original reports

Cell-Free DNA Alterations in the *AR* Enhancer and Locus Predict Resistance to AR-Directed Therapy in Patients With Metastatic Prostate Cancer

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abstract

PURPOSE Cell-free DNA (cfDNA) and circulating tumor cell (CTC)–based liquid biopsies have emerged as potential tools to predict responses to androgen receptor (AR)–directed therapy in metastatic prostate cancer. However, because of complex mechanisms and incomplete understanding of genomic events involved in metastatic prostate cancer resistance, current assays (eg, CTC AR-V7) demonstrate low sensitivity and remain underutilized. The recent discovery of *AR* enhancer amplification in > 80% of patients with metastatic disease and its association with disease resistance presents an opportunity to improve on current assays. We hypothesized that tracking *AR*/enhancer genomic alterations in plasma cfDNA would detect resistance with high sensitivity and specificity.

PATIENTS AND METHODS We developed a targeted sequencing and analysis method as part of a new assay called Enhancer and Neighboring Loci of Androgen Receptor Sequencing (EnhanceAR-Seq). We applied EnhanceAR-Seq to plasma collected from 40 patients with metastatic prostate cancer treated with AR-directed therapy to monitor *AR*/enhancer genomic alterations and correlated these events with therapy resistance, progression-free survival (PFS), and overall survival (OS).

RESULTS EnhanceAR-Seq identified genomic alterations in the *AR*/enhancer locus in 45% of cases, including a 40% rate of *AR* enhancer amplification. Patients with *AR*/enhancer alterations had significantly worse PFS and OS than those without (6-month PFS, 30% *v* 71%; $P = .0002$; 6-month OS, 59% *v* 100%; $P = .0015$). *AR*/enhancer alterations in plasma cfDNA detected 18 of 23 resistant cases (78%) and outperformed the CTC AR-V7 assay, which was also run on a subset of patients.

CONCLUSION cfDNA-based *AR* locus alterations, including of the enhancer, are strongly associated with resistance to AR-directed therapy and significantly worse survival. cfDNA analysis using EnhanceAR-Seq may enable more precise risk stratification and personalized therapeutic approaches for metastatic prostate cancer.

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INTRODUCTION

Metastatic castration-resistant prostate cancer (mCRPC) is the most aggressive form of prostate cancer.[1] Outcomes have improved significantly with the advent of androgen receptor (AR)–directed therapies such as abiraterone and enzalutamide.[2-4] Still, approximately 20%-40% of patients exhibit primary resistance to these treatments and have substantially worse survival (median, < 6 months).[5,6] Other patients develop secondary resistance to AR-directed therapy, responding well initially before eventually developing resistance.[7] There is thus an urgent need for molecular biomarkers that can detect resistance to AR-directed therapy early, especially primary resistance, which would enable clinicians to consider alternative treatments (ie, chemotherapy, immunotherapy, or systemic radiotherapy) and potentially improve patient survival.

The clinically validated circulating tumor cell (CTC) assay for detecting an aberrant *AR* splice variant (AR-V7), a predictive biomarker of resistance to AR-directed therapy, highlights the potential value of liquid biopsy analysis in patients with mCRPC.[5,6,8] However, the reported sensitivity of this test for detecting AR-resistant mCRPC remains low at only approximately 30%.[6,8] Thus, although indicated for clinical use, there is a need for more sensitive assays to detect resistance to AR-directed therapy.

Assessment of cell-free DNA (cfDNA) has recently emerged as a noninvasive means to assess relevant genomic alterations in multiple cancer types, including prostate cancer.[9-16] cfDNA assessment of circulating tumor DNA has been shown to be sensitive for identifying tumor-specific somatic mutations with capability to even detect molecular residual disease.[10,11,13,16,17] In mCRPC, detection sensitivities

ASSOCIATED CONTENT

Appendix

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

Can we predict resistance to androgen receptor (AR)–directed therapy in patients with metastatic prostate cancer by tracking genomic alterations in the *AR* enhancer in addition to the *AR* gene body (*AR*/enhancer) in plasma cell-free DNA (cfDNA)?

Knowledge Generated

We developed Enhancer and Neighboring Loci of Androgen Receptor Sequencing (EnhanceAR-Seq) to monitor *AR*/enhancer alterations via liquid biopsy and detected *AR* enhancer amplification in cfDNA of 40% of patients with metastatic prostate cancer, including 8% without *AR* gene body amplification. Patients with cfDNA-detected alterations in the *AR* enhancer or gene body ubiquitously exhibited resistance to AR-directed therapy and had significantly worse survival.

Relevance

AR/enhancer alterations are the most frequent somatic event in metastatic prostate cancer, which we show are detectable in plasma cfDNA and predictive of resistance to AR-directed therapy and poor survival. Therefore, cfDNA liquid biopsy analysis of the *AR*/enhancer locus has the potential to improve risk stratification and help guide clinical decision making for metastatic prostate cancer.

have been shown to be high before treatment initiation, and genomic alterations, including those that target the *AR* gene body, can be reliably measured.[9,12,15] Still, it remains to be seen if measuring these genomic alterations can reliably identify resistance to AR-directed therapy.

Although *AR* is the key player in mCRPC treatment resistance, our understanding of the genomic alterations affecting *AR* is incomplete. To address this, recent large whole-genome sequencing studies discovered a long-range noncoding enhancer upstream of *AR* that promotes *AR* expression and resistance to AR-directed therapies.[18-20] Indeed, the *AR* enhancer was found to be amplified in 81%-87% of patients and is the most frequent genomic alteration in mCRPC (11%-17% more than *AR* gene body amplification).[18,20] Although studies have shown detection of *AR* gene body alterations in plasma cfDNA of patients with mCRPC,[9,12,15] none of these tracked the *AR* enhancer. Here we present a liquid biopsy cfDNA technique to monitor genomic alterations, including the *AR* enhancer, called Enhancer and Neighboring Loci of Androgen Receptor Sequencing (EnhanceAR-Seq) and demonstrate the ability to detect resistance to AR-directed therapy with high sensitivity and specificity.

PATIENTS AND METHODS

Patient Enrollment

We prospectively enrolled 40 patients with metastatic prostate cancer treated with at least 1 month of standard-of-care AR-directed treatment (eg, abiraterone or enzalutamide). All patients were maintained on standard androgen deprivation therapy (ie, luteinizing hormone-releasing hormone receptor agonist or antagonist). Prior treatment with other systemic agents, including chemotherapy, was allowed. Patients with evidence of any active nonprostate malignancy other than localized skin cancer were excluded from the study. Eligible patients underwent blood collection for cfDNA analysis at the time of enrollment. All patients underwent continued clinical and laboratory follow-up as per the standard of care. In addition, healthy adult blood donors (n = 36) were recruited from the Washington University School of Medicine and the American Red Cross Blood Center in St Louis, Missouri. All samples were collected with informed consent and institutional review board approval in accordance with the Declaration of Helsinki.

Sequencing and Analysis of Plasma cfDNA

We developed EnhanceAR-Seq as a targeted sequencing assay of plasma cfDNA to monitor genomic alterations in the *AR* gene and *AR* enhancer loci and other frequently altered genes[9,18] in metastatic prostate cancer (Appendix Table A1). We performed EnhanceAR-Seq on plasma from all patients acquired at the time of enrollment and analyzed genomic alterations with respect to matched plasma-depleted whole blood and unmatched healthy donor samples (Fig 1; Appendix Tables A2 to A7). In four patients, we also performed EnhanceAR-Seq on serial time points, including at least one time point during AR-directed treatment.

Clinical Outcomes and Statistical Analysis

Resistance to AR-directed therapy was scored by a board-certified academic medical oncologist specializing in genitourinary cancers. Primary resistance was defined as prostate-specific antigen (PSA) progression, change of therapy, or death within 4 months of treatment initiation, or radiographic progression within 6 months. Secondary resistance was defined as PSA progression, change of therapy, radiographic progression, or death outside of this

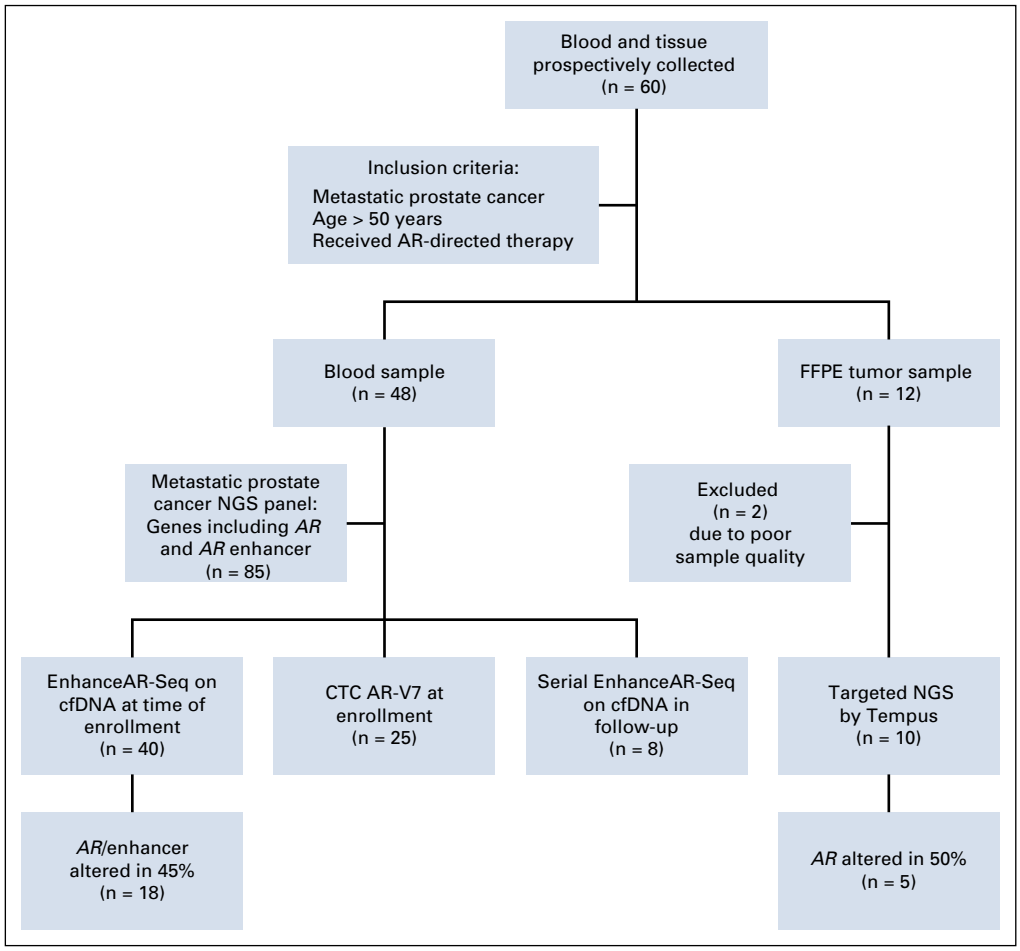

FIG 1. Patient enrollment and sample collection. Patients with biopsy-proven metastatic prostate cancer treated with androgen receptor (AR)-directed therapy were enrolled in the study and samples were collected for tissue, cell-free DNA (cfDNA), and circulating tumor cell (CTC) analyses. EnhanceAR-Seq, Enhancer and Neighboring Loci of Androgen Receptor Sequencing; FFPE, formalin-fixed paraffin-embedded; NGS, next-generation sequencing.

time frame. Associations between assay results and resistance to AR-directed therapy were assessed by Fisher's exact test. A progression-free survival (PFS) event was defined as the time to PSA progression by Prostate Cancer Clinical Trials Working Group 3[21] criteria or death, and an overall survival (OS) event was defined as the time to death. The Kaplan-Meier method and multivariate Cox proportional hazards models were used to analyze survival outcomes. Additional methodological details are provided in the Appendix.

RESULTS

Patient Characteristics

We prospectively enrolled 40 patients with metastatic prostate cancer treated with AR-directed therapy between November 2018 and November 2019 (Appendix Tables A2 and A3). The median age was 69 years, Eastern Cooperative Oncology Group performance status ranged between 0 and 2, and median follow-up time on study was 6.0 months. Among these patients, 11 were on their first line of systemic therapy, and the remaining 29 were on their second or greater line of systemic therapy for metastatic prostate cancer at the time of study enrollment.

EnhanceAR-Seq Detects Somatic Alterations in Plasma cfDNA

The most frequent genomic events detected in plasma cfDNA from our cohort were *AR*/enhancer alterations (most commonly copy number gain and tandem duplication), present in 18 patients (45%), including a 40% amplification rate in the *AR* enhancer region (Fig 2A; Appendix Tables A8 and A9). Three patients (8%) were found to have independent *AR*-enhancer amplification without *AR* gene body amplification, consistent with previous tissue-based results[18,20] (Fig 2A; Appendix Fig A1; Appendix Table A8). Other genes frequently found in cfDNA to be targeted by alterations included *TP53* and *PTEN*, which demonstrated copy number loss in 6 patients (15%) and Catalog of Somatic Mutations in Cancer (COSMIC)[22]–annotated nonsynonymous single nucleotide variants in 5 cases (13%;

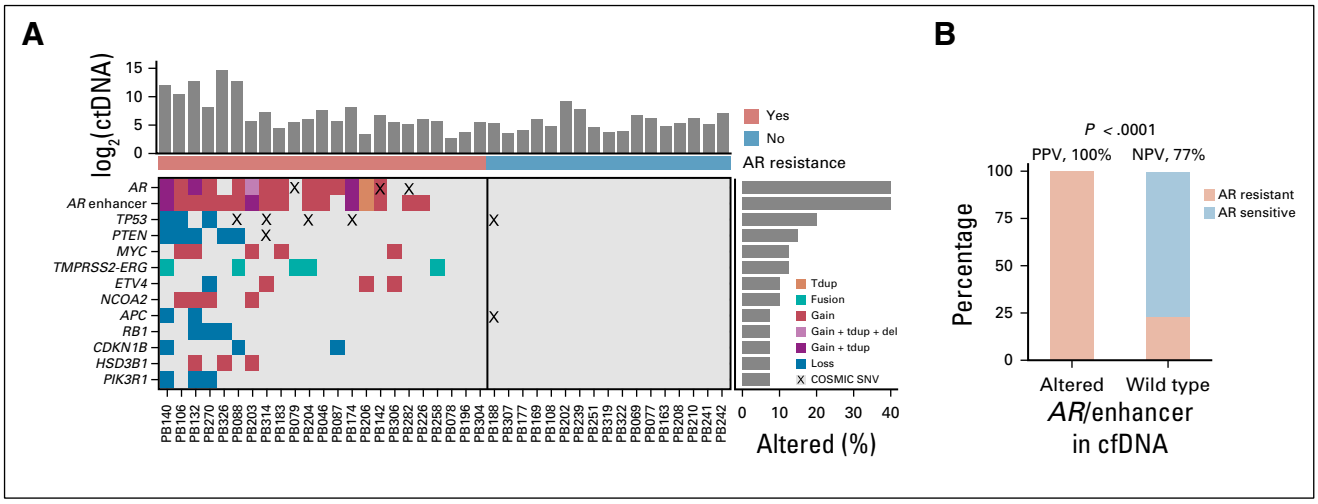

FIG 2. Genomic alterations in plasma cell-free DNA (cfDNA) in metastatic prostate cancer including the androgen receptor (*AR*)/enhancer locus. (A) Comutation plot based on cfDNA analysis of patients with metastatic prostate cancer treated with AR-directed therapy. Each column represents data from a single patient. Rates of queried genomic alterations are depicted by the bar graphs to the right. Only genes with > 5% alteration rate (considering tandem duplications, fusions, deletions, copy number changes, and Catalog of Somatic Mutations in Cancer [COSMIC]–indexed single-nucleotide variations [SNVs]) are displayed. Circulating tumor DNA (ctDNA) levels in haploid genome equivalents per milliliter of plasma are represented in the bar graph on top in $\log_2$ space. Resistance to AR-directed therapy is indicated below the bar graph as red (resistant) versus blue (sensitive). (B) Proportion of patients with *AR*/enhancer genomically altered (n = 18) or wild type (n = 22) in cfDNA, who developed resistance (n = 23) or not (n = 17) to AR-directed therapy. *P* value was calculated by Fisher's exact test. del, deletion; NPV, negative predictive value; PPV, positive predictive value; tdup, tandem duplication.

Fig 2A; Appendix Tables A8 and A10). We also detected *TMPRSS2-ERG* gene fusion in 5 cases (13%; Fig 2A; Appendix Table A9).

Ten patients consented to additional tissue-based analyses using metastatic biopsy samples. These samples were analyzed by targeted next-generation sequencing using the Tempus sequencing platform, which includes the *AR* gene body but not the enhancer.[23,24] Five patients had evidence of *AR* gene body alteration in tumor, with 4 of those having the same genomic changes evident in plasma. Overall, genomic alterations in *AR* were 80% concordant between tissue and plasma (Appendix Fig A2; Appendix Table A11), consistent with work published by others.[25]

AR/Enhancer Alterations in cfDNA Are Associated With Clinical Resistance

We observed the greatest concordance between genomic events and clinical resistance to AR-directed therapy for alterations in the *AR* locus including the enhancer (Fig 2). Alterations in the *AR*/enhancer locus predicted resistance with 78% sensitivity and 100% specificity (Fig 2B). There was a highly significant correlation between alterations detected in *AR*/enhancer in cfDNA and resistance to AR-directed therapy ($P < .0001$). Interestingly, all three patients with *AR* enhancer amplification in cfDNA in the absence of *AR* gene body amplification had disease progression to resistance at a median of 5.3 months (range, 0.6-8.0 months), indicative of improved sensitivity in identifying resistance when tracking the *AR* enhancer in addition to the gene body. The AR-V7 Nucleus Detect CTC assay was run at a median of 16 days from cfDNA analysis in 25 patients, including within 24 hours of cfDNA testing for 10 patients. AR-V7 was detected in CTCs from two patients (8%) and was negative in the remaining 23 (Appendix Fig A3; Appendix Table A3).

AR/Enhancer Alterations in cfDNA Portend Poor PFS

PFS was significantly shorter among men with detected *AR*/enhancer alterations in plasma cfDNA (18 patients) compared to those without (22 patients; hazard ratio [HR], 6.8; 95% CI, 2.5 to 18.6; $P = .0002$; Fig 3A). PFS remained significantly shorter with similar HR when restricting our analysis to just the *AR* enhancer region (HR, 8.1; 95% CI, 2.8 to 23.6; $P = .0001$; Appendix Fig A4A). cfDNA-detected alterations in the *AR*/enhancer locus or the *AR* enhancer alone remained highly significant by multivariate Cox proportional hazards regression, which included important baseline characteristics such as PSA concentration, circulating tumor DNA (ctDNA) level, number of lines of therapy received in the metastatic setting, prior enzalutamide versus abiraterone treatment, metastatic disease burden, and time since diagnosis (Appendix Tables A12-A15). We also found that overall ctDNA levels and mutational burden did not correlate with clinical outcomes, nor were they significantly different between patients who developed AR resistance versus those who remained AR sensitive (Appendix Fig A5; Appendix Table A16).

AR/Enhancer Alterations in cfDNA Portend Poor OS

Although median follow-up of our cohort from time of enrollment was only 6.0 months, we performed a

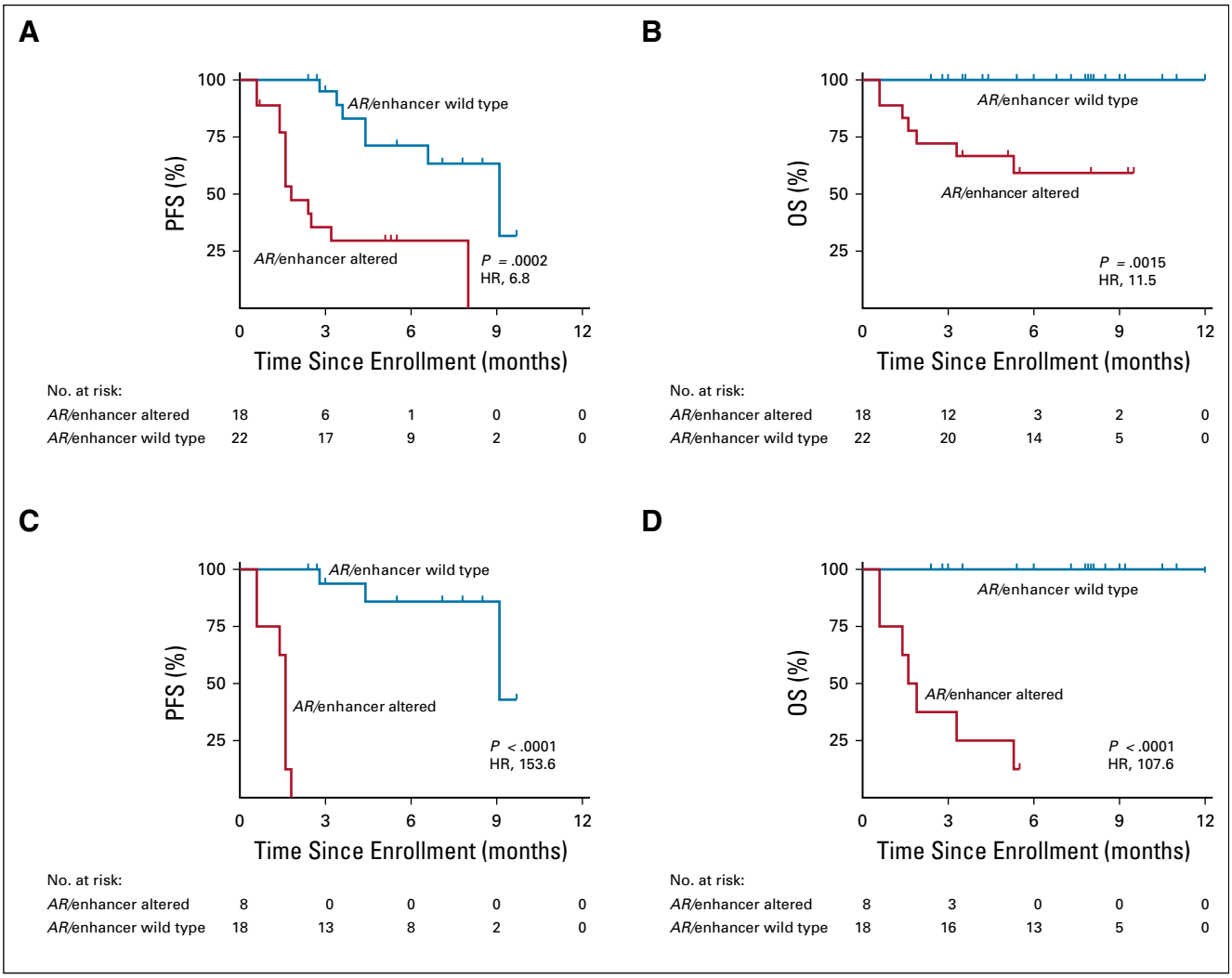

FIG 3. Progression-free survival (PFS) and overall survival (OS) according to androgen receptor (*AR*)/enhancer alteration status in cell-free DNA (cfDNA). (A) PFS, and (B) OS represent the full 40-patient cohort; (C) PFS, and (D) OS after excluding patients with secondary resistance to AR-directed therapy. Kaplan-Meier analyses were performed from the time of sample collection (time of enrollment), stratified based on the genomic alteration status of *AR*/enhancer measured in cfDNA. *P* values were calculated by the log-rank test and hazard ratios (HRs) by the Mantel-Haenszel method.

preliminary OS analysis. OS was significantly shorter among men with detected *AR*/enhancer alterations in plasma cfDNA compared to those without (HR, 11.5; 95% CI, 2.5 to 52.1; *P* = .0015; Fig 3B). OS remained significantly shorter with a high HR when ignoring *AR* gene body alterations and restricting our analysis to just the *AR* enhancer region (HR, 16.4; 95% CI, 3.5 to 77.2; *P* = .0004; Appendix Fig A4B).

AR/Enhancer Alterations in cfDNA in Primary Versus Secondary Resistance

Our cohort included 9 cases of primary resistance and 14 cases of secondary resistance to AR-directed therapy. In all cases of primary resistance, patients experienced no response, whereas in cases of secondary resistance, patients experienced a temporary treatment response before ultimately experiencing disease progression on AR-directed therapy. Notably, the previously published AR-V7 assay has only been shown to be capable of identifying primary resistance, albeit with limited sensitivity.[5,6] We thus decided to test EnhanceAR-Seq more exclusively in this space. Positive predictive value of cfDNA-derived *AR*/enhancer alterations for primary resistance was 100%, with every positive case progressing within 3 months and all but one patient dying within 6 months of study enrollment (Figs 3C and 3D). The sensitivity of our assay for detecting primary resistance was 89%, higher than the 71% we observed for secondary resistance, whereas specificity remained 100%.

We obtained serial samples from four patients with at least one time point being during AR-directed therapy (Fig 4). For patient PB078 (Fig 4A), EnhanceAR-Seq detected no evidence of *AR*/enhancer alterations at enrollment, and AR-V7 detection in CTCs was also negative. At 19 and 45 weeks

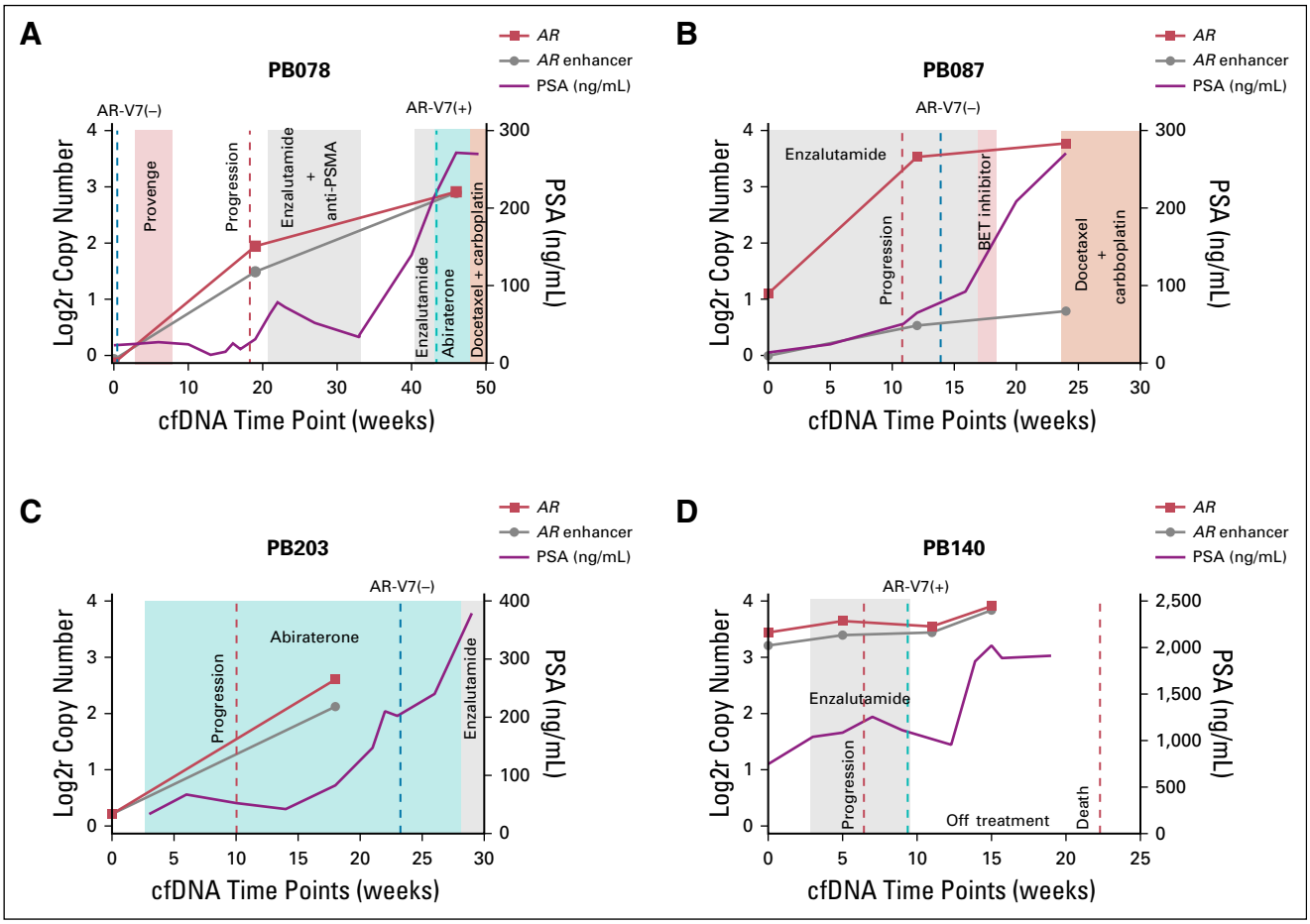

FIG 4. Serial time point liquid biopsy analyses of patients on androgen receptor (AR)-directed treatment. (A) The patient was negative for circulating tumor cell (CTC) AR-V7 and cell-free DNA (cfDNA) *AR*/enhancer alteration at the time of enrollment. At week 19, shortly before receiving enzalutamide and anti–prostate-specific membrane antigen (PSMA), he tested positive for amplifications in *AR* and its enhancer in cfDNA. The patient initially responded, then after a treatment break experienced rapid progression on both enzalutamide and abiraterone. Repeat testing at this final time point (approximately 45 weeks postenrollment) was positive, with additional amplification observed in *AR* and its enhancer in cfDNA and AR-V7 detected in CTCs. (B-D) Clinical vignettes of three more patients with metastatic castration-resistant prostate cancer (mCRPC) with serial cfDNA collected over time, with at least 1 time point occurring during AR-directed therapy. *AR* and *AR* enhancer copy number ratios in cfDNA are shown over time in $\log_2$ space, and prostate-specific antigen (PSA) concentrations in blood are shown in ng/mL. Treatments are indicated in colored boxes, time of progression or death as dashed red lines, and AR-V7 test results as dashed green lines (if positive) or dashed blue lines (if negative). Weeks since study enrollment are shown on the *x*-axis. BET, bromodomain and extraterminal domain; Log2r, logarithm base 2 ratio.

later, EnhanceAR-Seq revealed significantly elevated copy number amplification of both the *AR* gene body and enhancer, while the patient was actively developing resistance to enzalutamide followed by abiraterone. The CTC AR-V7 assay also became positive at approximately 45 weeks. Patients PB087 and PB203 similarly showed rapid increases in *AR*/enhancer copy number on enzalutamide and abiraterone, respectively, while AR-V7 testing remained negative (Figs 4B and 4C). Cell-free *AR*/enhancer amplification preceded increases in PSA and clinician-recognized resistance leading to therapy change. For patient PB140 (Fig 4D), *AR*/enhancer copy number increased more subtly on serial analysis; however, in this case the baseline copy numbers for *AR* and its enhancer were already > 8-fold elevated; reflective of this, the patient's disease progressed rapidly 6 weeks after study enrollment, and the patient died as a result of mCRPC at 22 weeks. These vignettes demonstrate the potential value of using cfDNA-based *AR*/enhancer analysis as a precision modality to monitor treatment resistance in patients with metastatic prostate cancer undergoing AR-directed therapy.

DISCUSSION

In this study, we developed and tested a cfDNA analysis method for assessing treatment resistance in metastatic prostate cancer, which we call EnhanceAR-Seq. Our results indicate that cfDNA analysis is a promising approach for detecting resistance to AR-directed therapy, with 100% positive predictive value and 78% sensitivity. Sensitivity increased to 89% when considering only

primary-resistant cases. EnhanceAR-Seq outperformed the CTC AR-V7 test used clinically, which was performed for a subset of patients in our study. In available cases, we also performed tumor sequencing and observed 80% concordance between *AR* genomic alterations in tumor and plasma.

We also factored in baseline ctDNA level in multivariate Cox regression analyses to determine if it might be a confounding variable, which we found did not correlate with clinical outcomes and was not significantly different between AR-resistant and AR-sensitive patients. Although other baseline differences between patients could have influenced our study's outcomes, we accounted for them through four separate multivariate Cox regression analyses (Appendix Tables A12-A15), where we found that only *AR*/enhancer alterations, including in the *AR* enhancer alone, were highly significantly associated with resistance to AR-directed therapy (HR $>$ 10; $P < .005$).

Within our cohort, every patient with detectable alterations in *AR* or its enhancer in cfDNA developed resistance and experienced progression despite a relatively short follow-up period. *AR*/enhancer alterations were associated with statistically significantly worse PFS and OS. In contrast, the Genomic Health (Redwood City, CA) CTC AR-V7 assay was positive in only 8% of tested cases and did not correlate significantly with outcomes. It is important to note, however, that larger studies have shown correlations of CTC AR-V7 detection with outcomes,[5,6,8] which may not have been evident here because of small cohort size, heterogeneous nature of our cohort, and CTC AR-V7 testing being performed in only 63% of our cohort. Still, the 8% positivity rate for the Genomic Health CTC AR-V7 assay in our cohort is similar to the 10% positivity rate of this assay in high-risk patients with mCRPC in the recently published PROPHECY trial,[26] suggesting our results may be in line with other prospective data.

Five cases of resistance to AR-directed treatment were not detected using our cfDNA assay. However, four of these represent secondary resistance to AR-directed therapy, where patients initially responded to treatment before eventually developing resistance. In this regard, we performed a serial time point analysis in a patient (PB078), where both EnhanceAR-Seq and CTC AR-V7 were negative at the initial responsive time point, but both assays became positive as the patient evolved resistance to enzalutamide followed by abiraterone. Serial time point analysis of two other patients without significant *AR*/enhancer amplification at baseline (PB087 and PB203), including one who received abiraterone followed by enzalutamide, also demonstrated dramatically increasing *AR*/enhancer copy numbers over time, which anticipated clinical progression and increasing PSA during AR-directed treatment. In contrast, a fourth case (PB140) of primary resistance demonstrated $>$ 8-fold amplification of *AR* and its enhancer at baseline, which remained highly elevated on serial analysis. This correlated with rapid early progression on enzalutamide and death from mCRPC at 22 weeks. These data support the potential value of serial time point analysis, especially in the secondary resistance setting where *AR*/enhancer amplification may not be apparent at baseline. These clinical vignettes also suggest that our assay could potentially inform clinicians when to trial a different AR-directed treatment (when *AR*/enhancer copy numbers remain low) or switch to a different therapy type altogether (when *AR*/enhancer copy numbers have risen high).

Resistant patients identified by *AR*/enhancer alterations may be completely distinct from those with AR-V7 messenger RNA splice variation.[27] Given assessment of different mechanisms of resistance, one at the DNA level (detected by EnhanceAR-Seq) and the other at the mRNA/protein level[26] (detected by CTC-based assays), it may be valuable to run both methods to more comprehensively assess multiple mechanisms of resistance in certain cases. In our cohort, CTC AR-V7 results did not improve on the sensitivity achieved with EnhanceAR-Seq; however, we note that AR-V7 testing was performed in only a subset of our patients.

To our knowledge, our assay is the first to monitor the *AR* enhancer in the cell-free compartment. In addition to showing that *AR* enhancer amplification can be detected in plasma cfDNA from patients with metastatic prostate cancer, we observed that 13% of resistant patients had *AR* enhancer amplification detectable in plasma cfDNA independent of gene body amplification. Although our cohort is small, the prevalence of independent *AR* enhancer amplification is consistent with prior studies.[18,20] Highlighting its clinical importance, *AR* enhancer amplification stratified patients by both resistance to AR-directed therapy and survival outcomes. All patients with independent *AR* enhancer amplification progressed to treatment resistance at a median of 5.3 months, highlighting the importance of monitoring the *AR* enhancer in addition to the gene body.

In addition to genomic alterations in *AR* and its enhancer, we assessed 84 other genes shown to be important in mCRPC.[9,18] In several cases, we observed multiple alterations involving different genes, including *TP53* and *PTEN*, consistent with prior work.[18] We also targeted a 13kb fusion hotspot in the *TMPRSS2* intronic region, on the basis of analysis of previously published whole-genome sequencing data in mCRPC.[18] This enabled us to identify a subset of *TMPRSS2-ERG* fusion events in our cohort. To monitor *TMPRSS2-ERG* fusions more comprehensively, we would have needed to target full lengths of *TMPRSS2* and *ERG* gene bodies and introns, which would have required a much larger targeted space and limited our sequencing depth of coverage.

Limitations of our study include a short follow-up period, reducing our ability to assess long-term clinical outcomes

such as PFS and OS. Despite this, hazard ratios for survival outcomes were high on Kaplan-Meier analysis. It is possible that with longer follow-up time, we would observe an even greater predictive and prognostic value of measuring *AR*/enhancer alterations in cfDNA. In addition, patients were enrolled while on different lines of therapy, leading to cohort heterogeneity, similar to clinical studies involving the CTC AR-V7 assay.[5,6,8] CTC AR-V7 testing was performed on only a subset of patients, which could have biased our ability to compare it to cfDNA analysis.

In conclusion, we developed a novel cfDNA assay, EnhanceAR-Seq, to detect genomic alterations in the *AR* locus including the enhancer. Our method effectively detected resistance to AR-directed therapy and stratified patients on the basis of PFS and OS despite short follow-up time. Assay performance improved further when considering only primary-resistant disease. Our results remained highly significant when accounting for baseline characteristics such as PSA concentration, ctDNA level, and metastatic disease burden. Serial time point analysis in four patients demonstrated the potential value of using our assay to monitor for AR resistance during treatment. Although our cohort was relatively small, EnhanceAR-Seq applied to a single time point predicted resistance to AR-directed therapy with high sensitivity and specificity. Our results suggest that cfDNA analysis through EnhanceAR-Seq can help improve risk stratification and clinical decision making for metastatic prostate cancer. Future clinical trials should be performed to validate our findings before clinical implementation.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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APPENDIX

Study Design

Our study was designed to determine whether assessment of genomic alterations in the *AR* enhancer and gene body (collectively referred to as *AR*/enhancer) in cell-free DNA (cfDNA) could predict resistance to AR-directed systemic therapy. The sample size of 40 was justified to achieve 90% power by a 2-sided normal test at a 5% α to detect a difference of 75% versus 25% rate of resistance for patients with positive versus negative cfDNA results, assuming a 50% rate of *AR*/enhancer alteration in cfDNA[15] and a 5% attrition rate. We obtained peripheral blood at the time of enrollment, which was processed within 6 hours of phlebotomy for cfDNA analysis. A separate blood sample was submitted for circulating tumor cell (CTC) AR-V7 analysis (Genomic Health) in a subset of patients at the discretion of the treating oncologist. Laboratory research investigators were unaware of the AR-V7 status of study participants at the time of cfDNA analysis. For 4 patients, blood was drawn serially for cfDNA analysis with time points being at least 2 weeks apart and at least 1 time point occurring during AR-directed treatment.

Specimen Collection and Processing

Between 10 and 20 mL of peripheral blood was collected in K_2EDTA Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) at the time of study enrollment. Tubes were centrifuged at 1,200*g* for 10 minutes, then plasma separated and centrifuged for another 5 minutes at 1,800*g*. Plasma was then frozen at –80°C before cfDNA processing and analysis. Leukocyte-enriched plasma-depleted whole blood (PDWB) was also collected and frozen at –80°C for isolation of germline genomic DNA as previously described.[14,17] Peripheral blood was separately collected in a subset of patients using collection tubes provided by Genomic Health (Redwood City, CA) for the Oncotype DX AR-V7 Nucleus Detect CTC assay. After collection, tubes were immediately sent to Genomic Health for analysis following their protocol.

DNA Isolation and Quantification

cfDNA was extracted from plasma using the QiaAmp Circulating Nucleic Acid Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. cfDNA concentration was measured with a Qubit 4.0 Fluorometer using the dsDNA High Sensitivity Assay Kit (Thermo Fischer Scientific, Waltham, MA). cfDNA fragment size was determined using an Agilent 2100 Bioanalyzer with the High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA). A median of 32 ng was inputted into sequencing library preparation on the basis of the percentage of cfDNA in the 70-450 bp region of the bioanalyzer electropherogram. The QIAamp DNA Micro Kit (Qiagen) was used to extract genomic DNA from 100 μL of PDWB. Genomic DNA from PDWB was fragmented before library preparation using an LE220 focused ultrasonicator (Covaris, Woburn, MA).

Development of EnhanceAR-Seq Gene Panel

To develop a next-generation sequencing (NGS) assay for metastatic prostate cancer cfDNA analysis, we designed a hybrid-capture gene panel to target the complete *AR* gene body (including introns), 30 kb of the *AR* enhancer, and exons of 84 other genes that have been shown to harbor genomic alterations in metastatic castration-resistant prostate cancer (mCRPC).[9,18] To gain finer detail for copy number analysis in the full *AR*/enhancer locus, we evenly placed 500-bp targeted regions (50 kb apart) between ~500 kb upstream of the *AR* enhancer and ~500 kb downstream of the *AR* gene body. Our panel also included the *TMPRSS2-ERG* gene fusion hotspot intronic region (13 kb) in the *TMPRSS2* gene to detect a subset of *TMPRSS2-ERG* gene fusions. In addition, 12 genes least frequently affected by copy number alteration in mCRPC (surveyed in prior whole-genome sequencing data[18]) were included in the panel as controls for copy number analysis, and three genes were included to assess clonal hematopoiesis (Genovese G, et al: N Engl J Med 371:2477-2487, 2014; Jaiswal S, et al: N Engl J Med 371:2488-2498, 2014). NimbleDesign was used to convert our desired gene panel into a SeqCap EZ Prime Choice probe set (Roche, Basel, Switzerland).

DNA Processing and Analysis

We performed cfDNA and PDWB DNA library preparation using the Cancer Personalized Profiling by deep Sequencing (CAPP-Seq) workflow[14] with duplex barcoded adapters,[17] then performed NGS on an Illumina HiSeq4000 with 2 × 150 bp paired-end reads, with 12 samples sequenced per lane, dedicating approximately 60 million reads per sample. We then applied a custom bioinformatics pipeline detailed in the sections below.

cfDNA Single-Nucleotide Variant and Indel Analysis

cfDNA sequencing results were analyzed for single-nucleotide variants (SNVs) and insertions/deletions (indels) using the CAPP-Seq bioinformatic pipeline.[10,14,17] Briefly, cfDNA sequencing reads were demultiplexed using sample-level index barcodes, mapped to the human reference genome, filtered for properly paired reads, filtered for bases with Phred quality score $\geq$ 30, then deduplicated using unique molecular identifiers. Background polishing using 12 healthy donor plasma samples was performed to reduce stereotypical base substitution errors as previously described using the integrated digital error suppression method.[17] Variant calling using the CAPP-Seq pipeline was then performed to call SNVs and indels from patient plasma using matched PDWB as the background reference, filtered further to remove potential single-nucleotide polymorphisms with variant allele fraction (vAF) $>$ 45%, loci with deduplicated depth $<$ 100, and mutations in the canonical clonal hematopoiesis genes *ASXL1*, *DNMT3A*, and *TET2* (Genovese G, et al: N Engl J Med 371:2477-2487, 2014; Jaiswal S, et al: N Engl J Med 371:2488-2498, 2014). Nonsynonymous SNVs and indels $\geq$ 2 bp in plasma, not present in matched PDWB, not present in the Genome Aggregation Database (gnomAD; Karczewski KJ, et al: bioRxiv 531210, 2019) at a $>$ 0.0001 frequency, and indexed in the Catalogue of Somatic Mutations in Cancer (COSMIC)[22] were reported in the final data set shown in Figure 2 and Appendix Table A10. Mutations in *AR* that met these criteria were considered positive by EnhanceAR-Seq. An additional SNV analysis using the filters described above but not requiring COSMIC indexing was performed to measure overall circulating tumor DNA (ctDNA) SNV burden (number of SNVs detected per patient) and levels (on the basis of mean vAF and cfDNA concentration), shown in Appendix Figure A5 and Appendix Table A16.

cfDNA Copy Number Analysis

Cell-free DNA sequencing results were demultiplexed using sample-level index barcodes, mapped to the human reference genome, then deduplicated using Picard (https://github.com/broadinstitute/picard) on the basis of identical start/end coordinates. Copy number analysis was performed based on a read depth approach. First, the genome was binned (larger bins for nontargeted regions and smaller bins for targeted regions) and read depth ratios for bins between plasma cfDNA and matched PDWB control samples were calculated and corrected for biases in GC content, sequence repeats, and target density using CNVkit (Talevich E, et al: PLOS Comput Biol 12:e1004873, 2016).

Subsequently, read depth ratios were centralized by subtracting the mean $\log_2$ ratios of all bins across chromosomes and normalized using read depth ratios from bins overlapping with copy number control genes. Copy number segmentation was performed using DNACopy (Seshan VE, et al: R package version 1.60.0, 2019). To obtain the background read depth ratios for individual genes/loci, we performed the same analysis on 24 pairs of plasma and matched PDWB control DNA samples from male healthy donors. Finally, a gain (or loss) event in patient plasma was called when the calculated $\log_2$ ratio was four standard deviations above (or below) the median $\log_2$ ratio of that locus in healthy plasma. Genes whose $\log_2$ ratios showed high variability or

deviation from 0 in healthy plasma samples (median > 0.2 or standard deviation > 0.2) were excluded from the copy number analysis.

cfDNA Structural Variation Analysis

Our targeted panel was designed to capture structural variation (SV) breakpoints targeting full-length *AR* (including intronic regions) and the *TMPRSS2-ERG* fusion hotspot in an intron of *TMPRSS2*. SVs including tandem duplications were called using Lumpy (Layer RM, et al: Genome Biol 15:R84, 2014) and Manta (Chen X, et al: Bioinformatics 32:1220-1222, 2016), using plasma samples with matched PDWB control samples. Subsequently, SVs with breakpoints overlapping the blacklist and low complexity regions (Li H: Bioinformatics 30:2843-2851, 2014) or those with both breakpoints falling in nontargeted regions were removed. Additional filtering was applied to retain only SVs with at least two supporting discordant read pairs or split reads and with high confidence regarding breakpoint positions (on the basis of the width of the confidence interval provided by Manta or Lumpy being < 5 bases) and filtering out SVs with abnormally high read support (> 150 discordant read pairs or split reads) in patient plasma cfDNA.

Tissue Molecular Analysis

For some cases, at the discretion of the treating oncologist, matched formalin-fixed paraffin embedded tumor tissue of a metastatic site was available for molecular analysis. Tissue was submitted to Tempus Laboratories, where DNA was isolated and targeted NGS performed with approximately 500× coverage using one of two panels—Tempus xO (Beaubier N, et al: Oncotarget 9:25826-25832, 2018; 1,714 genes) or Tempus xT (Beaubier N, et al: Oncotarget 10:2384-2396, 2019; 596 genes). Both panels included the *AR* coding region.

Clinical Outcomes and Statistical Analysis

The primary clinical end point, primary or secondary resistance to AR-directed therapy, was scored by a board-certified academic medical oncologist specializing in genitourinary cancers. Primary resistance was defined as prostate-specific antigen (PSA) progression, change of therapy or death within 4 months of treatment initiation, or radiographic progression within 6 months. Secondary resistance was defined as PSA progression, change of therapy, or radiographic progression or death outside of this time frame. PSA progression was defined as an increase of ≥ 25% above nadir and ≥ 2 ng/mL, with confirmation ≥ 3 weeks later (Prostate Cancer Clinical Trials Working Group 3 [PCWG3]; Scher HI, et al: J Clin Oncol 34:1402-1418, 2016). Secondary end points for our study were progression-free survival (PFS) defined as the time to PSA progression by PCWG3 criteria or death, or last known date of PSA measurement in nonprogressors, and overall survival (OS) defined as time to death or to last follow-up for alive patients. PFS and OS were calculated from the time of study enrollment.

We performed survival and statistical analyses using R version 3 (http://www.rproject.org) and Prism 8 (Graphpad Software, San Diego, CA). Fisher's exact test was used to assess the significance level of associations between assay results and resistance to AR-directed therapy. For PFS and OS Kaplan-Meier analyses, the log-rank test was used to estimate *P* values and the Mantel-Haenszel method used to estimate hazard ratios. Multivariate Cox proportional hazards models were fitted with incorporation of important baseline covariates including PSA concentration, ctDNA levels, number of lines of prior therapy, prior abiraterone versus enzalutamide treatment, metastatic disease burden, and time since diagnosis to further assess the independent impact of *AR*/enhancer alterations detected in cfDNA. Proportional hazards assumptions were confirmed for these analyses by evaluating the Schoenfeld residuals.

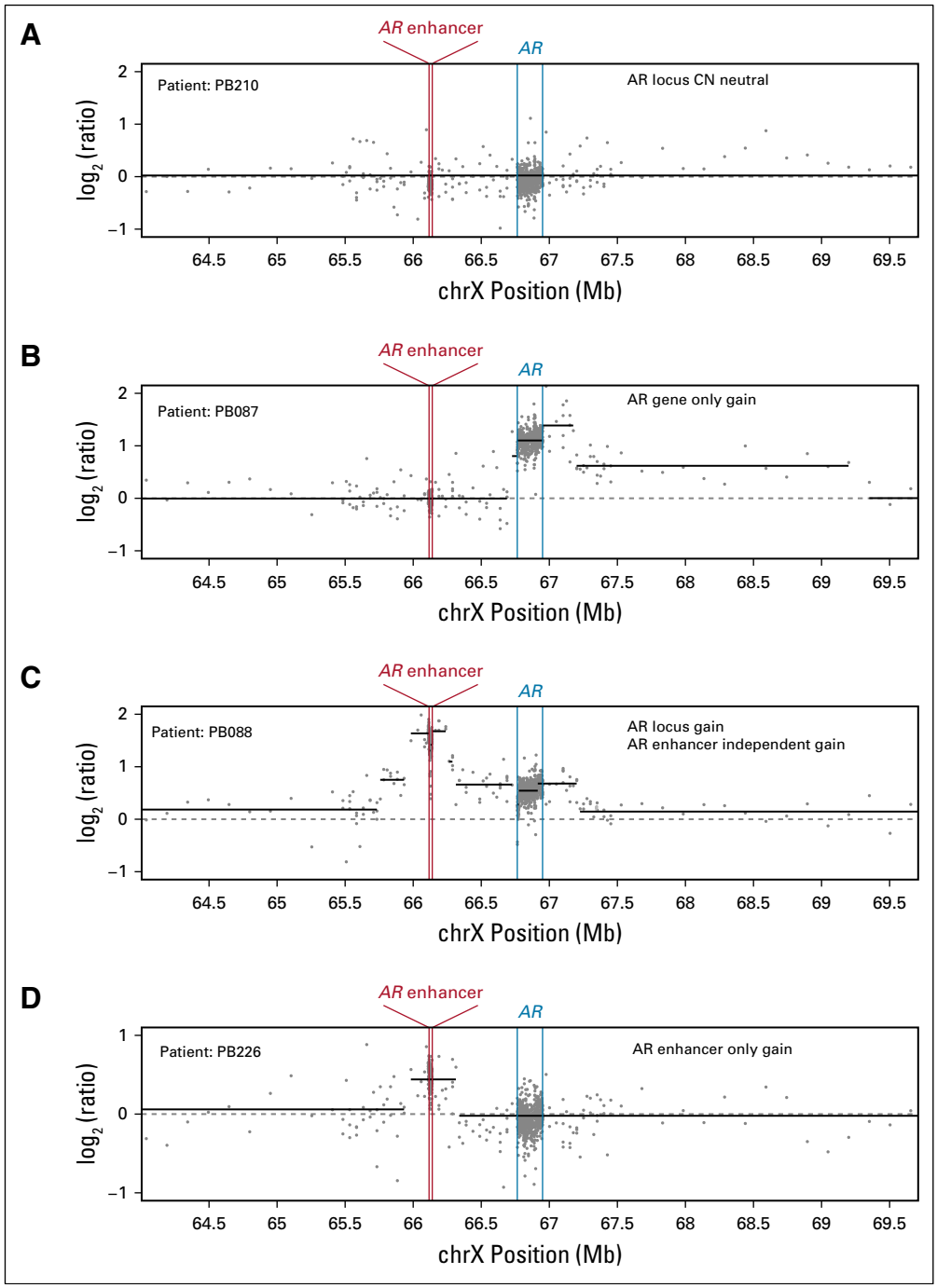

FIG A1. Examples of copy number determination in the androgen receptor (*AR*) enhancer and gene body from cell-free DNA (cfDNA). Each panel depicts the $\log_2$ copy number ratio of the *AR* locus and surrounding genomic space, from patient cfDNA normalized to matched plasma-depleted whole blood targeted next-generation sequencing. (A) Example of a patient with no copy number alterations in the *AR* enhancer or gene body. (B) cfDNA from a patient with copy number gain in the *AR* gene body but not enhancer. (C) Patient with cfDNA amplification of both the *AR* enhancer and gene body. (D) Patient with cfDNA copy number gain in the *AR* enhancer but not gene body. CN, copy number; chrX, chromosome X; $\log_2$, logarithm base 2; Mb, megabase.

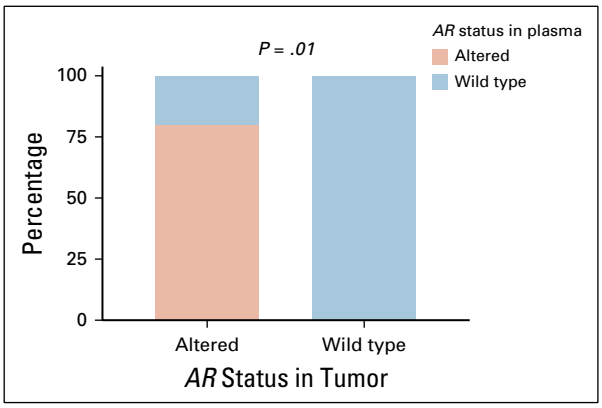

FIG A2. Comparison of androgen receptor (*AR*) gene body alterations detected by tumor and plasma cell-free DNA (cfDNA) sequencing. Ten patients had samples available for this analysis. Targeted next-generation sequencing (NGS) was performed on tumor DNA (extracted from formalin-fixed paraffin-embedded tissue) and plasma cfDNA. *AR* genomic alterations were detected in 5 cases by tumor NGS with these same alterations present in 4 cases by plasma NGS. *P* value was calculated by the Fisher's exact test.

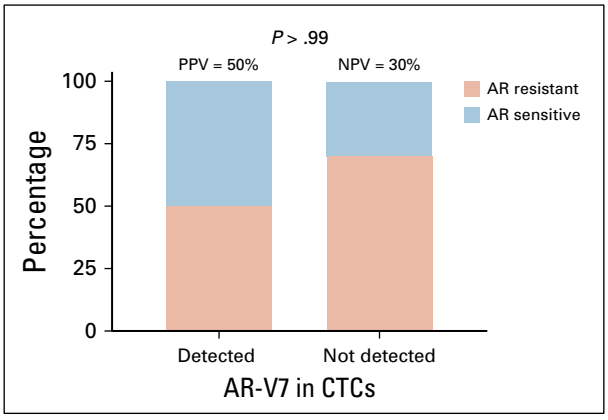

FIG A3. AR-V7 detection in circulating tumor cells (CTCs) and its association with resistance to androgen receptor (AR)–directed therapy in the present cohort. Proportion of patients with AR-V7 detected (n = 2) or not (n = 23) in circulating tumor cells who developed resistance or not to AR-directed therapy are shown. The positive predictive value (PPV) and negative predictive value (NPV) are displayed in each panel. *P* values were calculated using Fisher's exact test. *AR*, androgen receptor.

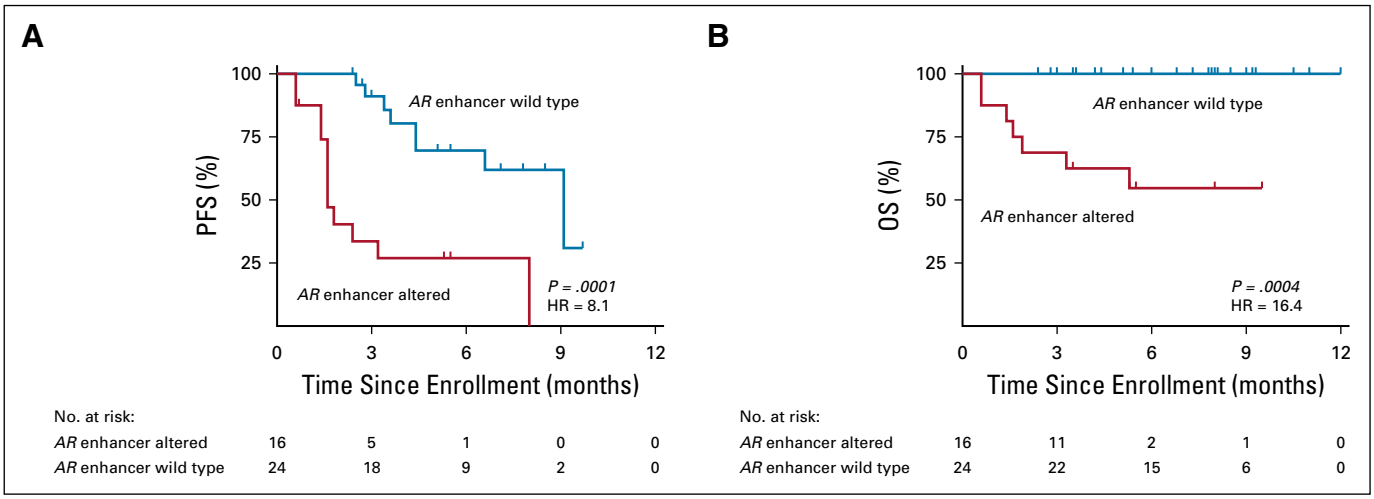

FIG A4. (A) Progression-free survival (PFS), and (B) overall survival (OS) according to androgen receptor (*AR*) enhancer status in cell-free DNA (cfDNA). For PFS, median was 1.6 months in patients with *AR* enhancer altered and 9.1 months in patients with wild-type *AR* enhancer in cfDNA. For OS, median was not reached in either arm. Hazard ratio (HR) for PFS was 8.1 (95% CI, 2.8 to 23.6; $P = .0001$) and 16.4 (95% CI, 3.5 to 77.2; $P = .0004$) for OS. *P* values were calculated by the log-rank test and HRs by the Mantel-Haenszel method.

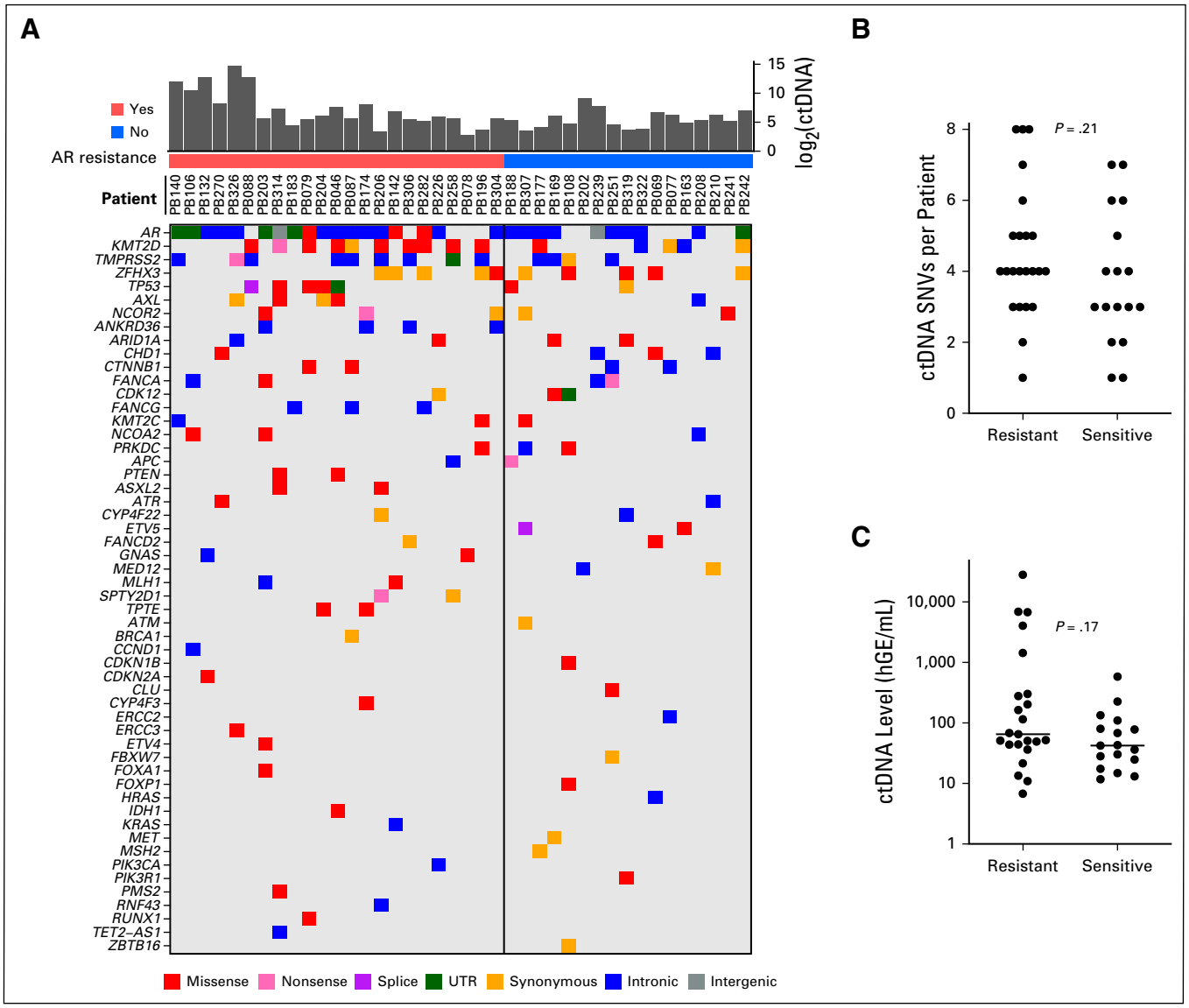

FIG A5. Single-nucleotide variant (SNV)–based analyses of mutational burden and circulating tumor DNA (ctDNA) levels at baseline. (A) Heat map of all somatic SNVs detected in cell-free DNA from each patient at time of enrollment. Genes are shown on the left and mutation types are indicated by color in the legend below. ctDNA levels are represented in the bar graph on top in $\log_2$ space. Resistance to AR-directed therapy is indicated below the bar graph as red (resistant) versus blue (sensitive). (B) Comparison of the number of SNVs and (C) ctDNA levels in AR-resistant versus AR-sensitive patients. ctDNA levels are in haploid genome equivalents (hGE) per milliliter; *P* values were calculated by Student's *t* test. UTR, untranslated region.

TABLE A1. Genes Included in the EnhanceAR-Seq Targeted Sequencing Panel

AKT1	*CDK4*	*ETV5*	*KDM6A*	*NFE2L2*	*SPOP*	*CYP4F3*
AKT2	*CDK6*	*FANCA*	*KMT2C*	*NKX3-1*	*TMPRSS2*	*ELF4*
AKT3	*CDKN1B*	*FANCC*	*KMT2D*	*PIK3CA*	*TP53*	*SLITRK2b*
APC	*CDKN2A*	*FANCD2*	*KRAS*	*PIK3CB*	*ZBTB16*	*SPANXN1*
AR	*CHD1*	*FANCE*	*MDM2*	*PIK3R1*	*ZFHX3*	*SPTY2D1*
AR Enhancer	*CLU*	*FANCF*	*MDM4*	*PMS1*	*ZNRF3*	*TPTE*
ARID1A	*CTNNB1*	*FANCG*	*MED12*	*PMS2*		*TRIM43*
ASXL2	*CUL1*	*FBXW7*	*MET*	*PRKDC*		*ACTRIB*
ATM	*ERCC1*	*FOXA1*	*MLH1*	*PTEN*		*AKAP7*
ATR	*ERCC2*	*FOXP1*	*MSH2*	*RAD51B*		*ANKRD36*
AXL	*ERCC3*	*GNAS*	*MSH3*	*RAD51C*		*APLN*
BRAF	*ERCC4*	*HDAC4*	*MSH6*	*RB1*		*CYP4F22*
BRCA1	*ERCC5*	*HRAS*	*MYC*	*RNF43*		*ASXL1*
BRCA2	*ERG*	*HSD3B1*	*NCOA2*	*RUNX1*		*DNMT3A*
CCND1	*ETV1*	*IDH1*	*NCOR1*	*RYBP*		*TET2*
CDK12	*ETV4*	*IDH2*	*NCOR2*	*SMARCA1*		

NOTE. Copy number and clonal hematopoiesis control genes are listed in the right-most column.
Abbreviation: EnhanceAR-Seq, Enhancer and Neighboring Loci of Androgen Receptor Sequencing.

TABLE A2. Patient Characteristics

Baseline Characteristic	All Patients (N = 40)
Age, years	69 (50-93)
Race	
White	32 (80.0)
African American	7 (17.5)
Other	1 (2.5)
ECOG performance status	
0	10 (25.0)
1	21 (52.5)
2	9 (22.5)
Time since diagnosis, years	4.2 (0.5-22.6)
Lines of systemic therapy	3 (1-11)
Baseline PSA, ng/mL	29.9 (0.1-1,343)
Metastatic burden	
High	31 (77.5)
Low	9 (22.5)
Presence of bone metastases	
Yes	34 (85.0)
No	6 (15.0)
Type of local treatment	
Surgery	13 (32.5)
Radiation	7 (17.5)
None	20 (50.0)
AR-directed therapy use	
Abiraterone	23 (57.5)
Enzalutamide	16 (40.0)

NOTE. Data are presented as No. (%) or median (range).

Abbreviations: ECOG, Eastern Cooperative Oncology Group; PSA, prostate-specific antigen.

TABLE A3. Clinical, Treatment, and Outcome Details for All Patients

Patient ID	Age (years)	Race	ECOG PS	Time Since Diagnosis[a] (years)	Line of Therapy in Metastatic Setting[b]	Baseline PSA[c]	Metastatic Disease Burden[d]	Primary Therapy	AR-Directed Therapy	Sequencing of Prior AR-Directed Therapy	Resistance to AR-Directed Therapy	Resistance Type	AR/Enhancer Alterations in cfDNA	AR-V7 CTC Assay	Progression Status[e]	PFS (months)	Survival Status[f]	OS (months)
PB046	61	White	0	17	11	214.7	High	Radiotherapy	Abiraterone enzalutamide	Abiraterone→enzalutamide	Yes	Secondary	Yes	Negative	None	5.5	Alive	5.5
PB069	75	White	0	2	1	0.1	Low	None	Abiraterone	Abiraterone	No	Sensitive	No		None	5.5	Alive	7.8
PB077	69	White	1	9	3	1.36	High	Surgery	Other	Other	No	Sensitive	No		None	9.7	Alive	10.5
PB078	58	White	1	3	6	23.18	High	Surgery	Abiraterone	Abiraterone	Yes	Primary	No	Negative	Progressed	4.4	Alive	12.0
PB079	77	White	2	17	5	55.88	Low	Surgery	Abiraterone enzalutamide	Enzalutamide→abiraterone	Yes	Secondary	Yes		None	5.1	Alive	5.1
PB087	62	White	1	3	5	13.61	High	None	Enzalutamide	Enzalutamide	Yes	Secondary	Yes	Negative	Progressed	2.5	Alive	9.3
PB088	77	White	2	14	4	467.9	High	Surgery	Abiraterone	Abiraterone	Yes	Primary	Yes	Negative	Died	1.6	Died	1.6
PB106	61	White	1	2	3	88.05	High	None	Abiraterone	Abiraterone	Yes	Primary	Yes	Negative	Progressed	1.6	Died	3.3
PB108	64	White	1	1	4	0.1	High	None	Other	Other	No	Sensitive	No	Negative	None	8.5	Alive	9.0
PB132	69	White	2	9	2	104.2	High	Radiotherapy	Enzalutamide	Enzalutamide	Yes	Primary	Yes		Died	1.4	Died	1.4
PB140	76	White	1	12	4	748.1	High	Surgery	Abiraterone enzalutamide	Enzalutamide→abiraterone	Yes	Primary	Yes	Positive	Progressed	1.6	Died	5.3
PB142	57	White	1	2	2	740.3	High	Radiotherapy	Abiraterone	Abiraterone	Yes	Secondary	Yes	Negative	None	5.5	Alive	5.5
PB163	66	African American	1	2	2	0.3	High	None	Enzalutamide	Enzalutamide	No	Sensitive	No		None	2.8	Alive	5.4
PB169	93	White	1	15	1	5.46	High	Radiotherapy	Other	Other	No	Sensitive	No	Positive	None	5.5	Alive	7.9
PB174	69	African American	1	5	5	1,343	High	None	Enzalutamide	Enzalutamide	Yes	Secondary	Yes	Negative	None	0.7	Alive	9.5
PB177	72	White	1	1	1	0.55	High	None	Abiraterone	Abiraterone	No	Sensitive	No		None	7.1	Alive	7.3
PB183	66	White	2	9	3	230.3	High	Surgery	Enzalutamide	Enzalutamide	Yes	Secondary	Yes		Progressed	2.4	Alive	5.5
PB188	66	White	0	4	2	27.99	Low	Radiotherapy	Other	Other	No	Sensitive	No		Progressed	2.8	Alive	11.0
PB196	64	White	1	12	7	117.3	High	Radiotherapy	Abiraterone enzalutamide	Enzalutamide→abiraterone	Yes	Secondary	No	Negative	Progressed	3.6	Alive	4.2
PB202	73	White	0	20	4	0.86	High	Surgery	Abiraterone enzalutamide	Enzalutamide/ abiraterone→enzalutamide	No	Sensitive	No	Negative	None	8.5	Alive	8.5
PB203	56	White	1	1	3	83.12	High	None	Abiraterone	Abiraterone	Yes	Primary	Yes	Negative	Progressed	1.8	Alive	5.5
PB204	79	Other/ non-Hispanic	1	4	3	56.45	High	None	Enzalutamide	Enzalutamide	Yes	Primary	Yes		Died	0.6	Died	0.6
PB206	72	White	0	5	4	7.85	High	None	Enzalutamide	Enzalutamide	Yes	Secondary	Yes	Negative	Progressed	3.2	Alive	3.5
PB208	71	White	0	5	1	0.1	High	Radiotherapy	Abiraterone	Abiraterone	No	Sensitive	No		None	7.8	Alive	8.1
PB210	50	African American	1	1	2	4.44	High	None	Other	Other	No	Sensitive	No		Progressed	9.1	Alive	9.2
PB226	88	White	1	23	5	220.4	High	None	Abiraterone enzalutamide	Abiraterone→enzalutamide	Yes	Secondary	Yes	Negative	Progressed	8.0	Alive	8.0
PB239	55	White	1	1	2	267.3	High	None	Other	Other	No	Sensitive	No	Negative	None	7.8	Alive	7.9
PB241	61	White	0	2	1	0.1	Low	Surgery	Abiraterone	Abiraterone	No	Sensitive	No		None	5.5	Alive	6.0
PB242	73	White	0	13	1	31.51	Low	None	Other	Other	No	Sensitive	No		None	7.8	Alive	8.0
PB251	88	White	1	1	1	28.27	High	None	Abiraterone	Abiraterone	No	Sensitive	No	Negative	None	2.8	Alive	2.8
PB258	83	White	2	11	5	233.5	High	ADT	Abiraterone	Abiraterone	Yes	Secondary	No	Negative	Progressed	6.6	Alive	6.8
PB270	64	African American	2	1	2	260.5	High	None	Other	Other	Yes	Primary	Yes		Progressed	1.6	Died	1.9
PB282	75	African American	2	14	3	380.6	Low	Surgery	Enzalutamide	Enzalutamide	Yes	Secondary	Yes		None	5.3	Alive	5.3
PB304	89	African American	2	22	2	6.47	Low	Surgery	Abiraterone	Abiraterone	Yes	Secondary	No	Negative	Progressed	4.4	Alive	4.4
PB306	61	African American	1	2	1	11.22	High	None	Abiraterone	Abiraterone	Yes	Secondary	No	Negative	Progressed	3.4	Alive	3.6
PB307	64	White	0	2	1	0.1	Low	Surgery	Abiraterone	Abiraterone	No	Sensitive	No	Negative	None	2.7	Alive	3.5
PB314	72	White	0	6	3	39.27	High	Surgery	Enzalutamide	Enzalutamide	Yes	Secondary	Yes	Negative	Progressed	1.4	Alive	3.5

(Continued on following page)

TABLE A3. Clinical, Treatment, and Outcome Details for All Patients (Continued)

Patient ID	Age (years)	Race	ECOG PS	Time Since Diagnosis[a] (years)	Line of Therapy in Metastatic Setting[b]	Baseline PSA[c]	Metastatic Disease Burden[d]	Primary Therapy	AR-Directed Therapy	Sequencing of Prior AR-Directed Therapy	Resistance to AR-Directed Therapy	Resistance Type	AR/Enhancer Alterations in cfDNA	AR-V7 CTC Assay	Progression Status[e]	PFS (months)	Survival Status[f]	OS (months)
PB319	68	White	1	1	1	0.2	High	None	Abiraterone	Abiraterone	No	Sensitive	No	Negative	None	2.4	Alive	2.4
PB322	78	White	1	20	1	0.28	Low	Surgery	Abiraterone	Abiraterone	No	Sensitive	No	Negative	None	3.0	Alive	3.0
PB326	67	White	2	3	4	68.55	High	None	Abiraterone enzalutamide	Abiraterone→enzalutamide	Yes	Primary	Yes	Negative	Died	0.6	Died	0.6

Abbreviations: ADT, androgen deprivation therapy; ECOG PS: Eastern Cooperative Oncology Group performance status; OS, overall survival; PFS, progression-free survival; PSA, prostate-specific antigen.

[a]Time since diagnosis of prostate cancer.

[b]Line of systemic therapy for metastatic prostate cancer.

[c]Baseline PSA at time of study enrollment.

[d]High metastatic burden: ≥ 4 bone metastases (≥ 1 outside axial skeleton) or visceral metastases.

[e]Progression status: during follow-up.

[f]Survival status: during follow-up.

TABLE A4. Plasma cfDNA, Library Preparation, and Sequencing Metrics for All Patients

Patient ID	Plasma cfDNA Concentration (ng/mL)	DNA Input Into Library Preparation (ng)	Total QC-Passed Reads	Total Deduplicated Reads	On-Target Rate (%)	Median On-Target Deduplicated Depth	Median Fragment Size (bp)
PB046	11.4	33.9	35,811,633	6,211,393	73.8	643	188
PB069	30.6	32.1	51,421,271	12,013,660	27.6	295	170
PB077	14.5	30.0	53,314,706	11,318,712	32.0	435	177
PB078	4.9	25.0	48,698,062	16,034,881	48.7	845	169
PB079	26.4	32.6	77,333,473	13,108,214	53.1	868	186
PB087	18.6	32.0	60,871,937	12,221,636	36.0	558	181
PB088	84.0	32.0	54,222,195	11,555,078	34.0	528	174
PB106	50.0	32.0	101,010,114	18,788,224	59.0	752	178
PB108	11.4	31.7	74,831,443	11,404,075	54.0	776	181
PB132	112.0	31.4	99,975,828	21,179,072	58.7	630	176
PB140	389.0	32.0	96,393,047	19,520,429	60.0	696	194
PB142	8.1	32.9	27,730,752	4,922,881	71.6	454	198
PB163	10.1	37.0	23,846,096	4,055,009	71.0	368	195
PB169	13.0	40.0	46,490,420	10,233,059	28.3	306	181
PB174	32.7	32.0	45,838,658	11,041,472	39.0	586	172
PB177	5.1	15.0	44,373,233	6,053,638	72.8	560	181
PB183	10.8	31.0	46,876,650	7,584,126	77.9	786	180
PB188	8.3	29.7	47,264,462	9,494,560	32.0	375	179
PB196	9.3	33.5	75,810,733	12,544,239	53.8	808	179
PB202	21.8	32.7	60,245,310	12,687,795	27.3	340	170
PB203	7.6	32.1	98,590,506	43,993,568	70.0	3,592	179
PB204	9.6	32.0	84,204,177	15,436,897	55.0	629	179
PB206	7.1	33.8	72,208,965	13,059,504	53.8	740	184
PB208	8.3	29.8	55,203,718	11,122,955	32.0	381	172
PB210	8.0	33.9	52,814,332	11,115,825	26.8	341	177
PB226	9.0	33.3	46,674,256	10,173,781	27.5	354	188
PB239	10.0	33.7	52,301,218	10,989,575	27.1	391	179
PB241	14.4	24.8	30,347,422	4,216,904	71.3	384	215
PB242	10.4	32.0	50,755,163	10,440,167	27.4	341	175
PB251	19.4	32.1	77,461,687	12,583,695	53.4	769	173
PB258	10.4	31.1	44,029,850	6,947,692	70.4	667	195
PB270	15.3	31.8	111,975,077	16,843,671	87.5	975	281
PB282	9.7	31.7	41,745,497	7,111,847	71.6	591	176
PB304	9.5	35.8	69,062,018	11,607,875	54.3	700	184
PB306	17.7	36.4	79,043,779	14,315,594	53.4	728	178
PB307	4.7	23.0	78,788,588	11,644,591	54.0	753	177
PB314	6.9	32.0	74,370,895	14,601,536	53.2	802	175
PB319	7.9	32.0	87,417,008	13,401,235	53.5	875	180
PB322	7.2	35.3	75,824,605	11,757,342	53.4	777	190
PB326	1,821.3	32.1	88,922,952	29,410,021	50.0	1,660	164

Abbreviations: cfDNA, cell-free DNA; QC, quality control.

TABLE A5. Plasma-Depleted Whole-Blood DNA, Library Preparation, and Sequencing Metrics for All Patients

Patient ID	DNA Input Into Library Preparation (ng)	Total QC-Passed Reads	Total Deduplicated Reads	On-Target Rate (%)	Median On-Target Deduplicated Depth	Median Fragment Size (bp)
PB046	32.0	85,237,216	14,783,577	60.8	1,170	253
PB069	32.0	50,897,072	9,056,165	36.3	397	265
PB077	32.0	50,399,746	11,853,588	19.0	289	263
PB078	32.0	80,408,114	17,731,588	20.0	415	259
PB079	32.0	78,018,102	11,026,989	51.3	680	250
PB087	32.0	74,963,429	18,212,674	21.0	448	285
PB088	32.0	69,184,474	16,258,371	20.0	406	268
PB106	32.0	77,281,996	17,555,510	20.0	433	264
PB108	32.0	82,766,728	11,374,292	52.1	709	262
PB132	32.0	37,387,751	6,982,870	36.5	314	275
PB140	32.0	75,379,042	17,115,275	20.0	419	251
PB142	32.0	79,441,191	12,476,012	59.7	945	244
PB163	32.0	81,090,614	13,195,556	62.6	922	257
PB169	32.0	48,783,426	8,764,724	36.9	391	282
PB174	32.0	74,223,787	16,598,133	20.0	396	268
PB177	32.0	78,021,222	12,137,245	60.2	932	244
PB183	32.0	63,723,091	10,134,883	58.7	416	217
PB188	32.0	75,401,145	17,065,679	20.0	417	260
PB196	32.0	74,235,558	10,593,351	54.0	693	250
PB202	32.0	45,454,494	8,581,803	35.9	370	300
PB203	32.0	73,534,208	16,715,752	20.0	404	259
PB204	32.0	73,992,782	16,476,682	20.0	385	255
PB206	32.0	72,220,182	10,252,594	52.6	668	281
PB208	32.0	66,176,742	14,891,640	20.0	355	257
PB210	32.0	48,522,841	8,303,726	36.6	371	272
PB226	32.0	35,175,225	6,600,914	36.7	290	284
PB239	32.0	51,558,911	9,117,534	37.0	411	286
PB241	32.0	79,341,551	11,910,576	59.3	887	232
PB242	32.0	44,904,894	8,083,876	36.0	355	263
PB251	32.0	74,310,887	10,331,431	53.3	668	260
PB258	32.0	72,064,235	11,910,457	60.1	919	236
PB270	32.0	77,261,496	12,022,280	61.6	933	247
PB282	32.0	78,279,327	11,978,777	60.3	887	250
PB304	32.0	76,687,065	11,279,705	54.8	676	250
PB306	32.0	82,086,562	11,250,019	52.1	700	245
PB307	32.0	78,923,278	11,201,071	51.5	690	253
PB314	32.0	73,574,614	10,702,493	53.6	695	266
PB319	32.0	82,145,254	11,404,620	54.0	740	268
PB322	32.0	82,649,282	11,636,855	53.7	757	275
PB326	32.0	57,570,878	26,953,612	49.0	1,569	251

Abbreviation: QC, quality control.

TABLE A6. Plasma cfDNA, Library Preparation, and Sequencing Metrics for Healthy Donors

Healthy Donor ID	Plasma cfDNA Concentration (ng/mL)	DNA Input Into Library Preparation (ng)	Total QC-Passed Reads	Total Deduplicated Reads	On-Target Rate	Median On-Target Deduplicated Depth	Median Fragment Size (bp)
58	11.2	32.3	83,829,366	11,217,622	61.7	758	213
66	6.6	32.0	46,679,488	5,487,842	74.3	529	206
67	10.4	32.0	60,411,003	8,472,113	70.3	601	266
69	12.0	31.6	86,267,380	10,381,184	63.2	765	178
70	22.1	32.0	50,721,606	7,533,110	73.5	446	165
71	4.0	33.7	54,707,352	6,134,886	74.2	455	198
80	8.1	32.0	57,897,360	10,456,916	50.0	448	170
83	9.0	32.0	55,919,904	7,749,514	73.1	530	197
85	5.4	32.0	53,708,903	6,444,048	75.4	557	179
86	8.4	32.0	58,844,473	7,146,649	74.8	527	179
87	10.6	32.0	51,817,078	6,910,462	73.7	466	191
88	4.6	32.0	56,212,610	12,953,203	55.4	328	176
89	6.5	32.0	37,543,560	4,245,844	74.2	396	194
90	4.9	32.0	54,378,738	8,230,087	72.7	461	184
91	7.1	32.0	48,679,262	6,024,691	73.1	515	302
92	11.3	45.3	75,389,265	9,724,499	61.8	737	193
93	4.6	22.5	72,106,903	9,574,187	62.6	619	188
94	7.8	38.5	78,839,228	16,788,284	56.8	504	175
95	13.8	35.2	88,239,561	11,517,292	58.7	692	181
96	4.8	23.5	70,470,845	9,871,316	63.1	544	170
98	8.6	40.7	75,125,520	9,578,494	62.3	663	191
99	6.7	33.0	73,112,878	10,113,673	62.2	682	190
101	4.2	20.5	76,098,033	9,498,951	62.6	648	181
102	4.6	22.5	71,873,908	8,909,315	62.5	600	186
103	6.7	33.0	77,607,164	11,082,711	61.7	667	223
PH1	10.4	32.0	31,024,346	7,375,811	52.7	355	187
PH2	7.8	32.0	61,161,500	10,155,898	56.6	719	185
PH3	4.0	32.0	39,972,207	9,796,724	53.6	381	297
PH6	3.8	32.0	77,852,625	17,952,619	53.3	790	181
PH7	2.6	32.0	58,292,188	9,592,109	48.2	483	179
PH8	1.0	32.0	56,870,770	9,663,762	55.7	654	187
PH9	4.0	32.0	47,838,537	8,687,004	50.7	458	203
PH13	4.0	32.0	56,985,843	10,954,981	52.0	622	187
PH14	2.6	32.0	46,221,350	11,734,327	52.0	352	174
PH16	3.8	32.0	51,800,892	9,349,639	56.1	610	237
PH17	3.3	32.0	58,264,553	11,170,858	50.5	542	177

Abbreviations: cfDNA, cell-free DNA; QC, quality control.

TABLE A7. Plasma-Depleted Whole Blood DNA, Library Preparation, and Sequencing Metrics for Healthy Donors

Healthy Donor ID	DNA Input Into Library Preparation (ng)	Total QC-Passed Reads	Total Deduplicated Reads	On-Target Rate (%)	Median On-Target Deduplicated Depth	Median Fragment Size (bp)
58	32.0	73,287,223	8,715,811	65.9	648	245
66	32.0	50,967,191	5,258,999	79.4	570	224
67	32.0	56,115,777	6,024,303	76.1	600	209
69	32.0	83,833,193	9,363,691	66.0	717	240
70	32.0	67,617,488	6,974,232	71.1	632	237
71	32.0	56,207,089	5,914,099	79.3	643	218
83	32.0	57,258,934	6,185,507	78.4	605	248
85	32.0	51,693,715	5,724,142	77.9	574	243
86	32.0	64,325,641	6,826,587	79.2	714	226
87	32.0	57,780,611	5,792,778	79.8	621	216
88	32.0	63,982,598	7,087,618	58.6	501	186
89	32.0	46,865,288	5,025,373	79.1	534	208
90	32.0	49,338,669	5,153,291	79.4	551	224
91	32.0	36,739,615	4,227,870	79.3	461	218
92	32.0	69,134,539	8,133,126	65.7	634	239
93	32.0	71,648,012	8,189,262	65.4	628	249
94	32.0	73,697,542	8,110,108	66.2	629	254
95	32.0	83,433,789	9,063,320	66.0	677	252
96	32.0	79,627,020	9,177,528	64.7	688	233
98	32.0	78,200,906	8,841,521	64.6	654	245
99	32.0	87,925,111	9,963,869	65.9	779	226
101	32.0	87,133,082	10,176,820	65.0	757	236
102	32.0	90,555,130	10,150,896	65.0	750	243
103	32.0	80,096,824	9,343,023	64.7	657	244

Abbreviation: QC, quality control.

TABLE A8. Copy Number Alterations Detected in Patient cfDNA by EnhanceAR-Seq

Patient ID	Gene	Copy Number log_2r[a]	Copy Number Call
PB046	*AR*	0.5822	Gain
PB046	*AR enhancer*	1.0969	Gain
PB087	*AR*	1.0124	Gain
PB087	*CDKN1B*	−0.5943	Loss
PB088	*AR*	0.465	Gain
PB088	*AR enhancer*	1.1594	Gain
PB088	*CDKN1B*	−0.7409	Loss
PB088	*PTEN*	−1.6989	Loss
PB088	*RAD51B*	−0.6891	Loss
PB106	*AR*	3.2017	Gain
PB106	*AR enhancer*	2.7963	Gain
PB106	*CDK6*	0.7232	Gain
PB106	*KMT2C*	−0.6396	Loss
PB106	*MET*	−0.813	Loss
PB106	*MYC*	0.4725	Gain
PB106	*NCOA2*	1.0267	Gain
PB106	*PIK3CA*	1.5989	Gain
PB106	*PRKDC*	0.8771	Gain
PB106	*PTEN*	−1.5795	Loss
PB106	*SPOP*	0.7323	Gain
PB106	*TP53*	−1.0869	Loss
PB132	*APC*	−0.4964	Loss
PB132	*AR*	4.4338	Gain
PB132	*AR enhancer*	4.0725	Gain
PB132	*CHD1*	−0.4964	Loss
PB132	*HSD3B1*	1.4571	Gain
PB132	*MYC*	1.2724	Gain
PB132	*NCOA2*	0.6142	Gain
PB132	*PIK3CB*	1.6086	Gain
PB132	*PIK3R1*	−0.5054	Loss
PB132	*PTEN*	−1.5449	Loss
PB132	*RB1*	−0.7089	Loss
PB140	*APC*	−0.5802	Loss
PB140	*AR*	3.3528	Gain
PB140	*AR enhancer*	3.1228	Gain
PB140	*CDKN1B*	−0.6325	Loss
PB140	*NCOR1*	−0.7864	Loss
PB140	*NFE2L2*	−0.7172	Loss
PB140	*PIK3R1*	−0.6554	Loss
PB140	*PTEN*	−1.2284	Loss
PB140	*RAD51B*	−0.7017	Loss
PB140	*TMPRSS2*	−0.6913	Loss
PB140	*TP53*	−0.8002	Loss
PB142	*AR*	0.414	Gain
PB142	*AR enhancer*	0.8903	Gain
PB174	*AR*	1.3927	Gain
PB174	*AR enhancer*	1.3584	Gain
PB174	*KDM6A*	0.5747	Gain
PB183	*AR*	2.3775	Gain
PB183	*AR enhancer*	2.3179	Gain
PB183	*CDK12*	0.4307	Gain
PB183	*MET*	0.5568	Gain
PB183	*MYC*	0.5628	Gain
PB203	*AR*	2.517	Gain
PB203	*AR enhancer*	2.0362	Gain
PB203	*HSD3B1*	0.5242	Gain
PB203	*MYC*	1.3512	Gain
PB203	*NCOA2*	0.8966	Gain
PB204	*AR*	2.9476	Gain
PB204	*AR enhancer*	2.7413	Gain
PB206	*ETV4*	0.3984	Gain
PB226	*AR enhancer*	0.3519	Gain
PB270	*AR*	4.1233	Gain
PB270	*AR enhancer*	4.1757	Gain
PB270	*BRCA1*	−0.7617	Loss
PB270	*ERG*	−0.784	Loss
PB270	*ETV4*	−0.7674	Loss
PB270	*NCOA2*	0.8025	Gain
PB270	*PIK3R1*	−0.5351	Loss
PB270	*RB1*	−1.0844	Loss
PB270	*RUNX1*	−0.6336	Loss
PB270	*TMPRSS2*	−0.7167	Loss
PB270	*TP53*	−0.7261	Loss
PB276	*CDK4*	0.7067	Gain
PB276	*ETV4*	0.6573	Gain
PB276	*MYC*	0.6367	Gain
PB282	*AR enhancer*	0.5748	Gain
PB306	*ETV4*	0.7187	Gain
PB306	*MYC*	0.7941	Gain
PB314	*AR*	0.3012	Gain
PB314	*AR enhancer*	0.5209	Gain
PB314	*ETV4*	0.5081	Gain
PB326	*AR enhancer*	0.99	Gain
PB326	*FOXP1*	−0.6056	Loss
PB326	*HSD3B1*	0.6184	Gain
PB326	*MDM2*	−0.6134	Loss
PB326	*NCOR1*	−1.4738	Loss
PB326	*PTEN*	−1.0807	Loss
PB326	*RB1*	−1.0556	Loss

(Continued in next column)

TABLE A8. Copy Number Alterations Detected in Patient cfDNA by EnhanceAR-Seq (Continued)

Abbreviations: cfDNA, cell-free DNA; EnhanceAR-Seq, Enhancer and Neighboring Loci of Androgen Receptor Sequencing.

[a]cfDNA copy number variation level with respect to matched plasma-depleted whole blood DNA.

TABLE A9. Structural Variations Detected in Patient Cell-Free DNA by EnhanceAR-Seq

Sample	From_ Chromosome	From_Chromosome Break Point Start Position[a]	From_Chromosome Break Point Stop Position[a]	To_ Chromosome	To_Chromosome Break Point Start Position[a]	To_Chromosome Break Point Stop Position[a]	From_ Chromosome Strand	To_Chromosome Strand	Structural Variation Type	Gene
PB132	chrX	66818706	66818707	chrX	67082381	67082382	–	+	Tandem duplication	*AR*
PB140	chrX	64431703	64431704	chrX	66823760	66823761	+	–	Tandem duplication	*AR, AR enhancer*
PB174	chrX	10719155	10719156	chrX	66931592	66931593	–	+	Tandem duplication	*AR, AR enhancer*
PB203	chrX	66935884	66935885	chrX	67279914	67279915	+	–	Deletion	*AR*
PB203	chrX	66109754	66109756	chrX	67790035	67790037	–	+	Tandem duplication	*AR, AR enhancer*
PB203	chrX	66835826	66835829	chrX	67365175	67365178	–	+	Tandem duplication	*AR*
PB203	chrX	66074341	66074343	chrX	66136602	66136604	+	–	Tandem duplication	*AR enhancer*
PB203	chrX	66867260	66867261	chrX	84180074	84180075	+	–	Tandem duplication	*AR*
PB206	chrX	66090231	66090234	chrX	66948715	66948718	+	–	Tandem duplication	*AR, AR enhancer*
PB079	chr21	39875816	39875829	chr21	42868014	42868028	+	–	Fusion	*TMPRSS2-ERG*
PB088	chr21	39883468	39883469	chr21	42867914	42867915	–	+	Tandem duplication	*ERG-TMPRSS2*
PB088	chr21	39883356	39883357	chr21	42870791	42870792	+	–	Fusion	*TMPRSS2-ERG*
PB140	chr21	39870366	39870367	chr21	42874479	42874480	+	–	Fusion	*TMPRSS2-ERG*
PB204	chr21	39858228	39858230	chr21	42869359	42869360	+	–	Fusion	*TMPRSS2-ERG*
PB258	chr21	39869149	39869150	chr21	42871036	42871037	+	–	Fusion	*TMPRSS2-ERG*

Abbreviation: EnhanceAR-Seq, Enhancer and Neighboring Loci of Androgen Receptor Sequencing

[a]Coordinates are per the GRCh37/hg19 genome assembly.

TABLE A10. COSMIC-Indexed Single Nucleotide Variants and Insertions/Deletions Detected in Patient Plasma, Not Detected in Matched Plasma-Depleted Whole Blood

Patient ID	Chromosome	Position[a]	Gene	Mutant Allele	Reference Allele	Mutation Type	Amino Acid Change	COSMIC70 Identifier	Locus NGS Depth	Mutant Allele Frequency (%)
PB046	chr2	208248388	*IDH1*	A	C	Missense	p.R132L	COSM28750	186	17.7
PB079	chr17	7676077	*TP53*	A	G	Missense	p.P98S/ p.P59S	COSM12296, COSM1386882, COSM1386881	1,818	0.71
PB079	chr3	41224612	*CTNNB1*	A	G	Missense	p.G34R	COSM5686	1,064	1.03
PB079	chrX	67711621	*AR*	A	T	Missense	p.L702H/ L170H	COSM238554, COSM238553	835	0.59
PB087	chr3	41224645	*CTNNB1*	C	T	Missense	p.S45P	COSM5663	478	1.25
PB088	chr17	7674291	*TP53*	T	C	Splice	NA	COSM131548, COSM131547, COSM3378445, COSM131549, COSM43751, COSM1725566	277	43.3
PB142	chrX	67711621	*AR*	A	T	Missense	p.L702H/ p.L170H	COSM238554, COSM238553	452	5.97
PB177	chrX	129523208	*SMARCA1*	—	CTT	Deletion	p.K57del	COSM1465521	400	5.5
PB188	chr5	112839990	*APC*	T	G	Stop-gain	p.G1448X/ p.G1466X	COSM23595	832	0.72
PB188	chr17	7674230	*TP53*	T	C	Missense	p.G245S/ p.G206S	COSM1640833, COSM121036, COSM6932, COSM121035, COSM121037, COSM3356965	490	2.04
PB204	chr17	7673787	*TP53*	A	G	Missense	p.P146L/ p.P119L/ p.P239L/ p.P278L	COSM129831, COSM3378341, COSM10863, COSM1646812	598	5.51
PB282	chrX	67711621	*AR*	A	T	Missense	p.L702H/ p.L170H	COSM238554, COSM238553	606	4.12
PB307	chr3	186105304	*ETV5*	T	C	Splice	NA	COSM446135, COSM446136	744	0.94
PB314	chr10	87933148	*PTEN*	A	G	Missense	p.R130Q/ p.R303Q	COSM5033	706	3.11
PB314	chr17	7675210	*TP53*	C	A	Missense	p.F2L/p.F95L/ p.F134L	COSM11319	1,392	9.05
PB326	chr2	127271381	*ERCC3*	A	G	Missense	p.R634C/ p.R570C	COSM203490	2,397	0.37

Abbreviations: COSMIC, Catalog of Somatic Mutations in Cancer; NA, not applicable; NGS, next-generation sequencing.
[a]Coordinates are per the GRCh38/hg38 genome assembly.

TABLE A11. *AR* Alterations Detected by Tumor Sequencing and Plasma Cell-Free DNA Sequencing

	***AR* Alterations**	
Sample ID	**EnhanceAR-Seq (plasma)**	**Tempus (tumor)**
PB078	None	None
PB087	Amplified	Amplified
PB108	None	None
PB132	Amplified	Amplified
PB183	Amplified	Amplified
PB203	Amplified	Amplified
PB208	None	None
PB226	None	Missense (26% vAF)
PB239	None	None
PB326	None	None

Abbreviations: EnhanceAR-Seq, Enhancer and Neighboring Loci of Androgen Receptor Sequencing; vAF, variant allele fraction.

TABLE A12. Multivariate Cox Regression for Progression-Free Survival Including Altered *AR/enhancer* Locus in cfDNA

Covariate	*P*	HR	95% CI
Patient age	.215	1.05	0.97 to 1.13
Non-White race	.162	2.84	0.66 to 12.30
ECOG PS	.317	1.69	0.60 to 4.76
Time since diagnosis	.108	0.88	0.75 to 1.03
Line of therapy	.673	1.08	0.75 to 1.56
Baseline PSA	.285	1.00	0.99 to 1.00
Metastatic disease burden	.150	4.06	0.60 to 27.37
Prior treatment with abiraterone	.484	1.62	0.42 to 6.30
Prior treatment with enzalutamide	.726	1.39	0.22 to 8.86
Baseline ctDNA concentration	.105	1.00	1.00 to 1.00
***AR*/enhancer alteration in cfDNA**	**.004**	**10.61**	**2.10 to 53.53**

Abbreviations: cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; ECOG PS: Eastern Cooperative Oncology Group performance status; HR, hazard ratio; PSA, prostate-specific antigen.

TABLE A13. Multivariate Cox Regression for Progression-Free Survival Including Amplified *AR*-Enhancer in cfDNA

Covariate	*P*	HR	95% CI
Patient age	.301	1.04	0.97 to 1.12
Non-White race	.420	1.81	0.43 to 7.70
ECOG PS	.077	2.58	0.90 to 7.35
Time since diagnosis	.062	0.86	0.73 to 1.01
Line of therapy	.493	1.13	0.80 to 1.60
Baseline PSA	.184	1.00	0.99 to 1.00
Metastatic disease burden	.394	2.27	0.35 to 14.86
Prior treatment with abiraterone	.337	2.01	0.48 to 8.36
Prior treatment with enzalutamide	.326	2.46	0.41 to 14.86
Baseline ctDNA concentration	.335	1.00	1.00 to 1.00
***AR-enhancer* amplified in cfDNA**	**.002**	**10.40**	**2.30 to 47.10**

Abbreviations: cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; ECOG PS: Eastern Cooperative Oncology Group performance status; HR, hazard ratio; PSA, prostate-specific antigen.

TABLE A14. Cox Regression for Progression-Free Survival With Baseline Covariates Including Altered *AR/enhancer* Locus in cfDNA

Covariate	*P*	HR	95% CI
Baseline PSA	.1743	1.00	1.00 to 1.00
Line of therapy	.5496	0.92	0.69 to 1.21
Metastatic disease burden	.2455	2.60	0.52 to 13.00
Time since diagnosis	.3003	0.96	0.88 to 1.04
***AR*/enhancer alteration in cfDNA**	**.0002**	**12.01**	**3.18 to 45.37**

Abbreviations: cfDNA, cell-free DNA; HR, hazard ratio; PSA, prostate-specific antigen.

TABLE A15. Cox Regression for Progression-Free Survival With Baseline Covariates Including Amplified *AR-enhancer* in cfDNA

Covariate	*P*	HR	95% CI
Baseline PSA	.1385	1.00	1.00 to 1.00
Line of therapy	.8403	0.97	0.76 to 1.25
Metastatic disease burden	.5405	1.66	0.33 to 8.40
Time since diagnosis	.2229	0.95	0.88 to 1.03
***AR-enhancer* amplified in cfDNA**	**.0002**	**11.69**	**3.25 to 42.08**

Abbreviations: cfDNA, cell-free DNA; HR, hazard ratio; PSA, prostate-specific antigen.

TABLE A16. Single-Nucleotide Variants Detected in Patient cfDNA, Not Detected in Matched Plasma-Depleted Whole Blood

Sample ID	Chromosome	Position[a]	Reference Allele	Mutant Allele	Gene	Mutation Type	Mutant Reads	Locus Depth	AF (%)	Mean AF (%)	cfDNA Concentration (ng/mL)	ctDNA Concentration (hGE/mL)[b]
PB046	chr2	208248388	C	A	*IDH1*	Exonic missense	33	186	17.74	5.90	11.42	204.1
	chrX	67685160	A	T	*AR*	Intronic	7	641	1.09			
	chr10	87864540	A	T	*PTEN*	Exonic missense	28	142	19.72			
	chr12	49054699	G	A	*KMT2D*	Exonic missense	6	785	0.76			
	chr21	41496729	G	A	*TMPRSS2*	Intronic	6	2,276	0.26			
	chr19	41243668	G	C	*AXL*	Exonic missense	4	602	0.66			
	chr17	7676514	C	T	*TP53*	UTR5	8	778	1.03			
PB069	chr3	10090385	G	T	*FANCD2*	Exonic missense	8	345	2.32	1.19	30.57	110.6
	chr11	533390	C	T	*HRAS*	Intronic	7	1,190	0.59			
	chr5	98893488	A	C	*CHD1*	Exonic missense	4	240	1.67			
	chr16	72795330	T	A	*ZFHX3*	Exonic missense	5	2,464	0.20			
PB077	chr12	49051487	C	T	*KMT2D*	Exonic synonymous	15	2,825	0.53	1.77	14.53	78.0
	chr19	45354917	T	A	*ERCC2*	Intronic	4	313	1.28			
	chr3	41224196	T	C	*CTNNB1*	Intronic	6	171	3.51			
PB078	chr20	58855134	T	C	*GNAS*	Exonic missense	7	1,529	0.46	0.46	4.91	6.8
PB079	chr21	34792205	C	T	*RUNX1*	Exonic missense	7	3,537	0.20	0.55	26.40	44.2
	chr17	7676077	G	A	*TP53*	Exonic missense	13	1,818	0.72			
	chr3	41224612	G	A	*CTNNB1*	Exonic missense	11	1,064	1.03			
	chrX	67711621	T	A	*AR*	Exonic missense	5	835	0.60			
	chr12	49044304	C	T	*KMT2D*	Exonic missense	7	3,215	0.22			
PB087	chr12	49051688	C	T	*KMT2D*	Exonic synonymous	8	2,781	0.29	0.91	18.56	51.1
	chr3	41224645	T	C	*CTNNB1*	Exonic missense	6	478	1.26			
	chr17	43051106	C	T	*BRCA1*	Exonic synonymous	6	384	1.56			
	chrX	67697175	C	T	*AR*	Intronic	9	1,032	0.87			
	chr21	41496738	T	C	*TMPRSS2*	Intronic	5	2,044	0.24			
	chr9	35075915	C	T	*FANCG*	Intronic	6	487	1.23			
PB088	chr12	49030973	T	C	*KMT2D*	Exonic missense	4	761	0.53	26.56	84.03	6,762.4
	chr21	41500552	G	C	*TMPRSS2*	Intronic	422	1,178	35.82			
	chr17	7674291	C	T	*TP53*	Splicing	120	277	43.32			
PB106	chr16	89746805	C	T	*FANCA*	Intronic	92	271	33.95	9.47	49.98	1,434.8
	chr11	69641536	T	A	*CCND1*	Intronic	9	10,776	0.08			
	chrX	67728617	T	A	*AR*	UTR3	25	780	3.21			
	chr8	70126872	A	C	*NCOA2*	Exonic missense	11	1,679	0.66			

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TABLE A16. Single-Nucleotide Variants Detected in Patient cfDNA, Not Detected in Matched Plasma-Depleted Whole Blood (Continued)

Sample ID	Chromosome	Position[a]	Reference Allele	Mutant Allele	Gene	Mutation Type	Mutant Reads	Locus Depth	AF (%)	Mean AF (%)	cfDNA Concentration (ng/mL)	ctDNA Concentration (hGE/mL)[b]
PB108	chr21	41494456	C	T	*TMPRSS2*	Exonic synonymous	7	2,954	0.24	0.82	11.37	28.2
	chr11	114063825	C	T	*ZBTB16*	Exonic synonymous	9	1,513	0.59			
	chr3	71198314	C	T	*FOXP1*	Exonic missense	8	2,668	0.30			
	chr16	72796666	G	A	*ZFHX3*	Exonic missense	7	2,119	0.33			
	chr8	47783759	C	T	*PRKDC*	Exonic missense	7	1,298	0.54			
	chr17	39531335	C	A	*CDK12*	UTR3	6	1,403	0.43			
	chr12	12718930	G	C	*CDKN1B*	Exonic missense	11	333	3.30			
PB132	chr9	21974733	A	C	*CDKN2A*	Exonic missense	441	1,574	28.02	20.28	112.00	6,883.4
	chrX	67710303	A	C	*AR*	Intronic	53	1,474	3.60			
	chr20	58899058	G	A	*GNAS*	Intronic	76	260	29.23			
PB140	chrX	67728617	T	A	*AR*	UTR3	25	766	3.26	3.46	388.97	4,074.1
	chr21	41500833	A	C	*TMPRSS2*	Intronic	4	496	0.81			
	chr7	152207258	C	A	*KMT2C*	Intronic	16	254	6.30			
PB142	chrX	67711621	T	A	*AR*	Exonic missense	27	452	5.97	4.68	8.10	1,14.9
	chr16	72793853	C	T	*ZFHX3*	Exonic synonymous	7	688	1.02			
	chr12	25245398	T	C	*KRAS*	Intronic	6	3,098	0.19			
	chr3	37047657	G	C	*MLH1*	Exonic missense	57	494	11.54			
PB163	chr3	186080054	G	A	*ETV5*	Exonic missense	7	996	0.70	0.99	10.13	30.2
	chr12	49031138	C	T	*KMT2D*	Intronic	8	631	1.27			
PB169	chr21	41502563	G	C	*TMPRSS2*	Intronic	7	7,346	0.10	1.73	13.00	68.3
	chrX	67696309	G	A	*AR*	Intronic	7	225	3.11			
	chr1	26761013	G	A	*ARID1A*	Exonic missense	7	939	0.75			
	chr7	116699564	C	T	*MET*	Exonic synonymous	7	511	1.37			
	chr17	39526220	G	A	*CDK12*	Exonic missense	13	388	3.35			
PB174	chrX	67686683	C	T	*AR*	Intronic	6	906	0.66	2.82	32.74	279.4
	chr19	15659377	C	A	*CYP4F3*	Exonic missense	17	405	4.20			
	chr21	10569708	T	C	*TPTE*	Exonic missense	6	209	2.87			
	chr12	124363705	G	A	*NCOR2*	Exonic stopgain	6	675	0.89			
	chr2	97146613	C	T	*ANKRD36*	Intronic	10	183	5.46			
PB177	chr21	41504799	G	A	*TMPRSS2*	Intronic	8	1,274	0.63	1.14	5.06	17.5
	chr12	49049905	C	T	*KMT2D*	Exonic missense	9	1,129	0.80			
	chrX	67702098	A	T	*AR*	Intronic	7	689	1.02			
	chr2	47414364	C	T	*MSH2*	Exonic synonymous	8	375	2.13			
PB183	chrX	67729170	C	T	*AR*	UTR3	13	2,162	0.60	0.66	10.80	21.5
	chr9	35075795	C	T	*FANCG*	Intronic	8	1,119	0.71			

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TABLE A16. Single-Nucleotide Variants Detected in Patient cfDNA, Not Detected in Matched Plasma-Depleted Whole Blood (Continued)

Sample ID	Chromosome	Position[a]	Reference Allele	Mutant Allele	Gene	Mutation Type	Mutant Reads	Locus Depth	AF (%)	Mean AF (%)	cfDNA Concentration (ng/mL)	ctDNA Concentration (hGE/mL)[b]
PB188	chr5	112839990	G	T	*APC*	Exonic stopgain	6	832	0.72	1.72	8.32	43.3
	chr17	7674230	C	T	*TP53*	Exonic missense	10	490	2.04			
	chrX	67599338	G	A	*AR*	Intronic	7	293	2.39			
PB196	chr7	152148363	A	T	*KMT2C*	Exonic missense	6	1,371	0.44	0.48	9.35	13.5
	chr21	41502171	G	A	*TMPRSS2*	Intronic	8	1,141	0.70			
	chr8	47902656	T	A	*PRKDC*	Exonic missense	6	1,541	0.39			
	chr12	49030379	C	T	*KMT2D*	Exonic missense	7	1,769	0.40			
	chr16	72798545	C	T	*ZFHX3*	Exonic synonymous	7	1,527	0.46			
PB202	chrX	71136792	T	C	*MED12*	Intronic	11	124	8.87	8.87	21.79	585.7
PB203	chr3	37047755	G	C	*MLH1*	Intronic	6	976	0.61	2.20	7.62	50.8
	chr14	37591986	G	C	*FOXA1*	Exonic missense	473	4,723	10.01			
	chr8	70141360	G	T	*NCOA2*	Exonic missense	10	7,956	0.13			
	chrX	67727678	G	C	*AR*	UTR3	152	13,458	1.13			
	chr16	89783062	C	T	*FANCA*	Exonic missense	9	5,387	0.17			
	chr17	43529586	G	T	*ETV4*	Exonic missense	9	5,130	0.18			
	chr2	97162045	T	C	*ANKRD36*	Intronic	16	307	5.21			
	chr12	124343206	C	T	*NCOR2*	Exonic missense	9	6,397	0.14			
PB204	chr19	41253688	C	T	*AXL*	Exonic synonymous	7	1,008	0.69	2.35	9.60	68.3
	chr17	7673787	G	A	*TP53*	Exonic missense	33	598	5.52			
	chrX	67712471	G	T	*AR*	Intronic	6	1,087	0.55			
	chr21	10567753	A	T	*TPTE*	Exonic missense	14	533	2.63			
PB206	chr17	58363498	C	A	*RNF43*	Intronic	6	1,441	0.42	0.51	7.13	11.0
	chr2	25749896	G	T	*ASXL2*	Exonic missense	7	1,844	0.38			
	chr21	41504586	G	T	*TMPRSS2*	Intronic	7	2,968	0.24			
	chr12	49032314	G	A	*KMT2D*	Exonic missense	6	1,201	0.50			
	chr19	15537398	C	T	*CYP4F22*	Exonic synonymous	13	1,348	0.96			
	chr16	72788259	T	C	*ZFHX3*	Exonic synonymous	6	1,118	0.54			
	chr11	18615691	G	A	*SPTY2D1*	Exonic stopgain	6	1,893	0.32			
	chrX	67683733	G	A	*AR*	Intronic	6	829	0.72			
PB208	chr8	70131829	G	A	*NCOA2*	Intronic	6	280	2.14	1.68	8.30	42.3
	chrX	67597021	T	A	*AR*	Intronic	5	323	1.55			
	chr19	41243552	G	T	*AXL*	Intronic	6	444	1.35			
PB210	chr3	142578628	G	C	*ATR*	Intronic	4	149	2.68	3.30	8.04	80.3
	chrX	71137770	A	C	*MED12*	Exonic synonymous	4	419	0.95			
	chr5	98876329	G	A	*CHD1*	Intronic	8	128	6.25			

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TABLE A16. Single-Nucleotide Variants Detected in Patient cfDNA, Not Detected in Matched Plasma-Depleted Whole Blood (Continued)

Sample ID	Chromosome	Position[a]	Reference Allele	Mutant Allele	Gene	Mutation Type	Mutant Reads	Locus Depth	AF (%)	Mean AF (%)	cfDNA Concentration (ng/mL)	ctDNA Concentration (hGE/mL)[b]
PB226	chr17	39530665	G	A	*CDK12*	Exonic synonymous	14	754	1.86	2.40	9.00	65.5
	chr3	179220975	G	C	*PIK3CA*	Intronic	6	148	4.05			
	chr1	26773613	G	C	*ARID1A*	Exonic missense	5	707	0.71			
	chrX	67613604	C	A	*AR*	Intronic	8	268	2.99			
PB239	chr5	98881237	T	A	*CHD1*	Intronic	8	161	4.97	7.51	10.00	227.7
	chrX	67980806	T	C	*AR,OPHN1*	Intergenic	17	208	8.17			
	chr16	89783175	C	T	*FANCA*	Intronic	14	149	9.40			
PB241	chr12	124372517	G	C	*NCOR2*	Exonic missense	4	481	0.83	0.83	14.40	36.3
PB242	chr12	49051958	T	A	*KMT2D*	Exonic synonymous	16	2,077	0.77	4.25	10.38	133.8
	chrX	67730720	G	C	*AR*	Downstream	28	320	8.75			
	chr16	72794654	G	A	*ZFHX3*	Exonic synonymous	23	712	3.23			
PB251	chrX	67657384	C	T	*AR*	Intronic	7	1,243	0.56	0.43	19.36	25.0
	chr4	152352587	T	C	*FBXW7*	Exonic synonymous	6	1,318	0.46			
	chr16	89791930	C	A	*FANCA*	Exonic stopgain	6	2,497	0.24			
	chr21	41504825	C	T	*TMPRSS2*	Intronic	7	2,097	0.33			
	chr8	27605160	C	T	*CLU*	Exonic missense	7	1,937	0.36			
	chr3	41225643	T	C	*CTNNB1*	Intronic	6	1,002	0.60			
PB258	chr21	41508109	C	T	*TMPRSS2*	UTR5	17	422	4.03	1.66	10.38	52.3
	chr11	18614693	T	C	*SPTY2D1*	Exonic synonymous	6	691	0.87			
	chr12	49041151	C	A	*KMT2D*	Exonic missense	7	1,427	0.49			
	chr5	112754858	T	C	*APC*	Intronic	6	478	1.26			
PB270	chrX	67723292	G	T	*AR*	Intronic	8	3,790	0.21	6.51	15.30	302.0
	chr3	142459369	C	T	*ATR*	Exonic missense	128	714	17.93			
	chr5	98856451	T	C	*CHD1*	Exonic missense	7	500	1.40			
PB282	chrX	67711621	T	A	*AR*	Exonic missense	25	606	4.13	1.24	9.72	36.5
	chr12	49027199	G	A	*KMT2D*	Exonic missense	9	2,743	0.33			
	chr16	72795125	C	T	*ZFHX3*	Exonic synonymous	8	5,121	0.16			
	chr9	35075757	G	A	*FANCG*	Intronic	7	2,061	0.34			
PB304	chr16	72787938	G	T	*ZFHX3*	Exonic missense	13	2,263	0.57	1.72	9.51	49.5
	chrX	67561898	G	A	*AR*	Intronic	9	532	1.69			
	chr12	124340612	T	A	*NCOR2*	Exonic synonymous	6	1,897	0.32			
	chr2	97146613	C	T	*ANKRD36*	Intronic	8	187	4.28			
PB306	chr12	49051780	T	A	*KMT2D*	Exonic missense	6	5,848	0.10	0.82	17.73	44.0
	chr2	97163221	T	C	*ANKRD36*	Intronic	6	309	1.94			
	chr21	41496589	C	T	*TMPRSS2*	Intronic	11	3,104	0.35			
	chr3	10090361	T	C	*FANCD2*	Exonic synonymous	9	1,029	0.87			

(Continued on following page)

TABLE A16. Single-Nucleotide Variants Detected in Patient cfDNA, Not Detected in Matched Plasma-Depleted Whole Blood (Continued)

Sample ID	Chromosome	Position[a]	Reference Allele	Mutant Allele	Gene	Mutation Type	Mutant Reads	Locus Depth	AF (%)	Mean AF (%)	cfDNA Concentration (ng/mL)	ctDNA Concentration (hGE/mL)[b]
PB307	chr3	186105304	C	T	*ETV5*	Splicing	7	744	0.94	0.83	4.69	11.8
	chrX	67603714	A	C	*AR*	Intronic	4	685	0.58			
	chr8	47933221	A	T	*PRKDC*	Intronic	6	321	1.87			
	chr11	108247118	C	T	*ATM*	Exonic synonymous	7	590	1.19			
	chr12	124356652	G	A	*NCOR2*	Exonic synonymous	7	1,182	0.59			
	chr7	152181799	T	A	*KMT2C*	Exonic missense	5	1,555	0.32			
	chr16	72959699	C	T	*ZFHX3*	Exonic synonymous	7	2,274	0.31			
PB314	chrX	66902224	G	C	*EDA2R,AR*	Intergenic	96	378	25.40	7.80	6.93	163.7
	chr10	87933148	G	A	*PTEN*	Exonic missense	22	706	3.12			
	chr4	105276616	G	A	*TET2-AS1*	Ncrna_intronic	17	194	8.76			
	chr2	25742503	T	A	*ASXL2*	Exonic missense	6	1,734	0.35			
	chr19	41219444	G	T	*AXL*	Exonic missense	8	1,922	0.42			
	chr7	5997333	G	A	*PMS2*	Exonic missense	9	108	8.33			
	chr17	7675210	A	C	*TP53*	Exonic missense	126	1,392	9.05			
	chr12	49040398	G	A	*KMT2D*	Exonic stopgain	254	3,658	6.94			
PB319	chr17	7673717	T	C	*TP53*	Exonic synonymous	6	1,433	0.42	0.54	7.95	13.1
	chr1	26774838	G	C	*ARID1A*	Exonic missense	4	1,184	0.34			
	chr16	72788710	G	A	*ZFHX3*	Exonic missense	9	2,355	0.38			
	chr19	15548101	T	C	*CYP4F22*	Intronic	6	539	1.11			
	chr5	68280629	C	A	*PIK3R1*	Exonic missense	6	1,378	0.44			
	chrX	67641809	T	C	*AR*	Intronic	6	1,036	0.58			
PB322	chrX	67717121	A	C	*AR*	Intronic	4	404	0.99	0.68	7.23	14.8
	chr12	49042533	C	A	*KMT2D*	Intronic	6	1,655	0.36			
PB326	chrX	67682465	T	A	*AR*	Intronic	5	113	4.42	5.10	1,821	28,121
	chr19	41256524	C	T	*AXL*	Exonic synonymous	7	2,872	0.24			
	chr1	26772992	C	T	*ARID1A*	Intronic	232	1,146	20.24			
	chr2	127271381	G	A	*ERCC3*	Exonic missense	9	2,397	0.38			
	chr21	41467843	C	T	*TMPRSS2*	Exonic stopgain	7	3,723	0.19			

Abbreviations: cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; hGE, haploid genome equivalent; vAF, variant allele fraction.

[a]Coordinates are per the GRCh38/hg38 genome assembly.

[b]ctDNA concentration in haploid genome equivalents per mL (hGE/mL)