Gene Expression Analysis of Human Prostate Carcinoma during Hormonal Therapy Identifies Androgen-Responsive Genes and Mechanisms of Therapy Resistance

Jeff Holzbeierlein,* Priti Lal,[†] Eva LaTulippe,[†] Alex Smith,[‡] Jaya Satagopan,[‡] Liying Zhang,[†] Charles Ryan,[§] Steve Smith,[†] Howard Scher,^{§¶} Peter Scardino,* Victor Reuter,[†] and William L. Gerald[†]

From the Departments of Urology,* Pathology,[†] Epidemiology and Biostatistics,[‡] and the Department of Medicine,[§] Genitourinary Oncology Service, Memorial Sloan-Kettering Cancer Center, New York; and the Department of Medicine,[¶] Joan and Sanford I. Weill College of Medicine of Cornell University, New York, New York

The androgen-signaling pathway is critical to the development and progression of prostate cancer and androgen ablation is a mainstay of therapy for this disease. We performed a genome-wide expression analysis of human prostate cancer during androgen ablation therapy to identify genes regulated by androgen and genes differentially expressed after the development of resistance. Six hundred and fifty-four of 63,175 probe sets detected significant expression changes after 3 months of treatment with goserelin and flutamide. This included 149 genes that were also differentially expressed 36 hours after androgen withdrawal in LNCaP cells. These genes reflect the physiological changes that occur in treated tumors and include potential direct targets of the androgen receptor. Expression profiles of androgen ablation-resistant tumors demonstrated that many of the gene expression changes detected during therapy were no longer present suggesting a reactivation of the androgen response pathway in the absence of exogenous hormone. Therapy resistance was associated with differential expression of a unique set of genes that reflect potential mechanisms of reactivation. Specifically an up-regulation of the androgen receptor and key enzymes for steroid biosynthesis suggest that resistant tumors have increased sensitivity to and endogenous synthesis of androgenic hormones. The specific pathways of reactivation provide opportunities for classification of resistant tumors and targeted therapies. (*Am J Pathol* 2004, 164:217–227)

Carcinoma of the prostate is the most common noncutaneous cancer of men in the United States and is expected to affect ~220,900 individuals in 2003.¹ More than 1 million men older than the age of 50 alive today are expected to die of this disease. The incidence of prostate cancer has increased sharply in the last decade as serum prostate-specific antigen testing has become widely available.² This is primarily because of detection of asymptomatic and early-stage disease. These organ-confined prostate cancers are potentially curable with surgery or radiation. On the other hand, locally advanced tumors and metastatic prostate cancers are much more difficult to treat and in many cases lethal.

Androgens play a critical role in normal prostate function and in the development and progression of prostate cancer.^{3–6} Androgenic hormones act as ligands and are able to bind to and activate the intracellular androgen receptor (AR). Ligand binding to the AR results in conformational changes, dissociation from chaperones, phosphorylation, homodimerization, and subsequent interactions with specific androgen response elements (ARE) in the promoter regions of androgen-targeted genes. Transcriptional activity is dependent on the release of repressors and the recruitment of co-activators. Other growth factor mediated signal transduction pathways may also influence androgen signaling.⁷ These critical interactions and multisubunit complexes are likely to be responsible for the diversity of androgen-regulated functions and cell context specificity. The expression of androgen-responsive genes determines the balance between cell proliferation, cell death, and differentiation of normal prostatic epithelia and is believed to similarly affect neoplastic prostate cells.⁸ Despite the importance of the androgen response pathway in prostate cellular

Supported by the National Institutes of Health (grant U01 CA84999 to W. G.).

Accepted for publication September 25, 2003.

Present address of J. H.: Department of Surgery, University of Kansas Medical Center, Kansas City, KS.

Present address of E. L.: Department de Pathologie, Hotel Dieu de Quebec, Quebec, P. Quebec, Canada, G1R 2J6.

Address reprint requests to Dr. William L. Gerald, Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021. E-mail: geraldw@mskcc.org.

function, relatively few downstream targets and pathways have been identified and characterized.

Prostate cancer cells are typically androgen-dependent and androgen ablation is the standard systemic therapy for this disease. Androgen deprivation induces programmed cell death in normal, hyperplastic, preneoplastic, and malignant prostatic epithelial cells.⁶ Ablation can be achieved surgically by orchiectomy or chemically with gonadotropin-releasing hormone analogues, exogenous estrogens, progestational agents, anti-androgens, or adrenal steroid synthesis enzyme inhibitors. Virtually all prostate cancer patients treated with androgen ablation respond but eventually develop resistance, an ominous clinical state for which no consistently effective therapy exists.⁸

The critical molecular mechanisms by which prostate cancer cells become resistant to hormone therapy are primarily unknown, but several hypotheses exist. Amplification or mutation of the *AR* occurs in 20 to 30% of androgen ablation-resistant prostate cancers (AARPCs) suggesting that tumor cells become hypersensitive and respond to low levels of androgens or become promiscuous and can be stimulated by alternative ligands with structural homology to androgen.^{6,9} An alternative model for therapy resistance suggests that recruitment of non-steroid receptor signal transduction pathways can activate the androgen response in the setting of clinical androgen deprivation.⁷ A more complete basic understanding of AARPC and new effective therapies are needed.

Study of the complex processes associated with androgen signaling and hormone-resistant progression in prostate cancer has proved difficult because few models exist that reproducibly mimic the clinical course of the disease in men. Many studies are based on a small repertoire of cell lines of uncertain relevance to primary disease. To characterize the androgen response program in human prostate cancer we performed a genome-wide analysis and identified gene expression changes that occur during androgen ablation therapy and in resistant disease. These expression profiles provide new insight into the biology of the androgen response program and the mechanisms of therapy resistance.

Materials and Methods

Samples

Prostatic tissues were obtained from therapeutic or diagnostic procedures performed as part of routine clinical management at the Memorial Sloan-Kettering Cancer Center, New York, NY. Samples included 5 benign prostate tissues from the peripheral zone of prostatectomies of cancer patients, 23 primary prostate cancers from patients undergoing radical prostatectomy with no therapy before surgery, 17 primary prostate cancers after 3 months of androgen ablation therapy (a monthly injection of 3.6 mg of goserelin and 250 mg of flutamide three times daily for 3 months before radical prostatectomy), and 9 metastatic prostate cancers, including 3 that were

Table 1. ARE Motifs Identified in Differentially Expressed Genes

Gene symbol	RefSeq accession	Putative ARE sequence	Position
Consensus		AGAACAAnnnTGTTC	
<i>DHCR24</i>	NM_014762	AaAAaAaacTGTTC	-3024
		AGAACAAtccTaTTCc	-2901
<i>LOC89944</i>	NM_138342	ActACAtcaTGTTC	-757
<i>NNMT</i>	NM_006169	AtAACAtgtTtTTC	-3851
<i>GSTM1</i>	NM_000561	TGAACcctgTGTTC	-3016
<i>UNC13</i>	NM_006377	AaAaGaatcTGTTC	-349
<i>BICD1</i>	NM_001714	AGAAtAttcTGTTCgT	-2293
<i>ENTPD5</i>	NM_001249	AGAACtggatGTTCgT	-259
<i>PFKFB3</i>	NM_004566	AGAACAAtagTcTTCc	-2404
<i>ARL7</i>	NM_005737	AGtACAtgcTaTTC	-3522
<i>FLJ22378</i>	NM_025078	AGcACAGacTGTTCaT	-2950
		AGAAaAcccGTTCa	-2186
<i>ATP2C1</i>	NM_014382	AGAACcacaTaTTC	-3263
		AcAACAgataGTTCT	-2385
<i>C20orf167</i>	NM_052951	AGctCaccaTGTTC	-2064
<i>SLC37A1</i>	NM_018964	AcAACAtctaGTTCT	-4642
		AcAtCagagTGTTC	-1091
<i>DOK4</i>	NM_018110	AGAACtggatGTTCtT	-591
<i>FLJ14249</i>	NM_022460	AGAACAActTtcTCT	-4864
<i>FLJ38482</i>	NM_152681	AGcACAtgaTgaTCT	-4855
<i>TMEPAI</i>	NM_020182	TGAaGaatgTGTTC	-913
<i>KLK3</i>	NM_001648	AGAACAgcaaGTgCT	-170
<i>ASAHI</i>	NM_004315	AGAACAActgTcaTCT	-1196
<i>UNC5H2</i>	NM_170744	AGAACAAtgcTGTgT	-2338

Sequence variations from the consensus ARE are shown in lower case. The position is relative to the start codon.

progressing after 5 to 10 years of androgen ablation (see Supplemental Table 1 at www.amjpathol.org for clinical details). Tissues were snap-frozen in liquid nitrogen and stored at -80°C . Each sample was examined histologically using hematoxylin and eosin-stained cryostat sections. Tissues of interest were manually dissected from the frozen block. Care was taken to remove nonneoplastic tissues from tumor samples. Tumor samples contained ~60 to 80% prostate cancer cell nuclei based on histological assessment. All studies were conducted under Memorial Sloan-Kettering Cancer Center Institutional Review Board-approved protocols.

LNCaP cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 media with 10% fetal bovine serum and streptomycin/penicillin to near confluence. Cells were then divided and allowed to proliferate for 24 hours before stimulation. Stimulation was performed with 0.1 nmol/L of R1881 (Sigma, St. Louis, MO), a synthetic androgen for 24 hours, then washed with phosphate-buffered saline and placed in phenol red-free RPMI 1640 with 10% charcoal-stripped fetal bovine serum (steroid-free conditions) for 36 hours to simulate androgen withdrawal conditions. Cells were then harvested for RNA extraction.

Gene Expression Analysis

Total RNA was extracted from frozen tissues by homogenization in guanidinium isothiocyanate-based buffer (Trizol; Invitrogen, Carlsbad, CA), purified using RNA easy (Qiagen, Valencia, CA) and evaluated for integrity by denaturing agarose gel. Complementary DNA was

synthesized from total RNA using a T7-promoter-tagged oligo-dT primer. RNA target was synthesized by *in vitro* transcription and labeled with biotinylated nucleotides (Enzo Biochem, Farmingdale, NY). Labeled target was assessed by hybridization to Test2 arrays (Affymetrix, Santa Clara, CA) and detected with phycoerythrin-streptavidin (Molecular Probes, Eugene, OR) amplified with anti-streptavidin antibody (Vector, Burlingame, CA). Gene expression analysis was performed using Affymetrix U95 human gene arrays with 63,175 features for individual gene/expressed sequence tag clusters using instruments and protocols recommended by the manufacturer. The U95 set consists of five distinct microarrays (A through E), each containing probes for ~12,000 gene/expressed sequence tag transcripts. Two response measures, the average difference and absolute call, were extracted for each gene on every sample, as determined by default settings of Affymetrix Microarray Suite 4.0. Average difference was used as the primary measure of expression level, and absolute call was retained as a secondary measure. Expression values on each array were multiplicatively scaled to have an average expression of 2500 across the central 96% percent of all genes on the array.

Data Analysis

For oligonucleotide arrays, scanned image files were visually inspected for artifacts and analyzed using Microarray Suite v4.0. Uniformly and strongly differentially expressed genes were determined by the following approach (fully described in supplementary information). The expression data set was first filtered to include only those probe sets detecting genes with mean expression values that differed by at least threefold between groups. Probes were then ranked based on the relative magnitude of the difference (*t*-test) between the means of any two sample sets. A Mann-Whitney test was used in place of the *t*-test when comparing small numbers of samples. Data sets used for hierarchical clustering were normalized by standardizing each gene and sample (array) to mean = 0 and variance = 1. Average linkage hierarchical clustering and result display were performed using Cluster and TreeView software.¹⁰ Specific genes corresponding to individual probe sets were identified using the Affymetrix database (<https://www.affymetrix.com/index.affx>) to identify the GenBank accession number of the clone used to produce the oligonucleotide probe set and annotated through review of internet resources (GeneCards, <http://nciarray.nci.nih.gov/cards/>; Locus Link, <http://www.ncbi.nlm.nih.gov/LocusLink/index.html>; Online Mendelian Inheritance in Man, <http://neptune.nlm.nih.gov/entrez/query.fcgi?db=OMIM>; Gene Ontology Browser, <http://cgap.nci.nih.gov/Genes>) and publications identified in Pubmed (<http://www.ncbi.nlm.nih.gov/PubMed/>).

Identification of ARE Motifs

Reference sequence accession numbers were identified for 80 genes with differential expression in both human

tumors and LNCaP cells after androgen withdrawal by search of the Affymetrix database and the RefSeq database at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/LocusLink/refseq.html/>). The reference sequences were used to query the assembled human genome sequence (<http://genome.ucsc.edu/>) and 5 kb of sequence 5' to the transcription start site of each gene was retrieved. Each sequence was analyzed for homology to the consensus ARE motif 5'-AGAACAnnnT-GTTCT-3' using the Filtered String-based Search Query of TESS (<http://www.cbil.upenn.edu/tess>). In the "Factor Filter" section, we choose class 2.1.1.3 for *AR* according to transcription factor classification (<http://www.gene-regulation.com/pub/databases/transfac/cl.html>). In the "Score Filters" section, the "Maximum Allowable String Mismatch %" was set to 20%, all of the other parameters were set the same as default. Motifs with at least 10 identities of the 12 consensus ARE nucleotides were reported.

Immunohistochemistry

Multitissue blocks of formalin-fixed, paraffin-embedded tissue corresponding to the samples used in this analysis were prepared using a tissue arrayer (Beecher Instruments, Silver Spring, MD). The blocks contained three representative 0.6-mm cores from diagnostic areas of each case. Immunohistochemical detection of prostate-specific antigen (1:2000; Biogenex, San Ramon, CA), *AR* (1:50; DAKO, Carpinteria, CA), squalene monooxygenase (1/4000; gift of Dr. Todd D. Porter, University of Kentucky, Lexington, KY) was performed with standard streptavidin-biotin-peroxidase methodology using formalin-fixed, paraffin-embedded tissue and microwave antigen retrieval as described.¹¹

Fluorescent in Situ Hybridization

Bicolor fluorescent *in situ* hybridization studies were performed on paraffin sections using commercially available probes for *AR* (Vysis, Downer's Grove, IL) and chromosome X centromere-specific alphoid region (Vysis). Fluorescent *in situ* hybridization was performed as described.¹² The number of hybridization signals for each probe was assessed in an average of 36 nuclei with strong and well-delineated signals. As controls, normal peripheral blood lymphocytes were simultaneously hybridized.

Results and Discussion

Gene Expression Analysis of Androgen Ablation-Treated Prostate Cancer

The development and progression of prostate cancer is dependent on androgenic hormones. Androgens normally regulate transcription of genes involved in critical cellular pathways controlling cell proliferation, differentiation, and programmed death. The specific transcrip-

Figure 1. Representative microscopic images of prostate cancers used for expression analysis. **A:** Typical histological appearance of hormone therapy naive (untreated) prostate cancer. **B:** Typical histological appearance of prostate cancer after 3 months of neoadjuvant androgen ablation therapy (treated).

tional targets of the AR that mediate these biological programs for prostate cancer are unknown. To identify genes that are responsive to androgens, we performed comprehensive expression analysis of resected prostate carcinomas and nonneoplastic prostate tissues from patients that had been treated with a standardized regimen of androgen ablation therapy and compared those to expression profiles of similar tissues from patients that had not been treated. All tumor samples were evaluated histologically and tissue blocks trimmed to provide a consistent prostate cancer cell content of between 60% and 80%. The morphological features typical of androgen ablation therapy were evident in neoadjuvant-treated cases (Figure 1 and Supplemental Table 1 at www.amjpathol.org) and resulted in slightly different nonneoplastic cell content between the sample groups.¹³ The trimming of frozen tissue blocks and stringent criteria for differential expression (described below) were designed to take this into account.

Samples were analyzed using hybridization of RNA target to oligonucleotide microarrays with 63,175 probe sets for ~54,000 genes and expressed sequence tags. Seventeen patients received neoadjuvant hormonal ablation therapy with goserelin and flutamide for 3 months (hereafter referred to as "treated" cases), and 23 patients received no neoadjuvant therapy (untreated). General levels of gene expression for the two groups were similar. Sixty-four percent of the 63,175 probe sets detected expression in at least 1 of the 23 untreated prostate cancer samples based on the Microarray Suite 4.0 present/absent algorithm. Of the 17 treated cases, 61.9% of the probe sets had a present call in at least one of the samples. In contrast, analysis of gene expression in the LNCaP prostate carcinoma cell line cultured in androgen-supplemented media demonstrated a present call for 50.2% of the probe sets, and with androgen withdrawal a present call was made 50% of the time. The greater

diversity of transcripts in native prostate cancer likely reflects cell type heterogeneity in human tissues.

Comparison of expression profiles using a hierarchical clustering algorithm demonstrated a strong tendency to subgroup the samples with respect to treatment status (Figure 2A). This suggests that distinct, consistent differences in transcript levels occur in prostate cancers as a result of androgen deprivation. We used a stringent filter to identify genes that were strongly differentially expressed with androgen ablation therapy. A total of 654 of the 63,175 probe sets detected a difference of at least threefold between the mean expression values for treated and untreated tumors with a *t*-test *P* value of less than 0.001 (see Supplemental Table 2 at www.amjpathol.org for entire gene list). Two hundred ninety genes were up-regulated with androgen ablation therapy and 364

Figure 2. Average linkage hierarchical clustering dendrograms of samples demonstrating relationship of gene expression profiles based on all 12,559 probe sets of U95A array. **A:** Untreated primary (green) and androgen ablation-treated primary prostate cancers (yellow). **B:** Untreated primary and androgen ablation-treated primary prostate cancers and androgen ablation-resistant metastatic prostate cancers (pink).

genes were down-regulated. Of these 654 probe sets, 512 (78.3%) detected similar directional alteration in gene expression after therapy in samples of adjacent nonneoplastic prostate enriched for epithelium and 336 of these further showed a threefold change. This suggests that many of the gene expression changes after therapy in prostate cancer represent a physiological response to androgen withdrawal that also occurred in nonneoplastic prostate. Others may be part of a tumor-specific androgen response program.

Among the genes that were highly responsive to 3 months of androgen ablation in human prostate cancer many were previously identified as androgen responsive in experimental model systems. Some of the differentially expressed genes are well known targets of the AR (*KLK3*, *KLK2*) and others have also been described as androgen regulated such as *DBI*, *FASN*, *IL6*, *SERPINB5*, *TGFBR3*, *TPRSS2*, *TUBA1*, *HOXC6*, *TRG*, and *FOLH1*.^{14–18} Therefore it appears that many of the gene expression changes detected in tumor samples after 3 months of androgen ablation therapy reflect modulation of AR-mediated transcriptional regulation and represent direct or indirect targets of the AR. However, some may represent a response to environmental stress that promotes survival of tumor cells during androgen-poor conditions. For example, *IL6* was dramatically up-regulated with androgen deprivation. This gene is believed to contribute to progression of prostate cancer and to androgen-independent growth.¹⁹ Gene expression changes such as these could reflect biological processes that contribute to the clinical phenomenon of androgen ablation-resistant tumor progression after long-term hormonal therapy as discussed below.

Identification of Genes Likely to Be Directly Regulated by Androgen

The data described above reflect gene expression in human prostate cancer after 3 months of androgen ablation and it is not possible to distinguish those changes that occur early and may correspond to direct targets of the AR, from those that occur at a later time and most likely represent subsequent events. To identify differentially expressed genes that were likely to be directly regulated by the activated AR we used the androgen-responsive LNCaP cell line to characterize changes in transcript levels that occur rapidly after androgen withdrawal and compared that to the data from human tumors. Expression profiles of LNCaP cells growing in R1881 (a synthetic androgen analog)-supplemented media were compared to profiles of cells 36 hours after transfer to androgen-depleted media. One hundred forty-nine of the 654 genes (22.8%) that were strongly differentially expressed in prostate cancer with androgen-ablation therapy also showed at least a twofold change in transcript level in LNCaP cells after androgen withdrawal (Supplemental Table 2 at www.amjpathol.org). In contrast, less than 1% of probe sets demonstrated a twofold change in a replicative hybridization of RNA from androgen-supplemented LNCaP. Genes with quantitative

mRNA changes occurring within hours of androgen withdrawal are more likely to be direct targets of the activated AR and include the prototypical androgen response gene *KLK3*. However some of the expression changes could be because of secondary events after androgen withdrawal.

To further explore the potential as a direct target gene of the AR, we analyzed the 5' regulatory region of 80 of the 149 genes that were strongly differentially expressed in both human tumors and LNCaP for which reference sequence information was available. Motifs with a high degree of homology to the consensus ARE were identified in 20 genes (Table 1) suggesting the potential for AR binding. This subset of differentially expressed genes is likely to represent direct and very early downstream targets of the androgen response program in human prostate cancer.

Several high-throughput methods have been previously used to address the androgen response program in LNCaP cells.^{14–18} This cell line expresses a functional although mutated AR, proliferates in response to androgens, and produces prostate-specific antigen in an androgen-dependent manner. Most studies have analyzed cells within hours of androgen manipulation and many differentially expressed genes have been identified in common. It is interesting to note that ~25% of the differentially expressed, named genes in our study of human prostate cancers have also been shown to have significant changes in expression after short-term androgen manipulation of LNCaP cells.^{17,18} This degree of concordance using diverse experimental systems and technical platforms serves to validate that many of the genes identified in this analysis are truly androgen regulated.

Changes in Gene Expression Related to Androgen Ablation-Resistant Progression of Prostate Cancer

After an initial response to therapy most patients develop progressive disease resistant to androgen ablation. The molecular mechanisms underlying this clinically important transition to androgen ablation resistance are poorly understood. To characterize gene expression changes that occur as part of the resistance mechanism we compared gene expression in resistant tumors to samples from patients that had not developed resistance. These are rare clinical samples, however the three AARPC samples represent tumors that were progressing in the setting of undetectable serum testosterone and long-term androgen ablation. Of particular interest, hierarchical cluster analysis revealed that the overall expression patterns for these androgen-independent tumors were more similar to that of the untreated, androgen-dependent primary cancers than to tumors under conditions of androgen deprivation such as the neoadjuvant androgen ablation-treated cases described above (Figure 2B). This suggested a reversal of many of the expression changes because of androgen ablation possibly through reactivation of the androgen response pathway as suggested by others.^{20–23} In fact the vast majority of genes that were

Table 2. Genes Differentially Expressed in Androgen Ablation-Resistant Prostate Cancer

Probe set ID	Gene name	Symbol	Location	Fold change
32527_at_HG-U95Av2	Adipose specific 2	<i>APM2</i>	10q23.32	0.03
1578_g_at_HG-U95Av2	Androgen receptor	<i>AR</i>	Xq11.2-q12	11.03
1577_at_HG-U95Av2	Androgen receptor	<i>AR</i>	Xq11.2-q12	9.00
64381_at_HG-U95C	ARG99 protein	<i>ARG99</i>	12p12.1	0.13
35803_at_HG-U95Av2	Ras homolog gene family, member E	<i>ARHE</i>	2q23.3	0.29
60226_at_HG-U95C	Ankyrin repeat and SOCS box-containing 5	<i>ASB5</i>	4q34.1	0.08
39565_at_HG-U95Av2	Bone morphogenetic protein receptor, type IA	<i>BMPR1A</i>	10q22.3	0.27
42988_at_HG-U95B	C1q and tumor necrosis factor related protein 6	<i>C1QTNF6</i>	22q13.1	5.17
33704_at_HG-U95Av2	Chromosome 21 open reading frame 108	<i>C21orf108</i>	21q22.11	3.16
36661_s_at_HG-U95Av2	CD14 antigen	<i>CD14</i>	5q31.1	0.16
38995_at_HG-U95Av2	Claudin 5	<i>CLDN5</i>	22q11.21	0.11
47466_i_at_HG-U95B	Contactin associated protein-like 2	<i>CNTNAP2</i>	7q35-q36	0.06
31773_at_HG-U95Av2	Cytochrome b-561	<i>CYB561</i>	17q11-qter	5.51
44596_at_HG-U95B	Likely ortholog of mouse and rat twist-related bHLH protein Dermo-1	<i>DERMO1</i>	2q37.3	0.17
31902_at_HG-U95Av2	Deiodinase, iodothyronine, type II	<i>DIO2</i>	14q24.2-q24.3	0.19
36834_at_HG-U95Av2	DKFZP564G202 protein	<i>DKFZP564G202</i>	6q23.1-23.3	0.10
51029_at_HG-U95C	Hypothetical protein DKFZp761D0614	<i>DKFZp761D0614</i>	10q23.33	0.23
36149_at_HG-U95Av2	Dihydropyrimidinase-like 3	<i>DPYSL3</i>	5q32	0.22
73128_at_HG-U95E	Dihydropyrimidinase-like 3	<i>DPYSL3</i>	5q32	0.21
59266_at_HG-U95B	Ephrin-A3	<i>EFNA3</i>	1q21-q22	3.33
36543_at_HG-U95Av2	Coagulation factor III	<i>F3</i>	1p22-p21	0.19
38430_at_HG-U95Av2	Fatty acid binding protein 4, adipocyte	<i>FABP4</i>	8q21	4.13
34848_at_HG-U95Av2	Farnesyl-diphosphate farnesyltransferase 1	<i>FDFT1</i>	8p23.1-p22	3.70
53115_at_HG-U95C	Fidgetin	<i>FIGN</i>	2q24.2	0.21
75147_at_HG-U95E	Hypothetical protein FLJ13848	<i>FLJ13848</i>	11q13.1	15.20
56574_at_HG-U95C	Hypothetical protein FLJ21069	<i>FLJ21069</i>	2p23.2	0.23
50310_at_HG-U95C	Hypothetical protein FLJ21308	<i>FLJ21308</i>	5q11.1	0.28
50001_at_HG-U95B	Hypothetical protein FLJ21986	<i>FLJ21986</i>	7q22.1	0.17
65852_at_HG-U95C	Duodenal cytochrome b	<i>FLJ23462</i>	2q31.1	0.33
38875_r_at_HG-U95Av2	GREB1 protein	<i>GREB1</i>	2p25.1	0.22
1260_s_at_HG-U95Av2	Glutathione S-transferase A2	<i>GSTA2</i>	6p12.1	36.20
46698_at_HG-U95B	Cervical cancer oncogene 4	<i>HCC-4</i>		0.07
37420_i_at_HG-U95Av2	Major histocompatibility complex, class I, F	<i>HLA-F</i>	6p21.3	0.19
34251_at_HG-U95Av2	Homeo box B5	<i>HOXB5</i>	17q21-q22	5.46
64676_at_HG-U95C	Testican 3	<i>HSJA1454</i>	4q32.3	0.14
1237_at_HG-U95Av2	Immediate early response 3	<i>IER3</i>	6p21.3	0.26
1736_at_HG-U95Av2	Insulin-like growth factor binding protein 6	<i>IGFBP6</i>	12q13	0.21
50240_at_HG-U95B	Similar to lymphocyte antigen 6 complex	<i>LOC112476</i>	16q13	0.08
52922_at_HG-U95B	LR8 protein	<i>LR8</i>	7q35	0.01
78442_at_HG-U95E	Hypothetical protein MGC4692	<i>MGC4692</i>	16	5.00
41854_at_HG-U95Av2	v-myb myeloblastosis viral oncogene homolog	<i>MYB</i>	6q22-q23	0.28
37369_s_at_HG-U95Av2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	<i>NFATC4</i>	14q11.2	0.14
82657_f_at_HG-U95D	Ninein (GSK3B interacting protein)	<i>NIN</i>	14q21.3	0.08
40732_at_HG-U95Av2	Nuclear protein, ataxia-telangiectasia locus	<i>NPAT</i>	11q22-q23	0.13
63315_at_HG-U95C	Netrin 4	<i>NTN4</i>	12q22-q23	0.13
36682_at_HG-U95Av2	Pericentriolar material 1	<i>PCM1</i>	8p22-p21.3	0.25
57214_at_HG-U95B	Hypothetical protein PP1044	<i>PP1044</i>	17p13.2	0.15
157_at_HG-U95Av2	Preferentially expressed antigen in melanoma	<i>PRAME</i>	22q11.22	3.20
38407_r_at_HG-U95Av2	Prostaglandin D2 synthase 21kDa (brain)	<i>PTGDS</i>	9q34.2-q34.3	0.24
80501_f_at_HG-U95E	Prostaglandin D2 synthase 21kDa (brain)	<i>PTGDS</i>	9q34.2-q34.3	0.21
38406_f_at_HG-U95Av2	Prostaglandin D2 synthase 21kDa (brain)	<i>PTGDS</i>	9q34.2-q34.3	0.09
216_at_HG-U95Av2	Prostaglandin D2 synthase	<i>PTGDS</i>	9q34.3	0.17
63846_at_HG-U95C	Paxillin	<i>PXN</i>	12q24	11.92
34419_at_HG-U95Av2	RAD51-like 1 (<i>S. cerevisiae</i>)	<i>RAD51L1</i>	14q23-q24.2	3.88
1042_at_HG-U95Av2	Retinoic acid receptor responder 1	<i>RARRES1</i>	3q25.31	0.28
31941_s_at_HG-U95Av2	Ret finger protein-like 3	<i>RFPL3</i>	22q12.3	0.3
531_at_HG-U95Av2	Glioma pathogenesis-related protein	<i>RTVP1</i>	12q14.1	0.11
87288_at_HG-U95E	Special AT-rich sequence binding protein 1	<i>SATB1</i>	3p23	0.33
37513_at_HG-U95Av2	Stearoyl-CoA desaturase (delta-9-desaturase)	<i>SCD</i>	10q23-q24	3.35
38113_at_HG-U95Av2	Synaptic nuclei expressed gene 1	<i>SYNE-1</i>	6q25	0.27
36807_at_HG-U95Av2	TED protein	<i>TED</i>	Xq13.1	5.30
35146_at_HG-U95Av2	Transforming growth factor beta 1 induced transcript 1	<i>TGFB111</i>	16p11.1	0.18
1814_at_HG-U95Av2	Transforming growth factor, beta receptor II	<i>TGFR2</i>	3p22	0.20
53712_at_HG-U95B	TIGA1	<i>TIGA1</i>	5q21-q22	0.16

Table continues

Table 2. *Continued*

Probe set ID	Gene name	Symbol	Location	Fold change
31410_at_HG-U95Av2	Tumor necrosis factor receptor superfamily, Member 13B	<i>TNFRSF13B</i>	17p11.2	0.26
36873_at_HG-U95Av2	Very low density lipoprotein receptor	<i>VLDLR</i>	9p24	5.90
69929_at_HG-U95E	Zinc finger protein 10 (KOX 1)	<i>ZNF10</i>	12q24.33	4.47
59570_at_HG-U95B	Homo sapiens cDNA FLJ40054 fis,			10.70
52853_g_at_HG-U95B	ESTs			11.01
52851_at_HG-U95B	ESTs			9.80
43436_g_at_HG-U95B	ESTs			7.59
72319_at_HG-U95D	ESTs			18.40
43435_at_HG-U95B	ESTs			6.60
68919_at_HG-U95E				5.65
49549_at_HG-U95B	ESTs, Weakly similar to cytokine receptor-like factor 2; cytokine receptor CRL2 precursor			5.11
71057_at_HG-U95E	ESTs			4.95
50739_at_HG-U95C				7.45
47205_at_HG-U95B	ESTs, Moderately similar to SMCY_HUMAN SmcY protein (Histocompatibility Y antigen) (H-Y)			24.30
79968_at_HG-U95D	ESTs			21.05
77237_at_HG-U95E	ESTs, Weakly similar to hypothetical protein FLJ20489			0.24
88940_at_HG-U95D	ESTs			8.45
54228_at_HG-U95B	Homo sapiens cDNA FLJ33400 fis, clone BRACE2009828			0.09
84506_i_at_HG-U95D				0.09
65817_at_HG-U95C	Homo sapiens cDNA FLJ38577 fis,			0.31
49321_at_HG-U95B	ESTs			0.29
43047_at_HG-U95B	ESTs			0.16
91768_at_HG-U95E	Homo sapiens cDNA: FLJ21333 fis,			0.25
72548_at_HG-U95D	ESTs			0.25
48874_f_at_HG-U95B	ESTs			0.24
52668_at_HG-U95B	ESTs			0.23
56880_at_HG-U95C	ESTs			0.21
48296_at_HG-U95B	ESTs			0.17
82445_at_HG-U95D	Homo sapiens cDNA FLJ39434 fis,			0.17
39324_at_HG-U95Av2	Homo sapiens cDNA FLJ10784 fis,			0.20
48071_at_HG-U95B	Homo sapiens cDNA FLJ36990 fis, c			3.78
82162_g_at_HG-U95D	ESTs			0.17
39273_at_HG-U95Av2	Homo sapiens, clone IMAGE:4811759			0.28
48637_at_HG-U95B	ESTs, Weakly similar to hypothetical protein FLJ20378			0.25
77410_r_at_HG-U95E	Homo sapiens cDNA FLJ33630 fis,			0.09
69668_at_HG-U95D	ESTs			0.11

identified as differentially expressed with short-term androgen ablation did not demonstrate changes to the same extent in AARPC even though these tumors were also in an androgen-poor environment. However, a unique set of genes was differentially expressed in resistant tumors. A total of 304 of the 63,175 U95 probe sets detected tumor-related differential gene expression between primary androgen-dependent and metastatic hormone-resistant tumors of at least threefold with a Mann-Whitney test P value <0.001 (see Supplemental Table 3 at www.amjpathol.org for entire gene list). Because there are marked differences in gene expression between primary and metastatic prostate cancer regardless of treatment status,¹¹ we further identified the subset of these genes that were also differentially expressed between metastatic AARPC and other metastatic prostate cancers. One hundred of the 304 genes were also differentially expressed threefold in hormone-resistant metastatic tumors ($n = 3$) compared to androgen-dependent metastatic tumors ($n = 6$) (Table 2). Thirty-three were up-

regulated and 67 were down-regulated in the resistant tumors.

The genes that are differentially expressed in AARPC represent many biological categories however several have functional attributes that could contribute to hormone therapy-resistant growth. One of the most notable was a marked increase in the level of the *AR* mRNA detected by two separate probe sets in all three AARPCs tested (Figure 3A). Analysis of a larger set of AARPCs by immunohistochemistry demonstrated a high level of nuclear-localized AR protein in 26 of 28 additional cases of AARPC (Figure 3, B and C). The level of expression was increased relative to that seen in the majority of primary untreated prostate cancers. Because gene amplification is a known mechanism by which *AR* could be overexpressed, we performed fluorescent *in situ* hybridization for the *AR* gene and found that none of the AARPC analyzed by microarray and only 8 of 29 additional AARPC cases with high levels of *AR* expression had increased gene copy number (Figure 3, D and E). This

Figure 3. AR expression in human prostate cancers. **A:** Bar graph representing *AR* transcript levels based on hybridization of cDNA target to oligonucleotide arrays. Two different probe sets for *AR* are presented. **B** and **C:** Immunohistochemical detection of the AR in representative primary untreated prostate cancer with low level of AR transcripts (**B**) and metastatic AARPC with high levels (**C**). **D** and **E:** Fluorescent *in situ* hybridization for *AR* (red) and chromosome X centromere (green) in metastatic AARPC with high levels of *AR* transcripts and no amplification of the *AR* gene (**D**) and metastatic AARPC with high level of AR expression and *AR* amplification (**E**).

Sterol and Steroid Hormone Biosynthesis

Figure 4. Schematic diagram of steroid biosynthesis with superimposed gene expression changes in androgen ablation-resistant prostate cancer.

data and other published series^{24–26} suggest that strong expression of the *AR* is present in most cases of AARPC and for many tumors the increase in *AR* transcript levels occurs by mechanisms other than gene amplification. Overexpression of the *AR* could contribute to reactivation of the androgen response pathway in resistant tumors through increased sensitivity to low levels of ligand or as a cooperative effect with ligand independent pathways of *AR* activation.²⁷

One of the strengths of an unbiased, comprehensive expression analysis is the potential to identify coordinated gene expression changes that reflect biological programs critical to specific tumor phenotypes. In this regard, it was intriguing that our expression analysis of AARPC detected increased expression of several genes that correspond to enzymes involved in steroid precursor synthesis (Figure 4). This included one of the rate-limiting enzymes in sterol synthesis, squalene monooxygenase.²⁸ By semi-quantitative immunohistochemistry we

were able to demonstrate that immunoreactive squalene monooxygenase was produced by prostate cancer tumor cells (Figure 5) and the level of immunoreactivity generally correlated with RNA levels in a small number of cases available for study (data not shown). In addition strong immunoreactivity for squalene monooxygenase was detected in a separate subset of AARPC in contrast to non-resistant tumors that in general had lower levels. This result implies that essential enzymes have been up-regulated in a manner that would facilitate endogenous production of steroids in some cases of AARPC. Increased availability of androgenic ligands would provide a compensatory mechanism for overcoming androgen deprivation that complements increased sensitivity as a result of *AR* overexpression. If in fact this pathway is proven to contribute to resistance, it is encouraging that drugs to inhibit squalene monooxygenase have been developed.²⁸

In addition to the gene expression alterations noted above, it is of interest that changes in expression levels

Figure 5. Immunohistochemical detection of squalene monooxygenase in prostate cancer samples. Liver serves as positive control.

for several genes that potentially participate in nonandrogen-mediated survival and proliferation pathways were also detected in AARPC. One of the genes down-regulated in all three cases was *PTEN* (3.7-fold reduced in AARPC), a phosphatase that is a key regulator of the PI3/AKT/FRAP pathway among others.²⁹ The *PTEN* gene is often lost or mutated in advanced prostate cancer³⁰ and is expected to result in activation of this critical pathway and may affect other biological systems that play a role in tumor biology. In fact significant changes in the expression of several members of this pathway have previously been detected in the CWR22 model of AARPC.²¹ CWR22 is a prostate cancer xenograft that exhibits androgen-dependent growth and expression of a mutated *AR*. When deprived of androgens, tumors undergo involution but recur as rapidly growing androgen-independent tumors in 3 to 12 months. In this model most of the gene expression changes that occur with androgen ablation are reversed after the development of androgen-independent tumors, similar to our finding in human cancers.^{20–22} Several genes of the PI3/AKT/FRAP pathway show significant changes in expression with the development of androgen-independent growth of CWR22. Several other genes identified in CWR22 androgen-independent tumors were considered stable up-regulated (*FKBP5*, *THRA*, *S100P*, *SDC1*, *NCOR1*, *APELIN*) and not androgen responsive, suggesting that they may

serve as markers for androgen-independent disease. Of these, *FKBP5* was overexpressed in two of three and *SDC1* in one of three of our AARPC cases relative to the mean of nonresistant tumors suggesting a role in some human prostate cancers.

Conclusion and Clinical Implications

The androgen response program is critical to the progression of human prostate cancer and is a central therapeutic target in this disease. Our studies of prostate cancers during androgen ablation therapy and the development of androgen independence highlight several important biological features of this clinically critical process. There were dramatic changes in gene expression during therapy that primarily reflect modulation of AR-mediated transcriptional regulation. These differentially expressed genes reflect the physiological changes that occur in treated tumors and may provide useful markers of response and targets for combination therapy. Expression profiles for androgen ablation-resistant tumors reflected an apparent reactivation of the androgen-responsive program. Of particular interest is that some of the genes that were differentially expressed in resistant tumors suggested pathways that could contribute to the process of reactivation. In addition to confirming the im-

portance of up-regulation of the AR, our studies suggest an important role for sterol and steroid synthesis in tumor progression independent of exogenous androgen and the activation of other transduction pathways that may have direct effects on AR signaling or activate other survival pathways. The altered expression profiles detected in this analysis suggest that several complementary mechanisms contribute to therapy resistance and that there is heterogeneity among tumors. The determination of specific contributions for individual differentially expressed genes, and mechanisms that regulate their expression may lead to improved targeted therapeutic strategies for prostate cancer patients.

Acknowledgments

We thank Lishi Chen, David Kuo, Sandra Levcovici, Muzaffar Akram, Faye Taylor, Michelle Pappas, and members of the Pathology and Genomics Core Facilities for technical and database assistance; and Dr. Todd D. Porter for antisera to squalene monooxygenase.

References

- Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ: Cancer statistics, 2003: further decrease in mortality rate, increase in persons living with cancer. *CA Cancer J Clin* 2003, 53:5–26
- Barry M: Prostate specific antigen testing for early diagnosis of prostate cancer. *N Engl J Med* 2001, 344:1373–1377
- Klocker H, Culig Z, Kaspar F, Hobisch A, Eberle J, Reissigl A, Bartsch G: Androgen signal transduction and prostatic carcinoma. *World J Urol* 1994, 12:99–103
- Logothetis CJ, Hoosein NM, Hsieh JT: The clinical and biological study of androgen independent prostate cancer (AI PCa). *Semin Oncol* 1994, 21:620–629
- Griffiths K, Morton MS, Nicholson RI: Androgens, androgen receptors, antiandrogens and the treatment of prostate cancer. *Eur Urol* 1997,32:S24–S40
- Feldman BJ, Feldman D: The development of androgen independent prostate cancer. *Nature Rev Cancer* 2001, 1:34–45
- Lee C: Role of androgen in prostate growth and regression: stromal-epithelial interaction. *Prostate* 1996, 6:S52–S56
- Abate-Shen C, Shen M: Molecular genetics of prostate cancer. *Genes Dev* 2000, 14:2410–2434
- Fenton MA, Shuster TD, Fertig AM, Taplin ME, Kolvenberg G, Bubley GJ, Balk SP: Functional characterization of mutant androgen receptors from androgen-independent prostate cancer. *Clin Cancer Res* 1997, 3:1383–1388
- Eisen MB, Spellman PT, Brown PO, Botstein D: Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 1998, 95:14863–14868
- LaTulippe E, Satagopan J, Smith A, Scher H, Scardino P, Reuter V, Gerald W: Comprehensive gene expression analysis of prostate cancer reveals distinct transcriptional programs associated with metastatic disease. *Cancer Res* 2002, 62:4499–4506
- Argani P, Antonescu CR, Illei PB, Lui MY, Timmons CF, Newbury R, Reuter VE, Garvin AJ, Perez-Atayde AR, Fletcher JA, Beckwith JB, Bridge JA, Ladanyi M: Primary renal neoplasms with the ASPL-TFE3 gene fusion of alveolar soft part sarcoma: a distinctive tumor entity previously included among renal cell carcinomas of children and adolescents. *Am J Pathol* 2001, 159:179–192
- Armas O, Aprikian AG, Melamed J, Cordon-Cardo C, Cohen DW, Erlandson R, Fair WR, Reuter VE: Clinical and pathobiological effects of neoadjuvant total androgen ablation therapy in clinically localized prostatic adenocarcinoma. *Am J Surg Pathol* 1994, 18:970–991
- Xu L, Su Y, Labiche R, Segawa T, Shanmugam N, McLeod D, Moul J, Srivastava S: Quantitative expression profile of androgen-regulated genes in prostate cancer cells and identification of prostate-specific genes. *Int J Cancer* 2001, 92:322–328
- Vaarama M, Porvari K, Kyllonen A, Vihko P: Differentially expressed genes in two LNCaP prostate cancer cell lines reflecting changes during prostate cancer progression. *Lab Invest* 2000, 80:1259–1268
- Moore SM, Nelson PS: Gene expression profiling of the human prostate androgen response program. *J Androl* 2002, 23:163–169
- Nelson P, Clegg N, Arnold H, Ferguson C, Bonham M, White J, Hood L, Lin B: The program of androgen-responsive genes in neoplastic prostate epithelium. *Proc Natl Acad Sci USA* 2002, 99:11890–11895
- DePrimo SE, Diehn M, Nelson JB, Reiter RE, Matese J, Fero M, Tibshirani R, Brown PO, Brooks JD: Transcriptional programs activated by exposure of human prostate cancer cells to androgen. *Genome Biol* 2002, 3:0032.1–0032.12
- Smith PC, Hobisch A, Lin DL, Culig Z, Keller ET: Interleukin-6 and prostate cancer progression. *Cytokine Growth Factor Rev* 2001, 12: 33–40
- Bubendorf L, Kolmer M, Kononen J, Koivisto P, Mousses S, Chen Y, Mahlamaki E, Schraml P, Moch H, Willi N, Elkahoul A, Pretlow T, Gasser T, Mihatsch M, Sauter G, Kallioniemi O-P: Hormone therapy failure in human prostate cancer: analysis by complementary DNA and tissue microarrays. *J Natl Cancer Inst* 1999, 91:1758–1764
- Mousses S, Wagner U, Chen Y, Kim J, Bubendorf L, Bittner M, Pretlow T, Elkahoul A, Trepel J, Kallioniemi O: Failure of hormone therapy in prostate cancer involves systematic restoration of androgen responsive genes and activation of rapamycin sensitive signaling. *Oncogene* 2001, 20:6718–6723
- Amler L, Agus D, LeDuc C, Sapinoso M, Fox W, Kern S, Lee D, Wang V, Leysens M, Higgins B, Martin J, Gerald W, Dracopoli N, Cordon-Cardo C, Scher H, Hampton G: Deregulated expression of androgen-responsive and nonresponsive genes in the androgen-independent prostate cancer xenograft model CWR22-R. *Cancer Res* 2000, 60: 6134–6141
- Zegar-Moro OL, Schmidt LJ, Huang H, Tindall DJ: Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res* 2002, 62:1008–1013
- Palmberg C, Koivisto P, Kakkola L, Tammela TL, Kallioniemi OP, Visakorpi T: Androgen receptor gene amplification at primary progression predicts response to combined androgen blockade as second line therapy for advanced prostate cancer. *J Urol* 2000, 164: 1992–1995
- Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL, Visakorpi T: Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res* 2001, 61: 3550–3555
- Gregory CW, He B, Johnson RT, Ford OH, Mohler J, French F, Wilson EW: A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer Res* 2001, 61:4315–4319
- Gregory CW, Johnson RT, Mohler J, French F, Wilson EW: Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. *Cancer Res* 2001, 61:2892–2898
- Chugh A, Ray A, Gupta JB: Squalene monooxygenase as hypocholesterolemic drug target revisited. *Prog Lipid Res* 2003, 42:37–50
- Yamada KM, Araki M: Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. *J Cell Sci* 2001, 114: 2375–2382
- Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV: Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 1997, 15:356–362