RNA-Seq of Single Prostate CTCs Implicates Noncanonical Wnt Signaling in Antiandrogen Resistance

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Abstract

Prostate cancer is initially responsive to androgen deprivation, but the effectiveness of androgen receptor (AR) inhibitors in recurrent disease is variable. Biopsy of bone metastases is challenging, hence sampling circulating tumor cells (CTCs) may reveal drug resistance mechanisms. We established single cell RNA-sequencing profiles of 77 intact CTCs isolated from 13 patients (mean 6 CTCs/patient) using microfluidic enrichment. Single CTCs from each individual display

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considerable heterogeneity, including expression of *AR* gene mutations and splicing variants. Retrospective analysis of CTCs from patients progressing on AR inhibitor, compared with untreated cases indicates activation of noncanonical Wnt signaling ($P=0.0064$). Ectopic expression of Wnt5a in prostate cancer cells attenuates the antiproliferative effect of AR inhibition, while its suppression in drug-resistant cells restores partial sensitivity, a correlation also evident in an established mouse model. Thus, single cell analysis of prostate CTCs reveals heterogeneity in signaling pathways that could contribute to treatment failure.

Following its initial response to androgen deprivation therapy (ADT), metastatic prostate cancer invariably recurs as castration-resistant disease (1). Second-line inhibitors of the androgen receptor (AR) have been shown to increase overall survival in castration resistant prostate cancer (CRPC), consistent with the reactivation of AR signaling in the tumor, but responses are heterogeneous and often short-lived, and resistance to therapy is a pressing clinical problem (1). In other types of cancer, molecular analyses of serial biopsies have enabled the study of acquired drug resistance mechanisms, intratumor heterogeneity, and tumor evolution in response to therapy (2), an approach that is restricted by the predominance of bone metastases in prostate cancer (3, 4). Thus, isolation of circulating tumor cells (CTCs) may enable noninvasive monitoring as patients initially respond and subsequently become refractory to therapies targeting the AR pathway (5). Here, we established single cell RNA-sequencing profiles of CTCs, individually isolated following microfluidic enrichment from blood specimens of men with prostate cancer, to address their heterogeneity within and across different patients and their differences from primary tumor specimens. Retrospective analyses of clinical and molecular data were then performed to identify potentially clinically relevant mechanisms of acquired drug resistance.

Building on earlier approaches for capturing and scoring CTCs (3), highly efficient microfluidic technologies enable molecular analyses (6–9). We applied the CTC-iChip to magnetically deplete normal hematopoietic cells from whole blood specimens (10). Untagged and unfixed CTCs were identified by cell surface staining for epithelial (EpCAM) and mesenchymal (CDH11) markers and absent staining for the common leukocyte marker CD45, and individually micromanipulated (Fig. S1, A and B). A total of 221 single candidate prostate CTCs were isolated from 18 patients with metastatic prostate cancer and 4 patients with localized prostate cancer (Fig. S1C and Table S1). Of these, 133 cells (60%) had RNA of sufficient quality for amplification and next generation RNA sequencing, and 122 (55%) had >100,000 uniquely aligned sequencing reads (Methods and Figs. S1C and S2A). While many cancer cells in the circulation appear to undergo apoptosis, the presence of intact RNA identifies the subset enriched for viable cells. In addition to candidate CTCs, we also obtained comprehensive transcriptomes for bulk primary prostate cancers from a separate cohort of 12 patients (macrodissected for >70% tumor content) (Table S2), 30 single cells derived from four different prostate cancer cell lines, and 5 patient-derived leukocyte controls (Fig. S1C). The leukocytes were readily distinguished by their expression of hematopoietic lineage markers and served to exclude any CTCs with potentially contaminating signals. Strict expression thresholds were used to define lineage-confirmed CTCs, scored by prostate lineage-specific genes (*PSA*, *PSMA*, *AMACR*, *AR*) and standard epithelial markers (*KRT7*, *KRT8*, *KRT18*, *KRT19*, *EPCAM*) (Methods and Fig. S2B).

Twenty-eight cells were excluded given the presence of leukocyte transcripts suggestive of cellular contamination or misidentification during selection, and 17 cells were excluded given low expression of both prostate lineage-specific genes and standard epithelial markers. The remaining 77 cells (from 13 patients; average of 6 CTCs per patient) were defined as categorical CTCs (Fig. S1C and Table S1).

Unsupervised hierarchical clustering analysis of single prostate CTCs, primary tumor samples, and cancer cell lines resulted in their organization into distinct clusters (Fig. 1A). Single CTCs from an individual patient showed considerably greater intercellular heterogeneity in their transcriptional profiles than single cells from prostate cancer cell lines (Fig. 1, B and C; mean correlation coefficient 0.10 vs. 0.44, $P < 1 \times 10^{-20}$), but they strongly clustered according to patient of origin, indicating higher diversity in CTCs from different patients (Fig. 1C and Fig. S2C; mean correlation coefficient 0.10 for CTCs within patient vs. 0.0014 for CTCs between patients, $P = 2.0 \times 10^{-11}$).

We examined gene markers of prostate lineage, epithelial, mesenchymal, and stem cell fates, and cellular proliferation (Fig. 2A). Epithelial markers were abundantly expressed (>10 reads per million; rpm) by nearly all CTCs analyzed (92%), whereas mesenchymal genes were not upregulated compared to primary tumors or prostate cancer-derived cell lines. Among robustly expressed transcripts were putative stem cell markers (11), including *ALDH7A1*, *CD44*, and *KLF4*, present in 60% of CTCs. In addition, 47% of CTCs expressed markers of cell proliferation. We performed differential gene expression analysis to identify genes that are upregulated in prostate CTCs compared to primary tumor samples. A total of 711 genes were highly expressed in CTCs compared to primary tumors, with the most enriched being the molecular chaperone *HSP90AA1*, which regulates the activation and stability of AR, among other functions (12), and the non-coding RNA transcript *MALAT1*, which has been implicated in alternative mRNA splicing and transcriptional control of gene expression (13) (Fig. 2B; Fig. S4A; Table S3; FDR < 0.1 and fold-change > 2). We used the Pathway Interaction Database (PID) (14) to identify key molecular pathways upregulated in CTCs versus primary tumors, as well as those upregulated in metastatic versus primary prostate tumors based on analyses of previously published datasets (see Methods; Fig. 2C; Fig. S5; Table S4). In total, 21 pathways were specifically enriched in prostate CTCs, with the majority implicated in growth factor, cell adhesion, and hormone signaling (Fig. 2D; Fig. S5).

The AR pathway constitutes the primary therapeutic target in prostate cancer, with specific mutations in *AR* (1, 15) and *AR* mRNA splice variants (16, 17) implicated in acquired resistance. The *AR* transcript was expressed (>10 rpm) in 60/77 (78%) CTCs (12/13 patients with prostate cancer). The T877A mutation in AR, previously associated with ligand promiscuity and resistance to antiandrogens (1), was identified in 5/9 CTCs from a single (1/13) patient with metastatic CRPC (Fig. 3A; Table S5). The F876L mutation in the ligand-binding domain, which converts the AR antagonist enzalutamide to a potential AR agonist (18, 19), was not detected in any of the CTCs ($<1/32$ CTCs with sufficient sequencing reads for mutational analysis). Thus, in our study, point mutations in *AR* known to be associated with altered signaling were uncommon in patients with CRPC, consistent with other reports (4, 20).

We then analyzed *AR* mRNA splice variants lacking a ligand-binding domain and encoding constitutively active proteins (1, 16). These alternative transcripts are not attributable to discrete genetic mutations, but they are commonly expressed in CRPC (4), and detection in bulk CTC preparations of the single splice variant *AR-V7* has been correlated to clinical resistance to antiandrogens (17). Our single cell analysis revealed far more complex and heterogeneous patterns of *AR* splice variant expression among individual CTCs from patients with CRPC: 33/73 (43%) expressed at least one type of *AR* splice variant (8/11 CRPC patients). Among these CTCs, 26/73 (36%) expressed *AR-V7* (8/11 patients); 18/73 (25%) had a distinct splice form *ARv567es* (*AR-V12*) (8/11 patients); and 7/73 (10%) had *AR-V1*, *AR-V3*, or *AR-V4* splice variants (5/11 patients), all of which are known to result in altered signaling (Fig. 3A; Table S6). Simultaneous expression of more than one type of *AR* splice variant was observed in 13/73 (18%) single CTCs (7/11 patients). In total, 7/11 (64%) CRPC patients had CTCs with more than one type of *AR* alteration (including *AR* splice variants and point mutations). In contrast, no such alterations were evident in 12 primary prostate tumors, and only 1/4 CTCs from 2 patients with previously untreated prostate cancer (CSPC) had low level expression of the *AR-V7* splice variant (Fig. 3A; Table S6). Aberrant alternative splicing is a recognized feature of many cancers (21), and indeed another prostate-specific transcript, *KLK3* (*PSA*) (22), showed many more alternative splice variants in CTCs from metastatic patients compared with primary tumors ($P=0.0088$) (Fig. S4B). Taken together, our observations indicate that intra-patient tumor heterogeneity is such that individual CTCs may have different or multiple mRNA splicing alterations.

Tumor heterogeneity is thought to increase further as second-line therapies exert additional selective pressure. We performed retrospective differential analyses in subsets of CTCs to identify mechanisms of resistance to enzalutamide, a potent AR inhibitor recently FDA-approved for CRPC (23). 41 CTCs from 8 patients with metastatic prostate cancer who had not received enzalutamide (Group A) were compared with 36 CTCs from 5 patients whose cancer exhibited radiographic and/or PSA progression during therapy (Group B) (Fig. 3A; Table S1). Gene Set Enrichment Analysis (GSEA) of candidate PID cellular signaling pathways showed significant enrichment for non-canonical (nc)-Wnt signaling in Group B compared to Group A CTCs (Fig. 3B and Fig. S6A; $P=0.0064$, FDR=0.239). This signaling pathway, activated by a subset of Wnt ligands, mediates multiple downstream regulators of cell survival, proliferation, and motility (Fig. S6B) (24–27). A separate analysis using a metagene for the PID ncWnt signature (Methods and Table S7) confirmed enrichment of the signature in Group B compared to Group A CTCs, at the level of both individual CTCs and individual patients (Fig. 3A; $P=0.0041$ (CTCs); $P=0.04$ (patients)). Among the downstream components of ncWnt, the most significantly enriched were *RAC1*, *RHOA*, and *CDC42*, signaling molecules involved in actin cytoskeleton remodeling and cell migration (Fig. 3A and Fig. S6B; $P=1E-06$ (*RAC1*), $P=0.0046$ (*RHOA*), $P=0.0097$ (*CDC42*)). In contrast, *AR* abnormalities were not significantly increased among either individual CTCs or patients, when comparing enzalutamide-resistant versus enzalutamide-naïve cases, using a similar analysis (Fig. 3A).

While most studies of CRPC have focused on acquired *AR* gene abnormalities, an alternative pathway, glucocorticoid receptor (GR) signaling, has recently been shown to contribute to antiandrogen resistance in a prostate cancer mouse xenograft model (28).

Within our human prostate CTC data set, *GR* transcripts and a metagene signature of GR signaling (Methods and Table S7) did not reach statistical significance between patients in Groups A versus B ($P = 0.35$ (CTCs); $P=0.59$ (patients), Fig. 3A), but an inverse relationship between *GR* expression and non-canonical Wnt signaling was evident. Among CTCs with low *GR* expression, GSEA analysis showed significant enrichment for non-canonical Wnt signaling in enzalutamide-progressing patients (Group B) ($P=0.025$), which was absent in CTCs with high *GR* expression ($P=0.34$) (Fig. 3B and Fig. S6D). Thus, these two AR-independent drug resistance pathways may predominate in different subsets of cancer cells.

Wnt proteins may be secreted by tumor cells as part of an autocrine loop, or they may be produced by surrounding stromal cells. We used RNA-in-situ hybridization (RNA-ISH) to identify the source of WNT production in tumor specimens and CTCs. Within primary untreated prostate cancers ($N=9$), the noncanonical *WNT5A* and *WNT7B* mRNAs were present in a subset of tumor cells (8.9%, 11.6%, respectively), but both were rare in surrounding stromal cells ($<0.2\%$, 0.5% , respectively) (Fig. 3C and Fig. S6C). Metastatic tumor biopsies from patients with CRPC ($N=24$) also had readily detectable *WNT5A* and *WNT7B* (8.0%, 6.1%, respectively) (Fig. 3C). Similarly, *WNT5A* or *WNT7B* mRNA was detected by RNA-ISH in a subset of CTCs from patients ($N=5$) with CRPC (6/180 CTCs; 3.3%) (Fig. 3C). Thus, a subset of prostate cancer cells express nc-Wnt ligands, which may provide survival signals in the context of AR inhibition.

To test whether activation of ncWnt signaling modulates enzalutamide sensitivity, we ectopically expressed the non-canonical ligands *WNT4*, *WNT5A*, *WNT7B*, or *WNT11* in LNCaP prostate cancer cells, which express low endogenous levels (Fig. S7, A and B). Survival of the AR-positive LNCaP cells in the presence of enzalutamide was enhanced by the ncWnt ligands, particularly *WNT5A* (Fig. 4A, $P=2.8\times 10^{-5}$; Fig. S7C). Remarkably, endogenous *WNT5A* was acutely induced upon treatment with enzalutamide, suggestive of a feedback mechanism, and its knockdown resulted in reduced cell proliferation (Fig. 4B; Fig. S7D; $P=6.6\times 10^{-4}$). We also generated stable enzalutamide-resistant LNCaP cells through prolonged *in vitro* selection (Fig. S7E). These cells also exhibited increased expression of endogenous *WNT5A*, whose suppression reduced proliferation in enzalutamide-supplemented medium (Fig. 4C; $P=0.005$; Fig. S7F). Finally, we tested the contribution of ncWnt to antiandrogen resistance in an independent dataset, interrogating the previously published mouse LNCaP xenograft model, in which aberrant activation of GR contributes to enzalutamide resistance (28). A significant association between enzalutamide resistance and ncWnt signaling was evident ($P=0.023$), again showing an inverse relationship between *GR* expression and ncWnt signaling ($P=0.032$ for *GR* low vs. $P=0.11$ for *GR* high) (Fig. 4D; Fig. S8, A and B). This independent dataset further validates the independent contributions of GR and ncWnt signaling to antiandrogen resistance.

In summary, by RNA profiling single prostate CTCs, we demonstrate their differences from primary tumors, as well as their heterogeneity within individual patients. The acquisition of AR-dependent and AR-independent alterations conferring resistance to antiandrogen therapies is also heterogeneous. Among *AR* alterations, over half of all patients had multiple *AR* splice variants present within different CTCs and about 1/6 of single cancer cells had simultaneous expression of several *AR* splice variants. Two AR-independent pathways,

activation of GR and ncWnt signaling, coexist in different subsets of cells. Wnt signaling has been implicated in multiple cellular functions linked to prostate cancer progression (4, 24–27), and ncWnt signaling may be targeted by suppression of its key downstream components, such as Rho kinase (29). Our study is limited by its retrospective nature and relatively small sample size (13 patients; average of 6 CTCs per patient), a consequence of the rarity of intact CTCs and inefficiencies inherent in manual single cell micromanipulation techniques, obstacles that might be overcome with future improvements in CTC isolation and single cell sequencing technologies. Nevertheless, the heterogeneity of CTCs in patients with CRPC stands in contrast to the striking homogeneity of AR signaling in single CTCs from untreated patients (5). Although these observations require validation in prospective trials, they point to complex and heterogeneous drug resistance mechanisms in advanced prostate cancer, which may impact therapeutic efficacy.

Supplementary Material

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Acknowledgments

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Fig. 1.

Single cell RNA-sequencing of prostate CTCs. **(A)** Heat map of unsupervised hierarchical clustering analysis of RNA-seq data from 77 single lineage-confirmed prostate CTCs, 12 primary tumor samples, and 30 single cells from four prostate cancer cell lines. **(B)** Heterogeneity, measured by mean correlation coefficient within individual samples with 3 or more cells available for analysis. **(C)** Heterogeneity analysis showing mean correlation coefficients from expression data for CTCs between and within patients (0.0013838 vs. 0.10055, Holm corrected $P=2.0 \times 10^{-11}$), and for prostate cancer cell lines between and within lines (0.11568 vs. 0.43534, Holm corrected $P=5.42 \times 10^{-14}$).

Interaction Database (PID) molecular pathways (14) enriched in CTCs compared to primary tumors and in metastases compared to primary tumors (based on analysis of multiple data sets; see Fig. S5 and Table S4). **(D)** Signaling pathways enriched in prostate CTCs. Molecular pathways from the Pathway Interaction Database (PID) upregulated in CTCs versus primary tumors (excluding those enriched in metastases compared to primary tumors), organized by PID categorization (14) (Fig. S5).

Fig. 3. Heterogeneity of treatment resistance mechanisms in prostate CTCs. **(A)** Heat map depicting androgen receptor (AR) abnormalities, selected signaling pathway signatures, and genes in radical prostatectomy specimens, prostate CTCs from enzalutamide-naïve patients (Group A), and prostate CTCs from patients who had radiographic or biochemical progression of disease while receiving treatment with enzalutamide (Group B). Non-canonical Wnt signature is from reference (14), glucocorticoid receptor (GR) signature is from reference (28), and AR signature is from reference (30) (Table S7). Numbers at top of heatmap

represent ID numbers (Pr numbers) for patients from which each CTC is derived. **(B)** Top, Gene Set Enrichment Analysis (GSEA) plots showing enrichment of non-canonical (nc) Wnt pathway in CTCs from Group B (patients with cancer progression on enzalutamide) compared to Group A (enzalutamide-naïve patients). Bottom, GSEA plots showing enrichment of ncWnt pathway in CTCs from Group B compared to Group A, stratified by GR gene expression. **(C)** Left: Representative micrograph (40X) of RNA-in situ hybridization assay in metastatic prostate tumors, probing for *WNT5A* and *KRT8/18*, scale bar = 50 μ m. Inset, high magnification, arrow points to *WNT5A* signal (red dot), arrowhead points to *KRT8/18* signal (blue dot), scale bar = 10 μ m. Adjacent tissue sections were probed for *WNT7B*, and quantification of RNA-ISH data are displayed in the table. Of 9 primary tumors examined, 5 had >1% *WNT5A* expression in KRT+ cells (range 0.3%–42%) and 7 had >1% *WNT7B* expression (range 0.5%–33.6%). Of 24 metastatic tumors examined, 16 had >1% *WNT5A* expression (range 0%–50.5%) and 15 had >1% *WNT7B* expression (range 0%–26%). Right: Representative fluorescence micrographs of RNA-in situ hybridization in prostate CTCs, probing for *WNT5A/7B* (yellow dots), and prostate CTC-specific markers (*EPCAM*, *KLK3*, *FOLH1*, *KRT8/18/19*) (red dots). DNA is stained with DAPI (blue). Scale bar = 10 μ m.

Fig. 4. ncWnt signaling and enzalutamide resistance. **(A)** Ectopic expression of ncWnt in LNCaP cells increases cell survival in the presence of enzalutamide (3 μ M). **(B)** Enzalutamide treatment induces *WNT5A* mRNA expression in LNCaP cells, and *WNT5A* suppression in enzalutamide-treated LNCaP cells results in decreased proliferation. **(C)** Stable enzalutamide-resistant cells (LN_EnzR) derived from LNCaP cells express increased levels of *WNT5A*, and *WNT5A* siRNA results in reduced proliferation in the presence of enzalutamide. Each experiment (**A–C**) was performed 3 times. Data are presented as mean $\pm$ SD. **(D)** GSEA plot showing enrichment of ncWnt pathway in mouse xenografts derived from enzalutamide-resistant LREX cells (28) compared to control LNCaP cells (data from

GEO GSE52169). Lower GSEA plots show ncWnt pathway enrichment in enzalutamide-resistant xenografts, when stratified by GR gene expression.