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Multiparametric liquid biopsy analysis in metastatic prostate cancer

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Molecular profiling of prostate cancer with liquid biopsies, such as circulating tumor cells (CTCs) and cell-free nucleic acid analysis, yields informative yet distinct data sets. Additional insights may be gained by simultaneously interrogating multiple liquid biopsy components to construct a more comprehensive molecular disease profile. We conducted an initial proof-of-principle study aimed at piloting this multiparametric approach. Peripheral blood samples from men with metastatic castrate-resistant prostate cancer were analyzed simultaneously for CTC enumeration, single-cell copy number variations, CTC DNA and matched cell-free DNA mutations, and plasma cell-free RNA levels of androgen receptor (AR) and AR splice variant (ARV7). In addition, liquid biopsies were compared with matched tumor profiles when available, and a second liquid biopsy was drawn and analyzed at disease progression in a subset of patients. In this manner, multiparametric liquid biopsy profiles were successfully generated for each patient and time point, demonstrating the feasibility of this approach and highlighting shared as well as unique cancer-relevant alterations. With further refinement and validation in large cohorts, multiparametric liquid biopsies can optimally integrate disparate but clinically informative data sets and maximize their utility for molecularly directed, real-time patient management.

Introduction

Prostate cancer is the second most prevalent malignancy in the US and the third highest cause of cancer mortality in men (1). Next-generation sequencing (NGS) studies of primary tumors and metastases have begun to map the genomic landscape of prostate cancer from early to late disease by identifying characteristic somatic alterations as well as disease-relevant transcripts and germline alterations (2–6). These studies have revealed extensive intratumor and interpatient heterogeneity and have identified somatic alterations that increase in frequency with exposure to therapies. Given this spatial and temporal heterogeneity, a single tissue biopsy from one disease site at one point in time is unlikely to fully represent the molecular profile of the cancer, yet obtaining sequential tissue biopsies from multiple sites is prohibitively invasive and costly.

Liquid biopsies enriched from a standard peripheral blood draw offer an alternative to solid tissue biopsies that is repeatable and minimally invasive, allowing "real-time" monitoring of a patient's treatment response and disease evolution over time (7). Blood-based biomarkers in advanced prostate cancer can be broadly divided into 2 main sample types: (a) the cellular component consisting of white blood cells (WBCs) and circulating tumor cells (CTCs) reflecting germline and somatic phenotypes, respectively, and (b) the plasma component containing cell-free DNA (cfDNA) and cell-free RNA (cfRNA) released from normal and malignant cells throughout the body.

To date, most studies have focused on the biomarker potential of individual liquid biopsies, yet each of these approaches yields orthogonal and potentially valuable cancer-relevant data. Therefore, a critical next step in liquid biopsy profiling is what we term a "multiparametric" approach, which integrates mul-

tiple blood-based tumor phenotypes to yield a maximally informative disease profile. To test the technical feasibility of such an approach, we undertook a pilot study using blood samples drawn from a cohort of 20 men with metastatic castrate-resistant prostate cancer (mCRPC). Each blood sample was analyzed simultaneously for tumor-relevant cfDNA, cfRNA, CTC DNA, and germline DNA, and these data were used to generate patient-specific, multiparametric liquid biopsy tumor profiles at sequential points.

Results

Patient cohort and liquid biopsies. Blood samples were collected from 20 men with mCRPC (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.125529DS1) with median age of 70 years (range: 46–81 years), median prostate-specific antigen (PSA) at blood draw of 48 ng/ml (range: 0–435 ng/ml,) and visceral metastases (poor prognostic factor) present in 60% of cases. At the time of sampling, patients had received an average of 3 lines of standard therapy: 85% with second-line hormonal therapy, 55% with chemotherapy (docetaxel, cabazitaxel), 40% with sipuleucel-T, and 30% with radium-223. Thirty percent of patients received additional therapies (e.g., poly [ADP-ribose] polymerase, or PARP, inhibitors; immune checkpoint inhibitors; tyrosine kinase inhibitors; and other chemotherapeutic agents).

At each liquid biopsy time point, 3 tubes of blood were processed simultaneously for CTC count, somatic single nucleotide variants (SSNVs) derived from CTCs and matched cfDNA, copy number variant (CNV) analysis derived from single CTCs, and androgen receptor (AR) expression profiles from cfRNA (Figure 1). In 40% of patients, a blood sample at a second time point was collected to assess changes in the tumor molecular profile; most of these samples were evaluated at disease progression, but some were still responding to the treatment being given at the first draw. An overview of data generated from all 20 patients at all time points is in Supplemental Table 2. Seventy-five percent of patients had detectable CTCs, with a median of 20 CTCs/7.5 ml (range: 1–692/7.5 ml). Single CTCs were recovered by dielectric manipulation for CNV analysis in 6 patients, revealing amplifications and deletions in multiple cancer-relevant genes, including *AR*, *MYC*, *TP53*, and *PTEN*. Matched CTC and cfDNA SSNV analyses were performed in 19 of 20 patients. Fourteen of nineteen (68%) had detectable SSNVs in CTC DNA or matched cfDNA, including prostate cancer–relevant genes, such as *TP53*, *PIK3CA*, *EGFR*, and *HRAS*. Matched CTC DNA and cfDNA were analyzed at a second time point in 8 patients, and these revealed previously observed as well as new SSNVs at disease progression. Nineteen of twenty patients had a detectable cfRNA AR transcript, 5 of whom had a detectable cfRNA *ARV7* transcript (*ARV7:AR* ratio range: 0.5%–33.2%).

Multiparametric molecular profiles. An integrated tumor profile was generated for 2 of the patients using their multiparametric molecular data. For example, patient 3 (Figure 2A) is a 76-year-old man with lymph node, bone, and brain metastases whose disease progressed after treatment with androgen deprivation therapy (ADT), abiraterone, enzalutamide, sipuleucel-T, and docetaxel. At the time of his liquid biopsy, he was responding to treatment with radium-223, and his PSA was 60 ng/ml. When his blood was analyzed, he was found to have a high CellSearch CTC count of 168/7.5 ml. Individual CTCs were analyzed for CNVs and were found to have *AR* amplification (commonly observed with progression on abiraterone or enzalutamide) as well as amplification in other cancer-related genes — *BCL6*, *CCND1*, *MYC*, *SOX2*, *STAT4*, *TERC*, *RUNX1T1*, and *TMPRSS2* — and losses in several tumor suppressor genes — *BRCA2*, *RB1*, and *TP53*. Some of these CNVs were concordant with FoundationOne genomic profiling (Foundation Medicine) from a concurrent lymph node biopsy, while others were unique to the CTCs. In addition, SSNV analysis of CTCs and matched plasma cfDNA revealed a concordant nonsense mutation in *TP53* detected in CTC DNA and solid tissue but not in cfDNA. Analysis of cfRNA was positive for *AR* transcripts but negative for *ARV7*.

In another example, patient 10 (Figure 2B) is a 46-year-old man with lymph node and bone metastases whose disease progressed after treatment with ADT, abiraterone, radium-223, and pembrolizumab. At the time of his first liquid biopsy, he was responding to treatment with a PARP inhibitor, and his PSA was 55 ng/ml. When his blood was analyzed, he was found to have a low CTC count of 3/7.5 ml by CellSearch. SSNV analysis of CTC DNA and cfDNA revealed a concordant missense mutation in *TP53* that was also detected in the FoundationOne profile of a primary tumor biopsy performed 38 months earlier. Analysis of cfRNA was positive for *AR* transcripts but negative for *ARV7*. A second liquid biopsy was drawn after progression on PARP inhibitors and a change in therapy to cabozantinib. At that time, patient 10's PSA had risen to 435 ng/ml and his CTC count had increased to 11/7.5 ml by CellSearch, both associated with poor prognosis. SSNV analysis revealed the same *TP53* missense mutation in both CTC DNA and cfDNA but also an additional *TP53* nonsense mutation detected in cfDNA only. This
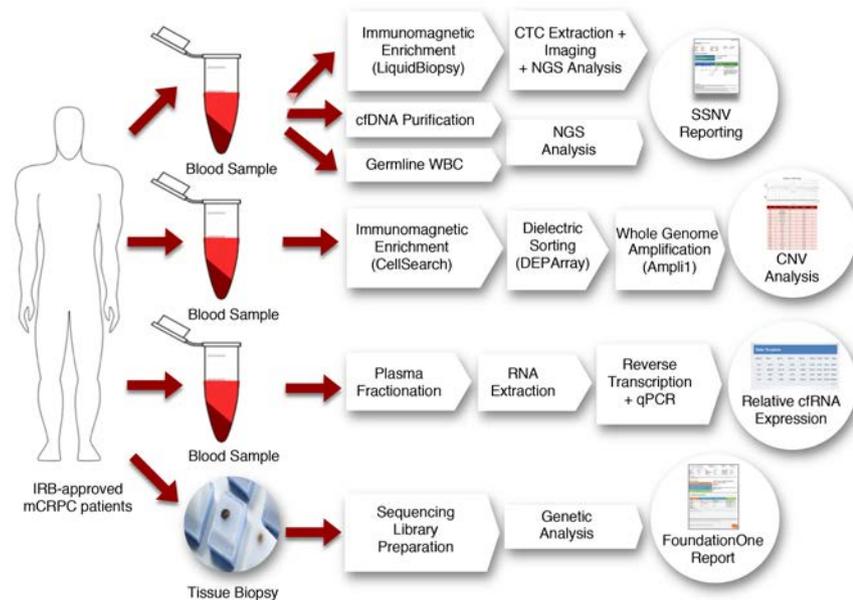
Figure 1. Multiparametric workflow. Three blood samples were collected and analyzed in parallel for cfDNA (SSNVs), CTC DNA (enumeration, SSNVs, CNVs), and cfRNA (*AR*, *ARV7* relative expression). Solid tissue biopsy data (SSNV, CNV) were available in a subset of patients (FoundationOne). qPCR, quantitative PCR.

mutation was not present in the first liquid biopsy or in the prostate tumor biopsy from 38 months prior. Analysis of cfRNA revealed a 200-fold increase in AR transcript compared with the liquid biopsy performed before progression, as well as newly detectable *ARV7*.

Comparative analysis of liquid biopsy versus tumor biopsy. Mutation profiles were compared between liquid biopsies and tumor biopsies in the subset of 10 patients with FoundationOne data (Figure 3A). Patient 7 had no detectable mutations in either panel. Among the other 9 patients, 3 had concordant mutations — all in *TP53* — detected in both tumor biopsy and liquid biopsy, and all other mutations were unique to either the tumor biopsy or the liquid biopsy. For the 3 patients with concordant mutations, the tumor and liquid biopsy samples were collected concurrently in patient 3, 5 months apart in patient 4, and 38 months apart in patient 10. Similarly, CNV profiles were compared between liquid biopsies and tumor biopsies in the subset of 6 patients with FoundationOne data (Figure 3A). Given the large number of potential genes assessed by whole genome amplification/low-pass (WGA/low-pass) sequencing (entire genome) and by FoundationOne (>300 genes), we focused on a subset of 58 prostate cancer–relevant genes curated from recently published prostate cancer genomic profiling studies (8–11) for these comparisons (Supplemental Table 3). Using this gene panel to compare CNVs from tumor biopsies and CTCs, we detected both shared and unique amplifications and deletions (Figure 3A).

Comparison of CTC DNA versus matched plasma. Mutation profiles were compared between CTC DNA and matched cfDNA fractions enriched from the same blood tube in the subset of 18 patients with available matched data (Figure 3B). We detected alterations unique to cfDNA (65.5%), unique to CTC DNA (20.7%), and shared in both (13.8%). For example, no *PIK3CA* mutations were detected in CTC DNA whereas 6 alterations were found in matched cfDNA samples.

Comparison of single CTCs from the same sample. CNV profiles were generated from multiple single CTCs recovered from 6 patients. For each, individual single-CTC CNV profiles were plotted and compared using the prostate cancer–relevant gene list described earlier (Supplemental Table 3 and Supplemental Figure 1, A–E). For example, patient 20 is a 65-year-old man with lymph node and bone metastases who was progressing on abiraterone and had a PSA of 82 ng/ml at the time of the liquid biopsy draw. The patient also had a concurrent biopsy of a bony metastasis analyzed with FoundationOne testing. His CTC count by CellSearch was 31/7.5 ml, and 2 of these cells were recovered and further analyzed for CNV analysis relative to a single WBC from the same sample (Figure 4A). As would be expected, the WBC-derived germline DNA had no detectable CNVs in the cancer-related genes interrogated. In contrast, there were multiple CNVs detected in the CTC-derived somatic samples. Deletion of exon 1 and exon 2 in CHD1 was
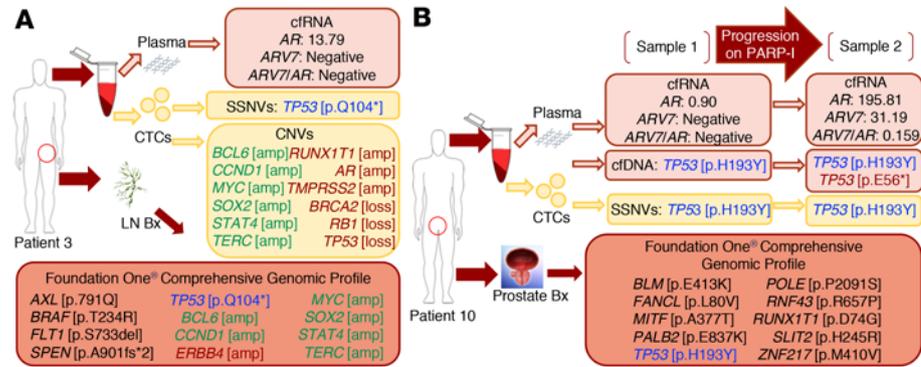
Figure 2. Multiparametric profiles of individual patients (ID numbers 3, 10). Blue = shared SSNVs in liquid and solid biopsies; green = shared amplifications or losses in liquid and solid biopsies; red = alterations interrogated in liquid and solid biopsy panels but detected in only 1; black = alterations or expression levels interrogated in only 1 panel. amp, amplification; LN, lymph node; Bx, biopsy; PARP-I, PARP inhibitor.

identified in the bone metastasis and in both CTCs, as a homozygous loss in CTC 1 and a heterozygous loss in CTC 2. The bone metastasis amplification in *CCNE1* was not mirrored in the CTCs because no call could be made at the gene locus. In addition, *AR* amplification was detected in the bone lesion and in CTC 1, which contained 9 copies of *AR*, but not in CTC 2. Additional CNVs were identified in the CTCs that were not detected in the bone tissue, including copy number gains in *BRCA1*, *MYC*, *PCA3*, *PIK3CA*, *TERC*, and *TP53* and copy number losses in *BRCA2*, *PDL1*, *PTEN*, and *RB1*. The number of shared versus distinct CNVs detected in individual CTCs from a single blood draw was analyzed for the 6 patients with CTC CNV data (Figure 4B). Shared CNVs were observed between individual CTCs in 5 of the patients, but the majority of CNVs detected in these patients were unique to one of their CTCs.

Discussion

Molecular analysis of liquid biopsies has become an important adjunct to more traditional tumor biopsies, and in fact blood-based biomarkers are emerging as stand-alone assays that provide unique information. CellSearch CTC enumeration was the first blood-based biomarker in prostate cancer with prognostic value, where high or increasing CTC counts were associated with worse outcomes and shorter overall survival (12–14). More recently, commercial assays such as Guardant360 (Guardant Health) and FoundationACT (Foundation Medicine/Roche) have emerged as circulating tumor DNA NGS-based liquid biopsies used to interrogate potentially actionable genes for alterations relevant to risk stratification and treatment selection in specific cancer settings (15, 16). However, most liquid biopsy strategies have been developed, analytically validated, and clinically tested in a manner that uses a single blood analyte (i.e., only circulating tumor DNA). Thus, although CTCs and CTC-derived nucleic acids can provide clinically relevant information, and plasma-derived cell-free nucleic acids (DNA and RNA) also yield valuable molecular data, little is known about the optimal way to leverage and integrate this multisourced information: To what degree can 1 assay corroborate the results of another or provide additional unique or complementary information, and how do these data compare with the results of more traditional tumor molecular profiling?

Here, we demonstrate the feasibility of a multiparametric approach that integrates and simplifies several workflows using 3 blood tubes simultaneously processed for CTC count, SSNVs derived from CTCs and cfDNA, CNVs from single CTCs, and AR expression profiles from cfRNA. The liquid biopsy assays chosen for this study were meant to encompass a broad array of analytically validated assays with well-developed CLIA workflows to generate as many unique blood-based phenotypes as possible from each sample (CTC mutations and CNVs, cfDNA mutations, and cfRNA transcripts). Our goal was to leverage our unique access to these technologies and patient samples to integrate and compare these disparate approaches. We aimed to demonstrate whether multiple liquid biopsy assays can be applied effectively to each sample to analyze and track molecular disease profiles as they evolve. This approach presented the challenge of integrating and streamlining workflows that had been developed independently and required different types, amounts, and purities of starting material.
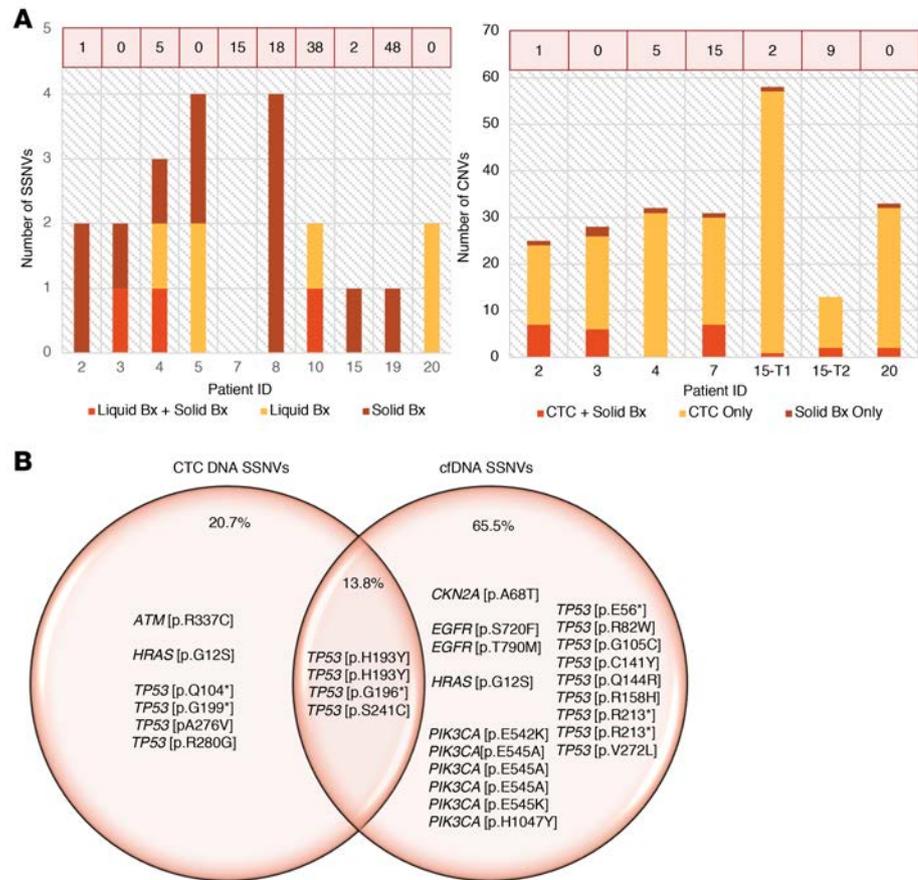
Figure 3. Distribution of genomic alterations by tissue source within individual patients. (**A**) Detection of SSNVs and CNVs in a patient's solid or liquid biopsy or in both. Analysis includes only alterations tested in both solid and liquid panels. Boxed numbers denote months elapsed between solid and liquid biopsies. (**B**) Detection of SSNVs in a patient's CTC DNA or cfDNA or in both (18 patients analyzed). Bx, biopsy.

One of the informatics challenges encountered unexpectedly in this pilot was how best to convey the large amounts of orthogonal data generated from each sample in a manner that was clear, was intuitive, and reflected similarities and differences in alterations. To do this, we generated pictorial profiles (Figure 2) that group the multiparametric results by source (plasma, whole blood, solid tissue), biopsy type (cfRNA, cfDNA, CTC DNA, FoundationOne), and assay (AR expression, SSNVs, and CNVs), with color-coding to highlight shared versus unique alterations. Whereas at this early stage, all the information is presented, one envisions an evolution toward more streamlined reports as the clinical utility of certain assays or of shared alterations is prospectively validated. The presence of specific alterations or transcripts may prove prognostic or predictive individually or as group, guiding treatment decisions. For example, patient 3 has a high CTC count of 168/7.5 ml by CellSearch, associated with poor prognosis. At this stage, this patient does not have detectable *ARV7*, suggesting he may still respond to AR-targeting therapy. At the same time, he exhibits losses in *RB1* and *TP53*. Cooperative losses of 2 or more tumor suppressor genes (*RB1*, *TP53*, *PTEN*) have been linked to an aggressive variant of castrate-resistant prostate cancer that is less susceptible to hormonal therapy and more responsive to platinum-based treatment (17, 18). Furthermore, the CTCs from this patient have a *BRCA2* loss, an alteration that has been associated with response to therapeutic PARP inhibition (19). Although these genomic signatures are still being clinically validated in large prospective trials, their detection in liquid biopsies such as these may in time help guide appropriate therapy.

This pilot was performed on men with advanced disease because they would be more likely to have detectable CTCs and genomic alterations and would also be more likely to progress on treatment, allowing for repeat liquid biopsy during the study period. The cohort's heterogeneity in age, PSA levels, and presence of visceral metastases reflects a diversity of advanced disease typical of a tertiary care center. As expected in this
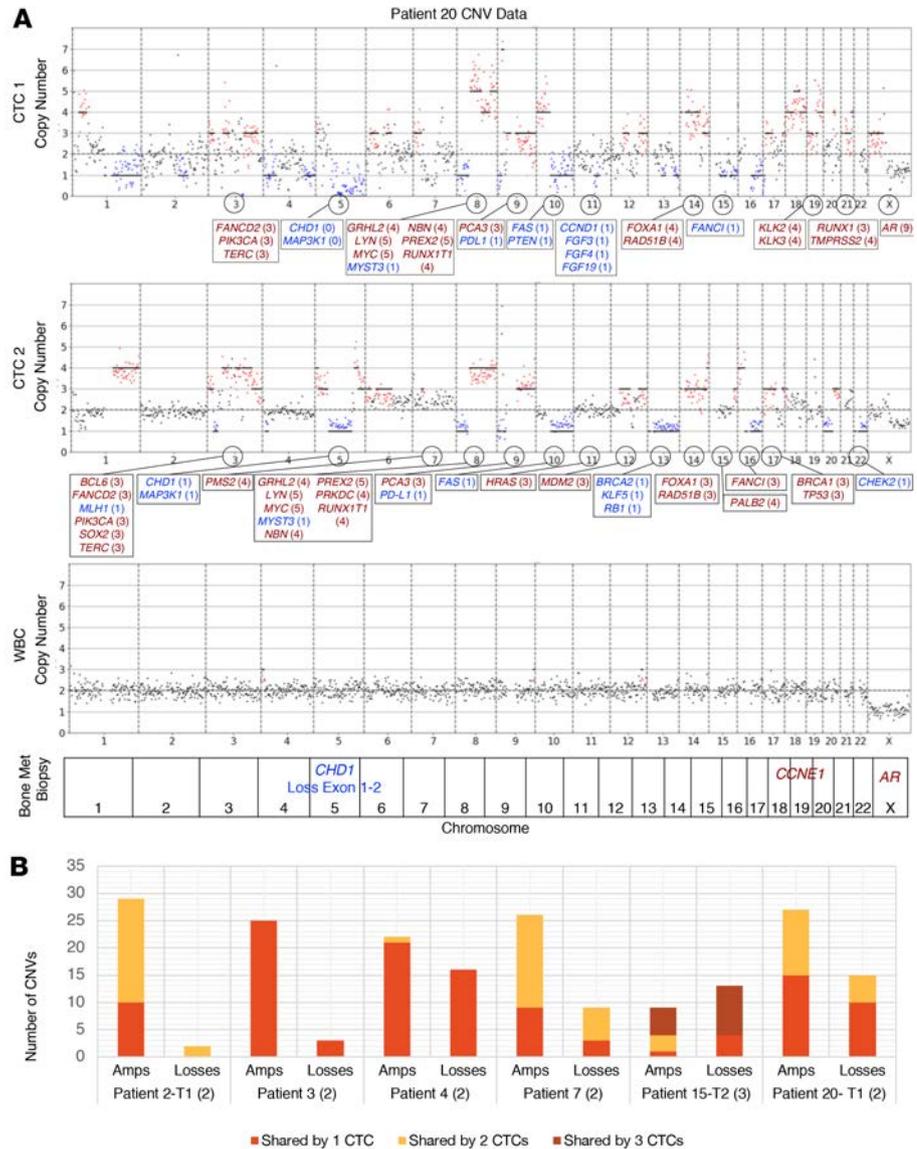
Figure 4. CNV distribution in individual CTCs. (A) CNVs identified in single CTCs, a WBC, and a bone metastasis biopsy obtained concurrently in patient 20 (copy number in parentheses). **(B)** CNVs shared by 1, 2, or 3 CTCs in a patient's sample (number of CTCs analyzed in parentheses).

setting, patients were heavily pretreated with an average of 3 lines of therapy consisting of standard as well as investigational agents, and 60% of patients had visceral disease compared with 20% in patients participating in first-line studies for mCRPC (20, 21). The disease severity and extent of pretreatment in this cohort likely affects tumor molecular profiles, because previous studies have demonstrated that heavily pretreated patients with advanced disease are more likely to have alterations in *AR*, *ERG*, *TP53*, *RB1*, *SPOP*, *CHD1*, and *BZTB16* as well as copy number gains and losses that emerge over time as clonal resistance adaptations (4, 8).

We detected CTCs in 75% of patients (median: 20/7.5 ml, range: 1–692/7.5 ml) reflecting a broad range also seen in prior larger cohort studies evaluating CTC counts as prognostic markers (13, 14, 22). These same studies demonstrated that increasing numbers of CTCs from baseline were associated with shorter overall survival and that changes in CTC count changes may be an indicator of response to treatment and improved survival, specifically conversion from greater than or equal to 5 to less than or equal to 4 CTCs or from greater than or equal to 1 to 0 CTCs at week 13 (12, 13, 23). In our cohort, 2 patients underwent such conversion, patient 9 (favorable, from 6 to 2 CTCs) and patient 10 (unfavorable, from

3 to 11), mirrored by a fall and rise in PSA, respectively. When matched CTC DNA and cfDNA were analyzed at a second time point in 8 patients, these revealed both shared and distinct SSNVs at disease progression, reflecting the potential emergence of resistant subclones. These findings are consistent with several recent large-scale genomic profiling studies in localized and metastatic prostate cancer, which identified alterations in *TP53*, *RB1*, *PTEN*, *AR*, *FOXA1*, *MYC*, *ERG*, *PI3K*, and *WNT* that emerged with progression from localized disease to metastatic castrate-resistant disease and increased in frequency with exposure to hormonal therapies (2, 4, 5, 8, 22, 24).

Ninety-five percent of our patients had a detectable cfRNA *AR* transcript, as expected from mCRPC patients for whom persistent AR signaling is a major driver of disease progression (25). AR has been extensively studied in liquid biopsies as a potential biomarker in late-stage studies. In metastatic disease, quantitative PCR detection of *ARV7*, a constitutively active truncated AR splice variant lacking the ligand-binding domain, in CTCs was associated with resistance to hormonal therapies, such as enzalutamide and abiraterone, but not chemotherapy, such as docetaxel and cabazitaxel (26–28). Subsequently, a variety of other liquid biopsy *ARV7* approaches were tested: *ARV7* was detected using an immunofluorescent protein staining assay, which showed that patients with nuclear *ARV7*+ CTCs had poor response to second-generation antiandrogens and better overall survival with taxane chemotherapy (29). In other studies, *ARV7* was also detected in whole-blood RNA (PAXgene tube) and was associated with poor prognosis (30–32). As demonstrated by this broad spectrum of assays, *ARV7* analysis continues to evolve rapidly, and recent data in fact suggest an imperfect concordance between *ARV7* positivity and resistance to AR-targeted therapy (33, 34). In our pilot, we evaluated *AR* and *ARV7* transcripts in cfRNA and found that 25% of patients had detectable *ARV7* (*ARV7*/*AR* ratio range: 0.5%–33.2%). All the *ARV7*+ patients had been treated with abiraterone at some point in their treatment regimen. However, the 2 patients who converted to being *ARV7*+ at a second time point were not progressing on second-line hormonal therapies but rather on PARP inhibition (patient 10) and cabazitaxel (patient 18), perhaps reflecting expansion of *ARV7*+ subclones that had not been previously detectable.

The central aim of this study was to test the feasibility of integrating multiple liquid biopsy workflows rather than to compare detection rates of blood-based versus tumor-based assays. However, because such comparisons are often made across tissues, it is important to recognize the biological and methodological factors that contribute to the profiles generated using these approaches. When possible, we normalized such differences by including only those genes that were queried by both assays (Supplemental Tables 3 and 4). Nevertheless, some differences in detected alterations may have been attributable to methodological differences between liquid biopsy–derived versus solid tumor–derived assays, such as DNA starting amounts, allele frequencies, and sequencing approaches. For example, whereas we used an amplicon-based enrichment assay for the liquid biopsies and called only mutations recorded in Catalogue Of Somatic Mutations in Cancer (COSMIC) using ×200 read depth, FoundationOne uses a probe-based enrichment assay on the solid tissue samples using median depth coverage of greater than ×500, and it calls additional mutations not identified in COSMIC. Beyond technical considerations, differences in liquid versus tumor biopsies may also reflect true biological distinctions, such as clonal evolution as disease progresses to new sites or develops resistance between the time of the tumor biopsy and the liquid biopsy.

In contrast, it was more feasible to compare SSNV profiles between CTC DNA and matched plasma cfDNA because the CTC fractions and plasma fractions were obtained concurrently from the same collection tube and analyzed using the same AmpliSeq workflow. Despite these similarities, cfDNA and enriched CTC DNA do present important differences: Plasma offers abundant starting material but usually a relatively low allele frequency, especially with lower volume disease (22); conversely, CTC DNA is less abundant but may have greater allele frequency if the CTCs are highly enriched and make up a significant portion of cells used to extract DNA. In this cohort, we detected no *PIK3CA* mutations in CTC DNA whereas we found 6 alterations in matched cfDNA samples. *AR* and *PIK3CA* alterations have been detected previously in cfDNA of mCRPC patients progressing on enzalutamide (35). Consistent with this, in our study 5 of the 6 patients with cfDNA *PIK3CA* mutations had been treated with second-line hormonal therapy — abiraterone, enzalutamide, or both.

We performed single-cell CNV analysis on individual CTCs recovered from a subset of patient samples. Rare single-cell recovery and analysis from a standard tube of blood is recognized as technically challenging. In this cohort, 7 of 23 patient samples met our predesignated criteria of having a minimum of 5 CTCs by CellSearch as well as passing WGA quality control requirements (Supplemental Methods). The CNV profiles generated from single CTCs (Supplemental Figure 1) were in some cases highly concordant,

with modest differences between cells likely reflecting technical variability, such as amplification bias. In other cases, the inter-CTC differences far exceeded any expected assay variability and more likely represented biologically distinct tumor subclones. Ultimately, single-CTC CNV analysis may provide a valuable high-resolution snapshot of advanced disease, but further studies characterizing larger numbers of single cells are needed to clinically validate the significance of these profiles

Conclusion. This pilot study to our knowledge is the first to integrate several liquid biopsy assays into a multiparametric tumor profile that can be repeated over time, demonstrating the feasibility and potential utility of this approach. The abundant CTC and cell-free DNA and RNA data generated from each sample comprise shared as well as unique cancer-specific alterations, which together produce a high-resolution snapshot of tumor biology on treatment and at progression. Though further refinement and validation in large prospective studies are necessary, this multiparametric liquid biopsy strategy may ultimately become a key instrument for minimally invasive yet comprehensive monitoring of disease phenotypes over time, helping better guide therapy.

Methods

Patient sample collection. The study was conducted at the USC NCCC between January 2017 and February 2018. Blood samples were obtained from 20 men with mCRPC encountered in the outpatient oncology clinic. For each patient, a total of three 7.5-ml blood collection tubes (EDTA; BD, Cell-Free RNA by Streck, and CellSave by CellSearch) were collected and processed per protocols.

CTC enumeration and CNV analysis. CTC enumeration was performed using the FDA-cleared CellSearch platform. Single CTCs were recovered by dielectric manipulation using the DEPArray V2 system (Menarini Silicon Biosystems), and DNA from isolated cells was extracted, amplified, and quality controlled per protocol. Sequencing libraries were prepared and sequencing was performed using the Ion Torrent Personal Genome Machine (Thermo Fisher Scientific), and Control-FREE Copy Number and Genotype Caller software (Control-FREEC) (http://boevalab.com/FREEC/index.html#downloads) was used to obtain copy number calls.

cfDNA and CTC DNA SSNV analysis. Blood samples were fractionated by centrifugation at 500 *g*. The upper layer of plasma was collected and cfDNA isolated; the remaining blood cell fraction was processed for CTC enrichment on the LiquidBiopsy CTC platform (Cynvenio Biosystems Inc.), followed by whole-cell lysis and multiplexed PCR with Cynvenio's 27-gene ClearID panel (Supplemental Table 4). Sequencing libraries were prepared and quantified, then sequenced on the Ion Torrent S5 XL sequencer. Single nucleotide variants were called using Everest Software.

Relative cfRNA expression. cfRNA was isolated, reverse transcribed, and stored as cDNA. Expression of *AR* and *ARV7* was measured by quantitative real-time PCR using gene-specific primers. Relative expressions were calculated using β-actin as the internal control and universal human reference RNA (*UHR*) as a positive control.

Statistics. Distribution of genomic alterations by tissue source (Figure 3) was provided as simple percentages (number from each tissue as a portion of total alterations). All other quantities in Figures 3 And 4 are described as direct counts of occurrences. Statistical cutoffs for variant calling are described for each assay in Supplemental Methods.

Study approval. The study was conducted at the USC NCCC under a protocol approved by the USC Health Science IRB in Los Angeles. All samples were collected after obtaining informed consent from patients before their participation in the study.

See Supplemental Methods for details.

Author contributions

EH analyzed the data and wrote the manuscript. GM, AC, DZ, TX, and YX contributed to sample collection and processing. PWD, PCP, FB, AK, SK, MT, PDC, MWM, SG, JU, SR, PD, and KD contributed to sample processing and analysis using their respective platforms. JC contributed to the final data analysis and manuscript writing. TD and DQ provided patient samples and reviewed the analysis and manuscript. AG designed the studies, analyzed the data, and wrote the manuscript.

Acknowledgments

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