pISSN 1598-2998, eISSN 2005-9256

https://doi.org/10.4143/crt.2022.1557 Cancer Res Treat. 2023;55(3):969-977

Original Article

Early Plasma Circulating Tumor DNA as a Potential Biomarker of Disease Recurrence in Non-metastatic Prostate Cancer

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Purpose In non-metastatic prostate cancer (nmPCa) setting, it is important to early identify the patients at risk of biochemical recurrence (BCR) for immediate postoperative intervention. Our study aimed to evaluate the potential clinical utility of circulating tumor DNA (ctDNA) for predicting disease recurrence.

Materials and Methods This real-world observational study evaluated 161 cases of nmPCa undergoing next-generation sequencing at our institution. A total of 139 ctDNA samples and 31 biopsied tumor tissue underwent genomic profiling. The study endpoint was BCR after radical prostatectomy. Relationships between the ctDNA status and the biochemical progression-free survival (bPFS) were analyzed by log-rank test and multivariate Cox regression.

Results Of 161 enrolled patients, 19 (11.8%) harbored deleterious alterations in *NCOR2*, followed by *BRCA2* (3.7%), *ATR* (2.5%), and *CDK12* (2.5%). Of available pre-operative blood samples (n=139), ctDNA was detectable in 91 (65.5%). Until last follow-up, 56 of 68 patients (85.3%) with detectable ctDNA had achieved BCR, whereas only eight of 39 patients (20.5%) with undetectable ctDNA had achieved BCR. Patients who had undetectable ctDNA experienced significantly longer bPFS compared with those who had detectable ctDNA (not available vs. 8.2 months; hazard ratio, 0.14; $p < 0.01$). Pre-operative ctDNA status was a significant prognostic factor of disease recurrence.

Conclusion Pre-operative ctDNA detection could identify patients at high risk of recurrence and has the potential to inform immediate postoperative interventions, but these approaches remain to be validated in prospective studies. ctDNA studies can provide insights into accurate monitoring and precise treatment rather than simply following routine clinical care.

Key words Circulating tumor DNA, Prostatic neoplasms, Biomarkers

Introduction

Prostate cancer (PCa) accounts for more than 20% of newly diagnosed cancer cases and continues to be the second cause of cancer death in American men in 2020 [1]. Although PCa is only the fourth most prevalent malignancies in Chinese men, the total number of Chinese patients is still large owing to the huge population size and aging society [2]. Attributed to the adoption of early screening, the trend of clinical stage migration towards localized PCa has been witnessed, resulting in the survival improvement in Chinese patients [3].

Nonetheless, of the patients with localized PCa undergoing radical prostatectomy (RP) or radiotherapy, around 27% and 53% will develop a rising prostate specific antigen (PSA) which is defined as biochemical recurrence (BCR) [4]. Once BCR has been confirmed, the disease is at an overwhelmingly increased risk of incurable distant metastases and overall mortality [5,6]. Hence it is of great importance to early identify the patients at risk of BCR for timely intervention.

Circulating tumor DNA (ctDNA) shed by tumor cells into bloodstream is highly specific and sensitive to reflect the genomic profile of the tumor without invasive biopsies [7-9]. Multiple recent studies have validated the prognostic value of ctDNA in predicting clinical outcomes and monitoring treatment response across distinct solid malignancies [10-15]. Especially, the detection of ctDNA in the perioperative period is strongly associated with increased risk of disease recurrence and progression [16,17]. However, it still remains unknown if the early detection of ctDNA before primary tumor surgery could predict the disease recurrence in PCa.

Thus, in this work, we reported the results of a biomarker study in patients with non-metastatic PCa (nmPCa), where

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Received November 28, 2022 **Accepted** February 27, 2023
Published Online March 2, 2023

the main objective was to elucidate the association between the detection of pre-operative ctDNA and the risk of disease recurrence.

Materials and Methods

1. Patients and samples

We conducted a retrospective study of 161 patients with nmPCa, who were treated at the Ren Ji Hospital (Shanghai, China). The study was approved by the Committee for Ethics of Ren Ji Hospital (approval number: KY2019-081) and informed consent was obtained from each patient. Peripheral blood samples were collected before surgical intervention from 139 patients. Biopsied tumor tissue samples were collected from 31 patients. All the samples were collected at the time of diagnosis and all the studied patients were treatment-naïve. As the sequencing platforms updated over time, two different multigene panels were included in the present study. Two different platforms had the same detection sensitivity, which was validated in our previous study [18].

2. Targeted gene sequencing and bioinformatics

Targeted gene sequencing of all collected samples was performed at GloriousMed Clinical Laboratory (Shanghai) Co., Ltd. Sequence data analysis, including identification of germline mutation, somatic mutation, copy number variant, and quality control, were performed as described in Supplementary Methods. Deleterious alterations were called when they were nonsense/stop-gains, frameshift insertions and deletions, and ±1, 2 splice-site variants, or were previously reported as pathogenic or likely pathogenic in the ClinVar database.

3. ctDNA fraction estimating

The mutant allele fraction (MAF) was first calculated using the somatic mutation profile from the sequencing data, followed by a correction model [19]. ctDNA fraction was defined as=2/(1/MAF+1) in diploid chromosomes because the MAF and ctDNA fraction are related: MAF=(ctDNA×1)/[(1−ctDNA)×2+ctDNA×1]. ctDNA detectable was defined as a ctDNA fraction > 0%.

4. The measurement of time to disease recurrence and the comparison of alteration frequency

The study endpoint was postoperative BCR. BCR was defined according to the European Association of Urology (EAU) Guidelines on Prostate Cancer (2017 edition): following RP, BCR is defined by two consecutive rising PSA values > 0.2 ng/mL [4]. Biochemical progression-free survival (bPFS) was defined as the time from initial RP to BCR. To evaluate the differences in genomics across nmPCa to metastatic PCa (mPCa), we compared the alteration frequency among the studied patients with ctDNA fraction > 2.0% with our previously published mPCa cohort [20].

5. Statistical analysis

All statistical analyses were completed using R ver. 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria). Clinical characteristics were summarized by different cohorts using descriptive statistics. The Kaplan-Meier method was used to estimate the bPFS of different groups of patients, and differences between groups were analyzed using the log-rank test in the survival package (v.2.44.1.1). Univariate and multivariate Cox regression analysis were used to calculate the hazard ratios (HR) and 95% confidence intervals (CIs). Only factors significant in univariate analysis were included in the subsequent multivariate analysis.

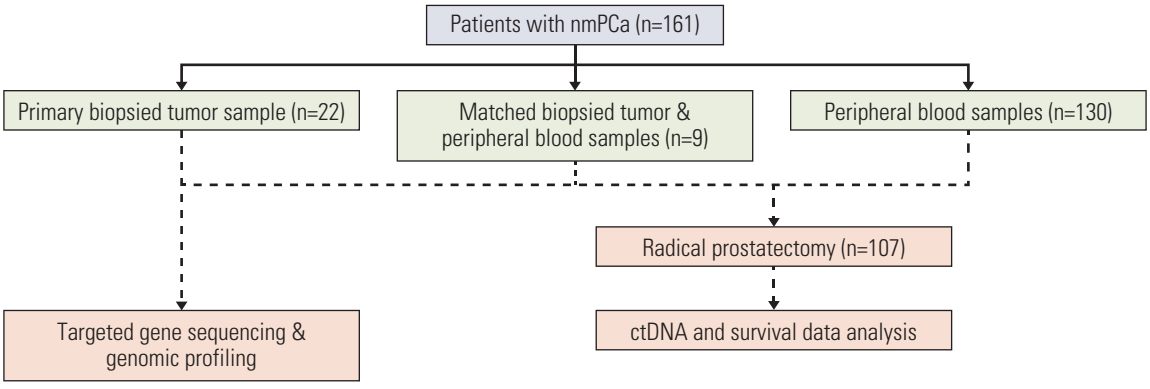

Fig. 1. The schema of the study. ctDNA, circulating tumor DNA; nmPCa, non-metastatic prostate cancer.

Table 1. Pre-operative clinicopathological characteristics of the studied patients with ctDNA analysis

Characteristic	nmPCa with ctDNA analysis (n=139)
Age (yr)	
Median (IQR)	66 (61.5-70.5)
PSA (ng/mL)	
0-20	63
20-100	47
> 100	20
Unknown	9
Gleason score	
6-7	72
8-10	61
Unknown	6
Clinical T category	
T1-2	93
T3-4	41
Unknown	5
Clinical N category	
N0	102
N1	32
Unknown	5
Radical prostatectomy	
Yes	107
No	32
Neoadjuvant therapy	
Yes	73
No	34
ctDNA fraction	
Median±IQR (%)	1.81 (0-3.18)

ctDNA, circulating tumor DNA; IQR, interquartile range; nmPCa, non-metastatic prostate cancer; PSA, prostate specific antigen.

Results

1. Patients' clinicopathological features

We conducted a retrospective genomic analysis on prospectively collected peripheral blood samples and biopsied tumor tissue samples from 161 patients with nmPCa involved in the present study. The schema of the study was presented in Fig. 1. At the time of writing, 107 of the 139 ctDNA sequenced patients received RP at the Ren Ji Hospital, of which 73 with high-risk PCa had received neoadjuvant hormonal therapy involving upfront subcutaneous injection of leuprorelin/goserelin and oral bicalutamide for a 3-month period. The remaining 32 of the 139 ctDNA sequenced patients were pragmatically addressed to active surveillance, hormonal therapy, or radiotherapy based on clinicians' judgment, which were excluded from the post-prostatectomy survival analysis. The clinicopathological features of the studied patients with ctDNA analysis were summarized in Table 1.

2. The genomic profiles of the studied patients

We successfully sequenced 139 ctDNA samples and 31 biopsied tumor tissue samples, among which nine patients had ctDNA and concurrent biopsied tumor tissue samples available. The genomic landscape of all the studied patients was shown in Fig. 2A. Among the patients with ctDNA samples, 91 (65.5%) had detectable ctDNA and 61 (43.9%) had ctDNA fraction > 2.0%. In the population as a whole, *NCOR2* (11.8%, n=19) was the most frequently altered gene, followed by *BRCA2* (3.7%, n=6), *ATR* (2.5%, n=4), and *CDK12* (2.5%, n=4). Among the nine patients with matched samples, except that three patients had undetectable ctDNA and did not have any deleterious alteration in tumor tissue samples, no shared alteration was found in ctDNA samples and tumor tissue samples of the remaining six patients (Fig. 2B). We compared the genomic landscape of deleterious alterations of our nmPCa cohort (n=161) to that of The Cancer Genome Atlas (TCGA) primary cancer cohort (n=333) [21]. As shown in S1 Fig., the alteration frequencies of *ATM* and *TP53* were significantly lower in our nmPCa cohort compared to the TCGA cohort (*ATM*, 0.62% vs. 3.90%, p=0.043; *TP53*, 1.86% vs. 6.91%, p=0.018), while other commonly altered genes showed similar frequencies.

3. The comparison of alteration frequencies between patients with nmPCa and mPCa

To further evaluate the genomic features in nmPCa, we generated the data set that contains genomic profiles of patients with ctDNA fraction > 2.0% across three distinct clinical states including nmPCa, metastatic castration-sensitive PCa (mCSPC), and metastatic castration-resistant PCa (mCRPC). Remarkably, we noticed that patients with nmPCa displayed lower mutation burden, whereas those with mPCa displayed much higher (Fig. 2C). *RB1* alteration was absent in the nmPCa cohort compared with 7.3% in mCSPC cohort and 7.9% in mCRPC cohort. *AR* alteration was obviously enriched in mCRPC cohort. The other genes including *FOXA1*, *SPOP*, *BRCA2*, *CDK12*, *TP53*, and *PTEN* were more frequently altered in both mCSPC cohort and mCRPC cohort.

4. The association between the clinicopathological features and ctDNA status

The overview of the clinicopathological features of the studied patients according to ctDNA status was shown in S2A Fig. The proportion of detectable ctDNA samples seemed to be higher among the patients with high Gleason score compared with those with low Gleason score (70.5%

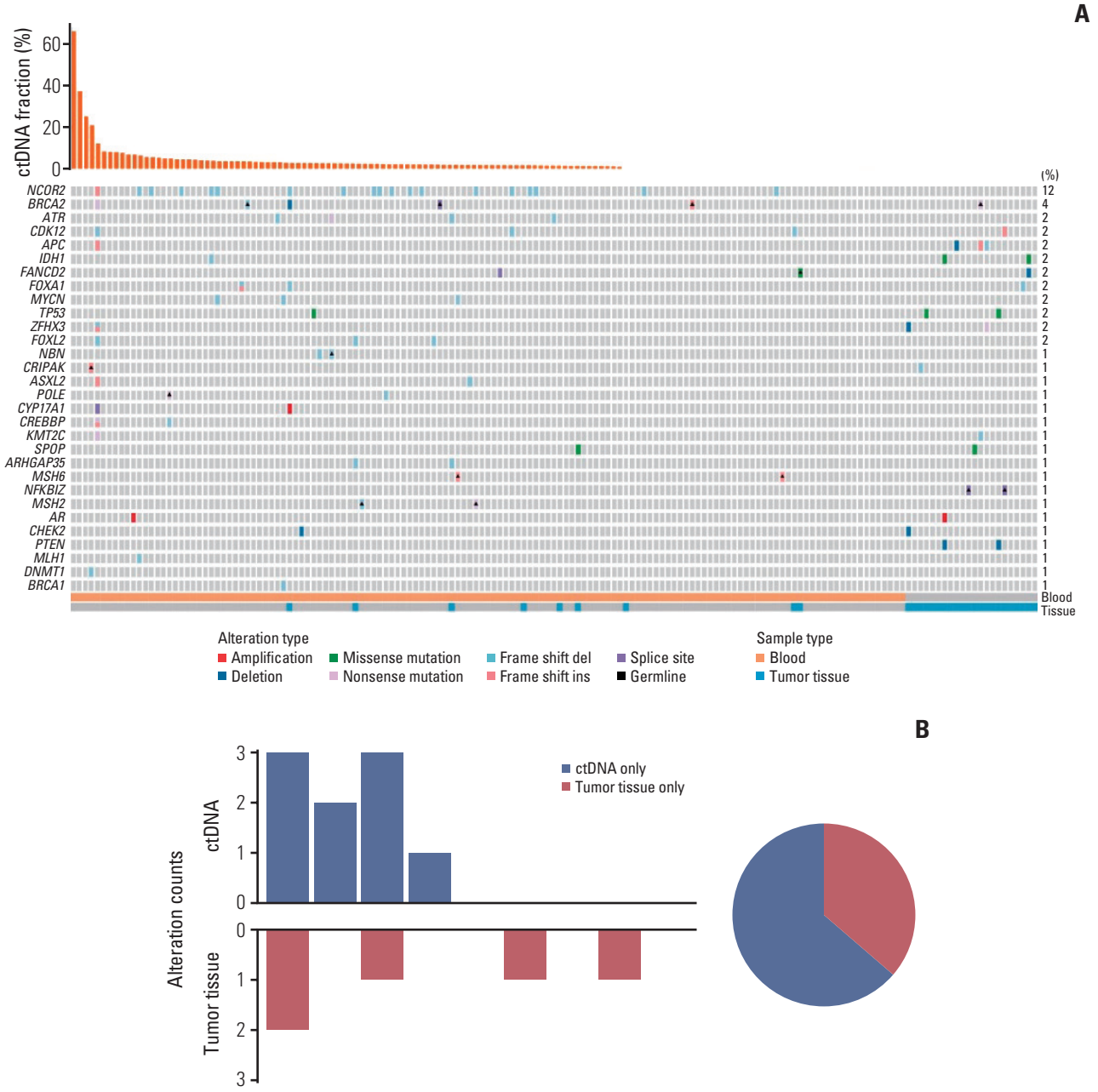

Fig. 2. The genomic profiles of the studied patients. (A) The genomic landscape of the studied patients. Each column represents alterations detected in individual sample. Upper track shows circulating tumor DNA (ctDNA) fractions. Frequencies of specific gene alterations are displayed on the right side. The color represents copy number variant, missense mutation, frame shift indel, nonsense mutation, splice and germline alteration. Cases with multiple variants in one gene are represented by split colors. (B) The somatic alteration count of ctDNA and matched tumor tissue samples in nine patients. *(Continued to the next page)*

vs. 58.3%, p=0.153). Patients at clinical T3-4 category or N1 category tended to have a higher ctDNA detection rate compared with those at clinical T1-2 category (65.9% vs. 62.4%, p=0.846) or N0 category (71.9% vs. 61.8%, p=0.399). The proportion of detectable ctDNA was lower among the patients with low PSA level (S2B Fig.).

5. ctDNA status before treatment is associated with increased risk of BCR

Overall, 107 patients had received RP and their recur-

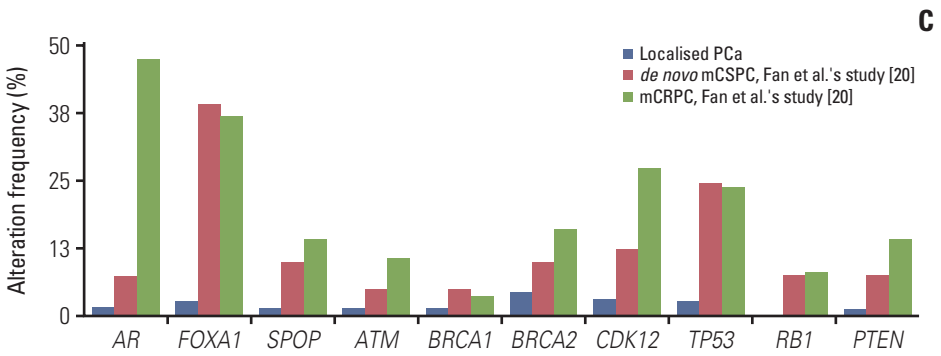

Fig. 2. *(Continued from the previous page)* (C) The comparison of the alteration frequencies between the patients with non-metastatic prostate cancer (PCa) and the patients with metastatic PCa. mCRPC, metastatic castration-resistant PCa; mCSPC, metastatic castration-sensitive PCa.

rence-free survival data according to ctDNA status were illustrated in Fig. 3A. Only eight of 39 patients (20.5%) with undetectable ctDNA had achieved BCR at the time of writing, whereas 56 of 68 patients (85.3%) with detectable ctDNA had achieved BCR. Patients who had undetectable ctDNA experienced significantly longer bPFS compared with those who had detectable ctDNA (not available [NA] vs. 8.2 mo; HR, 0.14; 95% CI, 0.09 to 0.24; p < 0.01) (Fig. 3B). Additionally, patients with or without pathogenic alterations had similar risk of BCR (12.0 vs. 14.1 months, p=0.579) (Fig. 3C).

6. The prognostic value of ctDNA status remains significant across subgroups

In the cohort at clinical T1-2 category, only 4 of 27 patients (14.8%) with undetectable ctDNA had experienced BCR and 36 of 47 patients (76.6%) with detectable ctDNA had relapsed at a median follow-up of 10.0 months (NA vs. 10.0 mo; HR, 0.13; 95% CI, 0.07 to 0.24; p < 0.01) (Fig. 4A). In patients at clinical T3-4 category who had detectable ctDNA, the median bPFS was 4.9 months. In the cohort with undetectable ctDNA, the median bPFS was 28.8 months (HR, 5.7; 95% CI, 2.5 to 13.0; p < 0.01) (Fig. 4B). Similar findings were noted in the cohorts at clinical N0 and N1 category. In the cohort at clinical N0 category, only six of 30 patients (20.0%) with undetectable ctDNA had experienced BCR and 40 of 50 patients (80.0%) with detectable ctDNA had relapsed at a median follow-up of 10.0 months (NA vs. 10.0 months; HR, 0.17; 95% CI, 0.09 to 0.29; p < 0.01) (Fig. 4C). In patients at clinical N1 stage who had detectable ctDNA, the median bPFS was 3.0 months. In the cohort with undetectable ctDNA, the median bPFS was 24.0 months (Fig. 4D). Univariate analysis of six variables was carried out in Table 2. Pre-operative ctDNA fraction as a continuous variate was not a statistically significant predictor (HR, 6.223; 95% CI, 0.842 to 45.967; p=0.073), while ctDNA status (undetectable vs. detectable) was significantly associated with the bPFS (HR, 0.136; 95% CI, 0.064 to 0.287; p < 0.001). HRs for other variables all trended in the expected direction but did not reach statistical significance.

Discussion

This is the first biomarker study that assessed the prognostic value of pre-operative ctDNA in predicting the disease recurrence of patients with nmPCa undergoing radical procedure. Our results revealed the genomic profiles of the patients with nmPCa and found low detection rate of deleterious alterations. Despite the limitations of ctDNA detection in a localized setting, we demonstrated the clinical utility of ctDNA as a reliable tool to reflect the overall mutational status, allowing for monitoring disease progression.

Although the target gene sequencing via liquid biopsy for nmPCa is hampered by the low overall abundance of ctDNA [22], we found that ctDNA was detectable 65.5% of the studied patients, which is consistent with a previous study that has reported the variant detection rate of 57% [23]. Additionally, the clinicopathological features including PSA, Gleason score, and clinical stage were not strongly associated with the ctDNA status in the present study, but the patients with aggressive disease subtypes tended to have detectable ctDNA. Our data might suggest the use of ctDNA analysis in the patients with more aggressive PCa at the time of diagnosis. Specially, we observed that ctDNA samples shared no concurrent alteration with tumor samples. As ctDNA proves to have the potential ability to capture both likely clonal and subclonal alterations from multiple tumor cell populations [23], our results substantially support that ctDNA reflecting the comprehensive mutational status could be used in the evaluation of heterogeneous nmPCa.

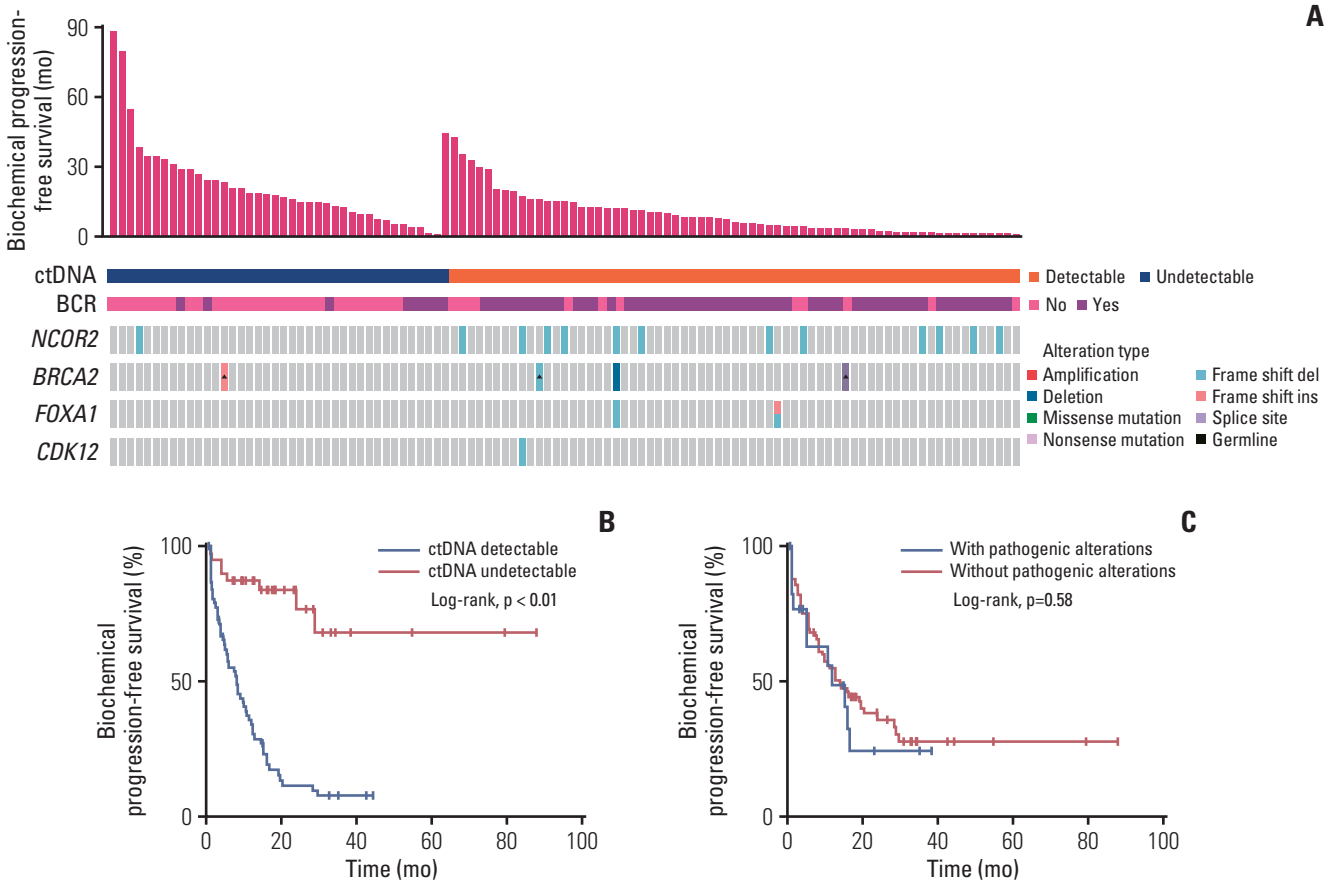

Fig. 3. Circulating tumor DNA (ctDNA) status and clinical outcomes. (A) The overview of the studied patients according to the time to biochemical recurrence (BCR) and ctDNA status. (B) Kaplan-Meier curves for time to BCR of the patients with detectable ctDNA and with undetectable ctDNA. (C) Kaplan-Meier curves for time to BCR of the patients with and without pathogenic alterations.

The landscape of deleterious alterations of our nmPCa cohort was overall similar to that of the TCGA primary PCa cohort. However, the frequencies of *ATM* and *TP53* were significantly lower in our cohort. Ethnic differences may contribute to these results, as over 80% of the TCGA cohort are Caucasians while our cohort consists of Chinese patients [21]. In line with our results, a report of genomic and epigenomic analyses in 208 Chinese primary PCa patients revealed a relatively lower frequency of *TP53* and *ATM* copy number variations compared to Western cohorts [24]. To better understand the genetic landscape in Chinese men with PCa, we compared the distribution of genomic alterations between the nmPCa cohort and the mPCa cohort from our previous study [20]. Interestingly, the genomic profiles of the patients with nmPCa revealed low detection rate of somatic deleterious alterations. In the localized setting, the genes involved in androgen receptor pathway, DNA damage repair pathway, and cell cycle pathway were less frequently altered compared with the mPCa cohort. This is possibly because of the low disease burden and low abundance of DNA shed by tumor, underscoring the distinct genomic basis of indolent and non-indolent disease. Serial studies have demonstrated that numerous genomic alterations in *ATM*, *FOXA1*, and *PTEN* were predictive of disease phenotype and progression [24-26]. However, the predictive power of ctDNA for the disease recurrence in nmPCa setting remains unclear.

A previous study highlighted that the *TP53* ctDNA status was strongly associated with BCR and metastasis [27]. Similarly, our preliminary results suggest a potential role of pre-operative ctDNA as a predictive biomarker for disease recurrence. In the present study, 85.3% of patients with detectable ctDNA have experienced BCR at a median follow-up of 8.2 months after RP regardless of the neoadjuvant treatment. This might suggest that pre-operative ctDNA is enough informative to filter out the population at an increased relapse risk where immediate postoperative therapy should be considered and individualized neoadjuvant therapy could be explored. Consistent with multiple ctDNA studies

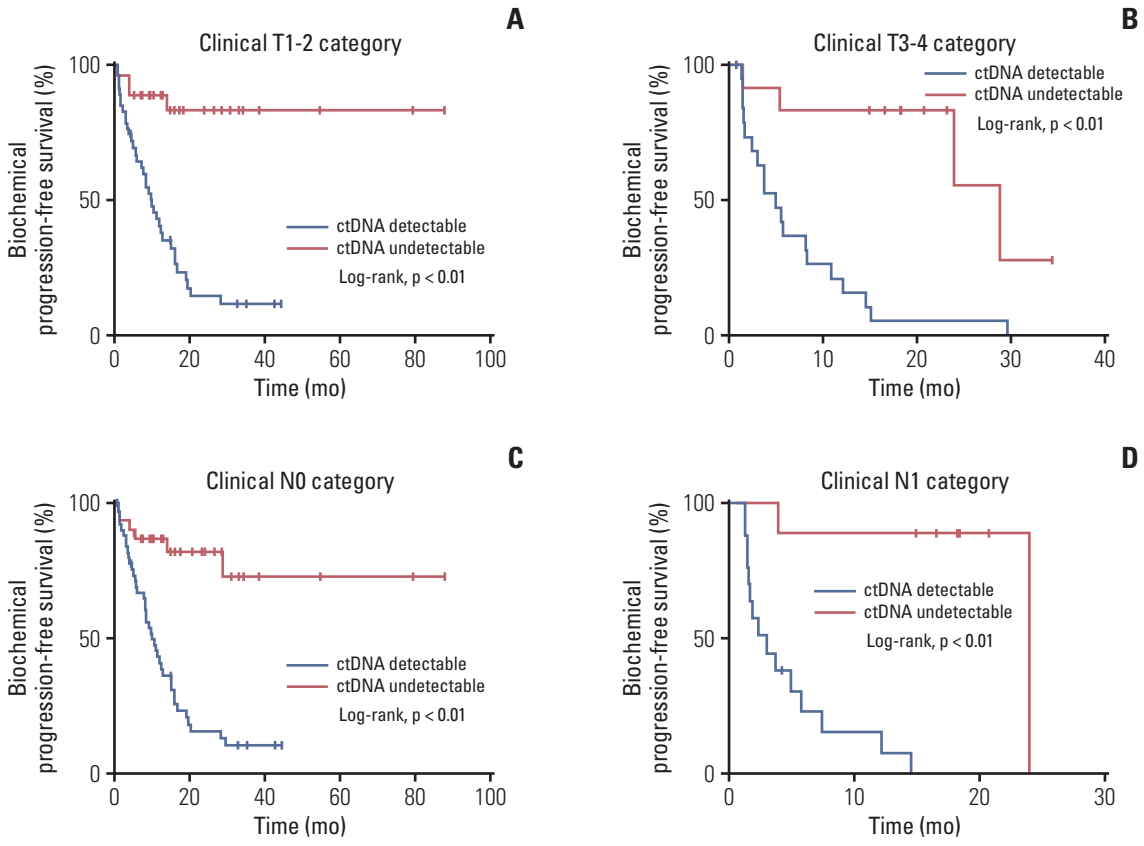

Fig. 4. The association between circulating tumor DNA (ctDNA) status and biochemical progression-free survival (bPFS). Kaplan-Meier curves for time to biochemical recurrence of the patients with detectable ctDNA and with undetectable ctDNA according to clinical T category (A, B) and clinical N category (C, D).

Table 2. Biochemical progression-free survival univariate analysis by clinicopathological variables and preoperative ctDNA status

Variable	Category	HR (95% CI)	p-value
PSA (ng/mL)	< 20 vs. ≥ 20	1.014 (0.614-1.677)	0.955
	Continuous	1.001 (0.997-1.004)	0.766
Gleason score	≤ 7 vs. > 7	0.761 (0.464-1.247)	0.278
	Continuous	1.133 (0.893-1.438)	0.303
Clinical T category	T1-2 vs. T3-4	0.680 (0.402-1.152)	0.152
Clinical N category	N0 vs. N1	0.673 (0.380-1.192)	0.174
ctDNA status	Undetectable vs. Detectable	0.136 (0.064-0.287)	0.001
ctDNA fraction	Continuous	6.223 (0.842-45.967)	0.073
Neoadjuvant therapy	No vs. Yes	0.861 (0.517-1.437)	0.568

CI, confidence interval; ctDNA, circulating tumor DNA; HR, hazard ratio; PSA, prostate specific antigen.

in the localized setting [10,11,15-17], our data support that ctDNA could detect the existence of micro-metastasis and minimal tumor-derived molecules. In the era of precision medicine, our study provides new insights into the clinical utility of ctDNA in nmPCa that would open new frontiers and perspectives for disease monitoring and individualized interventions.

The present study has several limitations including its retrospective nature and the absence of study design and validation cohort. In addition, we were unable to control the clinical features in a real-world setting. A subgroup of nmPCa patients with sequencing data was addressed to treatment

regimens other than surgery, which may cause potential selection bias in our survival analysis. Lastly, the postoperative ctDNA was not collected, since the serial changes in ctDNA were of great importance in reflecting treatment response [28,29]. We confirm that prospective ctDNA studies in the setting of resectable PCa would be conducted in a larger population in the near future.

To our knowledge, our study is one of the first studies to highlight the prognostic value of ctDNA in the nmPCa setting. Pre-operative ctDNA detection could identify the patients at high risk of recurrence and has the clinical potential to inform immediate postoperative interventions, but these approaches remain to be validated in prospective studies. It is of great importance that ctDNA studies provide insights into accurate monitoring and precise treatment rather than simply following routine clinical care.

In this study, we present a preliminary genomic atlas of nmPCa via liquid biopsy, which reveals the predictive power of pre-operative ctDNA for disease recurrence. Comparative analysis demonstrated low detection rate of somatic deleterious alterations in the nmPCa setting. Importantly, ctDNA reflecting the comprehensive mutational status could identify the patients at high risk of recurrence and has the clinical potential to inform immediate postoperative interventions, which provides insights into accurate monitoring and precise treatment.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The study was approved by the Committee for Ethics of Ren Ji Hospital (approval number: KY2019-081) and informed consent was obtained from each patient.

Author Contributions

Conceived and designed the analysis: Fei X, Dong B, Xue W.
Collected the data: Fei X, Wang J, Wang Y, Zhu Y, Pan J.
Contributed data or analysis tools: Fei X, Du X, Gong Y.
Performed the analysis: Fei X, Liu J, Gong Y, Fan L.
Wrote the paper: Fei X, Liu J, Fan L, Dong B, Xue W.
Revised the paper: Liu J.

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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

This work was supported by the Science and Technology Commission of Shanghai Municipality (19411967400, 19ZR1431000, 19XD1402300, 19YF1428400, 20YF1425300), Shanghai Municipal Health Bureau (201640247, 2019LJ11), Shanghai Municipal Education Commission (20152215, 20191906), Shanghai Jiao Tong University (YG2016ZD08, YG2017MS47, YG2017MS52), National Natural Science Foundation of China (81572536, 81672850, 81772742, 81702840, 82002710), Shanghai Jiao Tong University School of Medicine (TM201907), Renji Hospital Shanghai Jiao Tong University School of Medicine (PYZY 16-008, PYXJS16-015, RJZZ19-17).

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