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Characterization of an Abiraterone Ultraresponsive Phenotype in Castration-Resistant Prostate Cancer Patient-Derived Xenografts

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Abstract

Purpose—To identify the molecular signature associated with abiraterone acetate (AA) response and mechanisms underlying AA resistance in castration-resistant prostate cancer patient-derived xenografts (PDXs).

Experimental Design—SCID mice bearing LuCaP 136CR, 77CR, 96CR, and 35CR PDXs were treated with AA. Tumor volume and prostate-specific antigen were monitored, and tumors were harvested 7 days post-treatment or at end of study for gene expression and immunohistochemical studies.

Results—Three phenotypic groups were observed based on AA response. An ultraresponsive phenotype was identified in LuCaP 136CR with significant inhibition of tumor progression and increased survival, intermediate responders LuCaP 77CR and LuCaP 96CR with a modest tumor inhibition and survival benefit, and LuCaP 35CR with minimal tumor inhibition and no survival benefit upon AA treatment. We identified a molecular signature of secreted proteins associated

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with the AA ultrasensitive phenotype. Upon resistance, AA ultrasensitive LuCaP 136CR displayed reduced androgen receptor (AR) signaling and sustainably low nuclear glucocorticoid receptor (nGR) localization, accompanied by steroid metabolism alteration and epithelial-mesenchymal transition phenotype enrichment with increased expression of NF- κ B-regulated genes; intermediate and minimal responders maintained sustained AR signaling and increased tumoral nGR localization.

Conclusions—We identified a molecular signature of secreted proteins associated with AA ultrasensitivity and sustained AR/GR signaling upon AA resistance in intermediate or minimal responders. These data will inform development of noninvasive biomarkers predicting AA response, and suggest further inhibition along the AR/GR signaling axis may be effective only in AA-resistant patients who are intermediate or minimal responders. These findings require verification in prospective clinical trials.

Keywords

abiraterone acetate; prostate cancer; xenografts; biomarkers; androgen receptor; glucocorticoid receptor; castration resistance

Introduction

Androgen-deprivation therapy (ADT) has been the mainstay therapy for patients with advanced prostate cancer (1). Abiraterone acetate (AA), the prodrug of abiraterone, is a specific CYP17A1 inhibitor that blocks androgen biosynthesis, resulting in effective reduction of serum and intratumoral androgens (2–4). AA was the first second-generation ADT shown to improve survival in metastatic castration-resistant prostate cancer (mCRPC) patients (5–9). While dramatic decline in prostate specific antigen (PSA) was achieved in some patients, others exhibited a subtle PSA response or *de novo* resistance, and disease progression is universal (1, 5, 9).

Predictive biomarkers that distinguish ultrasensitive from intermediate or minimal responders to AA are critically needed. Early attempts using circulating tumor cells (CTCs) showed that TMPRSS2-ERG fusion did not predict the response to AA in CRPC patients (10). However, Antonarakis et al. recently showed that CRPC patients with positive androgen receptor transcript variant (ARv7) in their pretreatment CTC did not demonstrate PSA decline and 68% of a small cohort of patients with negative ARv7 demonstrated > 50% PSA decline after receiving AA (11), suggesting that the detection of positive ARv7 in CTCs may predict AA sensitivity.

De novo and acquired resistance to AA is emerging clinically, and there are preclinical and clinical efforts to investigate the mechanisms of resistance. In preclinical studies, resistance to AA was associated with an induction of full-length AR, ARv7, and CYP17A1 (12). In clinical studies, the presence of ARv7 in CTCs was associated with resistance to AA and shorter overall survival (11). In addition, acquired resistance to AA has been associated with the emergence of AR mutations that have been reported in up to 20% of patients who progressed (13–15). Recently, upregulation of glucocorticoid receptor (GR) has been shown to be a possible bypass mechanism to ADT, and CRPC patients with positive GR in their

bone marrow biopsies were less likely to have a durable response to enzalutamide, another second-generation ADT (16).

Currently, there is little information about biomarkers to identify patients who will durably respond to AA, and the mechanisms of resistance are diverse. In the present study we evaluated the AA response in a panel of LuCaP CRPC patient-derived xenografts (PDXs) that displayed differential responsiveness to AA and identified a molecular signature associated with AA ultrasensitiveness. We also provided evidence to support diverse resistance mechanisms upon AA treatment. This study highlights potential noninvasive biomarkers that may be used to select patients for durable AA therapy, and potential targeting of the epithelial-mesenchymal transition (EMT)/nuclear factor κ B (NF- κ B) pathway in AA ultrasensitive or AR/GR pathways in AA intermediate- or minimally responsive CRPC.

Materials and Methods

Prostate cancer PDX models

Animal procedures were carried out in accordance with National Institutes of Health guidelines and upon University of Washington Institutional Animal Care and Use Committee approval. Four different LuCaP human CRPC PDXs (LuCaP 136CR, LuCaP 77CR, LuCaP 96CR, and LuCaP 35CR) were used. All four PDXs express wild-type AR but exhibit differential expression of PSA, PTEN, and ERG (corresponding patient information is summarized in Supplementary Table S1). Two additional PDX models (LuCaP 70CR and LuCaP 86.2CR) were used for survival analysis upon AA treatment and assessment of gene signature.

Intact male CB-17 SCID mice (aged ~6 weeks; Charles River Laboratories, San Diego, CA) were implanted subcutaneously with tumor bits of LuCaP 136 or LuCaP 77. Mice were castrated when tumor volume was $\sim 100 \text{ mm}^3$. When tumor regrew to 1.5-fold the original volume, tumors were referred to as LuCaP 136CR or LuCaP 77CR (Fig. 1). LuCaP 96CR and LuCaP 35CR are castration-resistant PDXs that are propagated in castrated male mice. Castrated male CB-17 SCID mice were implanted subcutaneously with LuCaP 96CR or LuCaP 35CR tumor bits, and enrolled when tumor volume reached $\sim 100 \text{ mm}^3$ (Fig. 1). Upon enrollment, mice were randomized to vehicle (20% HPBCD/0.37N HCl/PBS) or AA treatment groups (0.5 mmol/kg; Janssen Pharmaceutical Companies, Beerse, Belgium). Animals were treated by oral gavage on a weekly schedule of 5 days on, 2 days off. Tumor volume and body weight were measured twice weekly, and blood samples were drawn weekly for PSA measurements using AxSym Total PSA Assay (Abbott Laboratories, Abbott Park, IL). Five animals in each group were sacrificed 7 days after the initiation of treatment (D7) and the remaining animals were followed and sacrificed when tumors exceeded $1,000 \text{ mm}^3$ (end of study, EOS) or sacrificed if animals became compromised. At sacrifice (D7 or EOS), half of the tumor was harvested for paraffin embedding and half was frozen for subsequent analyses. Treatment schemes for LuCaP 70CR and LuCaP 86.2CR are illustrated in Supplementary Fig. S1.

Intratumoral androgen measurement

Intratumoral androgen levels were measured using mass spectrometry as described previously (17, 18). Vehicle-treated tumors and AA-resistant tumors harvested at EOS were used for these analyses.

Immunohistochemistry

Hematoxylin and eosin staining of paraffin-embedded tissues was used to identify viable tumor cells in the tissues. Two cores (5–8 tumors per group) were punched and placed in tissue microarrays. The tissue microarray slides were stained for AR (F39.4.1, 1:100, BioGenex, Fremont, CA), GR (D6H2L, 1:100, Cell Signaling, Danvers, MA), chromogranin A (DAK-A3, 1:100, DAKO, Carpinteria, CA), and synaptophysin (D-4, 1:200, Santa Cruz Biotechnology, Dallas, TX) using standard procedures as described previously (19–21). All evaluations were performed in a blinded fashion and a quasi-continuous immunohistochemical (IHC) score was calculated by multiplying each intensity level (0 for no stain, 1 for faint stain, and 2 for intense stain) by the corresponding percentage of cells (0–100%) at the corresponding intensity and totaling the results. IHC scores ranged from 0 (no staining in any cell) to 200 (intense staining in 100% of the cells).

RNA extraction

Frozen pieces of tumor were embedded in Optimal Cutting Temperature Compound and 5- μ m sections were stained with hematoxylin and eosin. Areas of viable tumor cells were identified and macro-dissected for RNA extraction using a standard procedure with RNA STAT 60 (Tel-Test, Friendswood, TX). RNA was then purified using RNeasy Mini kit utilizing the optional DNase digestion in solution prior to purification (Qiagen, Hilden, Germany) for subsequent gene expression analyses. RNA integrity number was determined using the Agilent Bioanalyzer system (Agilent, Santa Clara, CA).

Gene expression analyses

For Affymetrix microarray analyses, biotin-labeled, amplified RNA (aRNA) was synthesized from 200 ng total RNA using the 3' IVT Express Kit (Affymetrix, Santa Clara, CA). The aRNA was purified using Agencourt RNAClean XP beads (Beckman Coulter Inc., Brea, CA) on the BioMek FX Workstation (Beckman Coulter Inc.). Biotin-labeled aRNA was fragmented using the 3' IVT Express Kit. A total of 4.5 μ g fragmented biotin-labeled aRNA was hybridized on an HT Human Genome (HG)-U219 96-array plate. The plate was washed, stained, and scanned with the GeneTitan Instrument. All reagents were from Affymetrix. Gene expression microarray data were normalized to minimize systematic technical variation using robust multichip average (22) and represented in the log₂ scale. Data were filtered to remove probes with mean signal intensities below the 25th percentile of signal intensities for all probes. The Significance Analysis of Microarrays (SAM) program (<http://www-stat.stanford.edu/~tibs/SAM/>) (23) was used to analyze expression differences between groups using unpaired, two-sample *t* tests and controlled for multiple testing by estimating *q*-values using the false discovery rate method. Gene family was manually curated from Gene Ontology and Uniprot databases. The AR score was determined by the expression of a 21-gene signature and calculated as described previously (24). Microarray

data are deposited in the Gene Expression Omnibus database under the accession number GSE85672.

Ingenuity pathway analysis

The differentially expressed genes between vehicle-treated and AA-resistant tumors at the EOS from each of the four LuCaP models were imported into Ingenuity Pathway Analysis (Ingenuity Systems; <https://www.ingenuity.com>) to identify molecular and cellular functions and regulator effect network involved in AA resistance as previously described (25, 26).

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) (27) was conducted to evaluate enrichment of differential expression patterns in canonical signaling pathways (Reactome) (28) or predefined gene signatures of prostate cancer core gene expression modules representing distinct biological programs (Compendia Bioscience, Ann Arbor, MI) and annotated signatures associated with EMT, AR activity, GR activity, and AA response.

Quantitative real-time polymerase chain reaction

Total RNA was reverse-transcribed to cDNA, and real-time polymerase chain reaction (PCR) was carried out as described previously (29). Species-specific primer sequences are presented in Supplementary Table S2. PCR reactions with SYBR GreenER PCR Master-Mix (Invitrogen, Carlsbad, CA) were monitored with the 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA). Individual mRNA levels were normalized to human RPL13a.

AR sequencing

Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) and PCR amplified using primer AR_exon8_c1-589_F: ATTGCGAGAGAGCTGCATCA and AR_exon8_c1-589_R: TGCTTGTTTTTGTGTTTGATTCC. Sanger Sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (# 4337454, Life Technologies, Carlsbad, CA) according to manufacturer's recommendations. Sequences were aligned to human AR genomic sequence NC_000023.11 and mRNA RefSeq NM_0044 using Sequencher Software (version 5.1, Gene Codes, Ann Arbor, MI). Mutations were verified using The Androgen Receptor Gene Mutations Database (McGill University).

Statistical analyses

Survival was determined using Kaplan-Meier estimation of time from start of treatment (vehicle or AA) to sacrifice and compared by log-rank (Mantel-Cox) test. Statistical analyses of tumor volume and PSA responses were performed as described previously (19). Briefly, longitudinal tumor measurements and PSA serum levels were log-transformed and modeled using linear mixed models conditional on the treatment group with random effects for each animal. Following standard diagnostic assessment of model fit, we simulated 1,000 datasets from each fitted model, calculated the empirical mean and 95% confidence limits at each time point, and refitted the models to these datasets. The final results represented means and 95% confidence limits of 1,000 bootstrap replicates. In addition, the rate of

change in serum PSA and tumor volume upon AA treatment was tested using estimated fixed effects for each LuCaP line. Student's *t* test and Pearson correlation coefficients were used for statistical comparisons between the groups in the intratumoral androgen measurements, gene expression analysis, and IHC analyses. For GSEA, a gene set that displayed false discovery rate <25% is considered significantly enriched.

Results

Heterogeneous AA responses and identification of an AA ultraresponder in LuCaP PDX models

CRPC was developed using four different models of LuCaP PDXs (Fig. 1). AA treatment improved survival and inhibited tumor progression in three of the four models. In mice bearing LuCaP 136CR tumors, survival was substantially improved in AA-treated compared with vehicle-treated mice ($P < 0.001$) and the median survival improved from 6.8 weeks (vehicle) to 21.8 weeks (AA; denoted as AA ultraresponder; 220% gain in survival; Fig. 2A). AA treatment resulted in statistically significant but modest improvement in survival in mice bearing LuCaP 77CR ($P = 0.05$) and LuCaP 96CR ($P = 0.02$) — both denoted as intermediate responders (36–74% gain in survival; Fig. 2A). AA did not significantly extend survival in mice bearing LuCaP 35CR (12% gain in survival; $P = 0.52$; denoted as minimal responder; Fig. 2A).

Both the AA ultraresponder LuCaP 136CR and intermediate responders LuCaP 77CR and LuCaP 96CR, but not the minimal responder LuCaP 35CR, demonstrated significantly delayed tumor and PSA progression (except for LuCaP 136CR, which has undetectable levels of serum PSA; Fig. 2, panels B and C), followed by both tumor and PSA recurrence. These results suggested the PDX models recapitulated clinical AA response phenotypes comprising ultraresponders with inhibition of tumor progression and a significant extension of survival followed by tumor recurrence, and intermediate and minimal responders with brief or limited AA effect on tumor growth inhibition followed by disease progression.

Gene expression associated with LuCaP 136CR ultraresponsiveness to AA

To identify the gene expression profiles associated with AA ultraresponsiveness, we conducted global transcriptome analyses of the PDX lines. We identified 531 differentially expressed genes between the AA ultraresponder LuCaP 136CR versus the intermediate responder LuCaP 96CR and minimal responder LuCaP 35CR at D7 ($P < 0.0001$, fold change ≥ 3 ; Fig. 3A). LuCaP 77CR D7 tumors were not included in the global analysis because the specimens were not available, but their EOS tumors were included in the gene expression validation. Of the 156 genes that were successfully mapped into known gene families, 68 (44%) were secreted proteins (Supplementary Fig. S2A). We observed that the differential expression of these 68 secretory proteins in LuCaP 136CR were consistent between early time point (D7; Supplementary Fig. S2B) and EOS (Supplementary Fig. S2C), suggesting the expression of these markers was not dependent on age of mice or tumor size. We then selected the top 10 upregulated and downregulated genes of secreted proteins (total 20 genes) in the AA ultraresponder LuCaP 136CR compared with the intermediate and minimal responders for qPCR validation (Fig. 3B and Supplementary Fig.

S3). Primers for 18 genes were available, and qPCR confirmed all of the eight upregulated genes (*CEL*, *ARMCX1*, *TNC*, *BMP7*, *IER3*, *FSTL5*, *SNTB1*, and *FBN2*; Fig. 3C) and 10 downregulated genes (*IL17RB*, *GDF15*, *ST6GAL1*, *SPOCK1*, *MSMB*, *INHBB*, *MINPP1*, *GALS3BP*, *C15orf48*, and *PLA2G2A*; Supplementary Fig. S4). However, the downregulated genes showed more variable expression in the intermediate (LuCaP 77CR, LuCaP 96CR) and minimal (LuCaP 35CR) responders and therefore were not included in the development of a stringent gene signature for AA ultraresponsiveness.

We next validated the highly consistent eight-gene signature that was upregulated in the AA ultraresponder LuCaP 136CR in an independent cohort of six LuCaP models that displayed different responses to AA. As expected, the signature positively correlated with the percentage gained survival on AA ($R = 0.95$, $P = 0.0002$; Fig. 3D and E), supporting the potential of this eight-gene signature in predicting AA ultraresponsiveness.

Mechanisms associated with the acquired resistance of individual AA-responsive phenotypes

To identify response and resistance mechanisms specific to different AA response phenotypes, we conducted global transcriptome analyses on the AA-treated (D7) and AA-resistant (EOS) tumors. Interestingly, upon AA resistance, a distinct set of genes was differentially expressed in each of the four models (vehicle vs. AA, $P < 0.01$, fold change 2), and there was virtually no overlap of genes between ultraresponders and intermediate/minimal responders or within the intermediate and minimal responders (Fig. 4A and Supplementary Table S5), suggesting that the AA-induced resistance mechanisms are largely diverse. Next, we conducted Ingenuity Pathway Analysis to identify molecular and cellular function involved in the AA resistance in individual models. For both ultraresponder LuCaP 136CR and the intermediate responder LuCaP 77CR, cell growth and proliferation represented 40% to 45% of genes that were associated with AA resistance. In LuCaP 96CR, a majority of AA differentially expressed genes were related to cell morphology (30%), whereas in the minimal responder, AA differentially expressed genes were principally mapped to cell-to-cell signaling (20%) or cellular death and survival (20%) (Fig. 4B).

GSEA analysis showed that AA treatment of LuCaP 77CR was negatively associated with signatures of cell growth and androgen-regulated genes upon resistance at EOS (Supplementary Fig. S5). Similarly, AA treatment of LuCaP 96CR was negatively associated with a cell cycle-associated signature that was previously reported to be decreased in a cell line-derived xenograft model of AA resistance (Supplementary Fig. S4) (30). Interestingly, in the AA ultraresponder LuCaP 136CR, we identified steroid metabolism as the top altered regulator effect network upon AA resistance (Fig. 4C), which, together with the high basal expression of the cholesterol esterase *CEL*, implies that alterations in the steroid availability and usage may contribute to the development of AA resistance in this model. Importantly, GSEA analysis showed that AA treatment of LuCaP 136CR was initially negatively associated with signatures of proliferation, cell growth, and a selected AR transcriptional program at D7, and this negative proliferation signature persisted but with fewer genes represented at the leading edge at EOS (Supplementary Fig. S5). Despite the negative association with the specific proliferation markers, LuCaP 136CR acquired AA resistance

that was enriched with genes associated with NF- κ B transcriptional activity, EMT, extracellular matrix, and prostate basal cells (Supplementary Fig. S5). These results suggest the diversity of resistance mechanisms to AA and specifically indicate potential mechanisms that drive AR-independent resistance in the AA ultraresponsive phenotype.

Low basal AR signaling and a further reduction of androgen signaling upon resistance in the AA ultraresponder LuCaP 136CR

We examined the AR signaling axis to gain insight into its role in AA resistance and tumor progression. Previous reports showed that AA treatment elevated serum levels of progesterone and other upstream steroids that activated mutant AR (e.g., L701H and T878A) leading to AA resistance (31–34). To elucidate whether AR mutation was involved in the differential AA responsiveness observed in our models, we sequenced the ligand-binding domain of AR and detected no mutation in the AA-treated LuCaP PDXs (data not shown), suggesting that the differential AA responsiveness was not due to AR mutation.

We next conducted targeted analysis on intratumoral androgens and androgen signaling pathways in AA-resistant tumors. We used a sensitive liquid chromatography–mass spectrometry method to detect intratumoral androgens that are sensitive to AA inhibition. In the ultraresponder LuCaP 136CR, AA treatment significantly reduced intratumoral levels of testosterone ($P = 0.009$), dihydrotestosterone ($P = 0.04$), androstenedione ($P = 0.03$; Fig. 5A), and androsterone ($P = 0.04$; Supplementary Fig. S6). Interestingly, LuCaP 136CR demonstrated the lowest basal AR signaling among the LuCaP lines tested, depicted by a low AR activity score (Fig. 5B) and a low AR signature score (Fig. 5C). Upon AA resistance, the decrease in intratumoral androgens was accompanied by a general downregulation of steroidogenic enzymes, including *LDLR* ($P = 0.004$), *STARD4* ($P = 0.005$), and *DUSP1* ($P = 0.01$) (Supplementary Table S3) (12), a further downregulation of AR activity (Fig. 5B), and a reduced AR signature score (Fig. 5C). These results suggested reduced AR signaling in the AA ultraresponder LuCaP 136CR upon resistance.

In contrast, despite decreasing testosterone in the intermediate responders LuCaP 77CR ($P = 0.03$) and LuCaP 96CR ($P = 0.02$) upon AA treatment, high variability in dihydrotestosterone levels was observed in LuCaP 77CR and a statistically insignificant reduction was observed in LuCaP 96CR ($P = 0.11$) (Fig. 5A). Upstream steroids, including pregnenolone ($P = 0.02$) and dehydroepiandrosterone (DHEA; $P = 0.056$), were increased in the intermediate responder LuCaP 77CR upon AA resistance (Supplementary Fig. S5), whereas progesterone was decreased in the intermediate responder LuCaP 96CR ($P = 0.02$; Supplementary Fig. S6). Consistent with the sustained level of intratumoral androgens, no reduction in the enrichment in AR-responsive genes (Fig. 5B) and AR signature (Fig. 5C) was detected upon AA resistance in the intermediate responders LuCaP 77CR and LuCaP 96CR. Similarly, in the AA minimal responder LuCaP 35CR, AA treatment showed an initial negative association with GSEA signatures of AR- and GR-regulated genes at D7 (Supplementary Fig. S5) and a reduction in our selected AR signature (Fig. 5C). However, the negative association was not observed upon AA resistance at EOS (Supplementary Fig. S5), and the AA-resistant tumor demonstrated a persistent expression of steroidogenic enzymes (Supplementary Table S3), AR-responsive genes (Fig. 5B), and AR signature (Fig.

5C). Due to the limited number of LuCaP 35CR AA-resistant tumors available, statistically significant change in the intratumoral androgens was not observed in these tumors upon AA resistance (Fig. 5A). Collectively, these results pointed to sustained AR signaling in the AA intermediate and minimal responders upon resistance. In all models, we also tested whether the AA-resistant tumors acquired a neuroendocrine phenotype. Our results showed that both neuroendocrine markers (chromogranin A and synaptophysin) were absent or minimally expressed (<0.1% in LuCaP 77CR) in the vehicle-treated tumors and the expression did not change upon AA resistance (data not shown).

Finally, we questioned whether AR and GR levels in the tumor may contribute to the downregulation of AR signaling in the AA-resistant tumors in the ultraresponder LuCaP 136CR and the sustained AR signaling in the intermediate or minimal responders. In the ultraresponder LuCaP 136CR, the gene expression of AR and ARv7 was increased upon castration (Supplementary Table S4) but remained unchanged upon further androgen ablation by AA (Fig. 5D), and the nuclear AR and GR localization was not altered upon AA resistance (Fig. 5, panels E and F). The nuclear GR level remained low even upon AA resistance in the ultraresponder LuCaP 136CR (Fig. 5F). In the intermediate and minimal responders, increased expression of AR and its variants was observed upon castration in LuCaP 77CR (Supplementary Table S4), but the expression of AR and ARv7 generally remained unchanged upon AA resistance except for LuCaP 96CR (Fig. 5D). Nuclear localization of AR remained high (i.e., H-score > 100) in the intermediate and minimal responders although a slight decrease in nuclear AR localization for LuCaP 77CR was observed upon AA resistance (Fig. 5, panels D and F). Collectively, these findings suggested active AR signaling in these AA-resistant tumors. Importantly, we observed a high basal level of nuclear GR in the AA minimal responder LuCaP 35CR (Fig. 5F) and a consistent upregulation of both GR gene expression (NR3C1, except for LuCaP 35CR) and nuclear localization for all intermediate and minimal responders (Fig. 5, panels D and F). These GR results may suggest that high basal nuclear GR localization is associated with AA minimal responsiveness, and that an increase in nuclear GR upon AA treatment is associated with rapid, acquired resistance. In summary, upon AA resistance, the ultraresponder LuCaP 136CR displayed lower intratumoral androgens and AR signaling accompanied by sustainably low nuclear GR localization. In contrast, the intermediate and minimal responders demonstrated a slight decrease in intratumoral androgens and sustained AR signaling associated with an increase in nuclear GR localization.

Discussion

AA is effective in a subset of patients, but responding tumors eventually develop resistance. We used PDX models that recapitulated the diverse clinical responses of CRPC to AA and identified heterogeneous response phenotypes, including ultraresponsive, intermediate, and minimal. The ultraresponsive phenotype represents not only AA sensitivity but also durability. We report for the first time that the AA ultraresponsive phenotype is represented by a molecular signature of secreted proteins and biochemical features, including low basal AR signaling and a low basal nuclear GR level, which is insensitive to AA-induced upregulation.

Mechanisms underlying acquired resistance to AA are diverse and have not yet been fully identified. GR was shown to compensate for reduced AR activity through activation of overlapping target genes (35). High GR expression was associated with enzalutamide insensitivity (16), and preliminary results of the COU-AA-203 study demonstrated that high GR may predict low AA sensitivity (36). Our results provided novel information to highlight the role of GR in response to AA: (a) a low level of nuclear GR, and sustainably low GR on AA therapy, was predictive of durable AA inhibition; (b) low to intermediate levels of GR, despite initial response, and increase in nuclear GR was associated with rapid, acquired resistance to AA; and (c) a high basal level of GR was associated with *de novo* resistance/minimal responsiveness. Notably, while we observed a concordant increase in both GR transcript and protein expression levels in some models, discordance was present in others. This result indicates that GR transcripts may not ideally reflect the protein level, especially the nuclear protein level indicative of active GR signaling. Retrospective clinical studies investigating response and resistance patterns have suggested cross-response/resistance between enzalutamide and AA (37–44). However, whether a sustainably low level of GR will lead to a durable response to either AA or enzalutamide in patients, and whether an increase in GR is attributable to rapid AA resistance, requires clinical confirmation.

Copy number gain of *AR* and *CYP17A1* has been shown to predict shorter progression-free survival with AA treatment (45). Our results supported, at a gene expression level, that the intermediate responders LuCaP 77CR/LuCaP 96CR and the minimal responder LuCaP 35CR demonstrated a higher AR level and enhanced androgen signaling when compared with the ultraresponder LuCaP 136CR. On the other hand, other preliminary studies on gene expression using pretreatment primary prostate cancer samples reported a significant association between proliferation-associated genes, androgen-regulated genes, and CYP17 cofactors with longer radiographic progression-free survival of patients receiving AA (46).

In our studies, the ultraresponsive phenotype demonstrated reduced AR signaling upon AA resistance, indicating an emergence of an AR-independent pathway to sustain survival. Upon AA resistance, the ultraresponders presented an enrichment of genes associated with EMT, prostate basal-type cells, and NF- κ B activity. This is consistent with a previous report showing an association between EMT induction and the emergence of prostate cancer stem-like cells (CSC)-like phenotype following androgen deprivation (47). Additionally, activation of the NF- κ B pathway is involved in the induction and maintenance of EMT (48, 49) and CSC-like characteristics in prostate cancer (50–52). These characteristics are concordant with the results of a preclinical study identifying a progenitor-like cell population with increased NF- κ B activity upon resistance to androgen depletion (53) and reduced AR signaling upon increased NF- κ B activity in prostate cancer (54). A recent report on NF- κ B as a potential resistance mechanism for enzalutamide independent of ARv7 may provide another cross-resistance mechanism for AA (55).

In view of the heterogeneity of patients' responses to AA therapy, identification of biomarkers of responses has important implications for treatment selection in the context of precision oncology. The preclinical eight-gene molecular signature of secreted proteins associated with AA durable response that we identified can potentially be developed into a fast, noninvasive test to predict AA response. However, our results at this point are limited to

the preclinical setting and by the number of PDX models representing each response phenotype. Validation in prospective clinical studies is needed to support translational value of this signature.

Collectively, the diverse resistant phenotypes associated with differential AA responses highlighted the need for a tailored next line of therapy. The resistance in the AA ultraresponsive phenotype was represented by low intratumoral androgens and AR signaling accompanied by a sustainably low nuclear GR localization, and alteration in gene expression associated with NF- κ B activity and a EMT/basal cell phenotype. In contrast, resistance in the intermediate and minimally responding phenotypes demonstrated sustained AR signaling and increased nuclear GR localization. Novel treatments may be explored to target NF- κ B activity with a rationale to prevent or revert an EMT basal cell phenotype in the ultraresponders and to target sustained AR/GR signaling in the intermediate or minimal responders upon AA resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of interest:

Peter S. Nelson has served on advisory boards for Janssen and received compensation. Ryan McMullin, Michael Gormley, Weimin Li, Deborah S. Ricci, Karin Verstraeten, and Shibu Thomas are employees of Janssen Research & Development and own stock in Johnson & Johnson.

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Translational Relevance

Abiraterone acetate (AA) improves survival in patients with metastatic castration-resistant prostate cancer (mCRPC); however, not all tumors respond and responding tumors eventually develop resistance. Currently there is no information available regarding how to stratify patients for durable AA therapy, and the mechanisms underlying AA resistance are diverse. We used patient-derived xenograft models that recapitulated the diverse clinical response of CRPC to AA and identified a molecular signature of secreted proteins associated with the AA ultraresponsive phenotype. The signature will provide the much needed information on noninvasive biomarker development to select AA-responsive patients. Upon resistance, our results suggested reduced androgen receptor (AR) signaling and sustainably low nuclear glucocorticoid receptor (nGR) localization in the AA ultraresponders. In contrast, sustained AR signaling and increased nGR localization was observed in the intermediate and minimal responders. Further inhibition along the AR/GR signaling axis may be effective in AA-resistant patients who are intermediate or minimal responders.

Figure 1.
Treatment scheme for AA on CRPC PDXs. Castration-resistant tumors were developed and mice were treated orally with either vehicle or AA (0.5 mmol/kg/day). Mice were sacrificed and tumors were harvested on D7 or when tumors reached $1,000 \text{ mm}^3$ (EOS).

Figure 2.

Ultraresponsiveness to AA in LuCaP 136CR PDX models. A, Kaplan-Meier curves showing survival benefits of AA treatment in different LuCaP PDX models. B, Linear model analyses of tumor volume. C, Serum PSA upon AA treatment. $n = 9\text{--}14$ per group.

Figure 3.

Gene expression associated with LuCaP 136CR ultraresponsiveness. A, Supervised clustering analyses showing 531 differentially expressed genes between LuCaP 136CR and LuCaP 35CR and LuCaP 96CR on D7. Yellow: high gene expression; blue: low gene expression. B, Schematic diagram on gene shaving to identify an eight-gene signature associated with the LuCaP 136CR AA ultraresponsive phenotype. C, qPCR confirmation on the eight-gene signature associated with LuCaP 136CR AA ultraresponsive phenotype (D7 and EOS). D, Heat map showing the microarray gene expression of the eight-gene signature

in multiple LuCaP models. E, Correlation between the enrichment of the eight-gene signature associated with AA ultraresponsive phenotype and percentage gained in survival upon AA treatment. Percentage survival gained was calculated based on median survival in AA-treated versus vehicle-treated mice in each xenograft model. Each data point or column represented an individual animal. $P < 0.05$ was considered statistically significant.

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Figure 4. Biological mechanisms underlying the acquired resistance to AA. A. Venn diagrams showing distinct gene alternations by AA upon treatment resistance at EOS among different LuCaP PDXs. B. Ingenuity Pathway Analysis identified the molecular and cellular functions associated with AA resistance in different LuCaP PDXs. C. Top regulator effect network in AA-resistant tumors in the AA ultrasponder LuCaP 136CR PDXs.

Figure 5. Reduction of androgen signaling upon treatment resistance in the AA ultraresponsive phenotype. A, Levels of intratumoral androgens in control and AA-resistant CRPC PDXs measured by mass spectrometry. *n* = 2–6 per group. B, Heat map showing the low AR activity (top row, pink squares) and low expression of genes involved in androgen signaling in LuCaP 136CR (*n* = 4–6 per group), and C, their respective AR signature score in LuCaP PDXs (*n* = 4–6 per group). D, qPCR analysis in vehicle versus AA-resistant tumors at EOS (*n* = 4–6 per group). E, Representative IHC pictures of AR and GR, and F, their respective

H-score in control and AA-resistant PDXs ($n = 6\text{--}12$ per group). Scale bar: 50 μm . Magnification: 200 $\times$. Each data point or column (heat map) represented an individual animal. $P < 0.05$ was considered statistically significant.