ANIMAL MODELS

Castration Induces Up-Regulation of Intratumoral Androgen Biosynthesis and Androgen Receptor Expression in an Orthotopic VCaP Human Prostate Cancer Xenograft Model

Matias Knuuttila,* Emrah Yatkin,† Jenny Kallio,* Saija Savolainen,* Teemu D. Laajala,‡ Tero Aittokallio,§ Riikka Oksala,‖ Merja Häkkinen,** Pekka Keski-Rahkonen,** Seppo Auriola,** Matti Poutanen,*‡ and Sari Mäkelä*†

From the Departments of Physiology* and Mathematics and Statistics‡ and the Turku Center for Disease Modeling,† Institute of Biomedicine, and the Functional Foods Forum,** University of Turku, Turku, Finland; the Institute for Molecular Medicine Finland,§ University of Helsinki, Helsinki, Finland; the Department of Oncology and Critical Care Research,‖ Orion Pharma, Turku, Finland; the School of Pharmacy,** University of Eastern Finland, Kuopio, Finland; and the Institute of Medicine,yy Sahlgrenska Academy, Gothenburg University, Gothenburg, Sweden

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Address correspondence to Sari Mäkelä, M.D., Ph.D., Institute of Biomedicine, University of Turku, Käpyläntie 10, 20520 Turku, Finland. E-mail: sarmak@utu.fi.

Androgens are key factors involved in the development and progression of prostate cancer (PCa), and PCa growth can be suppressed by androgen deprivation therapy. In a considerable proportion of men receiving androgen deprivation therapy, however, PCa progresses to castration-resistant PCa (CRPC), making the development of efficient therapies challenging. We used an orthotopic VCaP human PCa xenograft model to study cellular and molecular changes in tumors after androgen deprivation therapy (castration). Tumor growth was monitored through weekly serum prostate-specific antigen measurements, and mice with recurrent tumors after castration were randomized to treatment groups. Serum prostate-specific antigen concentrations showed significant correlation with tumor volume. Castration-resistant tumors retained concentrations of intratumoral androgen (androstenedione, testosterone, and 5a-dihydrotestosterone) at levels similar to tumors growing in intact hosts. Accordingly, castration induced up-regulation of enzymes involved in androgen synthesis (CYP17A1, AKR1C3, and HSD17B6), as well as expression of full-length androgen receptor (AR) and AR splice variants (AR-V1 and AR-V7). Furthermore, AR target gene expression was maintained in castration-resistant xenografts. The AR antagonists enzalutamide (MDV3100) and ARN-509 suppressed PSA production of castration-resistant tumors, confirming the androgen dependency of these tumors. Taken together, the findings demonstrate that our VCaP xenograft model exhibits the key characteristics of clinical CRPC and thus provides a valuable tool for identifying druggable targets and for testing therapeutic strategies targeting AR signaling in CRPC. (Am J Pathol 2014, 184: 2163–2173; http://dx.doi.org/10.1016/j.ajpath.2014.04.010)

In developed Western countries, prostate cancer (PCa) is a common and significant clinical problem. Almost all aging men develop prostatic intraepithelial neoplasia, precancerous lesions that frequently progress to locally invasive PCa.1 PCa growth can be attenuated by androgen deprivation therapy or by inhibition of androgen receptor (AR) action. In a considerable proportion of men, however, the disease progresses to castration-resistant prostate cancer (CRPC), which is a major challenge in the development of efficient therapies. Recent studies have demonstrated that the development of CRPC is associated with aberrant AR expression2 and activation of intratumoral androgen biosynthesis.3 These changes indicate that CRPC tumors remain androgen dependent, which is further supported by studies showing that AR target genes, such as KLK3 (alias PSA) and TMPRSS2,4 are frequently expressed in CRPC, and that transcription of AR is activated in CRPC.3 Mechanisms that

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contribute to androgen dependency in CRPC include non-gonadal (eg, adrenal) androgen synthesis, induction of intratumoral androgen synthesis, increase in AR expression in response to castrate levels of androgens, AR amplification and mutations, and ligand-independent or constitutive AR activity. Based on these findings, therapeutic strategies for CRPC have been developed to target androgen action by inhibition of androgen biosynthesis or AR-mediated signaling in CRPC. The CYP17A1 inhibitor abiraterone acetate (Zytiga) has been shown to prolong survival of men with CRPC and is currently used before and after docetaxel chemotherapy. The efficacy of abiraterone further supports the role of local intratumoral androgen production as an important growth-promoting factor in CRPC. In addition, enzalutamide (Xtandi; tested as MDV3100), a novel AR antagonist that blocks AR signaling and leads to AR degradation, was approved in 2012 by the U.S. Food and Drug Administration for treatment of patients with CRPC after docetaxel therapy. Despite the promising results with abiraterone and enzalutamide, therapies that achieve more efficient and long-term blockade of intratumoral androgen biosynthesis and/or AR action are needed for treatment of CRPC. A number of preclinical models in which androgen-responsive human PCa cell xenografts are grown in castrated immunodeficient mice have been used to elucidate the mechanisms involved in progression to CRPC, as well as for testing drug candidates. These include LNCaP, C4-2 derived from LNCaP, LuCaP 35, CW22-R, LAPC, and VCaP cell lines and xenografts, all of which exhibit CRPC-like properties but differ from each other in origin, tumorigenicity, growth rate, aggressiveness (metastatic potential), and expression of molecular markers typical of CRPC.

The VCaP cell line, originating from a vertebral PCa metastasis, is an attractive model for CRPC; it is androgen-sensitive and tumorigenic, and expresses AR, Rb, p53, prostate-specific antigen (PSA), and the TMPRSS2–ERG fusion gene, thus closely mimicking clinical CRPC. VCaP cells have also been shown to express enzymes involved in intratumoral androgen biosynthesis (eg, CYP17A1, AKR1C3, and HSD17B6). Serially passaged in castrated male SCID mice, these cells have been used to generate a subcutaneous model to study the transition from the androgen-responsive to the castration-resistant stage.

Despite considerable advances in understanding the biology of PCa, CRPC remains a major challenge for drug development, and relevant experimental preclinical models for CRPC are needed to identify the key molecular events regulating castration-resistant growth. To that end, we have established a preclinical in vivo model of CRPC based on orthotopic VCaP xenografts grown in castrated hosts. This mouse model exhibits the key characteristics of clinical CRPC, including up-regulation of intratumoral androgen biosynthesis and aberrant AR expression, as well as responsiveness to AR antagonists. Our model thus provides a valuable tool to identify druggable targets in CRPC and to test novel anticancer therapies.

Materials and Methods

Cell Culture

VCaP cells were purchased from ATCC (Manassas, VA) and were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), 2% l-glutamine (Gibco; Life Technologies, Carlsbad, CA), and 1% penicillin–streptomycin (Sigma-Aldrich). The cells were cultured at 37°C in a 5% CO2–enriched atmosphere on Corning CellBind flasks (Corning Life Sciences, Tewksbury, MA) and were split 1:2 once a week when subconfluent (approximately 20 × 10⁶ cells per 75-cm² flask); the medium was replaced every other day. Before inoculation, cells were counted using a Bürker chamber and suspended in serum-free medium.

Orthotopic VCaP Xenografts in Immunodeficient Mice

Adult male immunodeficient mice (HSD:Athymic Nude–Foxn1nu) were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed in individually ventilated cages under controlled conditions of light (12 hours light/12 hours dark), temperature (21 ± 3°C), and humidity (55% ± 15%). The mice were given irradiated soy-free natural-ingredient feed [RM3 (E); Special Diets Services, Witham, UK] and autoclaved tap water ad libitum, and were housed in specific pathogen–free conditions at the Central Animal Laboratory (University of Turku) in compliance with international guidelines on the care and use of laboratory animals. All animal handling was conducted in accordance with Finnish Animal Ethics Committee and institutional animal care policies, which fully meet the requirements defined in current NIH guidelines on animal experimentation (license number 1993/04.10.03/2011).

VCaP cells (10⁶ cells in 20 µL medium) were inoculated orthotopically into the dorsolateral prostate of nude mice (6 to 8 weeks of age) through an abdominal incision. For pain relief, mice were injected subcutaneously with 0.05 to 0.1 mg/kg buprenorphine (Temgesic; Reckitt Benckiser Healthcare, Slough, UK) before surgery and with 5 mg/kg carprofen (Rimadyl; Pfizer, New York, NY) after surgery. Isoflurane (Baxter, Deerfield, IL) was used for induction and maintenance of anesthesia. Tumor growth was monitored by serum PSA measurements. At 2 weeks after inoculation, approximately 100 µL of blood was collected for PSA measurement by saphenous vein puncture. After blood collection, mice were injected subcutaneously with 200 µL of 0.9% NaCl. This procedure was repeated once a week, until the mice were sacrificed. Serum PSA concentrations were determined using a time-resolved fluorometric assay as described previously, applying two antibodies in a direct sandwich technique. Fluorescence signal was measured using a Wallac 1420...
VICTOR2 microplate reader (PerkinElmer, Waltham, MA). Reagents for the PSA fluorometric assay were provided by Kim Petterson (University of Turku).

**VCaP Tumors Grown in Intact versus Castrated Mice**

In Study I, when the serum PSA reached ≥10 μg/L, the mice were divided into five groups. The first group of intact mice was sacrificed at the time when the serum PSA was approximately 10 μg/L (Intact 1; small tumors), and the second group when the serum PSA reached approximately 70 μg/L (Intact 2; large tumors). Mice in the remaining three groups (GNX, CRPC 1, CRPC 2) were castrated by removing the testes through a scrotal incision under isoflurane anesthesia; 0.05 to 0.1 mg/kg buprenorphine was used for analgesia. Mice in the gonadectomized (GNX) group were sacrificed 1 day after castration, and mice in the CRPC 1 (small tumors) and CRPC 2 (large tumors) groups were sacrificed when serum PSA reached 10 to 20 μg/L and 50 to 90 μg/L, respectively. At sacrifice, tumors were dissected, tumor weights were recorded, and tumor width, height, and length were measured using a calipers. Tumor volumes were calculated with the ellipsoid formula: Volume = (π/6) × Width × Height × Length. Samples from each tumor were both snap-frozen and stored in −80°C and fixed in 10% formalin for 24 hours and processed for paraffin embedding. Para-aortic lymph nodes were dissected, fixed in 10% formalin for 24 hours, and embedded in paraffin. Blood samples were collected by cardiac puncture before sacrifice; serum was separated by centrifugation at 4000 × g for 10 minutes and stored in −80°C.

**Effects of Antiandrogens on Castration-Resistant VCaP Tumors**

To investigate the effects of novel antiandrogen therapies in Study II, animals were treated with 20 mg/kg per day of enzalutamide (hereafter, MDV3100) (n = 15), ARN-509 (n = 15), or vehicle (n = 15). The compounds were synthesized at Orion Pharma (Espoo, Finland). The vehicle consisted of 50% polyethylene glycol 300 (Merck, Darmstadt, Germany), 35% 100 mg/mL glucose solution (Baxter), 10% Tween 80 (Merck), and 5% dimethylacetamide (Merck). Orthotopic VCaP tumors were generated as described for Study I and were allowed to grow for 4 to 5 weeks, until at least 60% of animals had reached a serum PSA level of >5 μg/L and the mean serum PSA value was approximately 15 μg/L. Thereafter, all mice with tumors were castrated within 2 weeks (weeks 4 and 5). Administration of vehicle and test compounds was begun when the serum PSA of all castrated mice had reached the precastration level (mean serum PSA of approximately 15 μg/L). The vehicle and the compounds were administered to the mice by gavage once a day for 28 days. The mice were allocated to multiple treatment arms at week 10, retaining a balance among the groups based on the PSA levels measured at week 10, the change of the PSA level from week 9 to 10, body weights at week 9, cage placement, and the week at which castration took place. A symmetric dissimilarity matrix was constructed using Mahalanobis distance on PSA levels and body weight measurements, and for each mouse the closest match was detected by minimizing the dissimilarities. Subsequently, matched mice were randomized to masked treatment arms to ensure that prognostically similar mice were available in each arm. All groups were constrained to have an equal number of mice castrated on week 9 or 10, to avoid stratification with respect to this factor. All of the tested baseline group differences for the PSA and body weight were highly statistically nonsignificant (all differences P > 0.8, one-way analysis of variance with Tukey’s honestly significant difference).

**Intratumoral and Serum Steroid Measurements**

Tumor samples were homogenized in sterile deionized water 1:10 (w/v) using an Ultra-Turrax homogenizer (IKA-Werke, Staufen im Breisgau, Germany; Wilmington, NC). Concentrations of androstenedione (A-dione), testosterone (T), and dihydrotestosterone (DHT) were measured in tumor homogenates and serum samples using a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method as described previously. Quantification limits for A-dione, T, and DHT were 0.02 ng/mL, 0.01 ng/mL, 0.1 ng/mL, respectively. For comparison of tumor homogenate concentrations to serum concentrations, 1 g of tumor sample was considered to correspond to 1 mL of serum.

**RT-qPCR**

Total RNA for quantitative RT-PCR (RT-qPCR) and micro-array analyses was extracted from tumor samples using an RNase mini kit (Qiagen, Venlo, the Netherlands; Valencia, CA) according to the manufacturer’s instructions. The RNA was then treated with DNase I (Invitrogen amplification grade; Life Technologies, Carlsbad, CA), and was reverse transcribed using M-MuLV Reverse Transcriptase (New England BioLabs, Ipswich, MA) and oligo(dT) primers (Promega, Madison, WI). RT-qPCR reactions were performed using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) and a 2× DyNAmo SYBR Green qPCR kit (Thermo Fisher Scientific, Waltham, MA). The expression of genes of interest were normalized to human ribosomal protein L19 (RPL19), and the amount of mRNA expressed was quantified using the Pfaffi method applied to relative quantification of the expression for reactions with diverse amplification efficiencies. The target genes and sequences of the primers used in RT-qPCR are listed in Table 1.

**Immunoblotting**

Tumor samples were homogenized using an Ultra-Turrax homogenizer (IKA-Werke) in ice-cold lysis buffer [150 mmol/L
Table 1  RT-qPCR Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR1C3</td>
<td>5'-GCCAGGTAGGAAACTTTCAC-3'</td>
<td>5'-CAATTATCTCCGCTGGATAGTGAC-3'</td>
</tr>
<tr>
<td>AR (FL)</td>
<td>5'-CTTACATTGGCAGACGACA-3'</td>
<td>5'-GGCTGTACCCAGGACCTTGTG-3'</td>
</tr>
<tr>
<td>AR (V1)</td>
<td>5'-GTTCATTGGGATGACGGGAC-3'</td>
<td>5'-CTGGTATGAGGACGTCGAGTCCT-3'</td>
</tr>
<tr>
<td>AR (V7)</td>
<td>5'-CTGGTATGAGGACGTCGAGTCCT-3'</td>
<td>5'-TTTGAATGGCAGTCGACCTTCTT-3'</td>
</tr>
<tr>
<td>CYP17A1</td>
<td>5'-TGTCTGGGCCTGCTCAA-3'</td>
<td>5'-AGGCGTAATCCCTTACGCTGT-3'</td>
</tr>
<tr>
<td>HSD17B6</td>
<td>5'-CTTCGACATCTGTCGAGAC-3'</td>
<td>5'-AAATATTGTGCTGGGCTCCTT-3'</td>
</tr>
<tr>
<td>KLK2</td>
<td>5'-TGTGGGCAATGCTTAAAGAAGA-3'</td>
<td>5'-GGCCTTTGTGGCTTGCAAGGC-3'</td>
</tr>
<tr>
<td>KLK3</td>
<td>5'-CCAAAGTTCACTGTGCTGCT-3'</td>
<td>5'-GGGTGCTTGTAGCTAACCCTTC-3'</td>
</tr>
<tr>
<td>KLK4</td>
<td>5'-GGCCTCTGTCATGGAGAAACAG-3'</td>
<td>5'-TCAAGACTGTGAGAGGCCACG-3'</td>
</tr>
<tr>
<td>RPL19</td>
<td>5'-AGGACCATAGCCTCTTACTTAAA-3'</td>
<td>5'-CCATGAGATCCGCTGCTTT-3'</td>
</tr>
<tr>
<td>TPMRSS2–ERG</td>
<td>5'-GGCGGCAGCTAAGCAGGAG-3'</td>
<td>5'-GTAGGCCACACTCAACARAGAGTGG-3'</td>
</tr>
</tbody>
</table>

Tris-HCl, 150 mmol/L, 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L SDS, 100 μmol/L sodium orthovanadate (Sigma-Aldrich) with a Complete Mini protease inhibitor tablet (Roche, Basel, Switzerland; Indianapolis, IN). Samples were centrifuged at 10,000 × g for 20 minutes at 4°C, and supernatants were collected for further analysis. Total protein concentrations were measured with a Pierce BCA protein assay (Thermo Fisher Scientific) using a Wallac 1420 VICTOR2 microplate reader (PerkinElmer). Protein lysates were denatured at 95°C for 5 minutes, and 50 μg of total protein of each lysate was separated on 4% to 10% SDS-PAGE gels and transferred onto Hybond ECL nitrocellulose membrane (GE Healthcare, Little Chalfont, UK) using a Trans-Blot Turbo transfer system (Bio-Rad Laboratories). The membrane (GE Healthcare, Little Chalfont, UK) using a Trans-Blot Turbo transfer system (Bio-Rad Laboratories). The membrane was blocked with 5% nonfat milk—phosphate-buffered saline containing 0.1% Tween (20 (Sigma-Aldrich), probed with rabbit antibody against AR (dilution 1:200; sc-816; Santa Cruz Biotechnology), phosphorylated histone (p-histone) H3 ser10 (dilution 1:1500; 06-475; EMD Millipore, Billerica, MA), and vimentin (dilution 1:500; M0725; Dako, Glostrup, Denmark) overnight at 4°C. After a washing, endogenous peroxidase activity was blocked by incubating the slides with 1% H2O2 for 20 minutes. Sections were then incubated with an anti-rabbit secondary antibody (Envision; Dako) for 30 minutes at room temperature, washed, and the immunocomplexes were visualized with a Liquid DAB+ substrate-chromogen system (Dako) and further counterstained with Mayer’s hematoxylin. The terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) method was used to determine apoptotic cells in sections and analysis was performed using an ApopTag peroxidase in situ apoptosis detection kit (Chemicon International; EMD Millipore) according to the manufacturer’s instructions.

Stained sections were digitized with a Pannoramic 250 slide scanner (3DHISTECH, Budapest, Hungary). The quantification of p-histone H3—positive cells in tumor sections was performed with algorithms customized by Quva (Tampere, Finland). The apoptotic index was calculated by quantifying TUNEL-positive cells in tumor sections from four randomly selected fields of 0.5 mm² from each slide.

Microarray Analysis

The microarray experiments were performed at the Finnish Microarray and Sequencing Center using a HumanHT-12 v4 Expression BeadChip array (Illumina, San Diego, CA), which contains nearly 48,000 probe types. The microarray data were normalized using quantile normalization. To study gene expression changes of androgen regulation in VCaP tumors, 85 androgen-regulated genes were selected based on an androgen pathway product analysis list provided by SwitchGear Genomics (Menlo Park, CA). The rank product method2 was used to test log-transformed differential expression of the androgen-regulated genes among the Intact 1, Intact 2, GNX, CRPC 1, and CRPC 2 groups. Genes with a false discovery rate below the 0.05 threshold in comparisons with the intact groups were considered to be differentially expressed. The within-group
average expression of the differentially expressed genes was subjected to hierarchical clustering with Euclidean distance for the sample and gene profiles. Microarray data have been deposited with the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo; accession number GSE56829).

Table 2  Serum PSA Levels at Different Stages of Study I, with Duration of Tumor Growth

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Before castration</th>
<th>1 Day after castration*</th>
<th>1 Week after castration</th>
<th>Castration-resistant stage*</th>
<th>Duration of tumor growth (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Inoculation to sacrifice</td>
</tr>
<tr>
<td>Intact 1</td>
<td>7</td>
<td>12.0 ± 1.36*</td>
<td></td>
<td></td>
<td></td>
<td>21−63</td>
</tr>
<tr>
<td>Intact 2</td>
<td>7</td>
<td>78.2 ± 10.57*</td>
<td></td>
<td></td>
<td></td>
<td>21−98</td>
</tr>
<tr>
<td>GNX</td>
<td>8</td>
<td>15.7 ± 3.74</td>
<td>7.6 ± 1.60</td>
<td></td>
<td></td>
<td>21−43, 21−42</td>
</tr>
<tr>
<td>CRPC 1</td>
<td>7</td>
<td>12.7 ± 1.87</td>
<td>1.8 ± 0.34</td>
<td>13.6 ± 1.37</td>
<td></td>
<td>56−126, 21−42</td>
</tr>
<tr>
<td>CRPC 2</td>
<td>7</td>
<td>16.4 ± 1.82</td>
<td>1.9 ± 0.24</td>
<td>73.5 ± 4.30</td>
<td></td>
<td>91−133, 21−42</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM.

*At sacrifice.

Statistical Analysis

Pearson’s correlation coefficient \( r \) was used to compare terminal PSA values and tumor volume measurements. Student’s \( t \)-test or one-way analysis of variance with Dunn’s post hoc test was applied to compare steroid and gene expression levels among intact, GNX, and CRPC groups. These analyses were performed using SigmaPlot version 12.0 statistical software (Systat Software, San Jose, CA). Data are expressed as means ± SEM. Differences were considered statistically significant at \( P < 0.05 \). R statistical software version 3.0.1 (http://cran.r-project.org) was used for the analysis of PSA growth curves. Mixed-effects models of the lme4-package version 1.1-6 (http://cran.r-project.org/package=lme4, last accessed March 25, 2014) were used for testing differences in the linear growth slope of PSA in response to an intervention:

\[
y_{i,j} = y_{i,j,\text{case}} - y_{i,j,\text{control}} = b_0 + b_1 \text{Week}_i + u_{i,j} + u_{i,j,\text{Week}} + e_{i,j} \tag{1}
\]

where case is either ARN-509 or MDV3100, \( i \) is the index of the treated mice, \( j \) is the index of the control mice, \( t \) is the treatment week, \( b_0 \) and \( b_1 \) are the population-specific fixed effects, \( u_i \) and \( u_j \) are the individual-specific random effects, and the response \( y \) is the PSA level difference for a matched pair of tumors at a given time \( t \); \( e \) is an error term. The pairs of \( i \) and \( j \) were constrained to the mice matched for randomization before interventions based on the prognostic variables (preintervention PSA level, PSA change, body weight, and castration week). Such a model captures paired differences in tumor growth patterns, with the growth pattern of a control tumor subtracted from the pattern of its matched treated tumor. By definition, the \( y = 0 \) level indicates no difference over time \((b_0 = b_1 = 0)\), whereas a negative slope \((b_1 < 0)\) indicates a less aggressive growth in treated tumors. The intercept term \( b_0 \) was effectively omitted in the modeling process because the difference in baseline PSA levels was negligible.

Results

Growth Properties of Orthotopic VCaP Xenografts

Serum PSA levels were used as an indicator of tumor take and growth. Mouse prostate does not produce PSA, and no PSA
was detected in tumor-free mice. Detectable levels (>0.5 μg/L) of PSA in mouse serum were observed 2 weeks after tumor inoculation, and 71% of mice exhibited significant levels of PSA, which increased constantly in the intact mice during the 14-week follow-up period (Figure 1A and Table 2). Castration (2 to 5 weeks after inoculation) caused a marked decline in PSA levels (Figure 1A and Table 2), confirming the importance of androgen production for VCaP tumor growth. At 1 week after castration, mean serum PSA in castrated mice was reduced sevenfold (Table 2). At 2 weeks after castration, PSA levels started to increase in 83% of the mice, reaching precastration levels within 3 to 12 weeks (Figure 1A and Table 2), indicating castration-resistant growth of the VCaP tumors. Serum PSA concentrations measured from terminal blood samples at sacrifice showed a significant correlation with tumor volume \( r = 0.85, P < 0.001 \) (Figure 1B), confirming that PSA is a reliable indicator of tumor volume in the orthotopic VCaP xenograft model. Moreover, PSA concentration, and thus also PSA production, was similar in large tumors collected from intact mice and from castrated mice (Figure 1C).

Cell proliferation and apoptosis in the tumors were quantified using p-histone H3-stained and TUNEL-stained tumor sections, respectively. Tumors from intact mice exhibited significantly higher proliferation rates, compared with intact 2, CRPC 1, and CRPC 2 mice). No difference was observed in apoptosis index among tumors grown in intact and castrated mice (Supplemental Figure S1). Furthermore, we used immunohistochemical staining for vimentin to detect the potential epithelial–mesenchymal transition in castration-resistant tumors, but no staining was detected in Intact 1 and 2 mice \( n = 13 \) or CRPC 1 and 2 mice \( n = 8 \) (data not shown). No signs of metastasis to the para-aortic lymph nodes were observed in any of the four groups of mice (Intact 1, Intact 2, CRPC 1, and CRPC 2; \( n = 33 \)).

**High Intratumoral Androgen Concentrations Are Recovered in Castration-Resistant VCaP Tumors**

Concentrations of A-dione, T, and DHT in serum and tumor samples were analyzed by LC-MS/MS. As expected, A-dione and T were detectable in the serum of intact mice, and the concentrations decreased to unmeasurable levels after castration (GNX and CRPC groups) (Figure 2). In contrast to A-dione and T serum DHT was below the quantification level (0.1 ng/mL) in all groups. We also analyzed the intratumoral concentrations of these androgens in intact and castrated mice. The data revealed that A-dione, T, and DHT were all present at measurable levels in tumors grown in both intact and castrated (CRPC) mice. Furthermore, intratumoral androgen concentrations were similar in small (Intact 1, CRPC 1) and large (Intact 2, CRPC 2) tumors. Despite the marked and permanent decrease in serum androgen concentrations after castration, no persistent decline was observed in the established castration-resistant tumors. There was a notable transient reduction in the intratumoral levels of the androgens at day 1 after castration, but the levels recovered to precastration levels in castration-resistant tumors analyzed at 3 to 14 weeks after castration (Figure 2). Thus, increases in the tumor/serum ratio concentration of A-dione and T in mice after castration, as well as the similar levels of DHT in tumors before and after castration, indicate up-regulation of local androgen production in castration-resistant tumors.

**Critical Steroidogenic Enzymes and Androgen-Regulated Genes Are Expressed in Castration-Resistant VCaP Tumors**

We observed significant induction of mRNA expression of the steroidogenic enzymes AKR1C3 (2.5-fold, \( P < 0.001 \), HSD17B6 (1.6-fold, \( P = 0.002 \)), and CYP17A1 (threefold, \( P < 0.001 \)) in tumors grown in castrated mice (GNX, CRPC), compared with tumors grown in intact mice (Figure 3A).
AKR1C3 and CYP17A1 were already up-regulated at 1 day after castration, whereas the increase in HSD17B6 was detected only later in the established castration-resistant tumors. Known androgen-regulated genes, such as the kallikreins KLK2, KLK3, and KLK4, were differentially expressed at different phases of tumor growth (Figure 3B). In the established castration-resistant tumors, expression of KLK2 and KLK3 was transiently decreased at 1 day after castration, but levels returned to pre-castration levels. By contrast, KLK4 expression in castration-resistant tumors was significantly higher than in tumors grown in intact mice or in GNX mice analyzed at 1 day after castration (Figure 3B). Furthermore, the TMPRSS2—ERG fusion gene was regulated in a manner similar to KLK2 and KLK3. The expression patterns of KLK2, KLK3, and TMPRSS2—ERG were thus in full accord with the intratumoral androgen concentrations: a transient decline at 1 day after castration, and thereafter a rise to levels similar to those observed in intact mice. These findings indicate that androgen signaling is maintained in castration-resistant VCaP tumors at a level similar to that observed in tumors grown in intact mice.

Results from microarray analysis support the idea that androgen-regulation is not significantly changed in castration-resistant tumors, compared with tumors from intact mice. In all, 49 probes out of the 85 selected androgen-regulated genes were differentially expressed in the five groups of mice (intact, CRPC, and GNX). Under unsupervised clustering, the Intact 1 and 2 groups and the CRPC 1 and 2 groups clustered together, suggesting that small and large tumors are similar in terms of androgen action. Moreover, the Intact and CRPC groups were found to be more similar to each other than to the GNX group (Figure 3C). These results further indicate a similar androgen signaling in the VCaP tumors before and after castration.

AR and Its Splice Variants Are Up-Regulated in Castration-Resistant VCaP Tumors

VCaP cells are known to express wild-type AR.19 Our RT-qPCR results indicate that the mRNA for the full-length AR
is increased in castration-resistant VCaP tumors approximately sixfold at 1 day after castration (GNX), and up to 20-fold in the established castration-resistant tumors (CRPC 1 and CRPC 2) at 3 to 14 weeks after castration (Figure 4A). Castration also markedly (>50-fold) induced expression of AR splice variants 1 and 7 (AR-V1 and AR-V7), which lack the ligand-binding domain, compared with the level detected in tumors grown in intact mice (Figure 4A). In accord, both Western blot analysis (Figure 4B) and immunohistochemistry (Figure 4C) performed with an antibody targeting an epitope at the N-terminus of AR. Brown color indicates positive immunostaining. Insets show a region of interest at higher magnification. Data are expressed as means ± SEM. A: n = 11, intact; n = 5, GNX; n = 10, CRPC. B: n = 4 per group. **P < 0.01, ***P < 0.001. Scale bar = 200 µm.

Castration-Resistant VCaP Tumors Respond to Antiandrogen Therapies

After establishing that castration-resistant VCaP tumors restore their intratumoral androgen concentrations to precastration levels and continue to express androgen-regulated genes, we investigated the response of castration-resistant tumors to two antiandrogen therapies, MDV3100 and ARN-509 (Figure 5). Treatment responses, as indicated by a reduced serum PSA concentration, were observable already at 1 week after initiation of treatment. At the end of the 4-week treatment period, mice receiving the antiandrogens had significantly lower serum PSA concentrations, compared with mice treated with vehicle alone (Figure 5). In all groups, independent of antiandrogen treatment, serum PSA and tumor volume correlated significantly (Supplemental Figure S2), suggesting that reduction of serum PSA is paralleled by reduction of tumor growth. Treatment effects were tested by analyzing differences in longitudinal PSA levels of paired tumors from the control and treatment groups, matched before interventions. The matched slope differences were estimated using equation 1, with $b_1 = -6.94$ ($P = 0.0036$) for ARN-509 and $b_1 = -5.73$ ($P = 0.0077$) for MDV3100. Only one case—control pair exhibited clearly greater growth in the treated tumor than in the vehicle tumor for both interventions (Figure 5). Our results thus indicate that both ARN-509 and MDV3100 exert a significant antitumorigenic (anti-androgenic) effect on castration-resistant VCaP tumors.

Discussion

We have established an orthotopic VCaP xenograft model that allows evaluation of cellular and molecular characteristics of castration-resistant growth of PCa, as well as study of intratumoral androgen biosynthesis in CRPC in vivo. Similar to clinical CRPC, in the VCaP model AR activity is restored despite low (undetectable) serum androgen levels, and AR-mediated signaling continues to play a key role in

Figure 4 AR and its splice variants are up-regulated in castration-resistant VCaP tumors. A: RT-qPCR analysis of relative mRNA levels of full-length AR (AR-FL) and AR splice variants V1 and V7, normalized to RPL19, in Intact 1 and 2 (black bars), GNX (gray bars), and CRPC 1 and 2 tumors (white bars). B: Relative expression of full-length AR protein by immunoblotting, normalized to GAPDH, in Intact 2 and CRPC 2 tumors. C: Representative images of immunohistochemical staining using an antibody targeting an epitope at the N-terminus of AR. Brown color indicates positive immunostaining. Insets show a region of interest at higher magnification. Data are expressed as means ± SEM. A: n = 11, intact; n = 5, GNX; n = 10, CRPC. B: n = 4 per group. **P < 0.01, ***P < 0.001. Scale bar = 200 µm.

Figure 5 Castration-resistant VCaP tumors respond to antiandrogen therapy. Paired differences in the growth rates (slopes) between vehicle and treatment during the 4-week antiandrogen treatment period for vehicle versus ARN-509 (A) and for vehicle versus MDV3100 (B). Data are expressed as estimated values for $b_1 + u_i(i - j)$ per match (ie, population differences between groups, adjusted for normally distributed variation for individual pairs). The $b_1$ and $u_i$ terms model differences in PSA levels per treatment week; negative values indicate lesser increase in PSA for the treated tumor than for the matching control tumor over time. The mean change ($b_1$ estimate) is indicated by a dotted line. **P < 0.01.
tumor growth, as indicated by response to antiandrogen treatments.

We have demonstrated by direct LC-MS/MS analyses that castration-resistant VCaP tumors produce significant levels of active androgens, independent of gonadal steroid synthesis. In contrast to marked decrease in serum androgens after castration, A-dione, T, and DHT were all present at detectable levels in tumors from castrated mice. There was a notable but transient reduction in the intratumoral levels of these androgens after castration, and the androgens returned to precastration levels in castration-resistant tumors. Thus, the increase in the tumor/serum ratio of A-dione and T, as well as the presence of DHT in tumor tissue after castration, indicates local androgen production in castration-resistant tumors, in accord with previous findings summarized above, results from murine xenograft models involving the conversion of 3α-androstane-3,17-dione to DHT via the backdoor pathway both in normal prostate and in development of CRPC.36–38 Furthermore, HSD17B6 (alias SDR9C6) is suggested to catalyze the conversion of 3α-androstenediol to DHT via the backdoor pathway both in normal prostate and in PCa.39,40 Consistent with these studies, we observed that AKR1C3 was already induced at 1 day after castration, and up-regulation of HSD17B6 was detected later in the established castration-resistant tumors. AKR1C3 has also been shown to catalyze the conversion of 3α-androstenediol to DHT via the backdoor pathway both in normal prostate and in development of CRPC.36–38 Furthermore, treatment of VCaP cells and castration-resistant VSC2 cells with the AKR1C3 substrate A-dione leads to a robust PSA expression, which can be blocked by an AKR1C3 inhibitor, indomethacin.41 In addition to the findings summarized above, results from murine xenograft models demonstrating elevation of AKR1C3 expression in androgen-depleted conditions41 suggest that AKR1C3 is one of the drivers of steroidogenesis in CRPC. Thus, it is plausible to suggest that selective inhibitors for AKR1C3 or HSD17B6 may be effective in management of CRPC, either used alone or in combination with other compounds targeting different steps of androgen biosynthesis or AR action.

In addition to intratumoral androgen production, several other mechanisms are likely to contribute to the persistent AR signaling in CRPC.8 These include, for example, i) genomic amplification and overexpression of AR,42 ii) gain-of-function mutations allowing AR to be activated by promiscuous ligands such as steroids or antiandrogens,43 and iii) AR splice variants encoding for ligand-binding domain–deficient receptors that are constitutively active but may be dependent and use a full-length receptor for functionality.44–46 Consistent with published results, we demonstrated that VCaP xenograft expression of the full-length androgen receptor is markedly up-regulated at 1 day after castration and is further increased in established castration-resistant tumors. Castration also markedly induced expression of AR-V1 and AR-V7, which lack the ligand-binding domain, compared with the level detected in VCaP tumors grown in the intact mice. Hu et al.47 reported 20-fold up-regulation of AR-V1 and AR-V7 in clinical CRPC samples, which is similar to our results with VCaP xenografts.

Expression of these AR variants increases in CRPC bone metastases, compared with hormone-naïve bone metastases, and is associated with a poor prognosis.48 Furthermore, it has been suggested that AR-V7 can regulate a unique set of genes that are not regulated by full-length AR.49 Using microarrays and RT-qPCR, we found that expression of the selected AR target genes in castration-resistant VCaP tumors (with marked up-regulation of AR variants) did not differ significantly from expression in VCaP tumors grown in intact mice. These findings suggest that differential expression of the AR splice variants does not affect the known AR-regulated genes. The increased relative expression of the full-length AR and the AR splice variants did not alter tumor growth properties in terms of serum PSA, tumor volume, invasiveness, and proliferation and apoptosis rates. The significance of the high expression of AR splice variants in castration-resistant VCaP xenografts thus warrants further studies.

We tested two AR antagonists, MDV3100 and ARN-509, to verify the androgen dependency of our CRPC model. MDV3100 blocks binding of the endogenous ligand and, after binding to the receptor, promotes AR degradation, whereas the high-affinity binding of ARN-509 to AR particularly inhibits the nuclear import and DNA-binding of AR.50 In phase I/II clinical trials with ARN-509, 43 of 47 patients with nonmetastatic PCa exhibited a PSA response to the drug, suggesting possible clinical applications.51 With the present study, we have demonstrated that both compounds significantly suppress PSA production of castration-resistant VCaP tumors.

In conclusion, the orthotopic VCaP xenograft model presented here exhibits the three key properties of clinical CRPC: i) up-regulation of full-length AR and AR splice variants; ii) activation of intratumoral steroidogenesis (as demonstrated by direct measurement of intratumoral concentrations of androgens by LC-MS/MS), and iii) increased expression of steroidogenic enzymes associated with intratumoral androgen production in clinical specimens, such as AKR1C3 and HSD17B6. The present findings demonstrate that this model is suitable for testing therapeutic strategies targeting AR signaling in CRPC.

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Supplemental Data

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