Increased Expression of Genes Converting Adrenal Androgens to Testosterone in Androgen-Independent Prostate Cancer

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Abstract

Androgen receptor (AR) plays a central role in prostate cancer, and most patients respond to androgen deprivation therapies, but they invariably relapse with a more aggressive prostate cancer that has been termed hormone refractory or androgen independent. To identify proteins that mediate this tumor progression, gene expression in 33 androgen-independent prostate cancer bone marrow metastases versus 22 laser capture–microdissected primary prostate cancers was compared using Affymetrix oligonucleotide microarrays. Multiple genes associated with aggressive behavior were increased in the androgen-independent metastatic tumors (*MMP9*, *CKS2, LRRC15, WNT5A, EZH2, E2F3, SDC1, SKP2*, and *BIRC5*), whereas a candidate tumor suppressor gene (*KLF6*) was decreased. Consistent with castrate androgen levels, androgen-regulated genes were reduced 2- to 3-fold in the androgen-independent tumors. Nonetheless, they were still major transcripts in these tumors, indicating that there was partial reactivation of AR transcriptional activity. This was associated with increased expression of *AR* (5.8-fold) and multiple genes mediating androgen metabolism (*HSD3B2, AKR1C3, SRD5A1, AKR1C2, AKR1C1*, and *UGT2B15*). The increase in aldo-keto reductase family 1, member C3 (*AKR1C3*), the prostatic enzyme that reduces adrenal androstenedione to testosterone, was confirmed by real-time reverse transcription-PCR and immunohistochemistry. These results indicate that enhanced intracellular conversion of adrenal androgens to testosterone and dihydrotestosterone is a mechanism by which prostate cancer cells adapt to androgen deprivation and suggest new therapeutic targets. (Cancer Res 2006; 66(5): 2815-25)

Introduction

Prostate cancer is the most common noncutaneous malignancy in men and is a leading source of cancer morbidity and mortality. Prostate cancer screening using serum prostate-specific antigen (PSA) has led to increased detection of early-stage prostate cancer that can be cured by radical prostatectomy or radiation therapy. Nonetheless, many patients still present with advanced disease, and a substantial fraction of patients who present with clinically localized prostate cancer and undergo primary therapy with curative intent will eventually recur with metastatic disease. The androgen receptor (AR) plays a central role in prostate cancer development, and androgen deprivation therapy is still the standard systemic treatment for metastatic prostate cancer (1). The majority of patients treated with androgen deprivation therapies, which suppress testicular androgen production [surgical castration or administration of luteinizing hormone–releasing hormone (LHRH) agonists] or block AR directly by treatment with AR antagonists, show clinical improvement and have decreases in serum PSA levels. Unfortunately, these patients invariably relapse with a more aggressive form of prostate cancer that has been termed hormone-refractory or androgen-independent prostate cancer.

Significantly, the AR is expressed at high levels in most cases of androgen-independent prostate cancer, with the *AR* gene being amplified in about one third of cases (2–5). Moreover, these androgen-independent prostate cancer resume their expression of multiple AR-regulated genes (such as *PSA*), indicating that AR transcriptional activity becomes reactivated at this stage of the disease (5–8). Studies using prostate cancer cell lines and xenografts similarly show that progression to androgen-independent prostate cancer is associated with increased levels of AR and resumed expression of androgen-regulated genes, and that AR down-regulation at this stage by small interfering RNA (siRNA) or other methods can suppress tumor growth (9–11).

In addition to increased AR expression, *AR* mutations in androgen-independent prostate cancer can enhance AR activation by weak androgens, other steroid hormones, or drugs (12, 13). Although the overall frequency of *AR* mutations in androgen-independent prostate cancer is low, mutant ARs that are stimulated by the AR antagonist flutamide are more frequent in patients treated long term with this drug, and these patients have increased responses to another AR antagonist (bicalutamide), indicating that there is positive selection for alterations that can enhance AR activity (12, 14). Further mechanisms that may stimulate AR transcriptional activity in androgen-independent prostate cancer include increased expression of transcriptional coactivator proteins and activation of signal transduction pathways that can enhance AR responses to low levels of circulating androgens, including the Ras/Raf/mitogen-activated protein kinase pathway, protein kinase A, and phosphatidylinositol 3-kinase (15, 16). Finally, direct measurements of intraprostatic androgens in castrated men with androgen-independent prostate cancer have shown that levels are not significantly reduced compared with normal prostate, indicating that increased testosterone uptake or synthesis may be a mechanism for reactivation of AR activity in androgen-independent prostate cancer (8, 17, 18).

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Several groups have used cDNA or oligonucleotide microarrays to elucidate molecular features of primary prostate cancer associated with metastatic potential, as patients with these tumors may benefit from adjuvant therapies, whereas others may be treated effectively with more conservative therapies (19–25). Comparable gene expression studies to identify mechanisms that mediate progression to androgen-independent prostate cancer have been more limited and used smaller numbers of tumors, largely due to difficulties in obtaining appropriate frozen androgen-independent prostate cancer samples (5, 26–28). In this study, we used Affymetrix oligonucleotide microarrays to examine gene expression in 33 metastatic androgen-independent prostate cancer samples derived from bone marrow biopsies, the predominant site for metastatic prostate cancer. These were compared with a group of 22 laser capture–microdissected (LCM) primary prostate cancer samples to identify genes that may contribute to metastatic behavior or androgen independence. This analysis identified a series of genes characterized previously as candidate biomarkers of more aggressive prostate cancer and additional genes that may contribute to metastatic growth. With respect to AR signaling, the data showed marked increases in *AR* message levels in the androgen-independent prostate cancer samples and increases in multiple genes involved in androgen metabolism, including aldo-keto reductase family 1, member C3 (*AKR1C3*, also called 17β-hydroxysteroid dehydrogenase type 5, *17βHSD5*), the prostatic enzyme mediating conversion of adrenal androstenedione to testosterone (29–33). The increased expression of *AKR1C3* in androgen-independent prostate cancer, in conjunction with increases in other enzymes mediating androgen synthesis and catabolism, indicates that enhanced intracellular production of testosterone and dihydrotestosterone from adrenal androgens is a mechanism for prostate cancer progression to androgen independence.

Materials and Methods

Tissue collection and LCM. Approximately 120 snap-frozen bone marrow biopsies from patients with androgen-independent prostate cancer were collected as a source of material for molecular studies addressing mechanisms of prostate cancer progression to androgen independence, including AR mutations and profiling of expressed kinases, as described previously (12). Frozen sections from these biopsies were carefully examined microscopically, which identified 33 independent biopsies (from 30 patients) that were largely replaced by tumor and had minimal residual normal bone marrow elements. To control for genes expressed at very high levels by residual bone marrow cells, four additional bone marrow biopsies from patients with androgen-independent prostate cancer that did not contain tumor were similarly analyzed. Total RNA was extracted from four to six adjacent 6-μm frozen sections using 1 mL of Trizol. High levels of *PSA* expression, as assessed by real-time reverse transcription-PCR (RT-PCR) amplification, confirmed the presence of androgen-independent prostate cancer in the samples (data not shown).

Primary prostate cancer was isolated by LCM from frozen biopsies or radical prostatectomies in hormone-naive patients. Frozen sections (8 μm) were stained with H&E and air-dried. LCM was done on sections using a PixCell II LCM System (Arcturus Engineering, Mountain View, CA), using about 4,000 laser pulses of 15-μm diameter and 25-mW pulse power to collect malignant epithelial cells. For biopsy samples with scant tumor tissue, multiple sections were used to reach a total of 4,000 laser pulses. Immediately following LCM, captured tissue was dissolved in lysis buffer from the Absolutely RNA Nanoprep kit (Stratagene, La Jolla, CA) and frozen at −20°C until RNA isolation.

RNA amplification and probe generation. Total RNA for the LCM samples was eluted into a final volume of 10 μL and used in its entirety for RNA amplification. For RNA from the above androgen-independent prostate cancer samples, 50 ng were suspended in a total volume of 10 μL for RNA amplification and probe generation in conjunction with the RNA from the primary tumors. Two serial rounds of double-stranded cDNA synthesis and *in vitro* transcription were carried out to obtain sufficient cRNA for microarray analysis. Briefly, first-strand cDNA was synthesized using a T7-(dT_{24}) primer and SuperScript III reverse transcriptase (Invitrogen, San Diego, CA). After second-strand cDNA synthesis, the double-stranded cDNA was phenol-chloroform extracted and subjected to *in vitro* transcription using a commercial kit (Ambion, Austin, TX). The resultant cRNA was RNAeasy column purified (Qiagen, Valencia, CA), and 600 ng of cRNA in 10 μL DEPC water were carried into the second round for further amplification.

For the second round of *in vitro* transcription amplification, the single-stranded cDNA was primed with random hexamers, whereas the double-stranded cDNA synthesis was primed with the T7-(dT_{24}) primer. The double-stranded cDNA was extracted as described above and subjected to *in vitro* transcription with the addition of biotinylated CTP and UTP in a 1:4 proportion to nonbiotinylated CTP and UTP. The cleanup of the labeled cRNA was done with RNeasy Mini Columns and eluted with 50 μL DEPC–treated water. RNA was quantified by spectrophotometer, and the quality was assessed by running a 1% denaturing agarose gel. After fragmentation, cRNA target from the androgen-independent prostate cancer and primary prostate cancer samples were hybridized to Affymetrix 133A oligonucleotide microarrays, with the primary and androgen-independent prostate cancer samples being analyzed at the same time using the same lot of chips.

Statistical analysis of expression data. Expression values were derived by probe set level analysis from the raw CEL files with R statistical software (R Development Core Team, 2004. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-00-3)[7] and BioConductor packages and libraries (34). Background correction, normalization, and expression value calculation of perfect match probe intensities was done with the RMA function of the BioConductor package "Affy." Chip-wide median signal intensities of the raw data ranged from 95 to 250, whereas the normalized median intensities of the 59 chips were close to 50 (interquartile range, 48.5-50.5; min, 47.0; max, 54.8). A comparison of these data with those produced by MAS5 (Affymetrix, Inc., Santa Clara, CA) and dChip (PM and PM-MM models) did not reveal great differences in the fold change ranking of the most highly expressed genes, but there was substantial variability in the expression values and statistical significance of weakly expressed genes. The PM-only models were less noisy at low signal intensities. A parallel analysis of this data set using dChip and dChip-generated expression values (PM-only and PM-MM models) gave very similar results.

Expression values were threshholded at the mean + 2 SDs to make the fold change of gene expression more robust against the influence of outliers. No further prefiltering for variance, expression level, or presence/absence call was used in the R-based analysis. The complete microarray data set consisted of these 59 samples and 22,283 probe sets on the microarray. A 22,066-member gene subset was formed by removing the control probes and 150 genes specifically expressed at high levels in the normal bone marrow samples (principally globins), which might otherwise contribute to the androgen-independent prostate cancer samples with even very low levels of normal marrow contamination. This subset was further reduced to 2,336 probe sets by removing genes that had minimal variability in the data set. Of these 2,336 probe sets, the majority (1,862 probe sets) were selecting based on high variability of expression, with SD/mean expression (coefficient of variance) >0.6. Also included were an additional 472 probe sets with a coefficient of variance between 0.3 and 0.6 and a fold change difference in the ratio of mean expression in androgen-independent prostate cancer versus primary tumors of >1.6. This second criterion was empirically found to be a concise means of identifying probe sets with extremely low t test adjusted Ps (third quartile Ps < 2.5×10^{-6}; maximum, 0.59×10^{-3}) and modest fold change ratios.

[7] http://www.R-project.org.

Statistically significant differences in gene expression/probe intensity between the local tumors and the androgen-independent prostate cancer bone marrow metastases were determined by calculation of Welch's t statistic for all probes in the array. Ps derived from the t statistics were adjusted with the Benjamini and Hochberg false discovery rate method as implemented in the BioConductor multitest package. Probe sets with adjusted Ps < 0.05 and a fold change in expression >1.8-fold were deemed significant.

A dendrogram of the gene expression results from the primary and metastatic androgen-independent prostate cancer tumors was prepared using divisive clustering with a Euclidean distance metric (*Diana* algorithm; ref. 35). The PAM algorithm was used to determine which genes were the most predictive classifiers of primary versus metastatic androgen-independent prostate cancer (36). The PAM analysis was done by randomly splitting the data set into training (41 samples) and test groups (14 samples), running the training and prediction programs, and collecting approximately the same number of predictive genes per run by adjusting the threshold within the zero prediction error range. In typical training runs, the number of predictive genes could be reduced to 2 to 3 before classification errors occurred. An estimate of the relative predictive power of an extended range of genes was produced by tabulating the results of 100 trials, randomly splitting the data set for each trial, and adjusting the threshold to yield 10 genes.

Real-time RT-PCR. Quantitative real-time RT-PCR amplifications were done on unamplified RNA extracted from additional metastatic androgen-independent prostate cancer samples and from primary prostate cancer samples containing high volumes of tumor, which were not LCM purified. The primers and probes (Biosource International, Camarillo, CA) were as follows: AKR1C3 forward, GAGAAGTAAAGCTTTGGAGGTCACA; AKR1C3 reverse, CAACCTGCTCCTCATTATTGTATAAATGA; AKR1C3 probe, FAM-ACTTATATGGCGGAACCCAGCTTCTATT-TAMRA; cyclin-dependent kinase subunit 2 (CKS2) forward, TCTTCGCGCTCTCGTTTCA; CKS2 reverse, AGATCTGCTTGTGGGCCATC; CKS2 probe, FAM-TTTCTGCAGCGCGC-CACGA-TAMRA; leucine-rich repeat containing 15 (LRRC15) forward, CGTAATCTGCGTTGTTGGGA; LRRC15 reverse, TCTCTGAACCACAGC-CATGG; LRRC15 probe, FAM-CCAGCAGTGGCCTTGGGAAGGAA-TAMRA; AKR1C2 forward, CCTAAAAGTAAAGCTCTAGAGGCCGT; AKR1C2 reverse, GAAAATGAATAAGATAGAGGTCAACATAG; AKR1C2 probe, FAM-CCGTTATCTTCGGCCCAAGGGT. Each reaction used 50 ng of RNA and was normalized by coamplification of 18S RNA. Reactions were carried out on an ABI Prism 7700 Sequence Detection System using Taqman Gold RT-PCR reagents (PE Applied Biosystems, Foster City, CA).

Immunohistochemistry. Tissue microarrays containing primary prostate cancer and metastatic androgen-independent prostate cancer from warm autopsies were prepared as described, and additional androgen-independent prostate cancer samples were obtained from transurethral prostate resections in patients with recurrent prostate cancer and bladder obstruction (26). AKR1C3 was detected using the mouse monoclonal anti-AKR1C3 antibody NP6-G6.A6, which does not cross-react with other AKR1C family members (33). For antigen retrieval, sections were microwaved for 20 minutes in 10 mmol/L citric acid (pH 6). The specific and control antibodies (nonimmune mouse IgG) were used at final concentrations of 5 μg/mL overnight at 4°C followed by a biotinylated anti-mouse secondary, horseradish peroxidase–conjugated streptavidin, and 3,3′-diaminobenzidine substrate.

Results

Primary and metastatic androgen-independent prostate cancer are distinguished by expression of AR-regulated genes. Affymetrix U133A oligonucleotide microarrays were used to assess gene expression in metastatic androgen-independent prostate cancer samples from bone marrow biopsies, as bone marrow represents the major site for metastatic prostate cancer. To minimize the contribution of RNA from erythroid and myeloid elements in normal bone marrow, we selected 33 samples (from >120 bone marrow biopsies) based on high tumor content and lack of normal erythroid and myeloid cells. A parallel analysis of bone marrow biopsies that did not contain tumor was also used to identify and exclude genes that were highly expressed in normal bone marrow. The metastatic androgen-independent prostate cancer samples were compared with 22 primary androgen-dependent prostate cancer samples, which were enriched for tumor cells by LCM.

Significantly, unsupervised clustering of the Affymetrix expression data (*Diana* algorithm) clearly distinguished the primary and metastatic androgen-independent prostate cancer (Fig. 1), and most of the predictive genes were expressed at higher levels in the primary tumors (Table 1; refs. 35, 36). Moreover, four of the top five predictive genes were androgen regulated (*ACPP, KLK3, KLK2*, and *NKX3.1*), and additional strongly androgen-regulated genes were also among the top 100 predictive genes (*PART1, SARG*, and *MSMB*). These findings indicate that decreased AR transcriptional activity (~2- to 3-fold) is the major single feature distinguishing the metastatic androgen-independent prostate cancer samples from the primary tumors, which is certainly consistent with the castrate androgen levels in the androgen-independent prostate cancer patients. However, whereas the levels of these AR-regulated genes were decreased relative to the primary tumors, they remained as major transcripts in metastatic androgen-independent prostate cancer. For example, the mean expression of *PSA* in the metastatic androgen-independent prostate cancer tumors was in the top 1% of all genes among these tumors. Taken together, these results indicated that the progression to androgen-independent

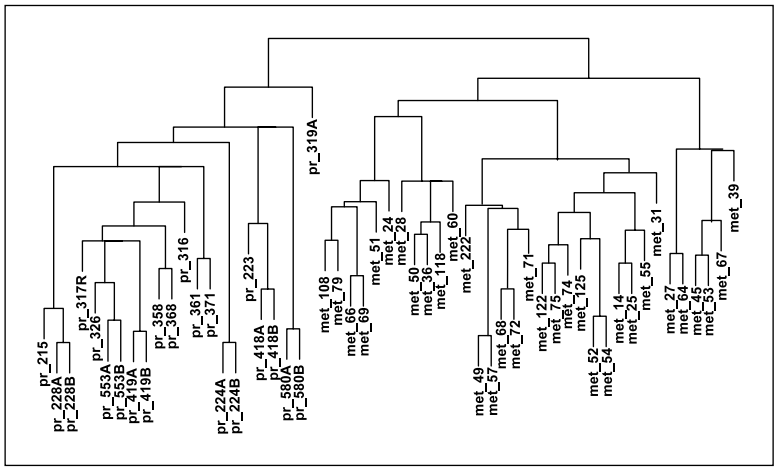

Figure 1. Dendrogram showing clustering of primary and metastatic androgen-independent prostate cancer samples. Affymetrix expression data were used to cluster primary prostate cancer (*pr*) and androgen-independent prostate cancer bone marrow metastases (*met*) using Diana algorithm.

prostate cancer entailed partial reactivation of AR transcriptional functions (despite castrate androgen levels), but not to the degree observed in primary androgen-dependent tumors with normal androgen levels.

The most highly predictive genes with increased expression in metastatic androgen-independent prostate cancer were *COL1A2* and *SET*. Importantly, expression of *COL1A2* in the metastatic androgen-independent tumors was not significantly higher than in normal bone marrow, indicating that the *COL1A2* transcripts may have been derived from normal bone stromal cells (data not shown). However, tumor cells can induce increased expression of *COL1A2* in their associated stromal cells, and this induced *COL1A2* expression has been identified as part of a molecular signature of metastatic tumors that could be identified in the corresponding primary tumors, including prostate cancer (21, 24, 25). Therefore, the positive correlation of *COL1A2* with metastatic androgen-independent prostate cancer likely reflects expression by tumor-associated stromal cells, in conjunction with the efficient depletion by LCM of stromal cells in the primary tumors. *SET* functions as an inhibitor of protein phosphatase 2A, but its potential role in prostate cancer is not yet unclear. It should be noted that some of the genes with increased expression in the androgen-independent tumors could reflect release from androgen repression, although there are no data indicating that *COL1A2, SET*, or other particular genes identified below as increased in the androgen-independent tumors are androgen repressed.

Genes differentially expressed in primary versus androgen-independent prostate cancer. The above analysis was focused on genes whose expression could most accurately classify the primary versus metastatic androgen-independent tumors, but did not identify genes that clearly contributed to metastatic or androgen-independent growth. Therefore, we next analyzed the data to identify genes expressed at significantly different levels in the two sets and ordered them based on fold difference. Genes whose expression was significantly higher ($P < 0.05$ after multiple testing adjustment) in the primary versus the metastatic tumors are shown in Table 2, whereas genes with higher expression in metastatic androgen-independent prostate cancer are shown in Table 3.

Many of the genes expressed at higher levels in the primary tumors encode smooth muscle proteins (Table 2). As the prostate stroma is comprised primarily of smooth muscle cells (myoepithelial cells), these smooth muscle transcripts were likely derived from small numbers of prostatic myoepithelial cells that contaminated the microdissected epithelium. As noted above, other genes with increased expression in the primary tumors are androgen regulated (*MSMB, PART1, ACPP, KLK3, KLK2, SARG, RAB3B*, and *NKX3.1*). Significantly, the higher expression of *KLF6* (2.2-fold) in the primary versus the metastatic androgen-independent tumors is consistent with this gene functioning as a tumor suppressor gene in prostate cancer and suggests that further loss of *KLF6* contributes to tumor progression (37).

Many of the genes most highly overexpressed in the metastatic androgen-independent prostate cancer tumors encoded collagens and other extracellular matrix proteins, which were potentially derived from normal bone (Table 3). Therefore, a further analysis was done to determine whether these extracellular matrix proteins were expressed at higher levels in the bone marrow metastases versus the normal bone marrow samples. Among the collagens, most were expressed at significantly higher levels in the metastatic prostate cancer samples (*COL11A1, COL5A2, COL3A1, COL6A3*, and

Table 1. Prediction analysis for microarrays of top 20 genes predictive of primary versus metastatic androgen-independent prostate cancer

Symbol	Frequency	Primary	AIPCa	Mean	Fold	Gene description
ACPP	100	69.30	−45.70	7,659	3.39	Acid phosphatase, prostate
KLK3	100	35.08	−23.15	4,642	2.85	Kallikrein 3, (prostate-specific antigen)
KRT18	98	17.42	−11.43	3,695	2.41	Keratin 18
KLK2	97	18.76	−12.49	1,657	2.69	Kallikrein 2, prostatic
NKX3.1	88	11.94	−7.97	5,583	2.08	NK3 transcription factor–related, l
RPL11	85	7.59	−5.25	2,896	1.71	Ribosomal protein L11
MYLK	80	10.93	−6.75	1,371	6.12	Myosin, light polypeptide kinase
MYH11	77	10.46	−6.45	1,781	10.1	Myosin, heavy polypeptide 11, smooth muscle
TAOK3	55	2.56	−1.57	842	3.92	TAO kinase 3
RDH11	40	3.05	−1.93	2,690	2.85	Retinol dehydrogenase 11
COL1A2	37	−2.68	2.01	6,611	9.03	Collagen, type I, α 2
CSRP1	27	2.24	−1.29	727	4.11	Cysteine and glycine-rich protein 1
SET	25	−1.10	0.87	392	1.55	SET translocation (myeloid leukemia associated)
PAFAH1B1	22	1.73	−1.05	2,597	2.75	Platelet-activating factor acetylhydrolase
TACSTD2	22	1.61	−1.13	23	1.2	Tumor-associated calcium signal transducer
ACTA2	22	1.12	−0.69	1,519	3.54	Actin, α 2, smooth muscle
DDAH1	17	1.76	−1.36	1,280	2.19	Dimethylarginine dimethylaminohydrolase 1
ALDH1A3	17	0.67	−0.43	1,526	3.64	Aldehyde dehydrogenase 1 family, member A3
SORD	15	0.85	−0.59	1,655	3.68	Sorbitol dehydrogenase
PART1	14	0.65	−0.045	423	3.62	Prostate androgen–regulated transcript 1

NOTE: For each gene is shown predictive power (frequency), sum of the shrunken centroids for primary tumors (primary) and androgen-independent prostate cancer metastatic tumors, mean expression (mean), and fold increased expression (fold) in primaries for all except COL1A2 and SET, which are mean expression and fold increase in the androgen-independent prostate cancer tumors.
Abbreviation: AIPCa, androgen-independent prostate cancer.

Table 2. Genes expressed at higher levels in primary prostate cancer

Symbol	Fold	Gene description
ACTG2	18.8	Actin, γ 2, smooth muscle
PCP4	11.1	Purkinje cell protein 4
MYH11	10.1	Myosin, heavy polypeptide 11, smooth muscle
MSMB	9.94	Microseminoprotein, β
MYLK	6.12	Myosin, light polypeptide kinase
TPM2	5.70	Tropomyosin 2, β
DHRS7	5.09	Dehydrogenase/reductase, SDR family
RPS11	4.81	Ribosomal protein S11
CALR	4.56	Calreticulin
ANXA3	4.39	Annexin A3
MEIS2	4.24	Meis1, myeloid ecotropic viral integration site
CNN1	4.17	Calponin 1, basic, smooth muscle
TAGLN	4.14	Transgelin
EGR1	4.12	Early growth response 1
CSRP1	4.11	Cysteine and glycine-rich protein 1
DMN	4.09	Desmuslin
ABAT	3.97	4-Aminobutyrate aminotransferase
TAOK3	3.92	TAO kinase 3
CUTL2	3.86	Cut-like 2 (*Drosophila*)
CYR61	3.84	Cysteine-rich, angiogenic inducer 61
ChGn	3.70	Chondroitin β1,4 *N*-acetylgalactosaminyltransferase
SORD	3.68	Sorbitol dehydrogenase
ALDH1A3	3.64	Aldehyde dehydrogenase 1 family, member A3
PART1	3.62	Prostate androgen–regulated transcript
PTPRN2	3.58	Protein tyrosine phosphatase, receptor type
GSTT1	3.57	Glutathione *S*-transferase theta 1
ACTA2	3.54	Actin, α 2, smooth muscle, aorta
GMPR	3.49	Guanosine monophosphate reductase
ZFP36	3.45	Zinc finger protein 36
ASAHL	3.41	*N*-acylsphingosine amidohydrolase
ACPP	3.39	Acid phosphatase
GHR	3.36	Growth hormone receptor
EPHX2	3.27	Epoxide hyrdrolase 2, cytoplasmic
ABCC4	3.23	ATP-binding cassette, subfamily C member 4
DHRS7	3.23	Dehydrogenase/reductase (SDR family) member 7
RPL37A	2.96	Ribosomal protein L37a
DPP4	2.93	Dipeptidylpeptidase 4 (CD26)
SFRP1	2.91	Secreted frizzled-related protein 1
MATN2	2.91	Matrillin 2
LAMP2	2.89	Lysosomal-associated membrane protein 2
PDE4B	2.85	Phosphodiesterase 4B
KLK3	2.85	Kallikrein 3, (prostate-specific antigen)
RDH11	2.85	Retinol dehydrogenase 11
RPL27	2.76	Ribosomal protein L27
PAFAH1B	2.75	Platelet-activating factor acetylhydrolase, isoform lb
CYP39A1	2.75	Cytochrome P450, family 39, subfamily A, polypetide 1
DMXL1	2.73	Dmx-like 1
HGD	2.71	Homogentisate 1,2-dioxygenase
C1orf24	2.71	Chromosome 1 open reading frame 24
KLK2	2.69	Kallikrein 2, prostatic
CPE	2.67	Carboxypeptidase E
FLJ14146	2.66	Hypothetical protein FLJ14146
TSPAN1	2.62	Tetraspan 1
GOLPH2	2.6	Golgi phosphoprotein 2
KIF5C	2.59	Kinesin family member 5C
TIPARP	2.59	TCDD-inducible poly(ADP-ribose) polymerase
PDZRN3	2.57	PDZ domain containing RING finger 3
PSCD2	2.55	Pleckstrin homology, Sec 7 and coiled-coil domains 2
CTBS	2.55	Chitobiase
SARG	2.54	Specifically androgen-regulated protein
ALDH6A	2.54	Aldehyde dehydrogenase 6 family, member A1
TRPM8	2.5	Transient receptor potential cation channel
LMOD1	2.49	Leimodin 1 (smooth muscle)
RPL27A	2.49	Ribosomal protein L27a
TMSNB	2.48	Thymosin, β
MT1F	2.47	Metallothionein 1F
GRP58	2.43	Glucose-regulated protein, 58 kDa
RAB3B	2.42	RAB3B, member RAS oncogene family
KRT18	2.41	Keratin 18
SPOCK	2.41	Sparc/osteonectin, cwcv and kazal-like domains
HERPUD	2.37	Homocysteine, endoplasmic reticulum stress-inducible
STAG2	2.34	Stromal antigen 2
ANKRD1	2.34	Ankyrin repeat domain 15
IQGAP2	2.33	IQ motif containing GTPase activating protein 2
PLN	2.32	Phospholamban
SCP2	2.32	Sterol carrier protein 2
CIRBP	2.31	Cold inducible RNA binding protein
TNS	2.3	Tensin
EIF5A	2.28	Eukaryotic translation initiation factor 5A
ALDH3A2	2.27	Aldehyde dehydrogenase 3 family, member A2
C6orf29	2.25	Chromosome 6 open reading frame 2
TMEM30B	2.25	Transmembrane protein 30B
ARGBP2	2.23	Arg/Abl-interacting protein
QDPR	2.23	Quinoid dihydropteridine reductase
PTGDS	2.23	Prostaglandin D2 synthase 21kDa
KLF6	2.2	Kruppel-like factor 6

COL4A1), indicating that they were derived from tumor or tumor-induced stromal cells. Most of the other extracellular matrix proteins were also significantly increased in the metastatic prostate cancer samples compared with normal bone, with the exceptions being *SPP1, IBSP, SPARC, CSPG2, FBN1, OMD*, and *LAMA4* (although the increases in *SPP1* and *IBSP* were borderline significant). Interestingly, previous studies have shown that *SPP1* and *IBSP*, whereas normally expressed by osteoblasts, are also expressed by tumor cells and at increased levels by bone cells in bone metastases from prostate as well as breast cancer. Moreover, their increased expression may play a role in tumor progression (38, 39). Taken together, these results indicated that most of the increases in transcripts encoding extracellular matrix proteins likely reflected tumor-induced stroma and new bone formation.

Among the tumor cell–derived transcripts, *AR* expression was increased in the majority of androgen-independent tumors

Table 3. Genes expressed at higher levels in androgen-independent prostate cancer bone marrow metastases

Symbol	Fold	Gene description
SPP1	30.19	Secreted phosphoprotein 1 (osteopontin)
COL11A1	19	Collagen,type XI, α 1
COL1A1	13.68	Collagen, type I, α 1
IBSP	12.48	Bone sialoprotein
COL5A2	9.62	Collagen, type V, α 2
MMP9	9.18	Matrix metalloproteinase 9
COL1A2	9.03	Collagen, type 1, α 2
CTSK	8.39	Cathepsin K
SMA4	7.14	SMA4
CaMKIIN	6.73	Calmodulin-dependent protein kinase I
SPARC	6.25	Osteonectin
POSTN	6.21	Periostin, osteoblast-specific factor
CKS2	6.05	CDC28 protein kinase regulatory subunit 2
WNT5A	6.05	Wingless-type MMTV integration site family
AR	5.84	Androgen receptor
FN1	5.8	Fibronectin 1
CSPG2	5.52	Chondroitin sulfate proteoglycan 2
DPT	5.52	Dermatopontin
COL3A1	5.43	Collagen, type III, α 1
AKR1C3	5.27	Aldo-keto reductase family 1, member C3
NRIP3	4.93	Nuclear receptor-interacting protein 3
LUM	4.84	Lumican
ASPN	4.82	Asporin
LRRC15	4.81	Leucine-rich repeat containing 15
DDIT4	4.65	DNA-damage-inducible transcript 4
COL6A3	4.53	Collagen, type VI, α 3
ENPP2	4.4	Pyrophosphatase/phosphpodiesterase 2
NNMT	4.36	Nicotinamide *N*-methyltransferase
THBS2	4.32	Thrombospondin 2
OLFML2B	4.2	Olfactomedin-like 2B
CMKOR1	3.9	Chemokine orphan receptor 1
SERPINF	3.87	Serine (or cysteine) proteinase inhibitor
APOE	3.79	Apolipoprotein E
KIAA0220	3.78	Phosphatidyinositol 3-kinase–related kinase SMG-1-like
COL4A1	3.73	Collagen, type IV, α 1
SULF1	3.51	Sulfatase 1
UGT2B15	3.45	UDP glycosyltransferase 2, B15
LAMB1	3.44	Laminin, β 1
SERPINE	3.39	Serine (or cysteine) proteinase inhibitor
AKR1C2	3.36	Aldo-keto reductase family 1, member C2
COL5A1	3.31	Collagen, type V, α 1
DCN	3.31	Decorin
TOP2A	3.28	Topoisomerase (DNA) II α 170 kDa
GPNMB	3.27	Glycoprotein (transmembrane) nmb
PLOD2	3.16	Procollagen-lysine, 2-oxogluturate 5-dioxy
AKR1C1	3.14	Aldo-keto reductase family 1, member C1
CDH11	3.11	Cadherin 11, type 2, OB-cadherin
FBN1	2.97	Fibrillin 1
CENPF	2.96	Centromere protein F, 350/400ka
RACGAP	2.95	Rac GTPase activating protein 1
RAB31	2.92	RAB31, member RAS oncogene family
NARG1	2.9	NMDA receptor–regulated 1
HSPA1B	2.89	Heat shock 70 kDa protein 1B
ADAMTS	2.85	A disintegrin-like and metalloprotease
ACTR2	2.85	ARP2 actin-related protein 2 homologue
CDKN3	2.8	Cyclin-dependent kinase inhibitor 3
KPNA2	2.78	Karyopherin α 2 (importin α)
HSPC163	2.76	HSPC163 protein
ZWINT	2.74	ZW10 interactor
HOXC6	2.71	Homeo box C6
PLS3	2.71	Plastin 3 (T isoform)
OMD	2.7	Osteomodulin
LAMA4	2.66	Laminin, α 4
CCNB1	2.63	Cyclin B1
MELK	2.61	Maternal embryonic leucine zipper kinase
KIF20A	2.6	Kinesin family member 20A
EIF2S3	2.58	Eukaryotic translation initiation factor 2
OLFML3	2.57	Olfactomedin-like 2B
SDC2	2.57	Syndecan 2
KIF4A	2.56	Kinesin family member 4A
TNFAIP6	2.56	Tumor necrosis factor α-induced protein6
LAPTM5	2.51	Lysosomal multispanning membrane protein
CFHL1P	2.5	Complement factor H-related 1 pseudogene
MRC1	2.5	Mannose receptor C type 1
PTTG1	2.5	Pituitary tumor-transforming1
MAP4K4	2.49	Mitogen-activated protein kinase kinase kinase kinase 4
RAFTLIN	2.48	Raft-linking protein
HPRT1	2.46	Hypoxanthine phosphoribosylatransferase 1
PHTF2	2.45	Putative homeodomain transcription factor 2
EZH2	2.44	Enhancer of zeste homologue 2
TRA2A	2.44	Transformer-2 α
E2F3	2.37	E2F transcription factor 3
LAMC1	2.37	Laminin, gamma 1
DDX39	2.35	DEAD box polypeptide 39
ATM	2.34	Ataxia telangiectasia mutated
CHN1	2.33	Chimerin 1
IFI30	2.29	IFN, gamma-inducible protein 30
CDC20	2.28	CDC20 cell division cycle 20 homologue
NEK2	2.28	NIMA (never in mitosis)–related kinase 2

(average 5.84-fold increase), with marked increases in a subset of the tumors (Fig. 2*A*). This finding is consistent with other studies showing that AR message and protein are highly expressed in androgen-independent prostate cancer, and that the *AR* gene is amplified in ~30% of cases (2–5). The increased expression of several other genes in Table 3, including *MMP9, WNT5A, EZH2*, and *E2F3*, has also been associated previously with higher grade local prostate cancer, disease recurrence, or metastatic prostate cancer (20, 23, 40, 41). Additional genes previously associated with more aggressive prostate cancer that were increased (~2-fold) in the metastatic androgen-independent prostate cancer samples were *SDC1, SKP2*, and *BIRC5* (42–45). Significantly, expression of *BIRC5/survivin* was also increased in a recent series of 13 androgen-independent prostate cancer samples compared with local prostate cancer (27).

Two of the most highly overexpressed genes in the androgen-independent prostate cancer samples, *CKS2* and *LRRC15*, have been linked to increased proliferation and invasion but have not been examined previously in prostate cancer. Therefore, their expression was further assessed by real-time RT-PCR using unamplified RNA from additional samples, which similarly indicated increased expression in metastatic androgen-independent

prostate cancer (Fig. 2*B*). *CKS2* associates with cyclin-dependent kinases and enhances their interaction with CDC25 phosphatases, which dephosphorylate them at the G_2-M transition (46). *LRRC15* is a cell surface glycoprotein normally expressed only in the invasive cytotrophoblast layer of the placenta. However, it was recently identified as the major transcriptional target of the Wilms tumor 1 (*WT1*; +KTS) isoform and is overexpressed due to a WT1 fusion protein in a rare, highly aggressive tumor type, desmoplastic small round cell tumor (47). Moreover, siRNA-mediated down-regulation of *LRRC15* was shown to block invasion in a breast cancer cell line (47).

Increased expression of genes regulating androgen metabolism in androgen-independent prostate cancer. In addition to increased *AR* expression in androgen-independent prostate cancer, there were increases in a series of genes mediating androgen synthesis (*AKR1C3, SRD5A1*, and *HSD3B2*) and catabolism (*AKR1C2, AKR1C1*, and *UGTB15*; Table 3; Fig. 3). *AKR1C3* (also called *17βHSD5*) has a number of substrates, including progesterone, estrone (converted to 17β-estradiol), and prostaglandin D2, but its role in androgen metabolism in prostate is reduction of androstenedione to testosterone (refs. 29–33, 48; Fig. 3). Type 1 5α-reductase (*SRD5A1*) and type 2 5α-reductase (*SRD5A2*, the major isoform in normal prostate) both convert testosterone to the higher-affinity dihydrotestosterone. Interestingly, levels of *SRD5A2* expression were moderately reduced (~50%) in the androgen-independent prostate cancer tumors, consistent with recent studies showing decreased *SRD5A2* in prostate cancer and a shift from *SRD5A2* to *SRD5A1* (49–51).

3β-Hydroxysteroid dehydrogenase type 2 (*HSD3B2*, also called *hydroxy-delta-5-steroid dehydrogenase* or *3β-hydroxysteroid dehydrogenase* and *steroid delta-isomerase 2*), which is expressed predominantly in adrenal, testis, and ovary, converts DHEA to androstenedione (Fig. 3; ref. 52). Therefore, its increased expression in androgen-independent prostate cancer would increase intracellular androstenedione levels, generating substrate for conversion to testosterone. These observations indicate that up-regulation of *AKR1C3*, in conjunction with *SRD5A1* and *HSD3B2*, is a mechanism for increasing intracellular androgen levels in androgen-independent prostate cancer.

Expression of the *AKR1C3*-related genes, *AKR1C2* and *AKR1C1*, was also increased in the androgen-independent prostate cancers (Table 3). In contrast to *AKR1C3, AKR1C2* reduces dihydrotestosterone to the inactive 5α-androstane-3α,17β-diol (3α-diol), which is subsequently glucuronidated to 5α-androstane-3α,17β-diol glucuronide (3α-diolG) and eliminated into the circulation (Fig. 3; refs. 32, 53, 54). *AKR1C2* can also catalyze the reverse oxidative reaction (3α-diol to dihydrotestosterone), but intracellularly, it seems to function primarily as a dihydrotestosterone reductase. The role of *AKR1C1* in androgen metabolism is similarly to reduce dihydrotestosterone, but to 5α-androstane-3β,17β-diol, a possible endogenous ligand for the estrogen receptor β in prostate (55). Therefore, increased expression of this enzyme may have effects on signaling through other receptors as well as AR. Finally, there was increased expression of UDP glycosyltransferase 2, B15 (*UGT2B15*), which in conjunction with *UGT2B17* mediates glucuronidation of dihydrotestosterone metabolites. Taken together, the increased expression of enzymes mediating dihydrotestosterone synthesis (*AKR1C3, SRD5A1*, and *HSD3B2*) and catabolism (*AKR1C2, AKR1C1*, and *UGT2B15*) indicates that intracellular conversion of weak adrenal androgens to testosterone and dihydrotestosterone is enhanced in androgen-independent prostate cancer cells and may contribute to prostate cancer relapse after androgen deprivation therapy.

Increased *AKR1C2* and *AKR1C3* expression by real-time RT-PCR and AKR1C3 by immunohistochemistry. Real-time RT-PCR using unamplified RNA, and primers shown previously to distinguish between *AKR1C3* and the related *AKR1C1, AKR1C2*, and *AKR1C4*, confirmed that *AKR1C3* message was increased in androgen-independent prostate cancer (Fig. 4*A*; refs. 32, 54). Consistent with the Affymetrix microarray data, there was an average increase of about 4-fold, with approximately one third of the androgen-independent prostate cancer showing particularly high expression levels (Fig. 4*A* and *B*). The increased expression of *AKR1C2* in the androgen-independent tumors was similarly shown by real-time RT-PCR (Fig. 4*C*), with both the RT-PCR and Affymetrix analyses indicating that there was particularly high expression in a subset of the androgen-independent tumors (Fig. 4*C* and *D*).

There was no correlation between increased expression of *AKR1C3* and *AR* in the androgen-independent prostate cancer samples (Fig. 4*E*). Indeed, the three tumors with the highest *AR* message levels had no increases in *AKR1C3* message, suggesting that very high level AR expression and AR gene amplification may be part of a distinct mechanism mediating androgen independence. In contrast, there was a positive correlation between expression of *AKR1C3* and *AKR1C2* in the androgen-independent tumors (Pearson correlation = 0.854), consistent with the increase in *AKR1C2* being in response to higher intracellular testosterone and dihydrotestosterone (Fig. 4*F*).

To determine whether the increased *AKR1C3* message expression was reflected in protein expression by androgen-independent prostate cancer tumor cells, we next assessed AKR1C3 protein by immunohistochemistry using a series of formalin-fixed and paraffin-embedded primary and androgen-independent prostate cancer samples. Previous studies by *in situ* hybridization and immunohistochemistry in nonneoplastic prostate showed AKR1C3 expression in blood vessels, stromal fibroblasts, and basal cells, whereas primary prostate cancer had increased staining in tumor and in endothelial cells (30, 33). We similarly found little or no AKR1C3 expression in nonneoplastic prostate luminal epithelium (Fig. 5*A*) and negative or heterogeneous weak staining in most primary androgen-dependent prostate cancers (Fig. 5*B* and *C*). In contrast, intermediate to strong AKR1C3 staining was observed in androgen-independent prostate cancer samples obtained from

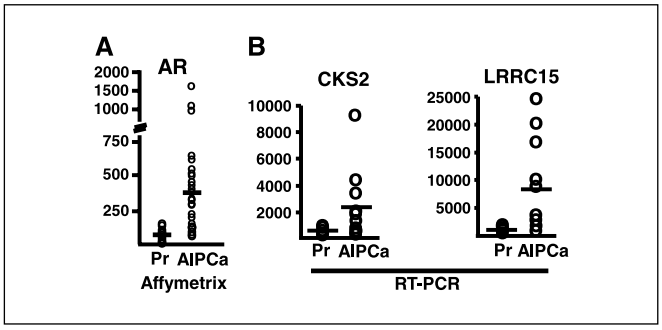

Figure 2. Range of *AR* expression and analyses of *CKS2* and *LRRC15* by quantitative real-time RT-PCR. *A,* range of *AR* expression in primary (*Pr*) and androgen-independent tumors (*AIPCa*) from Affymetrix data. *B, CKS2* and *LRRC15* expression assessed by quantitative real-time RT-PCR using unamplified RNA from 5 primary tumors and 10 androgen-independent prostate cancer bone marrow metastases. Expression levels are in arbitrary units based on the lowest level expression being set at 10. *Bars,* mean expression.

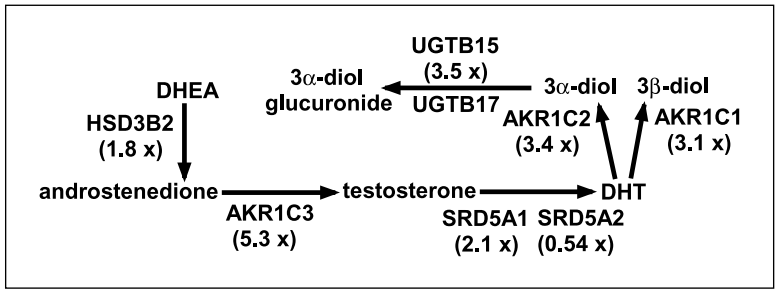

Figure 3. Increased expression of enzymes mediating androgen synthesis and catabolism in androgen-independent prostate cancer. Androgen synthesis (from adrenal DHEA and androstenedione) and catabolism are outlined, and fold increase for each enzyme is indicated. The indicated metabolites are 5α-androstane-3α,17β-diol (*3α-diol*), and 5α-androstane-3β, 17β-diol (*3β-diol*).

bone, prostate, and other sites (Fig. 5*D-J*). Overall, intermediate to strong staining was observed in only 1 of 18 primary androgen-dependent tumors versus 11 of 19 androgen-independent prostate cancer ($P < 0.001$).

Discussion

The majority of prostate cancer patients respond to androgen deprivation therapies, but invariably relapse with more aggressive tumors that have been termed hormone-refractory or androgen-independent prostate cancer. This study identified a series of genes expressed at higher levels in metastatic androgen-independent prostate cancer that have been associated previously with more aggressive behavior (including *WNT5A, CKS2, LRRC15, EZH2, E2F3, SDC1, SKP2*, and *BIRC5*), as well as decreased expression of *KLF6*, a candidate prostate cancer tumor suppressor gene. Consistent with previous data, this study also showed that *AR* expression was increased in androgen-independent prostate cancer, and that there was renewed expression of androgen-regulated genes, indicating that reactivation of AR transcriptional activity was associated with progression to androgen independence. Significantly, *AKR1C3* and other genes mediating testosterone and dihydrotestosterone production and catabolism from adrenal precursors (*HSD3B2, SRD5A1, AKR1C2, AKR1C1*, and *UGT2B15*) were also increased in androgen-independent prostate cancer. *AKR1C3* mediates the intaprostatic synthesis of testosterone from androstenedione and is distinct from the type 3 17β-hydroxysteroid dehydrogenase that catalyzes this reaction in the testes. The increased *AKR1C3* expression in androgen-independent prostate cancer is consistent with (and provides a mechanism for) the substantial levels of androgens found in prostate biopsies from castrated men with androgen-independent prostate cancer compared with normal prostate or nonneoplastic prostate after androgen deprivation therapy (8, 17, 18). More importantly, the increased expression of these genes that mediate androgen metabolism implicates enhanced intracellular conversion of adrenal androgens (DHEA and androstenedione) to testosterone and dihydrotestosterone as a mechanism by which prostate cancer adapts to androgen deprivation therapy.

The role of adrenal androgens in prostate cancer progression to androgen independence has been an important and controversial issue. Early studies using bilateral surgical adrenalectomy or hypophysectomy to ablate adrenal androgen production resulted in pain relief in the majority of patients and objective responses in up to 30%, but there was significant morbidity associated with this therapy, and it was replaced by medical adrenalectomy using aminoglutethimide or ketoconazole (56, 57). Response rates of about 40% have been reported to aminoglutethimide (usually given with hydrocortisone) in men with androgen-independent prostate cancer (58–60). Significantly, this treatment only partially suppresses adrenal androgen production (by about 50%), and responses have been correlated with the level of adrenal suppression (60). Similar results have been obtained using treatment with ketoconazole to suppress adrenal androgen production (61–64).

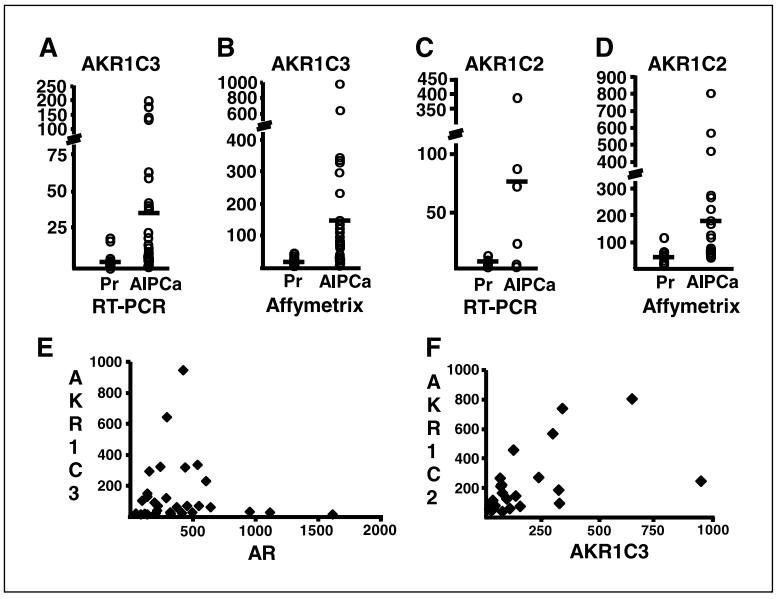

Figure 4. Expression of *AKR1C3* and *AKR1C2* by real-time RT-PCR. *A, AKR1C3* expression assessed by real-time RT-PCR using unamplified RNA from 10 primary tumors (*Pr*) and 32 androgen-independent prostate cancer bone marrow metastases (*AIPCa*). *B,* distribution of *AKR1C3* expression based on Affymetrix data. *C, AKR1C2* expression assessed by real-time RT-PCR using unamplified RNA from 5 primary tumors and 8 androgen-independent prostate cancer bone marrow metastases. *D,* distribution of *AKR1C2* expression based on Affymetrix data. Expression levels are in arbitrary units. *Bars,* mean expression (*A-D*). *E* and *F,* plots of Affymetrix expression data for (*E*) *AR* versus *AKR1C3* and (*F*) *AKR1C3* versus *AKR1C2*.

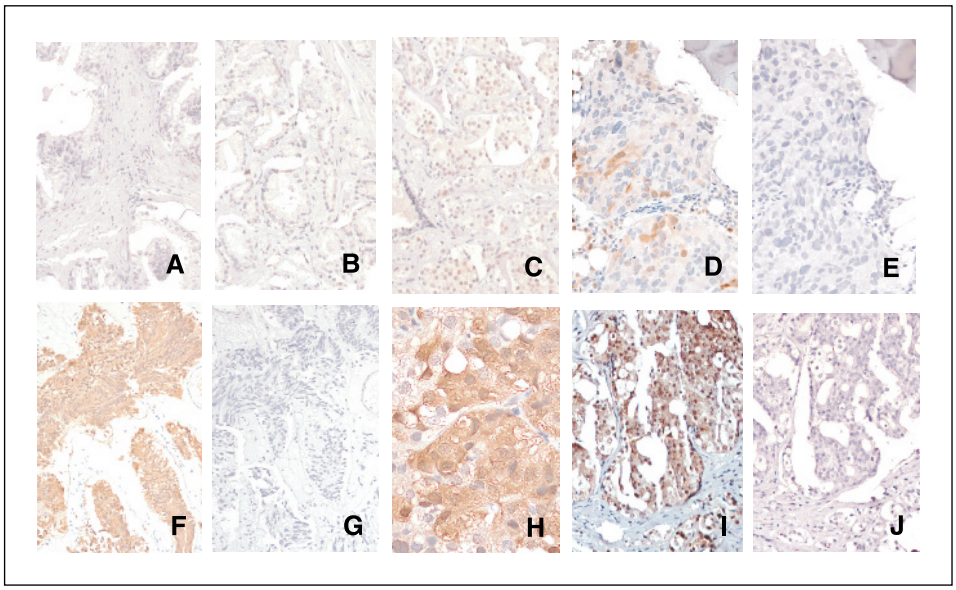

Figure 5. Expression of AKR1C3 protein by immunohistochemistry on paraffin sections. *A,* benign prostate. *B* and *C,* primary prostate cancer. *D* and *E,* androgen-independent prostate cancer bone marrow metastasis. *F* to *H,* recurrent androgen-independent prostate cancer in prostate (*H* is higher power). *I* and *J,* androgen-independent prostate cancer soft tissue metastasis. *A-D, F, H*, and *I,* stained with anti-AKR1C3; *E, G*, and *J,* stained with control antibody.

Taken together, the increased expression of *HSD3B2, AKR1C3*, and *SRD5A1* in androgen-independent prostate cancer, in conjunction with responses to therapies that decrease adrenal androgen production, supports the conclusion that adrenal androgens provide a stimulus for androgen-independent prostate cancer in at least a subset of patients. Nonetheless, data from a series of clinical trials show that the combination of castration (orchiectomy or LHRH agonist) and an AR antagonist to block AR stimulation by residual adrenal androgens results in only a very small improvement in disease-specific survival, despite more rapid responses and lower nadir PSA levels with the combined therapies (65). Importantly, the conclusion that can be drawn from these studies is that available AR antagonists do not have substantial activity against the androgen-independent tumor cells that emerge subsequent to androgen deprivation therapy. Consistent with this conclusion, the majority of prostate cancers that recur after orchiectomy or LHRH agonist monotherapy do not respond to secondary treatments with AR antagonists, including high-dose therapy with the relatively pure AR antagonist bicalutamide (14). Significantly, increased intracellular synthesis of testosterone and dihydrotestosterone may explain the poor response rate to direct AR antagonists versus antagonists of adrenal androgen production, as the direct AR antagonists have much lower affinities for the AR (micromolar range) than testosterone and dihydrotestosterone (high picomolar to low nanomolar range).

The increased expression of *SRD5A1* suggests that enhanced conversion of testosterone to the higher-affinity dihydrotestosterone may also contribute to AR activation in androgen-independent prostate cancer. However, there are no studies showing that 5α-reductase inhibitors are effective in androgen-independent prostate cancer. This may reflect the use of finasteride in most studies, as this inhibitor has more activity towards *SRD5A2*, which is the predominant 5α-reductase in normal prostate. Interestingly, in one reported small phase I study of a dual type 1 and 2 5α-reductase antagonist (LY320236), there were four responses among 15 patients with androgen-independent prostate cancer (66). Moreover, the drug caused a dramatic increase in serum estradiol levels (~10-fold) in these castrated patients, consistent with high levels of intracellular testosterone synthesis and catabolism to estradiol by aromatase in the absence of 5α-reductase activity. Importantly, as this finding illustrates, testosterone reduction by 5α-reducatse not only produces dihydrotestosterone but is also an initial step in testosterone catabolism. Indeed, the increased expression of *AKR1C2, AKR1C1*, and *UGT2B15* in androgen-independent prostate cancer suggests that dihydrotestosterone produced by 5α-reductase may be rapidly catabolized. In this case, 5α-reductase inhibitors may cause an increase in intracellular testosterone, which would render them less effective at suppressing AR activity. Further studies are clearly needed to determine the precise significance of increased *SRD5A1* in androgen-independent prostate cancer.

Previous studies found that expression of *AKR1C2* was decreased in primary prostate cancer relative to normal prostate, and this was proposed as a mechanism for increasing intracellular dihydrotestosterone in primary prostate cancer (53, 54). The current study is not inconsistent with these results, but indicates that increased testosterone synthesis becomes the more dominant mechanism for increasing androgen during the progression to androgen independence, with the increases in catabolic enzymes being part of a coordinated response to process the androgen signal. It should be noted that the extent to which this increase in testosterone synthesis is an early adaptation to androgen withdrawal therapy versus a late event occurring in a subset of tumor cells that triggers androgen-independent growth is not yet clear. Interestingly, a recent study examining selected genes in androgen-independent prostate cancer found increased expression of *HSD17B2*, which functions to catabolize testosterone and estrogen, and increased expression of multiple enzymes that catabolize androgens was observed in primary prostate cancer after neoadjuvant docetaxel treatment (27, 67). Finally, another study that examined three cases of androgen ablation–resistant prostate cancer found increased expression of several enzymes mediating early steps in sterol biosynthesis (5).

In summary, this study indicates that AR transcriptional activity is reactivated in androgen-independent prostate cancer and identifies the intracellular conversion of adrenal androgens to testosterone as a mechanism mediating this reactivation. The

increased intracellular conversion of adrenal androstenedione to testosterone by *AKR1C3* also provides an explanation for the relatively high levels of testosterone in androgen-independent prostate cancer biopsy samples (8, 17, 18). The clinical significance of this mechanism in androgen-independent prostate cancer is supported by responses to therapies that suppresses adrenal androgen production. However, it is not yet clear whether even total abrogation of adrenal androgens can completely suppress AR transcriptional activity in androgen-independent prostate cancer. Moreover, it is certainly unclear whether such complete suppression of AR activity would result in complete or durable clinical responses. These issues need to be addressed through the development of more potent AR antagonists or therapies that more effectively target adrenal androgens and their metabolism.

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References

1. Gelmann EP. Molecular biology of the androgen receptor. J Clin Oncol 2002;20:3001–15.
2. van der Kwast TH, Schalken J, Ruizeveld de Winter JA, et al. Androgen receptors in endocrine-therapy-resistant human prostate cancer. Int J Cancer 1991;48:189–93.
3. Visakorpi T, Hyytinen E, Koivisto P, et al. *In vivo* amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995;9:401–6.
4. Taplin ME, Bubley GJ, Shuster TD, et al. Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 1995;332:1393–8.
5. Holzbeierlein J, Lal P, LaTulippe E, et al. Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. Am J Pathol 2004;164:217–27.
6. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1:34–45.
7. Balk SP. Androgen receptor as a target in androgen-independent prostate cancer. Urology 2002;60:132–8.
8. Mohler JL, Gregory CW, Ford OH III, et al. The androgen axis in recurrent prostate cancer. Clin Cancer Res 2004;10:440–8.
9. Gregory CW, Hamil KG, Kim D, et al. Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. Cancer Res 1998;58:5718–24.
10. Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10:33–9.
11. Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. Cancer Res 2002;62:1008–13.
12. Taplin ME, Bubley GJ, Ko YJ, et al. Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Res 1999;59:2511–5.
13. Taplin ME, Rajeshkumar B, Halabi S, et al. Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. J Clin Oncol 2003;21:2673–8.
14. Joyce R, Fenton MA, Rode P, et al. High dose bicalutamide for androgen independent prostate cancer: effect of prior hormonal therapy. J Urol 1998;159:149–53.
15. Gregory CW, He B, Johnson RT, et al. A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. Cancer Res 2001;61:4315–9.
16. Weber MJ, Gioeli D. Ras signaling in prostate cancer progression. J Cell Biochem 2004;91:13–25.
17. Geller J, Albert JD, Nachtsheim DA, Loza D. Comparison of prostatic cancer tissue dihydrotestosterone levels at the time of relapse following orchiectomy or estrogen therapy. J Urol 1984;132:693–6.
18. Nishiyama T, Hashimoto Y, Takahashi K. The influence of androgen deprivation therapy on dihydrotestosterone levels in the prostatic tissue of patients with prostate cancer. Clin Cancer Res 2004;10:7121–6.
19. Dhanasekaran SM, Barrette TR, Ghosh D, et al. Delineation of prognostic biomarkers in prostate cancer. Nature 2001;412:822–6.
20. Varambally S, Dhanasekaran SM, Zhou M, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 2002;419:624–9.
21. Singh D, Febbo PG, Ross K, et al. Gene expression correlates of clinical prostate cancer behavior. Cancer Cell 2002;1:203–9.
22. LaTulippe E, Satagopan J, Smith A, et al. Comprehensive gene expression analysis of prostate cancer reveals distinct transcriptional programs associated with metastatic disease. Cancer Res 2002;62:4499–506.
23. Glinsky GV, Glinskii AB, Stephenson AJ, Hoffman RM, Gerald WL. Gene expression profiling predicts clinical outcome of prostate cancer. J Clin Invest 2004;113:913–23.
24. Lapointe J, Li C, Higgins JP, et al. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc Natl Acad Sci U S A 2004;101:811–6.
25. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. Nat Genet 2003;33:49–54.
26. Shah RB, Mehra R, Chinnaiyan AM, et al. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. Cancer Res 2004;64:9209–16.
27. Fromont G, Chene L, Vidaud M, et al. Differential expression of 37 selected genes in hormone-refractory prostate cancer using quantitative Taqman real-time RT-PCR. Int J Cancer 2005;114:174–81.
28. Best CJ, Gillespie JW, Yi Y, et al. Molecular alterations in primary prostate cancer after androgen ablation therapy. Clin Cancer Res 2005;11:6823–34.
29. Lin HK, Jez JM, Schlegel BP, Peehl DM, Pachter JA, Penning TM. Expression and characterization of recombinant type 2 3 alpha-hydroxysteroid dehydrogenase (HSD) from human prostate: demonstration of bifunctional 3 alpha/17 beta-HSD activity and cellular distribution. Mol Endocrinol 1997;11:1971–84.
30. El Alfy M, Luu-The V, Huang XF, Berger L, Labrie F, Pelletier G. Localization of type 5 17beta-hydroxysteroid dehydrogenase, 3beta-hydroxysteroid dehydrogenase, and androgen receptor in the human prostate by *in situ* hybridization and immunocytochemistry. Endocrinology 1999;140:1481–91.
31. Dufort I, Rheault P, Huang XF, Soucy P, Luu-The V. Characteristics of a highly labile human type 5 17beta-hydroxysteroid dehydrogenase. Endocrinology 1999;140:568–74.
32. Penning TM, Burczynski ME, Jez JM, et al. Human 3alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1–1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. Biochem J 2000;351:67–77.
33. Lin HK, Steckelbroeck S, Fung KM, Jones AN, Penning TM. Characterization of a monoclonal antibody for human aldo-keto reductase AKR1C3 (type 2 3alpha-hydroxysteroid dehydrogenase/type 5 17beta-hydroxysteroid dehydrogenase); immunohistochemical detection in breast and prostate. Steroids 2004;69:795–801.
34. Dudoit S, Gentleman RC, Quackenbush J. Open source software for the analysis of microarray data. Biotechniques 2003;Suppl:45–51.
35. Datta S, Datta S. Comparisons and validation of statistical clustering techniques for microarray gene expression data. Bioinformatics 2003;19:459–66.
36. Tibshirani R, Hastie T, Narasimhan B, Chu G. Diagnosis of multiple cancer types by shrunken centroids of gene expression. Proc Natl Acad Sci U S A 2002;99:6567–72.
37. Narla G, Heath KE, Reeves HL, et al. KLF6, a candidate tumor suppressor gene mutated in prostate cancer. Science 2001;294:2563–6.
38. Thalmann GN, Sikes RA, Devoll RE, et al. Osteopontin: possible role in prostate cancer progression. Clin Cancer Res 1999;5:2271–7.
39. Carlinfante G, Vassiliou D, Svensson O, Wendel M, Heinegard D, Andersson G. Differential expression of osteopontin and bone sialoprotein in bone metastasis of breast and prostate carcinoma. Clin Exp Metastasis 2003;20:437–44.
40. Foster CS, Falconer A, Dodson AR, et al. Transcription factor E2F3 overexpressed in prostate cancer independently predicts clinical outcome. Oncogene 2004;23:5871–9.
41. Hamdy FC, Fadlon EJ, Cottam D, et al. Matrix metalloproteinase 9 expression in primary human prostatic adenocarcinoma and benign prostatic hyperplasia. Br J Cancer 1994;69:177–82.
42. Yang G, Ayala G, De Marzo A, et al. Elevated Skp2 protein expression in human prostate cancer: association with loss of the cyclin-dependent kinase inhibitor p27 and PTEN and with reduced recurrence-free survival. Clin Cancer Res 2002;8:3419–26.
43. Krajewska M, Krajewski S, Banares S, et al. Elevated expression of inhibitor of apoptosis proteins in prostate cancer. Clin Cancer Res 2003;9:4914–25.
44. Zellweger T, Ninck C, Mirlacher M, et al. Tissue microarray analysis reveals prognostic significance of syndecan-1 expression in prostate cancer. Prostate 2003;55:20–9.
45. Chen D, Adenekan B, Chen L, et al. Syndecan-1 expression in locally invasive and metastatic prostate cancer. Urology 2004;63:402–7.
46. Spruck CH, de Miguel MP, Smith AP, et al. Requirement of Cks2 for the first metaphase/anaphase transition of mammalian meiosis. Science 2003;300:647–50.
47. Reynolds PA, Smolen GA, Palmer RE, et al. Identification of a DNA-binding site and transcriptional target for the EWS-WT1(+KTS) oncoprotein. Genes Dev 2003;17:2094–107.
48. Desmond JC, Mountford JC, Drayson MT, et al. The aldo-keto reductase AKR1C3 is a novel suppressor of cell differentiation that provides a plausible target for the non-cyclooxygenase-dependent antineoplastic actions of nonsteroidal anti-inflammatory drugs. Cancer Res 2003;63:505–12.
49. Thomas LN, Lazier CB, Gupta R, et al. Differential alterations in 5alpha-reductase type 1 and type 2 levels

during development and progression of prostate cancer. Prostate 2005;63:231–9.
50. Luo J, Dunn TA, Ewing CM, Walsh PC, Isaacs WB. Decreased gene expression of steroid 5 alpha-reductase 2 in human prostate cancer: implications for finasteride therapy of prostate carcinoma. Prostate 2003;57:134–9.
51. Titus MA, Gregory CW, Ford OH III, Schell MJ, Maygarden SJ, Mohler JL. Steroid 5alpha-reductase isozymes I and II in recurrent prostate cancer. Clin Cancer Res 2005;11:4365–71.
52. Simard J, Ricketts ML, Gingras S, Soucy P, Feltus FA, Melner MH. Molecular biology of the 3beta-hydroxysteroid dehydrogenase/delta5–4 isomerase gene family. Endocr Rev 2005;26:525–82.
53. Rizner TL, Lin HK, Peehl DM, Steckelbroeck S, Bauman DR, Penning TM. Human type 3 3alpha-hydroxysteroid dehydrogenase (aldo-keto reductase 1C2) and androgen metabolism in prostate cells. Endocrinology 2003;144:2922–32.
54. Ji Q, Chang L, VanDenBerg D, Stanczyk FZ, Stolz A. Selective reduction of AKR1C2 in prostate cancer and its role in DHT metabolism. Prostate 2003;54:275–89.
55. Steckelbroeck S, Jin Y, Gopishetty S, Oyesanmi B, Penning TM. Human cytosolic 3alpha-hydroxysteroid dehydrogenases of the aldo-keto reductase superfamily display significant 3beta-hydroxysteroid dehydrogenase activity: implications for steroid hormone metabolism and action. J Biol Chem 2004;279:10784–95.
56. Mahoney EM, Harrison JH. Bilateral adrenalectomy for palliative treatment of prostatic cancer. J Urol 1972; 108:936–8.
57. Geller J. Rationale for blockade of adrenal as well as testicular androgens in the treatment of advanced prostate cancer. Semin Oncol 1985;12:28–35.
58. Drago JR, Santen RJ, Lipton A, et al. Clinical effect of aminoglutethimide, medical adrenalectomy, in treatment of 43 patients with advanced prostatic carcinoma. Cancer 1984;53:1447–50.
59. Murray R, Pitt P. Treatment of advanced prostatic cancer, resistant to conventional therapy, with aminoglutethimide. Eur J Cancer Clin Oncol 1985;21:453–8.
60. Samojlik E, Lippman AJ, Kirschner MA, Ertel NH, Park Y, Szmal E. Medical adrenalectomy for advanced prostatic cancer: clinical and hormonal effects. Am J Clin Oncol 1988;11:579–85.
61. Trump DL, Havlin KH, Messing EM, Cummings KB, Lange PH, Jordan VC. High-dose ketoconazole in advanced hormone-refractory prostate cancer: endocrinologic and clinical effects. J Clin Oncol 1989;7:1093–8.
62. Small EJ, Baron AD, Fippin L, Apodaca D. Ketoconazole retains activity in advanced prostate cancer patients with progression despite flutamide withdrawal. J Urol 1997;157:1204–7.
63. Small EJ, Halabi S, Dawson NA, et al. Antiandrogen withdrawal alone or in combination with ketoconazole in androgen-independent prostate cancer patients: a phase III trial (CALGB 9583). J Clin Oncol 2004;22: 1025–33.
64. Scholz M, Jennrich R, Strum S, Brosman S, Johnson H, Lam R. Long-term outcome for men with androgen independent prostate cancer treated with ketoconazole and hydrocortisone. J Urol 2005;173:1947–52.
65. Eisenberger MA, Blumenstein BA, Crawford ED, et al. Bilateral orchiectomy with or without flutamide for metastatic prostate cancer. N Engl J Med 1998;339: 1036–42.
66. Eisenberger MA, Laufer M, Vogelzang NJ, et al. Phase I and clinical pharmacology of a type I and II, 5-alpha-reductase inhibitor (LY320236) in prostate cancer: elevation of estradiol as possible mechanism of action. Urology 2004;63:114–9.
67. Febbo PG, Richie JP, George DJ, et al. Neoadjuvant docetaxel before radical prostatectomy in patients with high-risk localized prostate cancer. Clin Cancer Res 2005;11:5233–40.