A, left Y-axis represents the frequency of GAs, and right Y-axis represents the number of samples that harbored the specified gene mutation listed on X-axis. **(D)** Volcano plot depicting GAs present in at least 10% of patients and association with liver/lung metastases (L/L Mets).

identifiable GAs had a poorer OS from the time of initial GA detection than those with only one GA. Median OS was not reached for patients with no GAs, was 16.4 months for patients with only one GA, and 9.4 months for patients with ≥2 GAs (Figure 3). Survival analysis was extended to include OS and FFS from the date of systemic treatment initiation nearest to the date of ctDNA analysis. OS and FFS were poorer in patients with ≥2 GAs than in those with only one or no GA. Median OS in patients with one GA was almost double that in patients with ≥2 GAs (16.7 vs. 9.0 months; p-value < 0.001) (Figure 4A). Median OS in patients with no GAs was not reached at the time of analysis. Median FFS was significantly different when comparing patients with 1 (4.3 months), or with ≥2 GAs (3.3 months), to patients with no GAs (median OS not reached, p-value < 0.001) (Figure 4B).

As demonstrated above, the majority of detected GAs were in *AR* (Figure 2A). Given our prior work demonstrating that presence of an *AR* GA being associated with worse prognosis (11), we next determined whether *AR* plus at least one other GA (designated as "*AR* Plus") had a difference in OS compared to patients having exclusively an *AR* GA. Patients with only an *AR* GA have a significantly better median OS compared to patients with "*AR* Plus" GAs (19.3 vs 8.9 months; p-value < 0.001) (Figure 5).

Cox regression univariate analysis of group A patients indicates that reduced OS may be present with amplifications in *MYC*, *BRAF*,

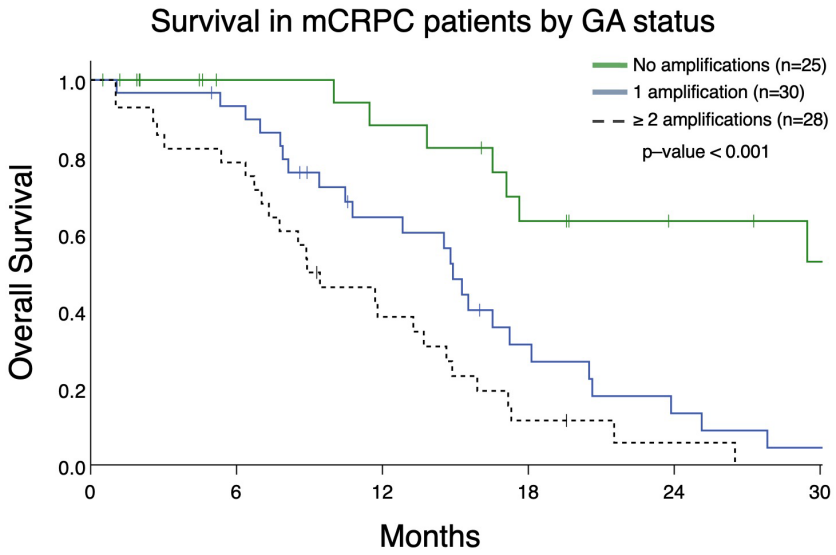

FIGURE 3
Overall survival in patients with mCRPC (Group A) from the date of initial detection by ctDNA assay in patients with no GAs, only 1 GA, or with ≥ 2 GAs. Patients with no GAs had greater survival (median survival not reached) compared to only 1 GA (median survival 14.9 months) or ≥ 2 GAs (median survival 8.9 months; p<0.001).

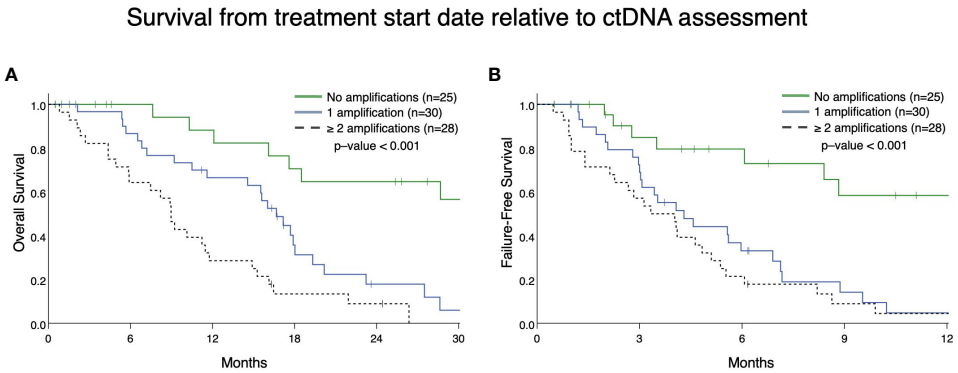

FIGURE 4
Overall survival **(A)** and failure-free survival **(B)** in Group A from the date of the nearest systemic therapy initialization. **(A)** Patients with mCRPC with ≥ 2 GAs had reduced survival (median survival in patients with one GA was 16.7 months vs. 9.0 months in patients with ≥ 2 GAs; p <0.001). Median survival for no GAs was not reached. **(B)** Patients with mCRPC with 1 or ≥ 2 GAs had reduced failure-free survival (median FFS in patients with one GA was 4.3 months vs. 3.3 months in patients with ≥ 2 GAs; p < 0.001) compared to patients with no GAs (median FFS not reached). There was no statistical difference in FFS between patients with only 1 GA compared to ≥ 2 GAs (p=0.28).

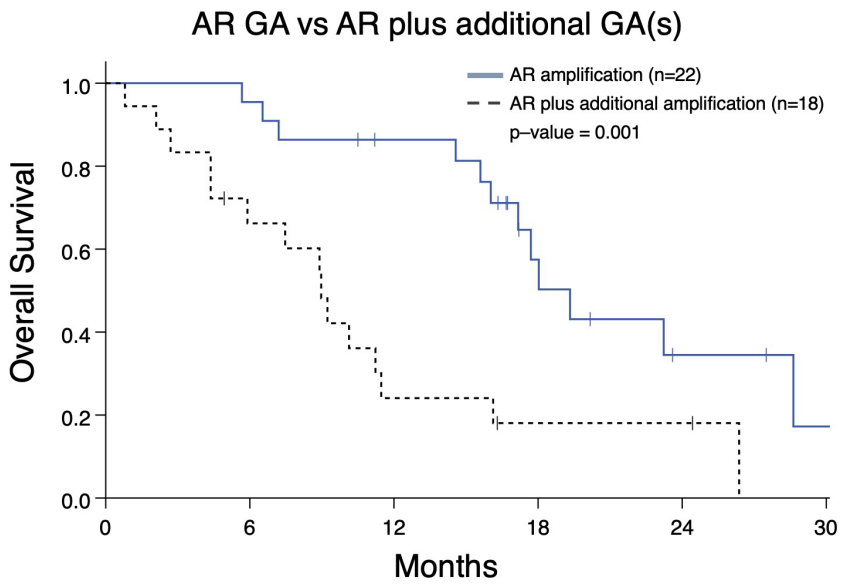

FIGURE 5
Overall survival in mCRPC patients (Group A) from the date of the nearest systemic therapy initialization with that have either an *AR* genomic amplification (GA) only vs patients with an *AR* GA as well as at least one additional GA in another gene. Patients with *AR* GA only had greater median survival (19.3 months) compared to patients with *AR* GA plus at least one GA (median survival 8.9 months; p=0.001).

PIK3CA, *CDK6*, *MET2*, *EGFR*, and *RAF1* (Table 2). However, when adjusted for significant covariates in a multivariate analysis, genomic amplification of *PIK3CA* remains significantly associated with worse survival in the final model (Table 2). Consistent with the Kaplan-Meier survival analysis, patients with one GA (HR 3.84 [1.45 – 10.16], p-value <0.01) and ≥2 GAs (HR 7.15 [2.50 – 20.46], p-value <0.001) were also significant in the final multivariate regression model as well. *PIK3CA* is associated with worse FFS in multivariate analysis as well (Table 3). Abiraterone prior to ctDNA analysis was shown to be prognostic for FFS (Table 3).

In light of *PIK3CA* GA association with increased liver and lung metastases (Figure 2D) and association with worse OS and FFS in multivariate analysis (Tables 2, 3), we determined the impact of a *PIK3CA* amplification on OS when compared to patients with GAs (regardless of number) without *PIK3CA* amplification (Figure 6). mCRPC patients that have at least one GA but lack any *PIK3CA* GA was associated with better OS compared to patients whose disease harbors a *PIK3CA* GA (16.0 vs 5.9 months; p-value < 0.001) (Figure 6). It is worth noting that a majority of patients with *PIK3CA* GA in this group did have at least one other GA as well (data not shown).

Reduction in *AR* and/or *MYC* PCN predicts improved OS

Serial ctDNA profiling permits assessment of PCN changes as they occur during a patient's treatment course. Therefore, we determined whether changes in PCN in patients with two consecutive ctDNA profiles might be prognostic. Analyzing the two most frequently occurring GAs, we identified patients with mCRPC who had *AR* and/or *MYC* GAs with an additional ctDNA profile completed afterwards to gauge PCN change. These patients were categorized as group B (Figure 1). Patients with ≥10% reduction in *AR* and/or *MYC* PCN (termed as "Response") on the second profile had better OS than those with ≤ 10% *AR* and/or *MYC* PCN decrease (termed as "No Response") (median OS, 25.1 months vs. 15.9 months, p = 0.008) (Figure 7).

The heatmap in Figure 8, which details the GAs identified in group B patients and the treatments those patients underwent prior to each ctDNA analysis, demonstrates PCN reduction following a variety of systemic therapies. Androgen axis-targeting agents such as abiraterone and enzalutamide were associated with detectable *AR*/*MYC* PCN reductions in seven out of 40 patients. Taxane-based chemotherapy (with or without carboplatin) resulted in responses in at least 10 patients. Interestingly, two of the responses were in patients who harbored either a *BRCA2* (PtID 152) or *ATM* mutation (PtID 205) and had *AR* PCN reduction following olaparib administration. In one patient who received Radium-223, *MYC* PCN was reduced while the PCN of other GAs increased (PtID 69), suggesting that perhaps the predominant subclone that was metastatic to the bone in this patient harbored the *MYC* GA and therefore had PCN reduction following Radium-223 treatment. One patient with no identifiable AR mutations was observed to have a reduction in the *MYC* GA-containing subclone following administration of the investigational agent TRC253 (PtID 18). In another patient with an identifiable *BRAF* mutation and amplification (PtID 35), *AR* PCN was reduced after administration of trametinib. Overall, a variety of systemic treatments resulted in PCN reduction of the two predominant GAs in this group and were significantly associated with increased survival.

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TABLE 2 Association of select variables in Group A with overall survival as determined by date of systemic treatment initiation nearest to time of ctDNA analysis.

Variable	Categorization	No.	Univariate Analysis		Multivariate Analysis	
			Hazard Ratio (95% CI)	p-value	Hazard Ratio (95% CI)	p-value
Age, y	Continuous	83	0.99 (0.96 – 1.03)	0.88		
Race	Caucasian or African-American	82	0.98 (0.56 – 1.72)	0.94		
Metastasis at diagnosis	Yes or No	83	1.07 (0.60 – 1.89)	0.83		
PSA, ng/ml	Continuous	82	1.0 (1.0003 – 1.0011)	<0.01		
HGB, g/dl	Continuous	79	0.80 (0.69 – 0.92)	<0.01	0.80 (0.69 – 0.93)	<0.01
ECOG Performance Status	≥1 or 0	81	2.54 (1.19 – 5.45)	<0.05		
Metastasis Site						
Bone	Yes or No	83	2.37 (0.74 – 7.62)	0.15		
LN/Soft Tissue	Yes or No	83	1.71 (0.92 – 3.19)	0.09		
Liver/Lung	Yes or No	83	1.31 (0.64 – 2.70)	0.47		
Bone + LN/Soft Tissue	Yes or No	83	1.81 (1.01 – 3.26)	<0.05		
All Sites	Yes or No	83	1.41 (0.60 – 3.32)	0.43		
Prior Systemic Therapy						
Abiraterone	Yes/No (%Yes)	83 (44.6)	1.87 (1.07 – 3.27)	<0.05		
Enzalutamide	Yes/No (%Yes)	83 (44.6)	1.00 (0.57 – 1.75)	0.99		
Docetaxel	Yes/No (%Yes)	83 (50.6)	1.31 (0.74 – 2.31)	0.35		
Cabazitaxel	Yes/No (%Yes)	83 (21.7)	1.95 (1.08 – 3.55)	<0.05		
Carboplatin	Yes/No (%Yes)	83 (18.1)	1.28 (0.66 – 2.51)	0.47		
Sipuleucel-T	Yes/No (%Yes)	83 (18.1)	0.90 (0.44 – 1.87)	0.79		
Radium-223	Yes/No (%Yes)	83 (13.3)	0.87 (0.34 – 2.22)	0.78		
Gene Amplification						
AR	Yes or No	83	1.43 (0.81 – 2.53)	0.22		
MYC	Yes or No	83	2.58 (1.40 – 4.73)	<0.01		
BRAF	Yes or No	83	2.31 (1.23 – 4.34)	<0.01		
PIK3CA	Yes or No	83	5.98 (2.87 – 12.46)	<0.01	3.05 (1.39 – 6.73)	<0.01
CDK6	Yes or No	83	3.24 (1.60 – 6.59)	<0.01		
MET2	Yes or No	83	2.51 (1.30 – 4.87)	<0.01		
EGFR	Yes or No	83	3.53 (1.56 – 8.02)	<0.01		
RAF1	Yes or No	83	2.28 (0.96 – 5.38)	0.06		
FGFR1	Yes or No	83	2.34 (0.84 – 6.54)	0.11		
KIT	Yes or No	83	2.64 (1.03 – 6.80)	<0.05		

Assessed by univariate and multivariate Cox proportional hazards models.
Only variables found to be statistically significant in the final multivariate model have reported hazard ratio and p-value.

TABLE 3 Association of select variables in Group A with Failure-Free Survival as determined by date of systemic treatment initiation nearest to time of ctDNA analysis.

Variable	Categorization	No.	Univariate Analysis		Multivariate Analysis	
			Hazard Ratio (95% CI)	p-value	Hazard Ratio (95% CI)	p-value
Age, y	Continuous	83	1.00 (0.96 – 1.03)	0.73		
Race	Caucasian or African-American	82	1.05 (0.60 – 1.84)	0.87		
Metastasis at diagnosis	Yes or No	83	1.12 (0.63 – 2.00)	0.70		
PSA, ng/ml	Continuous	82	1.00 (1.001 – 1.002)	<0.01	1.001 (1.001 – 1.002)	<0.01
HGB, g/dl	Continuous	79	0.83 (0.72 – 0.97)	<0.05		
ECOG Performance Status	≥1 or 0	81	2.87 (1.34 – 6.15)	<0.01	2.46 (1.14 – 5.33)	<0.05
Metastasis Site						
Bone	Yes or No	83	2.00 (0.62 – 6.42)	0.25		
LN/Soft Tissue	Yes or No	83	1.39 (0.75 – 2.59)	0.30		
Liver/Lung	Yes or No	83	1.41 (0.68 – 2.90)	0.35		
Bone + LN/Soft Tissue	Yes or No	83	1.60 (0.89 – 2.89)	0.12		
All Sites	Yes or No	83	1.48 (0.63 – 3.47)	0.37		
Prior Systemic Therapy						
Abiraterone	Yes/No (%Yes)	83 (44.6)	2.44 (1.37 – 4.34)	<0.01	2.34 (1.26 – 4.37)	<0.01
Enzalutamide	Yes/No (%Yes)	83 (44.6)	0.85 (0.49 – 1.49)	0.58		
Docetaxel	Yes/No (%Yes)	83 (50.6)	1.45 (0.82 – 2.56)	0.20		
Cabazitaxel	Yes/No (%Yes)	83 (21.7)	1.83 (1.01 – 3.33)	<0.05		
Carboplatin	Yes/No (%Yes)	83 (18.1)	1.52 (0.77 – 2.98)	0.22		
Sipuleucel-T	Yes/No (%Yes)	83 (18.1)	0.69 (0.34 – 1.43)	0.32		
Radium-223	Yes/No (%Yes)	83 (13.3)	0.84 (0.33 – 2.12)	0.71		
Gene Amplification						
AR	Yes or No	83	1.41 (0.80 – 2.48)	0.23		
MYC	Yes or No	83	2.26 (1.24 – 4.12)	<0.01		
BRAF	Yes or No	83	2.38 (1.27 – 4.43)	<0.01		
PIK3CA	Yes or No	83	3.62 (1.83 – 7.18)	<0.01	3.03 (1.5 – 6.11)	<0.01
CDK6	Yes or No	83	2.95 (1.46 – 5.94)	<0.01		
MET2	Yes or No	83	2.81 (1.46 – 5.42)	<0.01		
EGFR	Yes or No	83	4.41 (1.98 – 9.84)	<0.01		
RAF1	Yes or No	83	1.83 (0.78 – 4.31)	0.17		
FGFR1	Yes or No	83	3.48 (1.22 – 9.92)	<0.05		
KIT	Yes or No	83	1.82 (0.72 – 4.60)	0.21		

Assessed by univariate and multivariate Cox proportional hazards models.
Only variables found to be statistically significant in the final multivariate model have reported hazard ratio and p-value.

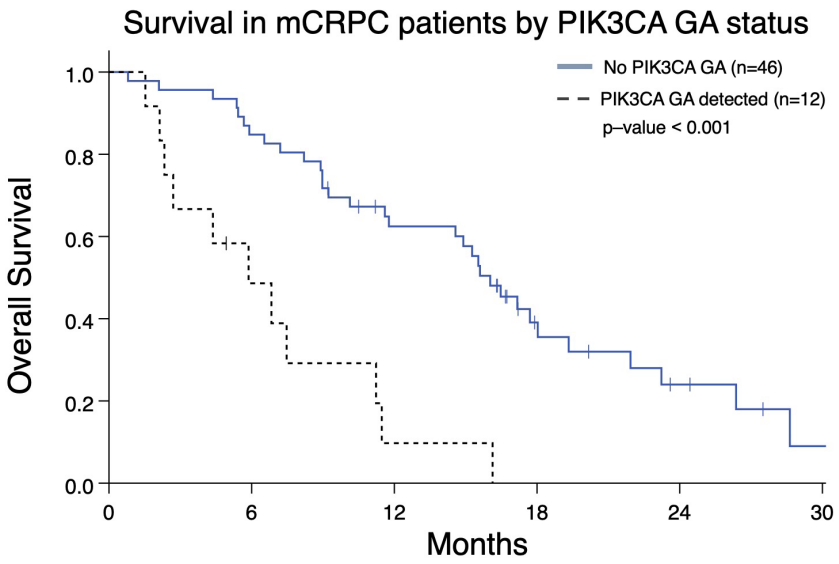

FIGURE 6
Overall survival from the date of the nearest systemic therapy initialization in Group A patients with mCRPC who have at least one GA (n=58) and either also have or lack a *PIK3CA* GA as well. Patients without *PIK3CA* GA have greater median OS (16.0 months) compared to patients with a *PIK3CA* GA (5.9 months; $p < 0.001$).

Discussion

Circulating tumor DNA analysis is increasingly being used in mPC and its role in decision making is being defined. While many commercially available assays examine the presence of single nucleotide variants, few ctDNA assays report GAs. Our data suggest that clinically useful information may be found in the additional analytes. Furthermore, our findings provide a basis for evaluating ctDNA somatic alterations at diagnosis of metastatic disease and throughout the course of treatment, with an emphasis on identifying GAs and continually assessing changes in their PCN. This paradigm of serial ctDNA analyses might be used not only to guide therapeutic decisions but also inform prognosis and serve as a biomarker of treatment response, complementing known disease markers such as PSA. Of note, prior studies have evaluated early changes of ctDNA across solid tumors and found that patients who achieve ≥50% decrease in their ctDNA ratio early in immunotherapy-based treatment as compared to baseline sampling have a superior PFS and OS advantage compared to patients who do not reach a 50% threshold for decrease (24, 25). While our cohort does contain serial sampling, the timepoints of collection are diverse and are not reflective of an early on-treatment change. Thus, we aimed to

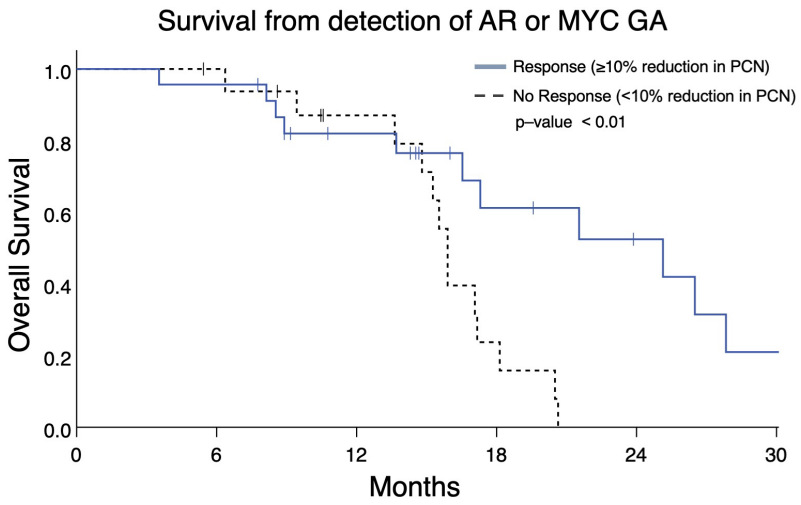

FIGURE 7
Overall survival in Group B patients with ≥10% reduction in *AR* and/or *MYC* PCN ("Response") following initial gene amplification had longer survival vs patients without PCN reduction (median OS 25.1 months vs 15.9 months, $p = 0.008$).

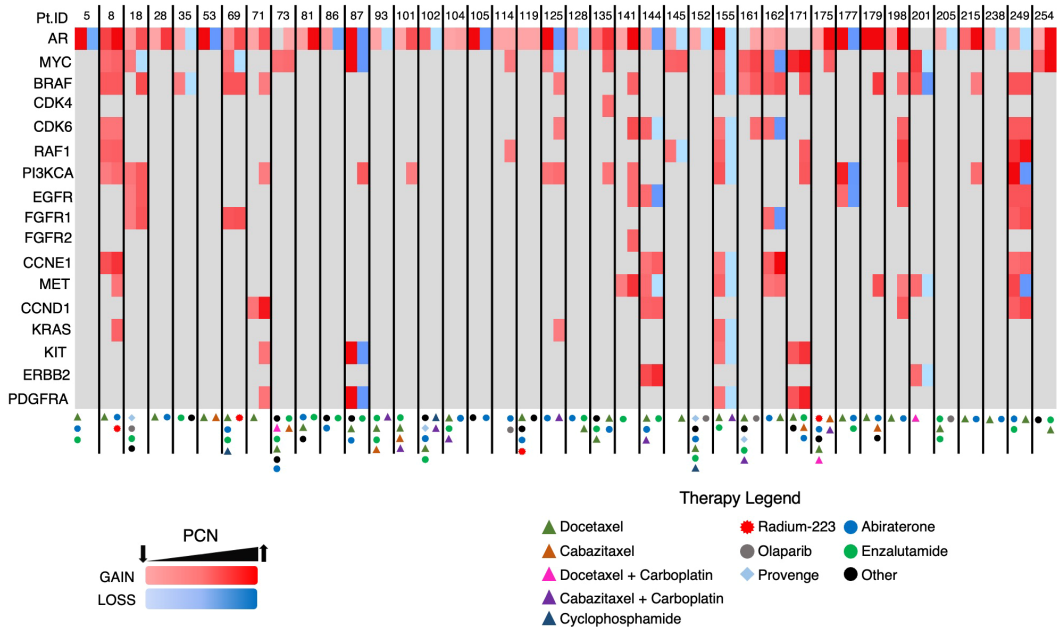

FIGURE 8
Heatmap of GAs in Group B patients. Each patient had two consecutive ctDNA analyses completed. Two columns are depicted for each patient with the initial *AR*/*MYC* detecting ctDNA analysis in the first column and the subsequent analysis in the second column.

demonstrate that any decrease in ctDNA or amplifications in patients with mCRPC, even at a low threshold of 10% reduction, is prognostic of clinical benefit to their therapy.

Previous studies have evaluated the clinical significance of specific mutations and the associations they may have in clonal development and treatment resistance (12–14). Although most patients in prior studies have had detectable co-existing mutations, our study emphasizes evaluation of clinical factors and outcomes in patients stratified based on specific GAs and changes in PCN in prostate cancer. Our approach is supported by the fact that GAs are commonly observed in patients with mPC, particularly when disease is castration-resistant. By identifying specific GAs, ctDNA analysis could prove a valuable management tool for patients seen at various points in their sequential treatment courses.

Inferior outcomes have been associated with alterations in *AR*, such as coding sequence mutations, splice variants, and amplifications (26). With regard to this study, it is important to note findings regarding presence of the number of GAs and which genes may be amplified are nuanced, and need to be evaluated in context (i.e. whether they co-occur with other GAs). For instance, prior work has demonstrated *AR* amplification being associated with reduced FFS and OS (11). However, in our study, "*AR* Plus" patients have worse OS compared to patients exclusively with an *AR* GA (Figure 5). This demonstrates *AR* amplification in the presence of other GAs is associated with worse clinical outcomes. In other words, tumor biology in mCRPC may not be as aggressive in a patient with only an *AR* amplification compared to those whose malignancy has multiple GAs that include *AR* (such as *MYC*, *BRAF*, *PIK3CA*, and others).

Increased numbers of GAs are seen in patients with *TP53* mutations (Figure 2C). This is consistent with *TP53* mutations being associated with increased aneuploidy frequency in malignancies (27, 28). Previous studies have identified putative associations and roles for *BRAF*, *PIK3CA*, and *FGFR1* in mCRPC (17, 29, 30). The association we found between *BRAF* or *PIK3CA* GAs, and liver or lung metastases, further prioritizes the need to identify potential treatment options for patients with these GAs (Figure 2D). This also corresponds to reduced OS seen in patients with *PIK3CA* amplification relative to other patients with GAs (Table 2 and Figure 6). A recent report from our group demonstrated CEA elevation is associated with a number of amplifications including *PIK3CA* (19). However, this was not associated with aggressive variant prostate cancer phenotype (19). Further evaluation regarding the biological input of *PIK3CA* amplification is needed.

Our study identified a high frequency of *BRAF* amplifications compared to the TCGA database. We feel this reflects the advanced disease of the subjects evaluated in this study. As multiple genes are co-amplified with *BRAF* such as *CDK6, EGFR*, and *MET* (also located on chromosome 7), this gives confidence that we are in fact detecting true *BRAF* amplifications. Related to this, we found that *CDK6* amplification was significantly associated with liver/lung metastases; however, it remains to be seen whether this association reveals a significant biological role for *CDK6* in disease pathogenesis or treatment. Finally, we observed an association between *FGFR1* amplification and liver/lung metastases (Figure 2D). Pre-clinical studies have demonstrated that *FGFR1* is involved in prostate cancer progression and has a role in the metabolic reprogramming of prostate cancer cells (29, 30).

Serial ctDNA analyses revealed the emergence of GAs in multiple patients following systemic therapy (Figure 8, PtID's 71,

101, 114, 135, and 175). In fact, some patients developed new GAs even after a reduction in *AR* and/or *MYC* PCN (i.e. PtID's 18, 69, 87, and 125). The latter phenomenon is likely reflective of tumor heterogeneity, with certain clones/subclones sensitive to treatment resulting in PCN reduction, while new subclones then emerge that are resistant to treatment. In the copy number analysis performed on the STAMPEDE patient cohort, sequencing was performed on multiple regions of the same prostate tumor, and intratumoral differences in GA burden, indicating increased heterogeneity, was associated with increased metastatic potential (18).

This study has a number of limitations. It is a single-institution retrospective study intended to be hypothesis-generating in nature and as such does not capture the breadth of geographic differences in patient clinical accessibility, outcomes, and provider bias for ctDNA testing and treatment recommendations. Furthermore, this study had a modest sample size and does not represent the full extent of tumor genomic heterogeneity among patients. Although 155 patients with mPC were evaluated in this cohort, the survival analysis was limited to the 91 subjects in Group A and Group B, (Figure 1), who had mCRPC and adequate treatment records for the entire disease course (Figures 3, 4, 7). Provider bias in the use or sequencing of abiraterone could have affected our finding that it was prognostic for FFS. Although the gene panel used in this study is meant to capture a wide variety of somatic alterations relevant to numerous malignancies, it evaluates a modest number of genes, ranging from 68-74. With regard to mPC, this panel did not evaluate somatic alterations such as *AR* variant 7 splice site changes, *SPOP* mutations, and *PTEN* loss (31, 32). Therefore, these are beyond the scope of this study. Moreover, in the analysis completed in group B, there is variability among patients in the timing between ctDNA analyses. This variability also extends to the timing and variety of systemic treatments administered prior to initial *AR*/*MYC* GA detection and between ctDNA analyses. Future work in controlled trial formats should investigate PCN changes in relevant GAs.

Nonetheless, this study provides significant support for the wider use of ctDNA evaluation to guide prognosis and mPC treatment selection. Additionally, it reinforces the role for serial monitoring of ctDNA to characterize changes in GAs and other somatic alterations. Ultimately, this raises the question whether ctDNA information should be incorporated into decision making regarding early treatment options. For instance, should mPC patients receive more aggressive initial systemic therapy such as the addition of docetaxel to ADT plus androgen axis targeting therapies (i.e. darolutamide or abiraterone as per the ARASENS or PEACE-1 studies) if they harbor GAs that are linked to increased likelihood of visceral metastases (33, 34)? Ongoing prospective clinical studies are evaluating the impact of pretreatment ctDNA evaluation on therapeutic decisions and clinical outcomes in metastatic disease. Additionally, it remains unknown whether specific amplifications are associated with increased expression of their respective gene products and enhanced downstream signaling of relevant oncogenic pathways. If such an association exists, protein products of select GAs would make attractive therapeutic targets.

Findings from our retrospective study demonstrate that GAs, as detected by ctDNA analysis, may play a significant role in informing potential risk for visceral metastases and overall prognosis in mCRPC. Furthermore, evaluation of serial PCN during mCRPC treatment provides an additional tool to determine putative responses to a variety of therapeutic interventions and, as such, can supplement clinical decision-making. Future studies are needed to determine the prognostic significance of specific GAs and the reliability of using PCN as a clinical tool to assess treatment responses.

Data availability statement

The datasets presented in this article are not readily available because of patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Medical University of South Carolina IRB. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

TD contributed to conceptualization, data analysis, study design, and was the primary manuscript writer/editor. ML contributed to conceptualization, data analysis, study design, and manuscript writing/editing. HL performed formal statistical analysis and manuscript review/editing. JK, LD, and AG contributed to data analysis, and manuscript writing/editing. TG and PH contributed to manuscript review/editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

LD is an employee of Guardant Health with stock and ownership interests in the company. AG is on the advisory board for Pfizer.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

Publisher's note

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