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Short Communication

Differentially Expressed Genes in Hormone Refractory Prostate Cancer

Association with Chromosomal Regions Involved with Genetic Aberrations

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Differential gene expression between the androgen sensitive human prostate cancer cell line LNCaP and an insensitive clonal variant, LNCaP-r, was demonstrated by suppression subtractive hybridization. Twenty-one sequences were identified of which 9 are homologous to known genes, 11 are represented by expressed sequence tags (ESTs), and 1 is novel. We present data for 5 of 7 sequences confirmed to be differentially expressed by Northern blot analysis and semiquantitative RT-PCR. Only one gene, fibronectin (*FN*), was highly overexpressed (>60-fold) in LNCaP-r cells, consistent with previously reported overexpression of *FN* in prostate cancer. Four sequences were down-regulated in LNCaP-r cells, including an inactive variant of the E2 ubiquitin conjugating enzyme (*UEV-1*), a novel metalloproteinase-related collagenase (*PM5*), and a potential tumor suppressor gene (breast basic conserved gene, *BBC1*). *UEV-1* is multifunctional, regulates the cell cycle via cdk1, has homology to MMS2 and likewise functions as a DNA protection protein, and also has homology to *TSG101*. Aberrant splice variants of *TSG101* occur frequently in both breast and prostate cancer, but its mechanism of action is unknown. *FN*, *BBC1*, and *UEV-1* localize to regions of chromosomal aberration (2q3.4, 16q24.3, and 20q13.2, respectively) associated with advanced prostate cancer and thus may be highly relevant to disease progression. *(Am J Pathol 1999, 154:1335–1343)*

Androgens stimulate growth of both the normal prostate and prostate cancer (CaP).[1] An initial response to antiandrogenic hormonal therapy is observed in 70–80% of patients with advanced CaP, but progression to an androgen-insensitive (AI) state occurs within 12–18 months[2,3] and after relapse there is no effective curative treatment.[4] Mechanisms underlying the androgen-sensitive (AS)-to-AI transition are unknown[5] but are thought to involve the androgen receptor (AR) gene and its downstream signaling pathways. There are numerous reports of AR mutations resulting in altered AR function (AR mutation database)[6] but controversy exists as to the frequency of their occurrence.[6–9] Overexpression of AR due to gene amplification is observed in 30% of hormone refractory tumors, which would enhance the ability of CaP cells to sustain growth at substantially reduced concentrations of androgens.[10,11] AR-mediated gene transcription is regulated not only by androgens but also by polypeptide growth factors,[12,13] retinoic acid receptors,[14] and cyclic AMP.[15] Each of these factors may also affect AS-to-AI transition. However, this transition may not be attributable exclusively to AR dysfunction but could result from an accumulation of other genetic aberrations that confer a relative growth advantage independent of AR.[5,16] Genetic changes in CaP have been analyzed by allelotyping, loss of heterozygosity, and comparative genomic hybridization to identify target genes underlying the transition of CaP from the AS to the AI state. In early stage CaP, loss of 8p is characteristic whereas loss of 2q, 5q, 10q, 11p, 13q, 16q, 17p, and 20q, gains of 8q and chromosome 7, and mutation or amplification of the AR gene are associated with the progression of metastatic AI CaP.[17,18] In addition to somatic mutations, germline defects account for 9% of all CaP, and one-third of these

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Table 1. Gene-Specific Primers Used for SQRT-PCR

Code	Gene	bp	Sequence (5′ → 3′)	Reference
G0934	AR	19	AGC TAC TCC GGA CCT TAC G	34
G0935		21	AGG TGC CAT GGG AGG GTT AG	
B504	GAPDH	20	GCC ACA TCG CTC AGA CAC CA	34
B505		20	GAT GAC CCT TTT GGC TCC CC	
PSA3	PSA	20	CAC AGA CAC CCC ATC CTA TC	59
PSA5		20	GAT GAC TCC AGC CAC GAC CT	
FIBS	Fibronectin	18	TCA GGA AGC ATC GTT GTG	55
FIBAS		21	ACA CTT TCC TTG TCA TCC TTG	
U2	*UEV-1A/1As*	24	ATG CCA GGA GAG GTT CAA GCG TCT	33
U4		23	TTA ATT GCT GTA ACA CTG TCC TT	
BBC1-F	*BBC1*	18	TTT CCG CTC GGC TGT TTT	35
BBC1-R		19	CGA CTG ATT CCA AGT CCC C	
PM5-F	*PM5*	20	ACC GAT TCT GCC TGT CCA AG	46
PM5-R		20	TTT TCT CCC TCT CCT GCC TG	
BS57		10	GGAAGCAGCT	60
BS58		10	CAGTGAGCGT	
GAP-S	GAPDH	20	ACC ACA GTC CAT GCC ATC AC	Clontech
GAP-AS	GAPDH	20	TCC ACC ACC CTG TTG CTG TA	
NP1		22	TCG AGC GGC CGC CCG GGC AGG T	Clontech
NP2R		20	AGC GTG GTC GCG GCC GAG GT	

AR, androgen receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PSA, prostate-specific antigen; *BBC1*, breast basic conserved gene; *UEV-1A/1As*, ubiquitin enzyme variant.

cases are coincident with the hereditary prostate cancer gene locus at 1q24–25.[19]

The androgen-sensitive human CaP cell line LNCaP[20] has been used to study the control of androgen-responsive genes.[21–24] A clonal variant, LNCaP-r,[25] exhibits androgen insensitivity while expressing AR levels similar to those of LNCaP.[26,27]

Our objective was to use the LNCaP/LNCaP-r *in vitro* CaP model reflecting the *in vivo* situation of CaP progression from the AS to the AI state and to identify candidate genes that were differentially expressed. We have identified three genes, fibronectin (*FN*), breast basic conserved gene-1 *(BBC1*), and ubiquitin enzyme variant-1 (*UEV-1),* which have not previously been reported to be associated with the development of AI CaP. These three genes map to 2q, 16q, and 20q, chromosomal regions known to be altered in advanced CaP.

Materials and Methods

Cell Culture

The prostate carcinoma cell lines LNCaP, PC-3, and DU-145 were purchased from the American Type Culture Collection (Manassas, VA). LNCaP-r[25] was a gift from Dr. van Steenbrugge (Erasmus University, Rotterdam, The Netherlands). The SV40 immortalized primary prostatic epithelial cell lines PNT1A, PNT1B, PNT2, and PSVF1 were a gift from Professor N. Maitland (York University, York, UK). All cell lines were maintained in Dulbecco's modified Eagle's medium without phenol red supplemented with 10% fetal bovine serum and cultured to 95% confluence for RNA and DNA extraction.

Isolation of DNA and Arbitrarily Primed-Polymerase Chain Reaction (PCR)

Genomic DNA was isolated from all cell lines using a standard proteinase K procedure[28] and used as a template for arbitrarily primed-PCR.[29] Amplification mixtures consisted of 50 ng DNA, 10 mmol/L Tris-Cl (pH 9.0), 50 mmol/L KCl, 0.01% gelatin (w/v), 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 1.5 U *Taq* polymerase (Promega, Madison, WI) and 0.2 μmol/L of either arbitrary primer BS57 or BS58 (Table 1) in a total volume of 25 μl. Thermal cycling conditions consisted of 1 minute at 94.5°C, 1 minute at 40°C, and 1 minute at 72°C for 45 cycles with a final extension at 72°C for 10 minutes. Amplified products were analyzed on a 2% agarose gel and visualized with ethidium bromide (0.5 μg/ml).

Isolation of RNA, poly(A)$^+$ RNA, and cDNA Synthesis

Total RNA was isolated from both LNCaP-r and LNCaP cells by the RNAzol B method (Biogenesis, Friendswood, TX) as previously described.[30] Poly(A)$^+$ RNA was purified from total RNA using the Oligotex Direct mRNA purification procedure (Qiagen Ltd., Crawley, UK). Double-stranded cDNA was synthesized from 2 μg of poly(A)$^+$ RNA as previously described,[31] except that no tracer reaction was used and the samples were incubated at 16°C for 2 hours, followed by a further 30 minutes after the addition of T4 polymerase.

Generation of Subtracted Libraries for both LNCaP-r and LNCaP by Subtractive Suppression Hybridization (SSH)

SSH was performed as previously described[31,32] using the PCR-Select Subtraction protocol (Clontech, Palo Alto, CA) according to the manufacturer's recommendations. Differentially expressed genes were amplified from the subtracted cDNA using a nested PCR protocol (PCR-Select Subtraction).[32] Nested primers (NP1 and NP2R, Table 1) specific to adapters ligated only to the tester cDNA were used to amplify differentially expressed sequences. Thermal cycling conditions were 94°C for 10 seconds, 68°C for 30 seconds, and 72°C for 1.5 minutes for 30 cycles (initial PCR) and 12 cycles (nested PCR). One-third of the subtracted and unsubtracted products were separated on a 1.5% agarose gel.

Evaluation of Subtraction Efficiency

Subtraction efficiency was determined by the PCR analysis of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression in subtracted and unsubtracted cDNA libraries of both LNCaP-r and LNCaP cell lines as described in the PCR-Select Subtraction protocol (Clontech).

Cloning and Colony PCR

Each positively subtracted cDNA library (LNCaP-r and LNCaP) was ligated into the pT-Advantage vector, a TA cloning system, and transformed into TOP10F′ super competent cells (Clontech). Aliquots (100 μl) of Lauria Bertani media supplemented with ampicillin (50 μg/ml) were inoculated with positive colonies and incubated overnight at 37°C. Plasmids containing cloned sequences from each subtracted library were identified using a colony PCR protocol based on the PCR-Select Differential Screening protocol (Clontech).[31] Briefly, inserts were amplified from each clone, 1× AmpliTaq Gold PCR buffer (10 mmol/L Tris (pH 9.0), 50 mmol/L KCl, 0.01% gelatin, 0.1% Triton X-100; Perkin Elmer, Foster City, CA), 1.5 mmol/L $MgCl_2$, 500 mmol/L, 200 μmol/L dNTPs, 0.3 μmol/L each nested primer (NP1 and NP2R) (Table 1) and 2.5U AmpliTaq Gold polymerase (Perkin Elmer) in a final volume of 20 μl. Thermal cycling was performed at 95°C for 10 minutes (×1), 94°C for 1 minute and 68°C for 3 minutes (×27 cycles). PCR products (5 μl) were separated on a 1.5% agarose gel.

Dot Blots of Positive Colony PCR Products

Each positive colony PCR product was denatured in an equal volume (10 μl) of 0.6 M NaOH and 0.5% bromophenol blue and 1 μl dot-blotted onto a nylon membrane (GeneScreen Plus, DuPont, NEN Research Products, Boston, MA). Two Clontech control cDNAs were used, C1 (340 bp) and C2 (200 bp), corresponding to a human homologue of the mouse testis specific protein (GenBank accession number X52128) and human seminal vesicle-specific semenogelin II mRNA (GenBank accession number M91652). Membranes were prepared in quadruplicate, soaked in 0.5 mol/L Tris (pH 7.4) for >5 minutes, washed with water, and UV cross-linked (120 mJ) in a Stratalinker (Stratagene).

Reverse RNA Dot Blot Analysis and Sequencing Positive Clones

Probes for both subtracted and unsubtracted libraries from both LNCaP-r and LNCaP cell lines were prepared as described in the PCR-Select Differential Screening protocol (Clontech). RNA dot blot membranes were prehybridized in Express Hybridization solution (Clontech) supplemented with 0.2× SSC, 0.01 volume of blocking solution (100 μg/ml denatured sheared salmon sperm DNA and 3 μg/ml primers NP1 and NP2R) (Table 1) at 72°C for >60 minutes. Probes were labeled by random priming with ^{32}P-dCTP, heat denatured, mixed with SSC and blocking solution (50 μl 20× SSC and 50 μl blocking solution), added to the membranes, and allowed to hybridize at 72°C overnight. Membranes were washed at 68°C four times (20 minutes each) with 100 ml of low-stringency (2× SSC and 0.5% sodium dodecyl sulfate (SDS)) washing solution followed by twice (20 minutes each) with high-stringency (0.2× SSC and 0.5% SDS) washing solution. Membranes were exposed to X-ray film from 1 to 12 hours at −70°C with intensifying screens.

Plasmid DNA was extracted from positive clones, identified by reverse RNA dot blot analysis using the Qiagen MiniPrep purification procedure, and then sequenced using an ABI 377 automatic sequencer (Perkin-Elmer). Sequences were compared with the GenBank, EMBL, dbEST, and THC databases using a BLAST search.

Northern Blot Analysis

Total RNA (30 μg/lane) was separated by denaturing (formaldehyde) gel eletrophoresis, transferred to GeneScreen Plus membranes, and hybridized with ^{32}P-labeled probes of either an *Eco*RI fragment of DNA from a positive clone or a 0.8-kb *Eco*RI/*Hin*dIII fragment of GAPDH. Membranes were prehybridized for ≥1 hour and hybridized for ≥12 hours in a buffer containing 2× SSC, 50% v/v formamide, 10% dextran sulfate, 0.1% SDS, and 100 μg/ml denatured sheared salmon sperm DNA at 42°C. Posthybridization washes consisted of 2× SSC for 10 minutes at room temperature, followed by 1 or 2 washes with 2× SSC and 1% SDS for 20 minutes at 65°C. In some cases in which higher stringency was required, two extra washes were performed with 0.2× SSC and 1% SDS for 1 hour at 65°C. Membranes were then exposed to film for 1–7 days at −70°C with intensifying screens and the resultant autoradiograms were analyzed on a Molecular Dynamics densitometer with Image Quant software.

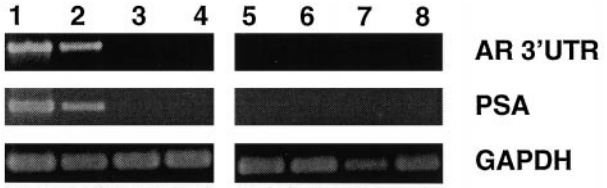


Figure 1. SQRT-PCR analysis of androgen receptor (AR), prostate specific antigen (PSA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Lane 1: LNCaP; Lane 2: LNCaP-r; Lane 3: PC-3; Lane 4: DU-145; Lane 5: PNT1A; Lane 6: PNT1B; Lane 7: PSVF1; Lane 8: PNT2.

Semiquantitative Reverse Transcriptase (SQRT)-PCR

Oligo $dT_{12–18}$-primed cDNA was synthesized from total RNA as previously described[30] and stored at −20°C until use. Aliquots of the cDNA were used for PCR amplification of either the 1.3-kb of the AR-3′ untranslated region or the 483-bp of GAPDH as previously described for 30 and 25 cycles, respectively.[30] All cDNAs were normalized to give the same relative expression of GAPDH before they were used to determine the relative expression of other genes of interest.

Prostate-specific antigen (PSA) PCR consisted of 1× AmpliTaq Gold PCR buffer (10 mmol/L Tris (pH 9.0), 50 mmol/L KCl, 0.01% gelatin, 0.1% Triton X-100; Perkin Elmer), 1.5 mmol/L $MgCl_2$, 500 mmol/L, 200 μmol/L dNTPs, 0.2 μmol/L each primer (PSA3 and PSA5; Table 1) and 2.5 U AmpliTaq Gold polymerase (Perkin Elmer) in a final volume of 25 μl. A hot start, touch down thermal protocol was used consisting of 1 cycle for 10 minutes at 95°C, 2 cycles for 1 minute at 95°C, 1 minute at 61°C, 2 minutes at 72°C, followed by a decrease in annealing temperature of 2°C every 2 cycles until 55°C, then continued for a further 27 cycles. Fibronectin (*FN*), basic breast conserved gene (*BBC1*), and ubiquitin enzyme variant (*UEV-1*) were amplified using the same reaction conditions as used for the PSA PCR; the primers for each gene are listed in Table 1. Thermal cycling for these genes consisted of a hot start protocol, 1 cycle at 95°C for 10 minutes, followed by 25–35 cycles at 95°C for 1 minute, 55–60°C for 1 minute, and 72°C for 2 minutes, with a final extension at 72°C for 5 minutes.

PCR products were separated on a 2% agarose gel and visualized by staining with ethidium bromide (0.5 μg/ml). To verify PCR products, bands migrating at the predicted sizes were gel-purified using the Hybaid II gel purification system (Hybaid, Ashford, UK) and sequenced on an ABI 377.

Results

Characterization of LNCaP and LNCaP-r Cell Lines

LNCaP and LNCaP-r cell lines express the full length AR message, although the expression is detectably higher in LNCaP than LNCaP-r as determined by SQRT-PCR normalized for GAPDH expression (Figure 1). PSA expression, an androgen responsive gene, is approximately threefold lower in LNCaP-r compared to the LNCaP cell line (Figure 1). In PC-3, DU-145, and all of the immortalized epithelial prostatic cell lines, AR and PSA expression were undetectable using these PCR conditions (Figure 1).

AP-PCR with primer BS58 resulted in a DNA profile that was similar in LNCaP, LNCaP-r, and PC-3 but different for DU-145 and the immortalized prostatic cell lines (data not shown). With primer BS57 the DNA profile for PC-3 was different than that of LNCaP and LNCaP-r (data not shown). Based on these data it is evident that LNCaP and LNCaP-r have similar predicted DNA profiles.

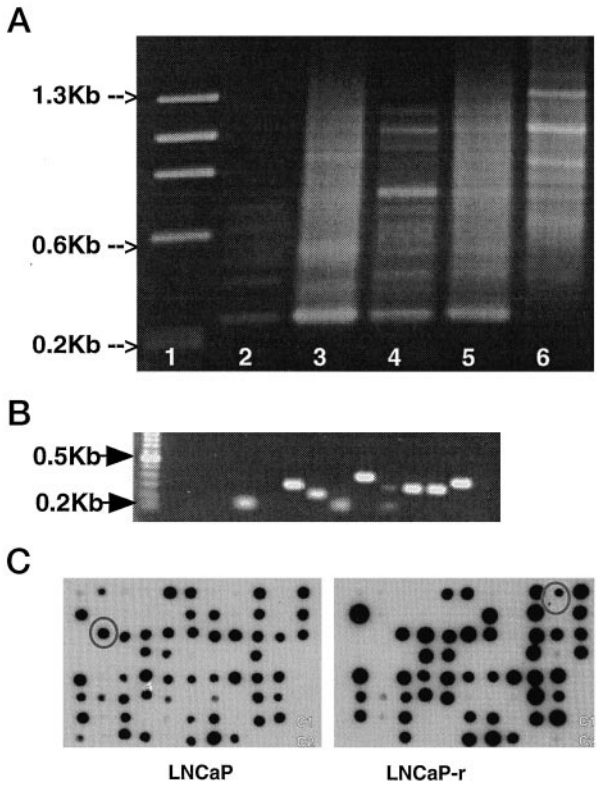


Figure 2. A: Identification of differential gene expression in LNCaP (L) (Lanes 2 and 3) and LNCaP-r (R) (Lanes 4 and 5) cells by SSH before cloning. PCR products of either subtracted (S) (Lanes 2 and 4) or unsubtracted (U) (Lanes 3 and 5) cDNA libraries are separated on a 1.5% TAE agarose gel. Lane 1: ϕX174 *Hae*III markers; Lane 6: positive control for subtraction (ϕX174 *Hae*III markers in placental cDNA). Primers and PCR conditions are described in Materials and Methods. B: An example of colony PCR, the products of which range from 0.2–0.8 kb. C: Reverse Northern screening assay for differentially expressed genes. Replica DNA dot blots were prepared and probed with either DNA from LNCaP (left panel) or LNCaP-r (right panel) subtracted libraries. Clones 49L and 17R are circled on left and right blots, respectively.

Identification of Differentially Expressed Genes by SSH

There are clear differences between the subtracted and unsubtracted cDNA libraries (Figure 2A). The observable PCR products in the LNCaP and LNCaP-r subtracted libraries ranged between 0.3–0.7 kb and 0.3–1.3 kb, respectively (Figure 2A).

Table 2. Characteristics of Differentially Expressed Genes in Androgen Dependent LNCaP and Androgen Independent LNCaP-r Prostate Cancer Cells

Clone code	Insert (bp)	Homology (bp/bp)	Mapped to	Homology (accession code)*	mRNA size	RNA species† detected (kb)	%‡	Reference
17R	450	352/371	3276–3647	fibronectin (HSFIB1)	:8kb	9–9.5 (R > L)	6000	55
12L	370	356/368	2066–2436	Hs*UEV-1* (HS49278)	:3.5kb	3.0 (L > R)	300	33
28R	300	298/300	410 –708	*BBC1* (HSBBC1)	:1.3kb	0.5 (R > L)	200	35
49L	643	634/643	1815–2449	*PM5* (HSPM5)	:4kb	6.0 (L > R)	168	46
85L	353	304/306	388 –692	proteasome subunit HsC10-II (HSPSH1)	:0.7kb	1.0 (L > R)	125	61
79L	284	277/279	925–1002	Histone H1.2 (HSH12)	:1.3kb	1.0 (L > R)	108	62
13L	284	284/284	1517–2028	$TAF_{II}30$ (HS13391)	:0.75kb	1.0 (L > R)	106	63

Differentially expressed genes were cloned and their expression verified by reverse Northern and Northern blot analysis. The inserts were sequenced and homology to other genes determined with a BLAST search of GenBank, EMBL, dbEST, and THC databases.

*All codes relate to the EMBL database.

†In which cell type the expression is greater, LNCaP (L) or LNCaP-r (R).

‡% increase normalized for GAPDH expression and the average of two samples for both LNCaP and LNCaP-r.

Estimation of Subtraction Efficiency

GAPDH is a nondifferentially expressed gene. Using a RT-PCR assay its level of expression in LNCaP/LNCaP-r libraries was used to determine the SSH subtraction efficiency. Its expression in both LNCaP and LNCaP-r subtracted libraries was detectable only after 33 cycles of PCR, whereas in the unsubtracted libraries higher levels were detected after only 18 cycles (data not shown).

Cloning and Colony PCR

A total of 140 colonies were isolated from both LNCaP-r and LNCaP subtracted libraries cloned into the pT-Advantage vector. The average insert size, as determined by colony PCR, was 300 bp (Figure 2B). Positive colony PCR products 59 and 28 from LNCaP and LNCaP-r subtracted libraries, respectively, were isolated for reverse Northern analysis.

Reverse RNA Dot Blot Analysis

Quadruplicate filters containing LNCaP selected (49L) and LNCaP-r selected (17R) sequences were probed with either LNCaP or LNCaP-r subtracted libraries (Figure 2C) and the unsubtracted LNCaP and LNCaP-r cDNA libraries (data not shown). After 4 hours' exposure at −70°C, 49L and 17R were clearly visible on both blots (Figure 2C). A total of 14 LNCaP-positive and 7 LNCaP-r-positive clones (ie, differentially expressed) were isolated on the basis of the reverse RNA dot blot analysis. Positive clones represented approximately 30% of the colony PCR products and the remainder were species common to both the tester and driver cDNA populations. The selective nature of these blots was confirmed by the low background of the filters and by the lack of detection of two negative control cDNAs (C1 and C2) included on each filter (Figure 2C) even after 12 hours' exposure (data not shown).

Northern Blot Analysis

To verify differential expression of clones identified by reverse RNA dot blot analysis, *Eco*RI-excised inserts were labeled and used to probe total RNA from LNCaP and LNCaP-r cells (Table 2 and Figure 3A). The expression levels of the differentially expressed genes between the two cell lines ranged from >6000% (17R) to only 6% (13L) (Table 2 and Figure 3A). The detected transcript sizes are shown in Figure 3A and the expected transcript sizes based on sequence homology for each clone are listed in Table 2. The levels of differential gene expression determined by the inserts from clones 79L and 13L encoding Histone H1.2 and the transcription factor $TAF_{II}30$, respectively, were below 10%, interpreted as within the range of experimental error. The lowest detectable change in expression, a 25% increase (LNCaP > LNCaP-r) using the insert from clone 85L, corresponded to a human proteasome subunit HsC10-II, which plays a role in the nonlysosomal pathways of protein turnover (Table 2 and Figure 3A).

SQRT-PCR analysis was used to verify specific differential expression of *FN, UEV-1*, *BBC1*, and *PM5* genes encoded by clones 17R, 12L, 28R, and 49L, respectively. Results are detailed below in order of decreasing differential expression as demonstrated between LNCaP and LNCaP-r cell lines.

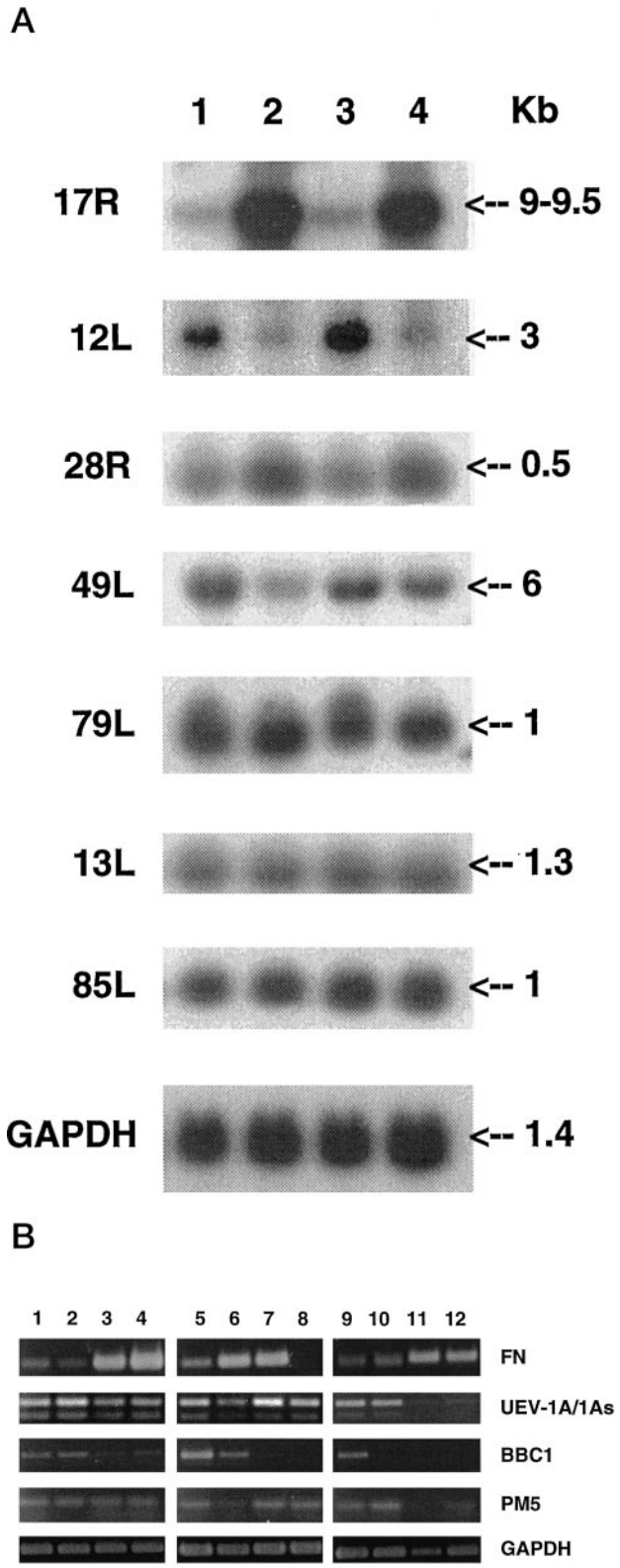
Fibronectin (Clone 17R)

There was a >60-fold increase in *FN* expression in LNCaP-r compared to LNCaP by Northern blotting (Figure 3A), verified by SQRT-PCR using two sets of primers spanning nucleotides 3323–3690 (Figure 3B) and the *FN* IIICS region (data not shown). The hormone-insensitive cell lines PC-3 and DU-145 were also analyzed and abundant expression of *FN* was detected in the PC-3 cell line but there was no detectable *FN* in DU-145 (Figure 3B). The SV40 immortalized cell lines PSVF1 and PNT2 expressed *FN* at levels comparable to those found in PC-3 and LNCaP-r, although lower *FN* levels were observed in PNT1A and PNT1B cells (Figure 3B).

UEV-1 *(Clone 12L)*

A threefold difference in expression, LNCaP > LNCaP-r, was demonstrated by Northern analysis using the insert

from clone 12L (Figure 3A), which maps to nucleotides 2066–2436 in the 3′UTR of *UEV-1*.[33] Published primer sequences were used to determine which of the four known splice variants is responsible for this differential gene expression.[33] Differential expression (LNCaP > LNCaP-r) was verified with primers specific for splice variants 1A/1As (Figure 3B) and not with primers for 1B/1Bs variants (data not shown). Expression was highest in PC-3, moderate in LNCaP, DU-145, PNT1A, and PNT1B, lower in LNCaP-r, and undetectable in PSVF1 and PNT2 (Figure 3B). Expression of *UEV-1*A does not appear to correlate with either AR or PSA gene expression in these cell lines.



BBC1 *(Clone 28R)*

The insert of clone 28R mapped to the 3′ coding region (nucleotides 410–709) of the 1.3-kb *BBC1*,[34] which shares homology with multiple ESTs of unknown function. Northern analysis using the *Eco*RI fragment from clone 28R revealed a twofold increase in expression (LNCaP-r > LNCaP) of a 0.5-kb gene (Figure 3A). SQRT-PCR analysis with *BBC1*-specific primers[35] indicated that *BBC1* (full coding sequence) was down-regulated in LNCaP compared with LNCaP-r (Figure 3B), in contrast to the Northern blotting data (Figure 3B), suggesting that the differential expression identified by SSH is due to the expression of one or more *BBC1*-like sequences. However, *BBC1* expression in PC-3, DU-145, and the SV40 immortalized cell lines was detectable only in PNT1A cells (Figure 3B).

PM5 *(Clone 49L)*

PM5 expression was 68% higher in LNCaP than LNCaP-r cells, as determined by Northern blot analysis, a difference that is difficult to verify by PCR (Figure 3B, lanes 1–6). Its levels varied in the other cell lines examined (Figure 3B). The *PM5* profile in the SV40 immortalized cells correlates directly with *UEV-1A* and inversely with *FN* expression.

Discussion

We have used SSH and identified five genes that are differentially expressed between LNCaP and LNCaP-r cell lines. SSH normalizes and positively selects differentially expressed genes in contrast to differential display, in which the relative abundance of genes affects the results.[36] In conjunction with reverse RNA dot blot analysis, SSH offers a high-throughput screening procedure for both high- and low-abundance transcripts.[31,32] The high background of clones common to both tester and

Figure 3. A: Northern blot analysis of differentially expressed sequences (Table1) in paired isolates of LNCaP and LNCaP-r. Total RNA (30 μg) for LNCaP and LNCaP-r was electrophoresed on a 1% denaturing gel, blotted onto nylon filters, and probed with *EcoR*I inserts isolated from the clones listed in Table. 1. All blots were reprobed with a control GAPDH cDNA probe (*EcoR*I-*Hind*III). Arrows, positions, and estimated sizes of detected mRNAs. Lanes 1 and 3: LNCaP; Lanes 2 and 4: LNCaP-r. B: SQRT-PCR analysis of fibronectin (FN), ubiquitin enzyme variants (UEV-1A/1As), breast basic conserved gene (BBC-1), PM5, and GAPDH. Lanes 1, 2, and 5: LNCaP; Lanes 3, 4, and 6: LNCaP-r; Lane 7: PC-3; Lane 8: DU-145; Lane 9: PNT1A; Lane 10: PNT1B; Lane 11: PSVF1; Lane 12: PNT2.

driver is due to limited differences between the tester and driver mRNAs, as LNCaP-r is a clone derived from LNCaP. From an initial 140 clones isolated, approximately 15% were positive at the reverse Northern stage and a total of 10% after Northern blot analysis.

We have demonstrated that LNCaP-r cells express AR mRNA at levels similar to those found in LNCaP, but the expression of PSA is dramatically reduced in the former. LNCaP-r cell growth is independent of steroids and not stimulated by the synthetic androgen R1881.[26] Therefore, genes differentially expressed between these two CaP cell lines are potentially representative of genes expressed *in vivo* in hormone refractory tumors which express functional AR but are not androgen-sensitive.[7,37–39]

FN-integrin interactions are important in tumor migration, invasion, and metastasis. Peptide and antibody inhibitors of *FN* and integrin interactions are effective inhibitors of metastasis[40] and reduced cellular expression of *FN* is associated with carcinogenesis in some cancers, such as colorectal and breast tumors.[41] In contrast, levels of *FN* are significantly greater in CaP tissue than in normal or hyperplastic prostate tissue[42,43] and MDA PCa2a prostate cancer cells have been shown to express high levels of *FN*.[44] We have demonstrated that LNCaP-r cells overexpress *FN* (>60-fold as compared with LNCaP). *FN*-integrin mediated cell adhesion triggers intracellular signaling events, such as activation of the *ERK2/MAP* kinase cascade via focal adhesion kinase (*FAK*), which modulate *FN*-mediated gene transcription.[45]

PM5 (clone 49L) is a 4-kb metalloproteinase-like collagenase that was isolated from a melanoma cDNA library by screening with oligonucleotide sequences to the metal-binding domain of human fibroblast collagenase. Expression in CaP has not been reported before but there is no difference in *PM5* mRNA expression in normal and malignant colorectal tissue.[46] In this study *PM5* expression was differentially expressed between LNCaP and LNCaP-r cells. The substrate for this enzyme is unknown and it is conceivable that it may degrade a matrix which promotes motility; its down-regulation in CaP would be consistent with tumor progression.

BBC-1, which is located at 16q24.3, was initially suggested to be a potential tumor suppressor gene in breast cancer,[34] although recent data have given rise to dispute about this role.[35] In CaP loss of heterozygosity at 16q has been observed in several studies[47–50] and 16q allelic loss is significantly higher in cancer death cases than in early stage tumors.[49] Loss of heterozygosity at 16q24.3 is significantly higher in metastatic than localized prostate tumors.[51] Expression of *BBC1* was down-regulated in LNCaP compared with LNCaP-r cells, supporting further study of *BBC1* as a tumor suppressor gene in CaP.

UEV-1 (or *CROC-1*),[33,52] an inactive variant of the E2-conjugating enzymes, is located on chromosome 20q13.2 and is expressed as four isoforms generated by alternative splicing.[33] *UEV-1A* is down-regulated in differentiating colon carcinoma cells and expressed in a cell cycle-dependent manner.[33] We have demonstrated that the threefold higher expression of *UEV-1* in LNCaP compared with LNCaP-r cells is due to variations in the 1A/1As and not the 1B/1Bs isoforms. Constitutive overexpression of the *UEV-1A* gene inhibits the activity of the mitotic kinase cdk-1, resulting in G_2-M growth arrest.[33] LNCaP-r cells possess a higher growth rate than LNCaP cells (data not shown), an effect which may be due to diminished inhibition of cdk-1 by reduced levels of *UEV-1A*. A similar effect *in vivo* would provide a growth advantage for hormone-insensitive CaP cells.

The role of *UEV-1* is not limited to regulation of the cell cycle. This gene was originally identified by its ability to transactivate a *c-fos* promoter.[52,53] Thompson et al[54] have demonstrated that *UEV-1A* protects cells from DNA-damaging agents and acts through a pathway common to the human *MMS2* gene.[53] Down-regulation of *UEV-1A,* as we have observed, could render LNCaP-r cells susceptible to mutation. *UEV-1* is structurally related to the tumor suppressor gene *TSG101*[55] and deletions and aberrant splicing patterns of the *TSG101* gene have been reported in both breast cancer[56,57] and CaP.[58] Cher et al[17] demonstrated that loss at chromosome 20q is frequently associated with advanced CaP, further implicating *UEV-1* as a candidate gene in the progression to AI.

In conclusion, we have identified genes (*FN*, *UEV 1*, and *BBC1*) that correspond to regions of genetic alteration found in both LNCaP-r cells and advanced CaP. The functional role of these genes in the development of AI CaP needs further evaluation.

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