Paired High-Content Analysis of Prostate Cancer Cells in Bone Marrow and Blood Characterizes Increased Androgen Receptor Expression in Tumor Cell Clusters

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Abstract

Purpose: Recent studies demonstrate that prostate cancer clones from different metastatic sites are dynamically represented in the blood of patients over time, suggesting that the paired evaluation of tumor cells in circulation and bone marrow, the primary target for prostate cancer metastasis, may provide complementary information.

Experimental Design: We adapted our single-cell high-content liquid biopsy platform to bone marrow aspirates (BMA) to concurrently identify and characterize prostate cancer cells in patients’ blood and bone and thus discern features associated to tumorigenicity and dynamics of metastatic progression.

Results: The incidence of tumor cells in BMAs increased as the disease advanced: 0% in biochemically recurrent (n = 52), 26% in newly diagnosed metastatic hormone-naïve (n = 26), and 39% in metastatic castration-resistant prostate cancer (mCRPC; n = 63) patients, and their number was often higher than in paired blood. Tumor cell detection in metastatic patients’ BMAs was concordant but 45% more sensitive than using traditional histopathologic interpretation of core bone marrow biopsies. Tumor cell clusters were more prevalent and bigger in BMAs than in blood, expressed higher levels of the androgen receptor protein per tumor cell, and were prognostic in mCRPC. Moreover, the patterns of genomic copy number variation in single tumor cells in paired blood and BMAs showed significant inter- and intrapatient heterogeneity.

Conclusions: Paired analysis of single prostate cancer cells in blood and bone shows promise for clinical application and provides complementary information. The high prevalence and prognostic significance of tumor cell clusters, particularly in BMAs, suggest that these structures are key mediators of prostate cancer’s metastatic progression. Clin Cancer Res; 23(7); 1722–32. © 2016 AACR.

Introduction

The approval of multiple life-extending treatment options for patients with prostate cancer (1, 2) has created a critical need for biomarkers to monitor disease behavior and assess benefit from therapy. Such markers could inform patient selection or optimize duration of treatment, ultimately leading to more effective treatment sequences or combinations. Traditionally, serial access to tumor deposits has been challenging in metastatic disease because of the complexity and morbidity of invasive biopsy procedures, and hence, attention has been directed to biomarkers in accessible sites, particularly the circulation but also bone marrow. The use of bone marrow aspirates (BMA) is of particular relevance to prostate cancer, as bone is the most frequent and often the only clinically detectable site of metastasis in this disease (3, 4).

The liquid biopsy approach can deliver single-cell resolution access to the tumor in routine peripheral blood samples through circulating tumor cells (CTC). A growing number of studies show that CTCs have diagnostic (5), prognostic (6–8), and predictive (9, 10) value in a variety of cancer settings. In metastatic castration-resistant prostate cancer (mCRPC), a semiautomated, epithelial cell enrichment and detection-based method has proven useful in prognosticating survival outcomes (11–13). Yet, this method and others that recognize CTCs based on size, density, or epithelial marker expression can miss CTC subpopulations, some of which may also be clinically relevant (14). In contrast to most CTC analysis approaches, the high-definition single-cell analysis (HD-SCA) platform used here allows for a flexible cell identification process, wherein all nucleated cells in a sample are stained and imaged without prior cell population enrichment (15). This direct analysis approach is designed to quantify and record morphometric parameters (such as size and shape) and protein expression levels in individual cells and cell clusters, and to

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Translational Relevance

To improve disease monitoring and facilitate early detection of therapy resistance, we expanded the combined phenotypic and genetic characterizing abilities of our single-cell high-content analysis platform to bone marrow aspirates and thus developed a strategy to concurrently profile tumor cells in peripheral blood and bone metastases of prostate cancer patients. This first simultaneous evaluation of prostate cancer cells in the two compartments revealed new insights into the prevalence and clinical significance of tumor cell clusters and inter/intrapatent and spatial heterogeneity in advanced disease.

provide routine and clinically consistent access to any cell for downstream analysis, including single-cell genomics and proteomics. By characterizing cancer at the single-cell level, we are aiming to link biology with the specific properties of an individual patient’s disease.

CTCs and single-cell high-content analysis may overcome critical limitations of tumor biopsies in dissecting temporal and spatial intratumor heterogeneity in the individual patient. Yet, CTCs as a population are also heterogeneous, molecularly and in their ability to form metastases (14, 16, 17), and dynamic, as their presence and composition can change over time and under treatment pressure (17, 18). As the primary target for metastasis, where tumor cells expand and adapt to therapy, bone is arguably the most relevant organ site to analyze the characteristics and behavior of advanced prostate cancer (4). We adapted the HD-SCA assay to fluid form BMAs to discern features most significant to the tumorigenicity and the dynamics of metastatic progression, and to enable repeat comparisons between tumor cells in the circulatory and bone marrow compartments in a clinically applicable manner in prostate cancer. We evaluated and present here the detection rate, organization, androgen receptor (AR) expression and distribution, and genomic architecture in tumor cells from peripheral blood and BMA from prostate cancer patients in various stages of progression. Our results demonstrate how the HD-SCA assay allows for paired interrogation of two types of liquid biopsies at the single-cell level, providing unique insights into the characteristics of the tumor’s circulatory and metastatic components.

Materials and Methods

Patients and specimen collection

All patients were treated for prostate cancer at the University of Texas MD Anderson Cancer Center (Houston, TX) and provided informed consent per an Institutional Review Board–approved prospective protocol between May 2013 and December 2014. Three patient cohorts were included: (i) biochemical (PSA) failure following definitive prostatectomy and/or radiation treatment, with no clinical metastasis [biochemically reactive prostate cancer (BRPC)]; (ii) newly diagnosed metastatic and hormone naïve (mCSPC); and (iii) mCRPC. All specimens were collected either before initiation of systemic treatment (i and ii) or while progressing on therapy by PSA or radiologic criteria (iii). Patients were prospectively followed from the time of inclusion until last visit or death.

Matched peripheral blood and bone marrow specimens [approximately 5 mL of BMA and separate core biopsies, respectively obtained through the posterior iliac crest with Illinois and Jamshidi bone marrow needles (CareFusion)] were simultaneously collected in preservative tubes (Streck) from individual patients at the Genitourinary Center at MD Anderson. The BMAs and core bone marrow biopsies were clinically evaluated for the presence of tumor cells by hematopathologists at MD Anderson using cell morphology and immunohistochemical stainings for epithelial (pan-cytokeratin) and/or prostate lineage markers (PSA, prostatic acid phosphatase, and/or protein).

Blood and BMA analysis

Blood specimen preprocessing has been described previously (15) and was applied in the same way to BMAs. Briefly, both peripheral blood and BMAs were drawn and placed into proprietary 10-mL tubes with preservative (Cell-Free DNA BCT, Streck) and shipped overnight to the central research laboratory. Upon receipt, red blood cells were lysed and the remaining cell population plated as a monolayer on a custom cell adhesion glass slide (Marienfeld) to achieve approximately 2.5 × 10^6 nucleated cells per slide. The cells were protected with a coverslip and slides were stored at −80°C before use.

Two to four slides were stained per specimen and time point, corresponding to an average sample volume of 1.3 mL for blood and 0.5 mL for BMA (the volume difference is due to the generally higher WBC count in the BMAs). The HD-SCA analysis allows for simultaneous evaluation of up to four fluorescent markers. Cell nuclei were identified through DAPI and epithelial origin (putative tumor cells) was detected with primary mAbs toward cyto-keratin 19 (1:100, Dako) and pan-cytokeratin (1:100, Sigma), and an Alexa Fluor 555 secondary (Invitrogen). An Alexa Fluor 647–conjugated anti-CD45 (1:125; AbD Serotec) was used as a leukocyte exclusion marker. AR levels were evaluated using a rabbit mAb (1:250, Cell Signaling Technology) as published previously (18, 19).

Tumor cell identification and characterization

The slides were imaged and tumor cell candidates identified using a computerized high-throughput fluorescence microscope at ×10 magnification. Candidate tumor cells were presented and manually classified by a pathologist-trained technician as DAPI and cytokeratin^+ and CD45^− (15). AR protein expression and subcellular localization were examined for each tumor cell and tumor cell cluster and quantified by averaging the fluorescent signal within a fixed size circle centered over each cell. Normalization between slides was performed automatically at the time of analysis by setting the exposure of the microscope to yield the same background intensity level. Clusters were defined as two or more tumor cells in direct contact. Disseminated tumor cells (DTC) and metastatic tumor cells (MTC) refer to cancer cells in BMAs of patients without or with clinical bone metastasis, respectively. All HD-SCA analyses were performed blinded to both disease state and bone marrow biopsy status.

Single-cell next-generation sequencing and analysis

Single cells were isolated and their genome amplified as described in our previous work (18). Briefly, cells were picked off the slide using a micropipette, and whole-genome amplification was performed on each cell individually. Libraries were

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constructed and sequenced as described previously (20, 21). The frequency of unique reads mapped to the human genome was used to reconstruct the copy number profile of each individual cell. Subclones were identified using unsupervised hierarchical clustering in R (Ward method with Euclidian distance).

Statistical analysis
The primary goals of this study were to detect and evaluate single-tumor cells and cell clusters in blood and BMA in different stages of prostate cancer progression and to compare the presence of tumor cells in BMA and bone marrow biopsies. Secondary goals were to compare AR expression and AR subcellular distribution in tumor cells in blood versus BMA and to evaluate the prognostic significance of tumor cell clusters. Progression-free (PFS) and overall survival (OS) were calculated from the date of specimen collection to the date of progression or death (or last follow-up if censored). For the Kaplan–Meier analyses (R *survival* package), log-rank tests were used to calculate P values for the significance of differences observed in survival.

Results

**HD-SCA for tumor cell detection in blood and BMAs of prostate cancer patients**

Patients’ characteristics are summarized in Table 1. The highest prevalence and number of tumor cells (cytokeratin+/CD45− cells with distinct morphology) in the blood per case was found in the mCRPC group (29/89 samples (33%) had at least 1 CTC; Fig. 1A; Table 1). Although relatively more specimens in patients with biochemical (PSA) failure (BRPC) than in patients with newly diagnosed metastatic and hormone naïve (mCSPC) were positive (i.e., had at least 1 CTC: 10/41, 24% vs. 7/38, 18%, respectively), the median number of cells per case was higher in mCSPC (Table 1).

Using the adapted HD-SCA assay, we additionally analyzed DTCs/MTCs in 157 BMAs (synchronously collected with blood) from patients with BRPC, mCSPC, and mCRPC (Table 1). Similar to blood, we identified cytokeratin-expressing cells in the bone marrow, but only in patients with metastasis. We did not find DTCs in any BRPC BMA (0/64 specimens, 0%; Fig. 1A), and all the corresponding core bone marrow biopsies obtained from the same iliac crest site were pathologically negative in independent assessment (Fig. 1C), suggesting high specificity for our assay. In contrast, MTCs were detected in 8 of 31 (26%) mCSPC and in 24 of 61 (39%) mCRPC BMAs (Fig. 1A), more frequently than in the corresponding core biopsies from the same patients [pathologically positive in 4/31 (13%) mCSPC and 18/61 (30%) mCRPC]. Tumor cells in BMA were hence exclusively found in metastatic patients, all of which had known bone disease. Considering all specimens from metastatic patients irrespective of clinical state, the overall concordance between BMA and core bone marrow biopsy status was 91% (142/156).

In the 14 discordant cases, 12 were positive in the BMA fraction and negative in the biopsy, and only two were the opposite (Fig. 1C). Tumor cells were detected in BMAs at 45% higher frequency than in the clinical bone marrow biopsies (32 vs. 22 positive cases, respectively). We randomly selected three of the core bone marrow biopsy−negative but HD-SCA BMA−positive cases (one mCSPC and two mCRPC samples with 3, 73, and 195 cells, respectively, and reviewed touch imprints and aspirate smears, and performed additional cytokeratin cocktail stains on the core biopsy materials. All three cases were confirmed biopsy negative for epithelial cells. The median number of cancer cells in the BMAs of the metastatic patients (536 cells/mL, range 2–4,381) greatly exceeded that in the blood (10 cells/mL, range 1–30).

Tumor cell clusters are more prevalent in BMAs than in blood and are enriched in AR expression in mCRPC

The HD-SCA assay not only detects fluorescent signal and intensity with accuracy but also measures physical cell parameters, such as nuclear size and shape and the number of cells in a cell cluster. As available experimental data suggest that cell clusters are more important contributors to metastasis than single CTC (16), we sought to evaluate the presence, distribution, and characteristics of tumor cell clusters in our patients’ sets. Presence of clusters was least abundant in BRPC (7% patients had them in blood, none in BMA) and became more frequent in mCSPC (13% in blood, 16% in BMA) and mCRPC patients (11% in blood, 31% in BMA). Furthermore, as expected from a tumor that often grows in gland form in the bone marrow, clusters were found to be more abundant and larger in BMA than in blood (Fig. 1B). In 14 informative (those with at least one tumor cell found in both sample sources) patient-matched and synchronously collected blood and BMA specimens, we found 10 (71%) with clusters in the BMA (13–357 clusters/case, with the exception of one case that had one cluster), whereas only three (21%) had CTC clusters in the blood (2–4 clusters/case; P = 0.0213, two-tailed Fisher exact test). The four cases that had no clusters in the marrow also had no clusters in the blood. These results were confirmed and expanded in a larger cohort of nonpaired bone marrow (n = 32) and blood specimens (n = 47). Specifically, 24 of 32 (75%) informative BMAs had clusters, whereas only 17 of 47 (36%) of blood specimens were cluster positive (P = 0.0012, two-tailed Fisher exact test.)

As part of the tumor cell characterization, we evaluated and quantified the expression of AR in each individual case and cells in clusters. We found a positive correlation between AR expression and cluster size in blood [Pearson correlation r = 0.23; 95% confidence interval (CI), 0.17–0.29; P = 10−15] and BMAs (r = 0.24; 95% CI, 0.22–0.26; P < 10−15) only in mCRPC patients, but not in those with BRPC or mCSPC disease (Fig. 2 and Supplementary Figure S1).

**Phenotypic and genotypic comparison of tumor cells in blood and BMAs**

To further compare tumor cells in paired blood and BMA compartments, we first performed a manual classification of AR expression in addition to the systematic recording of raw intensity level. Figure 3 shows an intrapatient comparison of the fraction of AR+/AR− cells in matched blood and BMA samples from 10 of the 14 informative patients, where tumor cells were identified in both compartments. We found that, with a few exceptions, the proportions of AR+/AR− cells were similar between compartments. However, the subsequent evaluation of whole-genome copy number profiling in single cells from 3 patients in whom tumor cells were synchronously present in both the blood and BMA compartments showed a more complex picture. We identified distinct clonal patterns and distribution in BMA/blood through unsupervised hierarchical clustering of the segmented copy number variation (CNV) profiles in all 3 patients (Fig. 4).
For patient A, 14 of 14 and 31 of 32 isolated cells were successfully sequenced from the blood and BMA, respectively. The genomic architecture of cells was highly clonal, with subclonality mainly identified in the X-chromosome, as illustrated by three distinct subclones. One subclone from patient A had a simple structure, with two full-length copies of the X-chromosome, an amplification (three copies) of nearly the entire Xq arm, and a breakpoint immediately centromere proximal to the AR locus, and was found exclusively in the BMA (23% frequency). A second subclone, present in both compartments (57% blood, 27% BMA), had a more complex architecture, whereas a third one, characterized by a smaller AR amplification and multiple amplifications and deletions of the q arm, was found exclusively in blood (14%). For patient B, 19 of 26 and 45 of 67 isolated cells were successfully sequenced from the blood and BMA, respectively.

### Table 1. Patients' characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>BRPC</th>
<th>mCSPC</th>
<th>mCRPC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>52</td>
<td>26</td>
<td>63</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65 (42–85)</td>
<td>62 (47–76)</td>
<td>66 (51–81)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Caucasian</td>
<td>46 (88)</td>
<td>22 (85)</td>
<td>54 (85)</td>
</tr>
<tr>
<td>African American</td>
<td>3 (6)</td>
<td>1 (4)</td>
<td>6 (10)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>3 (6)</td>
<td>3 (11)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>ECOG PS, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>51 (98)</td>
<td>20 (77)</td>
<td>21 (33)</td>
</tr>
<tr>
<td>1</td>
<td>1 (2)</td>
<td>6 (23)</td>
<td>36 (57)</td>
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<tr>
<td>2</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>6 (10)</td>
</tr>
<tr>
<td>Gleason score at diagnosis, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>7</td>
<td>26 (50)</td>
<td>4 (15)</td>
<td>7 (11)</td>
</tr>
<tr>
<td>8–9</td>
<td>25 (48)</td>
<td>21 (81)</td>
<td>46 (71)</td>
</tr>
<tr>
<td>10</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Unknown</td>
<td>0 (0)</td>
<td>1 (4)</td>
<td>7 (11)</td>
</tr>
<tr>
<td>Prior localized treatment, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery</td>
<td>47 (90)</td>
<td>3 (11)</td>
<td>33 (52)</td>
</tr>
<tr>
<td>Radiation</td>
<td>5 (10)</td>
<td>1 (4)</td>
<td>13 (21)</td>
</tr>
<tr>
<td>None</td>
<td>0 (0)</td>
<td>22 (85)</td>
<td>17 (27)</td>
</tr>
<tr>
<td>PSA (ng/mL), median (range)</td>
<td>1.3 (0.3–43.3)</td>
<td>20.9 (12.1–1,739.9)</td>
<td>13 (0.3–772.9)</td>
</tr>
<tr>
<td>LDH (IU/L), median (range)</td>
<td>—</td>
<td>—</td>
<td>473 (257–2,967)</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/L), median (range)</td>
<td>—</td>
<td>—</td>
<td>98 (44–1,192)</td>
</tr>
<tr>
<td>Site of metastases, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>N/A</td>
<td>11 (42)</td>
<td>59 (94)</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>18 (69)</td>
<td>28 (44)</td>
<td></td>
</tr>
<tr>
<td>Visceral</td>
<td>1 (4)</td>
<td>11 (18)</td>
<td></td>
</tr>
<tr>
<td>Metastasis volume, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>N/A</td>
<td>14 (54)</td>
<td>18 (29)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>6 (23)</td>
<td>22 (35)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>6 (23)</td>
<td>23 (36)</td>
<td></td>
</tr>
<tr>
<td>Prior treatment, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADT</td>
<td>N/A</td>
<td>0 (0)</td>
<td>63 (100)</td>
</tr>
<tr>
<td>First-generation antiandrogens</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>48 (76)</td>
</tr>
<tr>
<td>Sipuleucel-T</td>
<td>N/A</td>
<td>8 (13)</td>
<td></td>
</tr>
<tr>
<td>Abiraterone</td>
<td>N/A</td>
<td>15 (24)</td>
<td></td>
</tr>
<tr>
<td>Enzalutamide</td>
<td>N/A</td>
<td>3 (5)</td>
<td></td>
</tr>
<tr>
<td>Abiraterone + Enzalutamide</td>
<td>N/A</td>
<td>4 (6)</td>
<td></td>
</tr>
<tr>
<td>Docetaxel</td>
<td>N/A</td>
<td>13 (21)</td>
<td></td>
</tr>
<tr>
<td>Cabazitaxel</td>
<td>N/A</td>
<td>1 (2)</td>
<td></td>
</tr>
<tr>
<td>Radium 223</td>
<td>N/A</td>
<td>1 (2)</td>
<td></td>
</tr>
<tr>
<td>Clinical bone marrow biopsy status, n (%)</td>
<td>0 (0)</td>
<td>4 (15)</td>
<td>17 (27)</td>
</tr>
<tr>
<td>Positive</td>
<td>52 (100)</td>
<td>22 (85)</td>
<td>46 (73)</td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>7 (18)</td>
<td>29 (33)</td>
</tr>
<tr>
<td>HD-SCA assay results</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood: positive, n (%)</td>
<td>10 (24)</td>
<td>7 (18)</td>
<td>29 (33)</td>
</tr>
<tr>
<td>BMA: positive, n (%)</td>
<td>0 (0)</td>
<td>8 (26)</td>
<td>24 (39)</td>
</tr>
<tr>
<td>Blood: tumor cells/mL, mean (range)</td>
<td>1.2 (0–36)</td>
<td>4.5 (0–123.5)</td>
<td>7.2 (0–190.4)</td>
</tr>
<tr>
<td>BMA: tumor cells/mL, mean (range)</td>
<td>0 (0–0)</td>
<td>19.4 (0–375.3)</td>
<td>365.7 (0–5,545.6)</td>
</tr>
</tbody>
</table>

**NOTE:** First-generation antiandrogens include bicalutamide, nilutamide, and/or flutamide.

**Abbreviations:** ADT, androgen deprivation therapy; ECOG PS, Eastern Cooperative Oncology Group performance status; N/A, not applicable.

*Pretreatment.

*Hepatic, pulmonary, and/or pleural.

*Metastasis volume: low, ≤4 bone metastases and/or nonbulky lymphadenopathy and no visceral metastases or active primary; intermediate, 4–10 bone metastases and/or bulky lymphadenopathy and/or active primary and/or limited visceral metastases; high, >10 bone metastases and/or >3 visceral metastases and/or bulky active primary with lymphadenopathy of any size.

*Not including hormonal treatment adjuvant to radiation.
clone, whereas the remaining 60% of cells in BMA and all cells in blood had no or only minor copy number abnormalities. For patient C, 10 of 12 and 24 of 30 isolated cells were successfully sequenced from the blood and BMA, respectively. In contrast to the other two patients, all cells in both compartments belonged to a single clone.

Prognostic significance of individual tumor cell and tumor cell cluster presence in blood and bone marrow
Considering the limited maturity in monitoring time of the BRPC and mCSPC patients in the current dataset [median follow-up 576 days (range 574–909) and 466 days (range 374–746), respectively, with the disease of only 9 mCSPC patients becoming castration resistant], we performed survival analysis on the patients in the mCRPC cohort, where progression rate is highest and survival time shortest. Consistent with other studies (11, 12), we found the presence of any number of tumor cells in blood to be associated with shorter PFS (median 151 vs. 335 days in patients with no cells, \(P < 0.001\)) and OS [median 415 days vs. not reached (NR), \(P = 0.002\)], but we additionally confirmed a similar prognostic association in BMAs (PFS 140 vs. 347 days, \(P = 0.003\); OS 438 vs. NR, \(P < 0.001\); Fig. 5A and B). We further stratified the BMA-positive patients into two groups based on the fraction of MTCs found in clusters and identified those with a high proportion of tumor cells organized in clusters in BMA (relative to the median 73%) as the ones with the shortest PFS (high 106 days vs. low 246, \(P = 0.002\)) and OS (high 338 days vs. low NR, \(P = 0.03\); Fig. 5C). Patients with tumor cells simultaneously in blood and BMA also had shorter PFS than those with cells in only one compartment (111 vs. 182 days, \(P = 0.02\)).

Discussion
Although bone is the most frequent and often only site of clinically detectable metastasis (3), the patterns of metastatic dissemination in prostate cancer are increasingly recognized as
heterogeneous in organization and progressively complex over time (18, 22–24). Recent studies demonstrate that tumor clones from distinct metastatic foci are dynamically represented in the blood of patients at a given time and that differences may exist between those in tissue and circulation, especially under the selective pressure of therapy (17, 18, 24, 25). In this context, methods that allow for serial monitoring of the disease composition not just in the blood (as the necessary route for distant metastasis) but also in the bone marrow (as the most frequent site of metastatic progression and therapy resistance) are likely to provide valuable biological and therapeutic insights into the most relevant determinants of such progression. As part of efforts to complement and enhance the relevance and depth of our observations in blood (15, 18, 19) and to develop a technique to serially profile metastatic deposits with potential for clinical application, we decided to expand the combined phenotypic and genetic profiling abilities of our HD-SCA platform to BMAs in prostate cancer patients.

By profiling specimens across both castration-sensitive and -resistant metastatic disease, we were able to estimate a tumor cell detection rate in BMA samples of approximately 25% in mCSPC and 40% in mCRPC, which, for metastatic patients, was 45% higher than in corresponding bone marrow biopsies. When tumor cells were found in a BMA, their number often greatly exceeded that in the paired blood, but both the distribution of AR-expressing cells and the clonality as defined by CNV were similar across compartments, supporting that CTCs often provide an adequate representation of cells in the metastatic deposit. However, our initial observations comparing the subclonal composition between compartments in 3 patients revealed that large inter- and intrapatient differences can exist as well. All three cases differed in how the subclones were distributed between blood and bone, ranging from a single clone in both compartments to parallel clones, where some were shared and others unique to either compartment. Yet, perhaps our most intriguing and novel findings were the frequent cooccurrence of tumor cell clusters in the blood and liquid BMA fractions of individual metastatic patients, their prognostic significance in the subset of mCRPC patients, and especially how AR expression increased with the number of cells in clusters.

Even though almost 25% of the BRPC patients had at least one CTC in blood, we found no DTC in the corresponding BMAs (or biopsies). Although detection strategies based on cell enrichment (of tumor cells) and/or depletion (of hematopoietic or other nontumor cells) in relatively large volumes of BMA (6–20 mL) usually result in increased sensitivity (13%–72% BMAs have been reported DTC+ in nonmetastatic preprostatectomy patients; refs. 26–30), our emphasis is on unbiased high-content single-cell characterization. An obvious advantage of this approach is that by avoiding selection steps, we obtain a representation of all potential cancer cell phenotypes and other benign cells present in the circulation or bone marrow environment of a patient.
However, a lower positive sample fraction level can be expected due to our smaller effective sample volume and high stringency in tumor cell calling. In this study, we only include tumor cells defined by their relatively large size, nuclear morphology, and cytokeratin\(^\text{+/}^\text{-}\)/CD45\(^\text{-}\)/CD10 expression profile (15), but other less well-characterized tumor cell populations exist (14, 30, 31).

Our data support the clinical potential of profiling tumor cells in BMA together with blood in metastatic prostate cancer. Although more invasive than blood draws, BMAs are less uncomfortable than bone marrow biopsies, relatively easy and quick to obtain by trained personnel with minimal complications, thus allowing for low-frequency serial collection in a prostate cancer clinic. We found the fraction of positive BMAs [35% (25% in mCSPC and 39% in mCRPC)] in metastatic patients to be slightly higher than that of blood specimens [28% (18% in mCSPC and 33% in mCRPC)].

More significantly, of 38 cases with paired BMA and blood available in which at least one of the two was positive for tumor cells, in 17 (45%), the BMA was the only positive, while only 7 (18%) had detectable CTCs but no MTCs in the corresponding BMA. In the remaining 14 patients (37%), both MTCs and CTCs were identified. Likewise, although CTCs and MTCs generally correlated in terms of proportions of AR\(^\text{+/}\) cells and clonal diversity in the paired specimens available, the much higher median number of tumor cells in the positive BMAs than in the blood (536 vs. 10 cells/mL, respectively) allowed for more detailed characterization of the tumor’s composition.

Figure 3.
Four-fold plots for intrapatient comparison of AR expression between cells in blood and bone marrow. The colored fields show the proportion of cells in blood (red) and bone marrow (yellow) that are AR positive (dark) and negative (light) within each compartment. Dotted lines, 95% CIs. Of the 10 patients with more than one cell in both compartments, only patients A, B, and C (to a lesser degree) show a significantly shifted AR-positive/negative balance between the two compartments.
patients with only single CTCs or no CTCs at all, but we further extended it to the fraction of cells in clusters in BMA at the time of disease progression, where a significant signal was also detected for both PFS and OS. It will be of interest to assess whether a similar association exists for circulating or marrow clusters within the BRPC and mCSPC cohorts that are actively being followed, for use along PSA doubling time and other clinical factors, to improve determination of risk for metastatic progression and need for treatment.

Recent experimental model data suggest that clusters arise from aggregation of neighboring cells in tumor deposits rather than from proliferation and/or aggregation of single cells in the circulatory stream (16). Along these lines, the frequent occurrence of tumor cells organized as clusters we identified in the BMAs could indicate that an important proportion of those in the circulation of metastatic patients originates from bone. Clusters developing in the bone marrow from multiplying cells may have a relatively “easy pass” into the circulation through the bone marrow sinusoids. Our observation of a positive correlation between cluster size and AR expression is also original, and of probable biological and therapeutic significance. AR regulates a number of critical genes that promote proliferation and cell survival in prostate cancer, and its activity regularly increases over time through different mechanisms after androgen deprivation therapy, driving progression in the castration-resistant setting (35). Our findings support the hypotheses that the growth of clusters in the marrow,
their viability in the circulation, and/or their ability to colonize new environments are linked to increased AR expression. The biological basis of the observed AR overexpression in clusters, and whether it affects the effectiveness of AR signaling targeted treatments, remains to be established.

The inclusion of BMAs as part of a feasible and widely applicable liquid biopsy approach opens new opportunities for the analysis of advanced prostate cancer. Compared with image-guided biopsies, BMAs are easier and more cost-effective to obtain and can be collected repeatedly during cancer progression and treatment cycles, providing, as shown here, reliable access to the most relevant metastatic component of the tumor that can complement and expand the observations made in blood. The high concordance with the traditional histopathologic bone marrow biopsy interpretation and the fact that the discordant observations were exclusively found in patients with known clinical bone disease serve as strong validation of the technology and approach. The ability of analysis platforms, such as the HD-SCA, to characterize protein expression together with morphometric, organizational, and genomic features at the single-cell level greatly improves the power and resolution of disease monitoring. This could be leveraged to provide answers to important biological questions on cancer progression, including the clinical significance of clonal heterogeneity, and reveal determinants of

Figure 5.
Kaplan–Meier curves for mCRPC patients. Patients were stratified as no cells (A; blue, n = 41) versus any number of cells (red, n = 19) in the blood (PFS, P < 0.001; OS, P = 0.002). B, No cells (blue, n = 28) versus any number of cells (red, n = 23) in the bone marrow (PFS, P = 0.003; OS, P = 0.0005). C, Patients with any number of cells in the marrow (n = 23) were further stratified by the fraction of tumor cells found in clusters (the median, 73%, used as cut-off point; n = 11 and 12, respectively). Patients with larger average cluster size did significantly worse both in terms of PFS (P = 0.002) and OS (P = 0.03).
response and resistance to therapy, toward the ultimate goal of personalized medicine.

Disclosure of Potential Conflicts of Interest

P. Kuhn holds ownership interest (including patents) in and is a consultant/advisory board member for Epic Sciences, Inc. A. Kolatkar holds ownership interest (including patents) in Epic Sciences, Inc. J.B. Hicks is a consultant/advisory board member for Epic Sciences, Inc. C. Logothetis reports receiving commercial research grants from Astellas, Bayer, Bristol-Myers Squibb, Janssen, Medivation, and Sanoft and is a consultant/advisory board member for Astellas, Bayer, Janssen, and Sanoft. No potential conflicts of interest were disclosed by the other authors.

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Paired High-Content Analysis of Prostate Cancer Cells in Bone Marrow and BloodCharacterizes Increased Androgen Receptor Expression in Tumor Cell Clusters

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