Clinical Impact of Detecting Low-Frequency Variants in Cell-Free DNA on Treatment of Castration-Resistant Prostate Cancer

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ABSTRACT

Purpose: Although cell-free DNA (cfDNA) testing is expected to drive cancer precision medicine, little is known about the significance of detecting low-frequency variants in circulating cell-free tumor DNA (ctDNA) in castration-resistant prostate cancer (CRPC). We aimed to identify genomic profile including low-frequency variants in ctDNA from patients with CRPC and investigate the clinical utility of detecting variants with variant allele frequency (VAF) below 1%.

Experimental Design: This prospective, multicenter cohort study enrolled patients with CRPC eligible for treatment with abiraterone or enzalutamide. We performed targeted sequencing of pretreatment cfDNA and paired leukocyte DNA with molecular barcodes, and ctDNA variants with a VAF ≥0.1% were detected using an in-house pipeline. We investigated progression-free survival (PFS) and overall survival (OS) after different ctDNA fraction cutoffs were applied.

Results: One hundred patients were analyzed (median follow-up 10.7 months). We detected deleterious *ATM*, *BRCA2*, and *TP53* variants even in samples with ctDNA fraction below 2%. When the ctDNA fraction cutoff value of 0.4% was applied, significant differences in PFS and OS were found between patients with and without defects in *ATM* or *BRCA2* [HR, 2.52; 95% confidence interval (CI), 1.24–5.11; $P = 0.0091$] and *TP53* (HR, 3.74; 95% CI, 1.60–8.71; $P = 0.0014$). However, these differences were no longer observed when the ctDNA fraction cutoff value of 2% was applied, and approximately 50% of the samples were classified as ctDNA unquantifiable.

Conclusions: Detecting low-frequency ctDNA variants with a VAF <1% is important to identify clinically informative genomic alterations in CRPC.

Introduction

Plasma cell-free DNA (cfDNA) testing is emerging as an important tool to drive cancer precision medicine. Although next-generation sequencing (NGS) of tumor tissue is the optimal method for comprehensive genomic profiling, in castration-resistant prostate cancer (CRPC), accessing metastatic sites for tissue biopsies is a challenge. In addition, the analysis of tumor tissue from only one region might fail to capture intraindividual tumor heterogeneity. In contrast, cfDNA can be obtained less invasively and repeatedly, and circulating cell-free tumor DNA (ctDNA) can capture the current genomic profile of the tumor, encompassing its heterogeneity (1–3). However, ctDNA is highly diluted by abundant cfDNA from noncancer cells. The key challenge is to distinguish low-frequency ctDNA variants from background errors of sequencing (4). Because the intrinsic error rate of current NGS technology is around 1%, comparisons of multiple ctDNA variant detection platforms have revealed low concordance between the platforms especially for the variants with a VAF below 1% (5). To date, several comprehensive NGS-based ctDNA analyses have revealed the molecular profiles of CRPC and potential prognostic

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Translational Relevance

Cell-free DNA (cfDNA) testing is emerging as a tool for cancer precision medicine. However, little is known about the clinical significance of low-frequency variants detected in circulating cell-free tumor DNA (ctDNA) from patients with castration-resistant prostate cancer (CRPC). We conducted targeted sequencing of cfDNA and paired leukocyte DNA from patients with CRPC using molecular barcodes, and ctDNA variants with a variant allele frequency ≥ 0.1% were detected using an in-house pipeline. Several truncating *ATM* and *BRCA2* variants and deleterious *TP53* variants were detected at the ctDNA fraction of <2%. Defects in *ATM* or *BRCA2* and *TP53* were significantly associated with shorter progression-free survival and overall survival with a 0.4% ctDNA fraction cutoff value, but not with 2%. Several variants associated with clonal hematopoiesis of indeterminate potential were also detected in druggable genes, emphasizing the importance of sequencing paired leukocyte DNA to avoid inappropriate drug administration.

biomarkers, but the cutoff values of ctDNA fraction in the studies were commonly set at 2% to avoid false positive calls (6–9). However, this approach led to about 30% to 40% of patients being classified as having no ctDNA. This cutoff may not be sensitive enough to capture the full spectrum of cancer genome to aid the development of "precision" oncology.

In recent years, several highly sensitive methods using molecular barcoding have been developed to detect low-frequency variants (10–12). We also developed eVIDENCE, an approach that uses molecular barcodes and optimized bioinformatics methods to identify low-frequency variants from cfDNA sequencing data targeting about 80 genes (13). Several studies have performed comprehensive ctDNA analysis with commercially available low-frequency variants detection systems in CRPC (14, 15), but little is known about the clinical utility of identifying variants with the VAF of <1%. Therefore, we sought to perform targeted sequencing of cfDNA from patients with CRPC and detect ctDNA variants including those with the VAF below 1% using eVIDENCE.

For an accurate interpretation of cfDNA sequencing results, biological background noise as well as sequencing errors should be removed. Recently, clonal hematopoiesis of indeterminate potential (CHIP) is gaining attention as a biological background noise that can confound cfDNA analysis (16–18). Hematopoietic stem cells accumulate somatic variants during aging, which can lead to clonal expansion of variants in blood cells, and these variants are known as CHIP (19). To accurately discern CHIP-associated variants and true ctDNA variants, sequencing of paired leukocyte DNA and cfDNA using the same sequencing platform is necessary; however, this is not commonly performed due to increased cost of sequencing.

In this study, we collected cfDNA and matched leukocyte DNA from patients with CRPC who were eligible for abiraterone or enzalutamide treatment. The ctDNA variants with the VAF ≥0.1% were identified to evaluate the association of clinical outcomes with clinical and genomic variables. Next, we assessed the clinical utility of detecting low-frequency variants in ctDNA by comparing the results from different ctDNA fraction cutoffs. We also examined the importance of assessing the clonality of detected variants. Furthermore, CHIP-associated alterations were identified by comparing cfDNA and leukocyte DNA sequencing data. This study shows the clinical utility of detecting low-frequency ctDNA variants in CRPC. In addition, we raise several key factors to be considered when interpreting the data of ctDNA analysis for detecting low-frequency variants.

Materials and Methods

Patient cohort

Patients with CRPC eligible for treatment with either abiraterone or enzalutamide were prospectively enrolled from September 1, 2015, to November 30, 2020, at 17 institutions in Japan. All the institutions provided institutional review board approval. This study was conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from all participants. All patients received abiraterone or enzalutamide, and blood samples were collected prior to the medication administration. Plasma cfDNA and leukocyte DNA were extracted from the blood samples (Supplementary Materials and Methods) for targeted sequencing. Baseline clinicopathologic information were collected at enrollment, and prostate-specific antigen (PSA) levels were measured every 1 to 3 months after treatment initiation. During the enrollment, we collected tumor tissue and paired plasma cfDNA from 10 patients with CRPC to validate the performance of eVIDENCE to detect low-frequency ctDNA variants from patients with CRPC because the pipeline was initially developed using plasma cfDNA samples from patients with hepatocellular carcinoma. We compared the whole exome sequencing (WES) results of the tumor tissue DNA with the cfDNA analysis results and confirmed the high performance of the pipeline in detecting low-frequency ctDNA variants from patients with CRPC. For details, see Supplementary Materials and Methods, Supplementary Results, Supplementary Table S1, and Supplementary Fig. S1.

Targeted sequencing of cfDNA and paired leukocyte DNA

Briefly, 10 ng of cfDNA and 50 ng of leukocyte DNA per patient were used for sequencing library preparation, and unique molecular identifiers were added using ThruPLEX Tag-seq (Takara Bio). Targeted sequencing was performed using a KAPA HyperChoice system (Roche) and Illumina NovaSeq. Our custom gene panel captures the exonic regions of 88 genes (Supplementary Table S2). For details about library preparation, see Supplementary Materials and Methods. All the sequencing data from cfDNA and leukocyte DNA were processed by eVIDENCE. Single nucleotide variants (SNV) and short insertions and deletions (indels) with a VAF ≥ 0.1% were extracted and functionally annotated with ANNOVAR (20). Among the cfDNA variants, those detected in paired leukocyte DNA with the VAF ≥ 1% were discarded. If the same variants were detected in matched leukocyte DNA with the VAF < 1%, we measured the difference in the VAF between leukocyte and cfDNA samples by one-sided Fisher exact test. When the *P* value was less than 0.001 and the VAF in cfDNA was significantly higher than that in matched leukocyte DNA, those variants were regarded as tumor-derived cfDNA variants, but otherwise discarded. Additionally, variants repeatedly identified in more than three samples were removed unless they were known as hotspot variants in prostate cancer in Catalogue of Somatic Mutations in Cancer (COSMIC; ref. 21) or classified as pathogenic or likely pathogenic in ClinVar (22). Among ctDNA variants, all loss-of-function variants such as nonsense variants, frameshift, or splice-site variants were considered deleterious. Any missense variants identified in COSMIC as recurrent variants in prostate cancer or classified as pathogenic/likely pathogenic in ClinVar were also considered deleterious. In addition, missense variants that met both of the following criteria were defined as deleterious; (i) predicted to be deleterious in FATHMM (23); and (ii) CADD (24) phred score of ≥15.

Analysis of germline variants

We extracted variants with VAF ≥ 40% from the targeted sequencing data of leukocyte DNA and functionally annotated with ANNOVAR. From these variants, those registered in dbSNP (25) or observed in the 1,000 Genomes Project (26) were ignored. Loss-of-function variants and pathogenic/likely pathogenic variants in ClinVar were regarded as deleterious germline variants.

Identification of copy number alterations (CNA)

CNAs were analyzed from targeted sequencing data of cfDNA and leukocyte DNA using the software CNVkit (v0.9.6; ref. 27). Pooled reference data were created by assessing all leukocyte samples, and coverage log ratios were calculated against the reference data. Copy number segments were determined by using the circular binary segmentation algorithm (28), and segments were annotated to genes. We called a deletion for a gene with the coverage log ratio of ≤−0.3 and a gain for a gene with that of ≥0.3. Deep deletions and amplifications were called when the coverage log ratios were ≤−1.0 and ≥0.6, respectively.

AR copy number analysis by digital PCR

AR copy number analysis was performed by the QuantStudio 3D Digital PCR system for 83 cfDNA samples. Seven samples with known *AR* gain from the sequencing data analysis and 10 samples with insufficient sample volume were not assessed. We used Taqman Copy Number Assay for *AR* (Assay ID: Hs04107225), Taqman Copy Number Reference assay for *RNaseP* (Assay ID: 4403326), and 5 ng of cfDNA, and PCR amplification was performed as described before (29). On the basis of this previous report, the value of *AR* copy number of >1.54 copies was considered *AR* gain.

ctDNA fraction estimation

For each cfDNA sample, ctDNA fraction was estimated using the highest allele frequency in somatic variants in the sample as 2/([1/VAF]+1) (for autosomes) or VAF (for chromosome X) as described previously (6). If variants were detected in genes with copy number gain, those were excluded from this analysis. Since several samples with no somatic variants showed CNAs, we also estimated ctDNA fraction based on heterozygous SNP allele frequencies in genes with monoallelic deletion. In each leukocyte sample, we first detected heterozygous SNPs that are registered in dbSNP, which had 50 or more total consensus reads. From the heterozygous SNPs, those in the regions with monoallelic deletion in matched cfDNA sample were extracted, and the total consensus reads at any of these SNP loci in cfDNA had to be at least 100. Then, for each SNP, we tested whether the allele frequency in cfDNA is statistically significantly deviant from 0.5 by binomial tests. For each cfDNA sample, the median frequency of the dominant allele (AF) among those fulfilling the P value of < 0.05 were calculated. The samples with less than four loci to be examined by the above criteria were removed from this evaluation. Finally, the ctDNA fraction was calculated as 2−(1/AF). The ctDNA fraction obtained by this estimation was prioritized over that obtained from the other method when both could be calculated.

Clonal and subclonal variant analysis

For each somatic ctDNA variant, tumor fraction harboring the variant was calculated as 2/([1/VAF]+1) (for autosomes) or VAF (for chromosome X). Then, if the fraction was less than 25% of the ctDNA fraction of the sample, the variant was defined as "subclonal."

Detection of CHIP-associated alterations

Somatic variants that were detected in both cfDNA and matched leukocyte DNA and filtered out when detecting ctDNA variants as described above were extracted. Repeatedly identified variants were excluded, and the remaining were considered CHIP-associated alterations. CHIP variants were also annotated using ANNOVAR.

Statistical analysis

The following outcome measures were evaluated: PSA response, progression-free survival (PFS), and overall survival (OS). Progression was defined as PSA progression according to Prostate Cancer Working Group 3 (PCWG3; ref. 30) criteria or clinical/radiological progression. "*AR* aberrations," "*TP53* defect," "HRR-related genes defect," "*RB1* defect," "PI3K pathway defect," "WNT pathway defect," "*SPOP* variants and/or *CHD1* defect," "*KMT2C*/*KMT2D* variants," and "*ZFHX3* defect" were defined as harboring "deleterious *AR* variants and/or *AR* gain," "deleterious *TP53* variants and/or *TP53* deletion," "somatic/germline truncating variants or biallelic deletion in *ATM* or *BRCA2*," "deleterious *RB1* variants and/or *RB1* deletion," "deleterious variants and/or deletion in *PTEN* or *PIK3R1*, or deleterious *PIK3CA* variants," "deleterious *CTNNB1* variants or truncating variants in *APC*," "deleterious *SPOP* variants and/or loss-of-function variants or biallelic deletion in *CHD1*," "deleterious variants in *KMT2C* and/or *KMT2D*," and "truncating variants and/or deletion in *ZFHX3*," respectively. Factors predictive of PSA response were assessed using logistic regression models. The association of clinical and genomic variables with PFS and OS was examined using univariate Cox proportional hazards models. We then evaluated the independent prognostic biomarkers using a multivariate Cox regression model comprising variables, which were statistically significant in univariate analysis. PFS and OS were evaluated using the Kaplan–Meier method and differences were analyzed by the log-rank test. All tests were two-sided and P values of less than 0.05 were considered statistically significant. R software (version 4.0.1; R Foundation) was used for statistical analysis.

Data availability

Sequencing data presented in this article have been deposited at the Japanese Genotype-phenotype Archive (JGA, http://trace.ddbj.nig.ac.jp/jga), which is hosted by the DNA Data Bank of Japan (DDBJ), under accession number JGAS000330.

Results

Patient cohort

A total of 111 patients with CRPC were enrolled in this study. Eleven patients were excluded from subsequent analysis because either cfDNA yields were insufficient for library construction ($n = 8$) or they were lost to follow-up ($n = 3$), leaving 100 for the analysis. Fifty-one patients were treated with abiraterone and the remaining 49 with enzalutamide. Thirteen patients (13%) had received prior treatment with androgen receptor pathway inhibitor (ARPI). Median follow-up time was 10.7 months [interquartile range (IQR), 6.1–20.8 months]. Patient characteristics are described in **Table 1**.

Genomic landscape from cfDNA sequencing

The average depth of raw reads and de-duplicated reads for cfDNA was 21,700 and 800, and that for leukocyte DNA was 6,700 and 800, respectively (Supplementary Table S3). A total of 521 somatic variants were detected in this cohort, and 184 (35%) were deleterious (Supplementary Table S4). Of these 184, 86 (47%) showed VAF of <1.0% (range, 0.16%–0.98%). In the 100 samples, 88 (88%) had one or more detectable aberrations, and the remaining 12 samples (12%) were ctDNA unquantifiable (**Fig. 1**). Among the 88 ctDNA quantifiable samples, 42 patients (48%) showed ctDNA fraction below 2% (median

Table 1. Patient characteristics at baseline.

Characteristic	All ($n = 100$)	ABI ($n = 51$)	ENZ ($n = 49$)	*P* value
Age (years)	76 (70–82)	76 (69.5–82)	76 (71–82)	0.86
ECOG PS 0–1	87 (87.0%)	45 (88.2%)	42 (85.7%)	0.77
PSA (ng/mL)	10.3 (3.9–27.7)	6.6 (3.7–16.5)	16.8 (4.0–31.8)	0.087
Hemoglobin (g/dL)	12.6 (11.3–13.5)	12.6 (11.5–13.5)	12.4 (11.2–13.5)	0.71
LDH (IU/L)	190.0 (167.0–225.8)	190.0 (164.5–220.5)	190.0 (170.0–229.0)	0.62
Lymph node metastasis	36 (36.0%)	22 (43.1%)	14 (28.6%)	0.15
Bone metastasis	70 (70.0%)	37 (72.5%)	33 (67.3%)	0.66
Visceral metastasis	14 (14.0%)	5 (9.8%)	9 (18.4%)	0.26
Prior ARPI	13 (13.0%)	7 (13.7%)	6 (12.2%)	>0.99
cfDNA yield (ng/mL plasma)	10.6 (6.4–16.9)	9.8 (6.2–16.1)	11.0 (7.6–17.2)	0.25

Note: Shown are counts (percentages across columns) or median (interquartile range). *P* values for difference between the ABI and ENZ arm were calculated using Wilcoxon rank-sum test (continuous attributes) or Fisher exact test (categorical attributes).
Abbreviations: ABI, abiraterone; ARPI; androgen receptor pathway inhibitor; ECOG PS, Eastern Cooperative Group performance status; ENZ, enzalutamide; IU, International unit; LDH, lactate dehydrogenase; PSA, prostate-specific antigen.

fraction 2.2%; Supplementary Table S5). Several deleterious somatic variants in *TP53*, *ATM*, and *BRCA2* were identified even in samples with ctDNA fraction <2%, suggesting the significance of detecting low-frequency variants (**Fig. 1**; Supplementary Table S4). Among the deleterious variants, the most frequently mutated genes were *TP53* (18% of patients), *AR* (13%), and *ATM* (8%). The prevalence of *TP53* alteration was found to be lower than that in a previous WES analysis based study of metastatic CRPC tissue samples from Western population (31), but consistent with another primary prostate cancer analysis from Chinese patients showing lower *TP53* somatic variants than that detected in Western patients (32, 33). Coincidently, the rates of *AR* ligand-binding domain variants and *ATM* truncating variants were consistent with those reported previously (6, 31, 34).

CNAs were mostly detected in samples with ctDNA fraction >20% (**Fig. 1**). Although there were only 20 such samples, the landscape of CNAs showed frequent deletion of *ZFHX3*, *CHD1*, and *NKX3–1*, and gain of *AR*, *MYC*, and *NBN*. *AR* copy number analysis using digital PCR could detect *AR* gain in 16 additional samples (Supplementary Table S6). Nonetheless, the prevalence of *AR* gain (30%) was still lower than that reported previously (6, 29, 31, 34), probably due to the performance limit of digital PCR in detecting weak *AR* gain in samples with low ctDNA fraction.

When divided into clonal and subclonal variants, the clonal variants were more likely to be deleterious, whereas subclonal variants were more frequently benign (Supplementary Table S4; Supplementary Fig. S2). The proportion of clonal and subclonal deleterious variants in *AR*, *TP53*, genes related to homologous recombination repair (HRR), *RB1*, genes in PI3K and WNT pathway, *SPOP*/*CHD1*, *KMT2C*/*KMT2D*, and *ZFHX3* are shown in Supplementary Fig. S3. Among the *TP53* and *KMT2C*/*KMT2D* altered cases, there were four and three patients with a subclonal deleterious variant alone, respectively, but no case had subclonal deleterious variants alone in the other genes or pathways.

Clinical and genomic predictors of clinical outcomes

In this cohort, 57 patients (57%) achieved at least a 50% PSA response (PSA50) within 3 months (Supplementary Fig. S4). Clinical and genomic factors associated with PSA response were assessed (Supplementary Table S7), and multivariate analysis revealed that prior treatment with ARPI was the only factor significantly associated with PSA response [OR, 13.2; 95% confidence interval (CI), 2.28–76.8; $P = 0.0040$]. Twenty-five patients (49.0%) in the abiraterone arm demonstrated a PSA50 decline, and 32 (65.3%) in the enzalutamide arm achieved a PSA50 response, showing no significant difference between the two arms (OR, 1.94; 95% CI, 0.81–4.74; $P = 0.11$). Although there are several studies (35–38) which have reported that *AR* status in ctDNA is associated with resistance to abiraterone or enzalutamide, none of *AR* variants, *AR* gain, or *AR* aberrations were associated with PSA response in both arms in our study (Supplementary Table S8).

Median PFS was 8.8 months (95% CI, 5.9–14.2) in the full cohort and it did not differ between the abiraterone and the enzalutamide arm (14.3 months vs. 8.6 months; HR, 1.22 for enzalutamide; 95% CI, 0.71–2.09; $P = 0.47$; Supplementary Fig. S5). Multivariate analysis for PFS revealed that prior treatment with ARPI (HR, 5.35; 95% CI, 2.28–12.6; $P < 0.001$), defect in HRR-related genes (*ATM* and *BRCA2*; HR, 2.57; 95% CI, 1.05–6.31; $P = 0.040$), and *MYC* gain (HR, 5.42; 95% CI, 1.22–24.0; $P = 0.026$) were significantly associated with earlier progression (**Table 2**). Median OS was 33.2 months (95% CI, 23.0–not reached), and multivariate analysis showed that *TP53* defect (HR, 3.59; 95% CI, 1.16–11.1; $P = 0.027$) was the only significant adverse risk factor for OS (**Table 2**). Defects in HRR-related genes were not associated with early mortality even in univariate analysis. Furthermore, we performed univariate and multivariate analysis for OS including subclonal deleterious variants in *TP53* and found that they were no longer statistically significantly associated with OS in multivariate analysis (HR, 2.10; 95% CI, 0.76–5.83; $P = 0.15$; Supplementary Table S9).

To assess the clinical utility of detecting low-frequency ctDNA variants, we set two different thresholds for ctDNA detection: ctDNA fraction of 0.4%, which is the smallest value in our data, and 2%, which is a threshold value in previous studies (6–9). Upon applying the former threshold, there was a significant difference in PFS between those with and without HRR defect (HR, 2.52; 95% CI, 1.24–5.11; $P = 0.0091$; **Fig. 2A**), which was not seen with the latter threshold (HR, 1.92; 95% CI, 0.82–4.50; $P = 0.11$; **Fig. 2B**). Similarly, we found statistically significant difference in OS between those with and without *TP53* defects when the former threshold was applied (HR, 3.74; 95% CI, 1.60–8.71; $P = 0.0014$; **Fig. 2C**), but no difference with the latter threshold (HR, 2.26; 95% CI, 0.91–5.64; $P = 0.13$; **Fig. 2D**). We also compared treatment response between men harboring HRR defect with ctDNA fraction of 0.4% to 2% and 2% or higher, which showed no difference in PSA response between the groups (Supplementary Fig. S6A). In addition, we analyzed PFS in patients bearing HRR defect with ctDNA fraction of <2% and ≥2%, and there was again no statistically significant difference between the two groups (HR, 1.47; 95% CI, 0.36–5.91; $P = 0.59$; Supplementary Fig. S6B). Although the

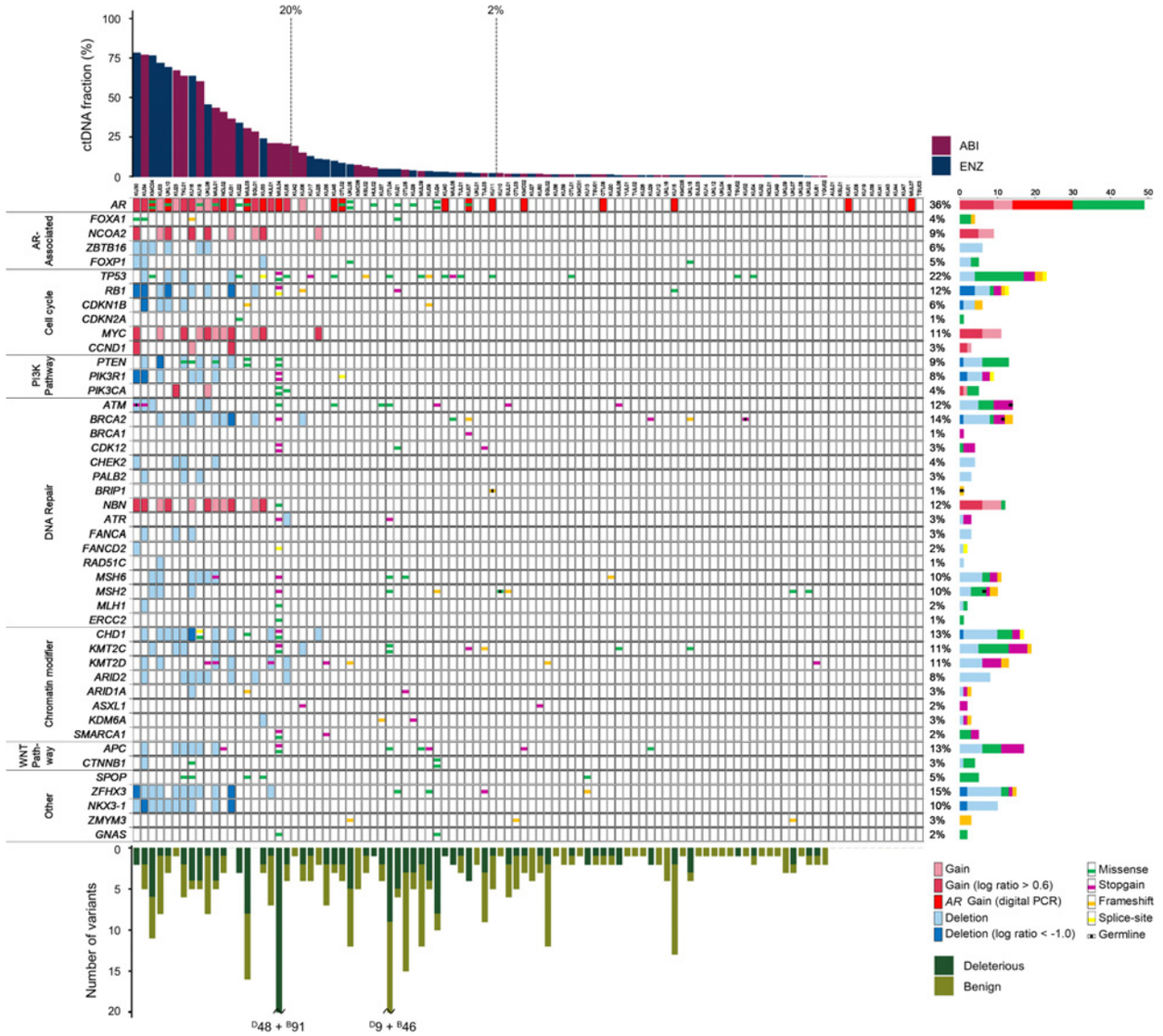

Figure 1.

Integrative landscape analysis of genomic aberrations in CRPC. Matrix shows genomic aberrations identified through cfDNA and paired leukocyte DNA sequencing and digital PCR for *AR* copy number analysis. Deleterious somatic and germline variants and copy number alterations along with the type of aberrations are color-coded. Columns represent individual patient samples ($n = 100$) and are sorted by ctDNA fraction (top). Rows represent specific genes grouped by pathway, and frequency of aberrations within the cohort is shown on the right. The number of benign/deleterious variants per individual is shown at the bottom. ABI, abiraterone; ENZ, enzalutamide; D, deleterious; B, benign.

number of patients included was small, these results support the clinical significance of detecting very low-frequency ctDNA variants.

CHIP-associated alterations

We detected a total of 81 CHIP-associated alterations. Among these 81, 40 (49.4%) were deleterious variants, showing CHIP interference with cfDNA testing (**Fig. 3A**; Supplementary Table S10). Importantly, the median VAF of CHIP interference clones in cfDNA and leukocyte DNA were 0.86% (IQR, 0.65–2.0%) and 0.89% (IQR, 0.62–1.5%), respectively. In addition, there were several CHIP interference clones in which VAF in cfDNA was over 1% and that in leukocyte DNA was below 1% (**Fig. 3B**; Supplementary Table S10). These results indicate that detecting low-frequency variants in leukocyte DNA is also important to avoid CHIP interference when detecting low-frequency ctDNA alterations. In total, 42 patients (42%) had one or more CHIP-associated alterations, and 24 patients (24%) harbored CHIP interference clones. The proportion of patients with CHIP-associated variants and CHIP interference clones increased with age (**Fig. 3C**). CHIP interference clones were detected most frequently in *ATM* ($n = 11$, 27.5%), followed by *TP53* ($n = 7$, 17.5%) and *CHEK2* ($n = 3$, 7.5%; **Fig. 3A**). Twenty-three patients had deleterious somatic, germline, or CHIP-associated variants in six genes that are targets of PARP inhibitors due to a direct or indirect association with HRR (**Fig. 3D**). Of the 23 patients, six (26.1%) had CHIP interference clones alone, which could potentially lead to inappropriate use of PARP inhibitors if paired leukocyte DNA was not analyzed. We also

Table 2. Univariate and multivariate Cox proportional hazard analysis of predictors of PFS and OS.

	Progression-free survival				Overall survival			
	Univariate		Multivariate		Univariate		Multivariate	
Variable	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (years)	0.99 (0.95–1.02)	0.40	NA	NA	1.01 (0.97–1.06)	0.66	NA	NA
ECOG PS, 2–3 vs. 0–1	2.53 (1.26–5.08)	0.0091	1.97 (0.63–6.15)	0.24	7.30 (3.35–15.8)	<0.001	3.87 (0.82–18.3)	0.088
PSA (ng/mL)	1.002 (1.001–1.002)	<0.001	1.000 (0.998–1.002)	0.89	1.002 (1.001–1.003)	<0.001	0.999 (0.997–1.003)	0.93
Hemoglobin (g/dL)	0.81 (0.70–0.94)	0.0059	1.05 (0.84–1.30)	0.68	0.61 (0.50–0.75)	<0.001	0.996 (0.69–1.36)	0.84
LDH (IU/L)	1.002 (1.001–1.003)	<0.001	1.001 (0.998–1.003)	0.67	1.004 (1.002–1.005)	<0.001	1.001 (0.997–1.005)	0.64
Visceral metastasis, yes vs. no	2.62 (1.37–5.00)	0.0035	2.12 (0.80–5.59)	0.13	5.16 (2.10–12.7)	<0.001	1.87 (0.27–13.2)	0.53
Treatment, ENZ vs. ABI	1.22 (0.71–2.09)	0.47	NA	NA	0.89 (0.41–1.92)	0.76	NA	NA
Prior ARPI, yes vs. no	3.73 (1.95–7.14)	<0.001	5.35 (2.28–12.6)	<0.001	2.35 (0.92–5.98)	0.074	NA	NA
cfDNA yield (ng/mL plasma)	1.002 (1.000–1.003)	0.033	0.999 (0.997–1.002)	0.83	1.003 (1.001–1.005)	0.0017	0.999 (0.995–1.002)	0.47
No. of variants, ≥3 vs. ≤2	1.50 (0.88–2.54)	0.13	NA	NA	2.29 (1.06–4.94)	0.034	1.06 (0.32–3.47)	0.93
ctDNA fraction (%)	1.02 (1.01–1.03)	<0.001	0.98 (0.95–1.02)	0.34	1.04 (1.03–1.06)	<0.001	1.02 (0.97–1.07)	0.47
AR aberrations, yes vs. no	2.47 (1.44–4.25)	0.0011	1.19 (0.54–2.62)	0.67	4.07 (1.85–8.95)	<0.001	1.60 (0.45–5.62)	0.47
TP53 defect, yes vs. no	2.55 (1.33–4.89)	0.0048	2.13 (0.90–5.07)	0.086	4.05 (1.76–9.32)	0.0010	3.59 (1.16–11.1)	0.027
HRR genes defect, yes vs. no	2.83 (1.40–5.73)	0.0039	2.57 (1.05–6.31)	0.040	1.86 (0.76–4.59)	0.18	NA	NA
RB1 defect, yes vs. no	3.20 (1.63–6.29)	<0.001	1.67 (0.54–5.22)	0.38	3.21 (1.40–7.36)	0.0059	0.52 (0.11–2.60)	0.43
PI3K pathway defect, yes vs. no	2.47 (1.23–4.97)	0.011	0.24 (0.052–1.11)	0.067	6.77 (2.91–15.8)	<0.001	0.33 (0.030–3.74)	0.37
WNT pathway defect, yes vs. no	0.58 (0.18–1.89)	0.36	NA	NA	1.36 (0.41–4.51)	0.62	NA	NA
SPOP variants and/or *CHD1* defect, yes vs. no	2.14 (0.85–5.42)	0.11	NA	NA	8.72 (3.37–22.6)	<0.001	0.88 (0.077–10.1)	0.92
MYC gain, yes vs. no	4.63 (2.23–9.64)	<0.001	5.42 (1.22–24.0)	0.026	6.53 (2.78–15.4)	<0.001	2.89 (0.77–10.9)	0.12
NBN gain, yes vs. no	3.63 (1.72–7.65)	<0.001	0.71 (0.12–4.28)	0.70	6.19 (2.48–15.5)	<0.001	1.36 (0.19–9.94)	0.76
KMT2C/D variants, yes vs. no	1.51 (0.64–3.56)	0.34	NA	NA	1.62 (0.49–5.40)	0.43	NA	NA
ZFHX3 defect, yes vs. no	3.76 (1.88–7.55)	<0.001	3.74 (0.77–18.2)	0.10	11.9 (4.99–28.6)	<0.001	3.30 (0.20–53.6)	0.40

Abbreviations: ABI, abiraterone; ARPI; androgen receptor pathway inhibitor; ECOG PS, Eastern Cooperative Group performance status; ENZ, enzalutamide; IU, international unit; LDH, lactate dehydrogenase; NA, not assessed; PSA, prostate-specific antigen.

examined clinical outcomes in patients with and without CHIP-associated alterations or CHIP interference clones. There were no significant differences between those with and without CHIP interference clones as well as CHIP variants in any of PSA50, PFS, or OS (Supplementary Table S11; Supplementary Figs. S7A–S7F). Furthermore, we evaluated PFS in patients with HRR defect in ctDNA and those with CHIP interference clones in HRR-related genes. ctDNA HRR defect was significantly associated with shorter PFS compared with deleterious CHIP variants in HRR genes (HR, 5.41; 95% CI, 1.13–25.9; $P = 0.018$; Supplementary Fig. S7G).

Discussion

Comprehensive analysis of cfDNA using NGS is an attractive platform for promoting precision medicine in CRPC as obtaining tissue samples is difficult. Although the key challenge of a comprehensive ctDNA analysis is to distinguish low-frequency ctDNA variants from sequencing errors, to our knowledge, few NGS-based comprehensive studies on CRPC have examined ctDNA fraction below 2%. However, this cutoff value might not be sufficient for identifying molecular profiles of patients with CRPC because about half of the patients in this study cohort showed ctDNA fraction of <2%. This proportion was comparable to a previous study that explored the utility of ctDNA analysis in a similar clinical setting, that is, before treating patients with CRPC with ARPI (6). Importantly, we detected truncating *ATM* and *BRCA2* variants and deleterious *TP53* variants in samples with ctDNA fraction below 2%. Similar to a previous study exploring the association between genomic aberrations in ctDNA and clinical response to ARPI (6), our study showed HRR-related gene defects and *TP53* aberrations to be significantly associated with shorter PFS and OS respectively with a 0.4% ctDNA fraction cutoff value. However, there was no statistical significance when the cutoff value was raised to 2%, and the number of patients with no ctDNA detection increased. In addition, treatment response and PFS did not differ between patients bearing HRR defect with ctDNA fraction of <2% and ≥2%, although the number of cases in each group was small. Patients who harbor deleterious *ATM* or *BRCA2* variants are eligible for treatment with PARP inhibitors (39). Therefore, to maximize the utility of cfDNA testing in a clinical setting, it is important to decrease the number of patients in whom ctDNA cannot be detected, which can be achieved by lowering the threshold of ctDNA fraction cutoff value.

Despite its clinical utility, interpretation of low-frequency ctDNA variants needs some attention. ctDNA variants with low VAF in samples with high ctDNA fraction are considered subclonal, which might not affect treatment response or patients' prognosis. Indeed, we showed subclonal variants were more likely to be benign compared

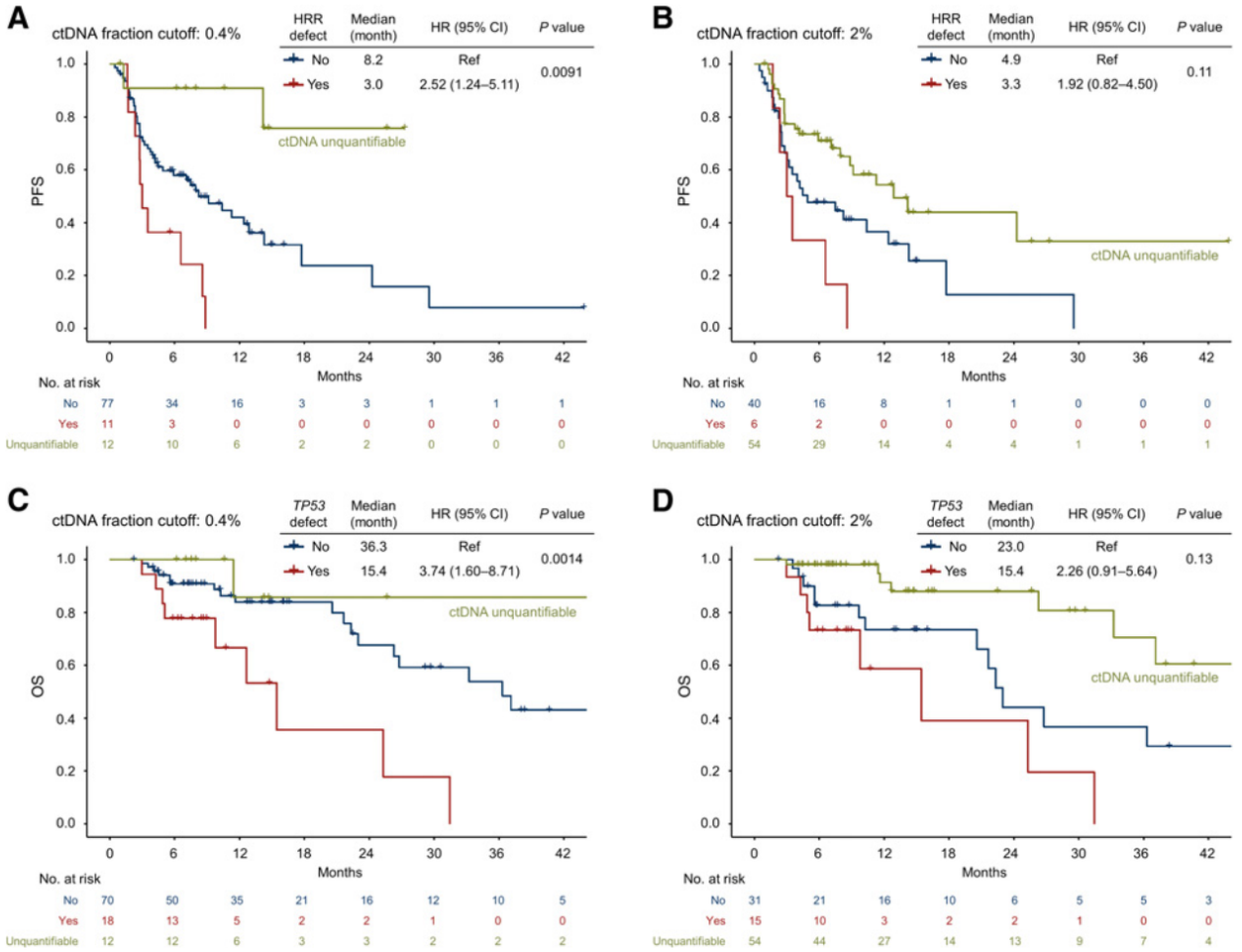

Figure 2.

Kaplan–Meier plot of PFS and OS using different ctDNA detection thresholds. **A,** Kaplan–Meier plot of PFS in patients with and without defect in *ATM* or *BRCA2*, and patients with unquantifiable ctDNA. ctDNA detection threshold of 0.4% was applied. **B,** Kaplan–Meier plot of PFS in patients with and without HRR defect, and patients with unquantifiable ctDNA. ctDNA detection threshold of 2% was applied. Statistically significant difference in PFS between patients with and without HRR defect was no longer observed. **C,** Kaplan–Meier plot of OS in patients with and without *TP53* defect, and patients with unquantifiable ctDNA. ctDNA detection threshold of 0.4% was applied. **D,** Kaplan–Meier plot of OS in patients with and without *TP53* defect, and patients with unquantifiable ctDNA. ctDNA detection threshold of 2% was applied. Significant difference in OS between patients with and without *TP53* defect disappeared. Ref, reference.

with clonal ones. Although it would be possible that subclonal population with deleterious variants exhibit clonal expansion during the clinical course, in the present study, multivariate analysis for OS demonstrated *TP53* defect including four subclonal deleterious variants was no longer a significant risk factor for early mortality. Therefore, assessment of clonality is necessary when analyzing the association of ctDNA aberrations with clinical outcomes using a low-frequency variants detection method.

Several studies targeting *AR* aberrations alone via sequencing or digital PCR correlate these with treatment resistance and poor outcomes in patients with CRPC (40, 41). In addition, a recent study showed *AR* aberrations to be a significant biomarker for PFS using a comprehensive cfDNA assay similar to ours (42). However, in the current study, multivariate analyses demonstrated that *AR* status in ctDNA was not an independent predictor for either PSA response, PFS, or OS, although it reached significance for PFS and OS in univariate analyses. This could be attributed to the lack of sensitivity for detecting *AR* gain due to the low median ctDNA fraction, or small sample size of the present study. In addition, we did not include *AR* enhancer in our analysis, which has also been reported to be associated with response to ARPI (43). On the other hand, this result was consistent with that of a previous comprehensive ctDNA analysis (6), possibly indicating that *AR* aberrations might not be a strong predictor for prognosis of patients with CRPC compared with HRR-related genes defect or *TP53* defect. In the future, a larger more comprehensive study analyzing the *AR* and its surrounding region as well as major *AR* cofactors may reveal the true predictive or prognostic impact of *AR* aberrations in this setting.

Furthermore, we examined CHIP-associated alterations, which potentially affect the interpretation of cfDNA analysis. We detected CHIP variants and CHIP interference clones in 42% and 24% of the patients, respectively. These proportions were higher than those reported previously in metastatic prostate cancer (18, 44), probably because we identified variants with a VAF below 1% in both cfDNA and leukocyte DNA. A recent study examined 469 cfDNA samples from prostate cancer patients and detected inferred clonal

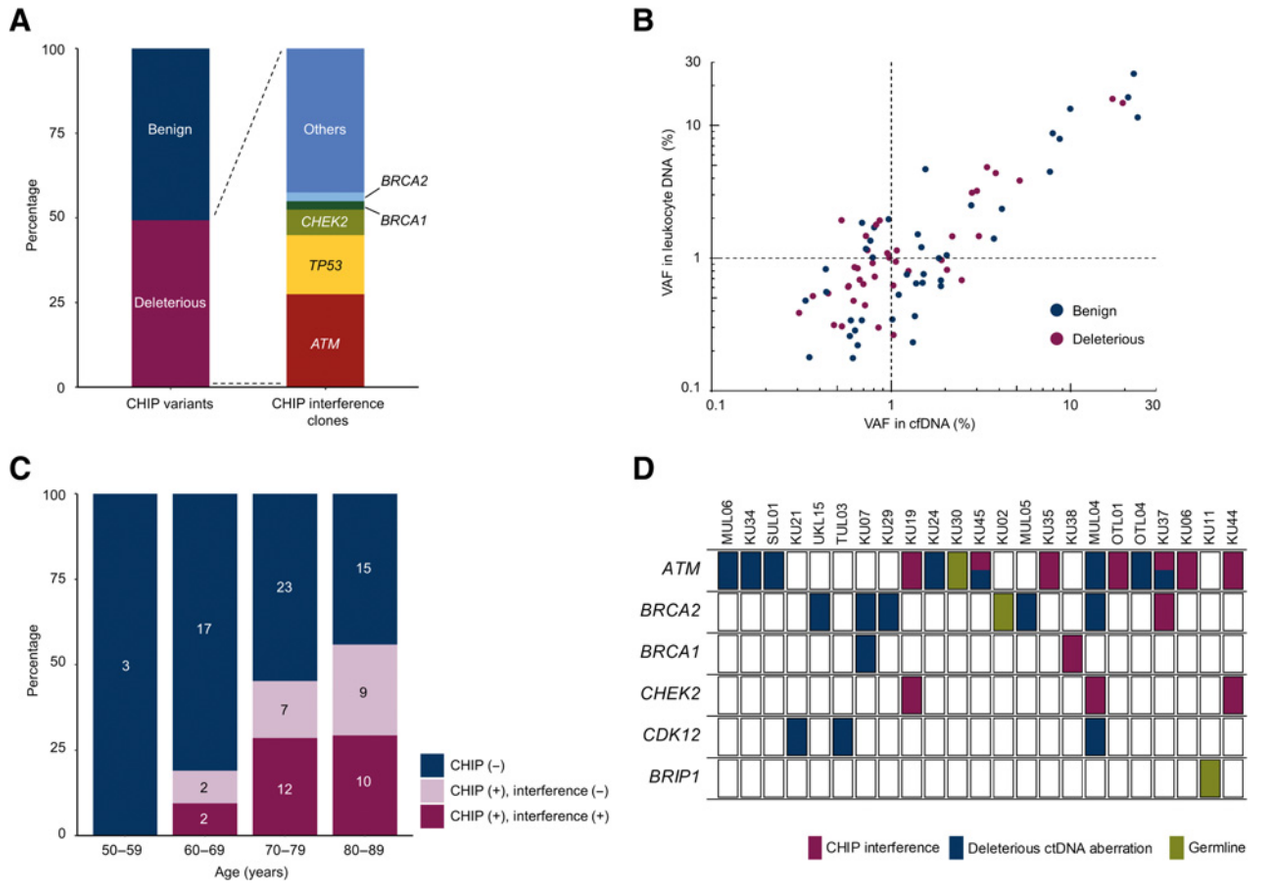

Figure 3.

CHIP variants in CRPC. **A,** Proportion of deleterious versus benign CHIP-associated alterations (left). Frequency of CHIP interference clones by gene is shown on the right. **B,** Scatter plot showing VAF in cfDNA versus VAF in leukocyte DNA for each CHIP variant. Pathogenicity prediction of CHIP variants is color-coded. **C,** Prevalence of CHIP variants and CHIP interference clones stratified by age. Numbers in blocks indicate the actual numbers of patients in each group. **D,** Matrix shows the 23 samples with deleterious somatic, germline, or CHIP-associated variants in six PARP inhibitor target genes. Type of variant is color-coded. Columns represent individual patient samples and are sorted by age from left to right.

hematopoiesis (CH) in 268 samples (57%) in 10 genes that are known to be associated with CH (45). They also showed 56% of the inferred CH were detected at a VAF of <2%, indicating that more CHIP variants can be detected when exploring low-frequency variants. Indeed, 26 (62%) and 15 (36%) cases in the present study had CHIP-associated alterations with a VAF below 2% and 1% in cfDNA, respectively. We identified several CHIP variants with a VAF of <1% in leukocyte DNA, showing the importance of detecting low-frequency leukocyte DNA variants when we explore low-frequency ctDNA variants. We also demonstrated that CHIP interference clones were most frequently identified in *ATM* as previously reported (18), followed by *TP53* and *CHEK2*. Although the high frequency of CHIP-associated alterations in these genes among CRPC patients has been reported by another study using a high sensitivity assay (17), it is important to note that defect in *ATM* and *TP53* were both shown to be prognostic biomarkers in the current study, and these CHIP variants may cause misjudgment of the patients' prognosis and negatively influence clinical decision making. Importantly, CHIP interference clones as well as CHIP-associated variants were not significantly associated with clinical outcomes. In addition, PFS in those with CHIP interference clones in HRR-related genes was significantly longer than that in patients with HRR defect in ctDNA. These results indicate that CHIP variants may not affect the prognosis of CRPC patients, even if they are deleterious and support the importance of discerning CHIP variants from true ctDNA variants. Recently, the FDA approved the FoundationOneLiquid CDx (Foundation Medicine), a cfDNA-based comprehensive genomic profiling assay, to identify patients with *BRCA1*, *BRCA2*, and/or *ATM* variants in patients with metastatic CRPC eligible for PARP inhibitor administration. However, this assay does not analyze paired leukocyte DNA, which could result in false positive findings associated with CHIP and lead to inappropriate administration of PARP inhibitors. Indeed, 26% of the patients with aberrations in genes associated with HRR harbored CHIP-associated alterations alone in the current cohort, which might lead to misdiagnosis and the incorrect use of PARP inhibitors without leukocyte DNA assessment. Although plasma cfDNA testing for detecting aberrations in genes related to HRR could become more common in the management of CRPC in clinical practice, simultaneous detection of variants in paired leukocyte DNA would be mandatory to avoid inappropriate drug administration.

The limitations of this study include relatively small sample size and short follow-up time. Although truncating *ATM* and *BRCA2* variants and deleterious *TP53* variants were associated with inferior PFS and OS, the number of patients who harbored those variants was comparatively small in this study. It is possible that with larger sample size and/or longer follow-up time with more events, statistical significance in clinical outcomes could have been found even using a ctDNA detection cutoff of 2%. However, it would still be important to set a lower ctDNA detection cutoff value because this could largely decrease the number of cases classified as ctDNA unquantifiable and enable more precise capture of genomic profile to personalize treatment. Second, CNAs including *AR* gain and deep deletion in HRR-related genes might have been under-detected, because the patient number with enough ctDNA fractions for CNA analysis (>20%) was low. In addition, we were not able to assess CHIP in some genes for which frequencies of CHIP-associated alterations are reported to be high such as *DNMT3A* and *TET2*, because our custom capture panel did not include these genes. Finally, the average depth of consensus reads was relatively low for detecting very low-frequency variants. Nonetheless, our study methods could be easily applied in a larger cohort, and the findings in this study could be validated to develop precision oncology with cfDNA testing.

In conclusion, this study demonstrated that identifying low-frequency ctDNA variants with VAF below 1% is important for clinically informative genomic alterations detection in CRPC. Assessment of clonality would be useful for detecting clinically actionable low-frequency variants. In addition, matched leukocyte DNA analysis is required in cfDNA testing to remove CHIP-associated variants and detect ctDNA variants accurately.

Authors' Disclosures

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Authors' Contributions

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