AD_____

Award Number: DAMD17-98-1-8565

TITLE: Protein Interaction with the N-Terminus of the Androgen Receptor

PRINCIPAL INVESTIGATOR: Paul H. Gumerlock, Ph.D.

CONTRACTING ORGANIZATION: The University of California
Davis, California 95616

REPORT DATE: March 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2002	Final (1 Sep 1998 - 28 Feb 2002)

4. TITLE AND SUBTITLE
Protein Interaction with the N-Terminus of the Androgen Receptor

5. FUNDING NUMBERS
DAMD17-98-1-8565

6. AUTHOR(S)
Paul H. Gumerlock, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of California
Davis, California 95616

E-Mail: paul.gumerlock@ucdmc.ucdavis.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

This project, which began with the goal of identifying proteins that bind to the human androgen receptor (hAR) differentially based on the length of the CAG repeat sequence, has been instrumental in revealing new biological findings of the role of the androgen receptor in prostate cancer progression to androgen-independence. Three significant findings were these: 1. Failure to identify any binding proteins for the Gln repeat region of the human androgen receptor. Our conclusion was that the continued search for such binding proteins is not warranted at this time, however further study of ARA24 is warranted. 2. The role of the RB pathway growth arrest in the androgen response in prostate cancer. The occurrence of cell death following the growth arrest, may provide a biology that can be exploited for prostate cancer patient therapy. 3. Bag1L binds to hAR is induced by gain-of-function p53 mutants, and contributes to androgen-independent growth of prostate cancer cells. We believe these findings make important and extremely valuable contributions toward understanding the role of the androgen receptor in hormone-refractory prostate cancer. These multiple findings provide clearer foundations on which to build and propose new projects for study of this important aspect of human prostate cancer.

14. SUBJECT TERMS
Androgen receptor, hAR, CAG repeats, RB, Bag1L, gain-of-function p53

15. NUMBER OF PAGES
56

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

FINAL REPORT

DAMD17-98-1-8565
Protein Interactions with the N-terminus of the human androgen receptor
P.I.: Paul H. Gumerlock, Ph.D.

I. INTRODUCTION

This specific goal of this project was to identify proteins that bind to the N-terminus of the human androgen receptor (hAR) including the region encoded by the CAG repeat sequence. The length of the CAG repeat sequence inversely correlates with the aggressiveness of prostate cancer in different ethnic groups, and somatic shortening of the CAG repeat length has been seen in the tumor cells compared to normal constitutional DNA from some prostate cancer patients. This led to our *hypothesis* that there would be a protein(s) that binds differentially to the hAR proteins containing different numbers of glutamine (Gln) residues encoded by the CAG repeat. The identification of a protein that down-regulates hAR activity through the Gln repeats may lead to more effective approaches to complete androgen blockade than the currently used approaches that target the carboxy-terminal hormone-binding domain of the receptor. This may then lead to improved therapy for prostate cancer patients. The scope of the research was the following: 1) to identify N-terminal binding proteins using a yeast two-hybrid system, 2) to confirm the interaction of the candidate proteins with the androgen receptor in mammalian cells using a mammalian two-hybrid system, 3) to investigate differential binding of these proteins to fragments of hAR with varied numbers of glutamine residues, and 4) to examine resultant levels of gene transcription activation by the different Gln length hAR molecules. Despite multiple attempts, including necessary modifications of protocols, we were unable to identify CAG repeat binding proteins using the yeast two-hybrid approach. Thus the focus of the project was on hAR-binding proteins and there role in the biology of androgen-independence, including the tumor suppressor and cell cycle regulator RB and the anti-apoptosis-associated and potentially hAR sensitizing protein Bag1L. These studies implicate both of these proteins as playing key roles in the androgen-independence of prostate cancer.

II. BODY

Our *hypothesis* was that unique proteins exist that bind preferentially to the longer polyglutamine repeat sequences and function to negatively regulate the transcription activity of the androgen receptor. The hypothesis was to be tested using the following technical objectives through the specific tasks listed below.

Objective 1: Identification of proteins that bind to the androgen receptor exon 1 encoded polypeptide with a glutamine repeat length of 25.

Task 1: To identify proteins encoded by prostate-derived cDNAs that bind the N-terminus of the androgen receptor with 25 glutamine repeats using the yeast two-hybrid system.

Due to the goal to isolate unique proteins with binding specificity to a small N-terminal region including the Gln repeat region, we chose to use as bait the region of hAR upstream of that where the transcription factor TFIIF had been shown to bind (**1**). This necessitated the cloning of the region from known hAR variants to obtain wild-type sequence clones of the region with different CAG repeat lengths. RT-PCR was used to amplify the target sequences for cloning the DNA encoding the N-terminus of the receptor. To identify androgen receptors with long and short glutamine repeats, RNAs from several prostate cancer cell lines were examined.

PCR cloning of the N-terminus of androgen receptor and construction of the bait plasmids. To construct bait plasmids for yeast two-hybrid screening, we cloned the DNA fragment encoding the N-terminal 90 amino acids plus the Gln repeat sequence of hAR (**Figure 1, Appendix**). The yeast MACTHMAKER two-hybrid system was purchased from Clontech.

The prostate cancer cell lines which express hAR were used to obtain the N-terminus of hAR with longer (> 25 x CAG) and shorter (< 18 x CAG) repeats. Total RNA was prepared from LNCaP cells and PC3-AR2 cells transfected with hAR, respectively. RT-PCR was performed with primers designed to create the restriction enzyme sites for cloning. Agarose gel electrophoresis analysis of the PCR products showed that the DNA fragments generated by the RNA from LNCaP cells were slightly larger than that from the PC3-AR2 cells (**Figure 2**). The PCR products were subcloned into the two-hybrid vector pAS2-1, which created the fusion product of the Gal4 DNA binding domain and the N-terminus hAR region. Subsequent DNA sequencing revealed that the N-terminus of hAR from LNCaP and PC3/hAR cells contained 26 and 16 CAG repeats, respectively. The yeast strain containing reporter genes, beta-galactosidase and *His3* genes, was transformed with these bait plasmids individually. **Figure 3** shows X-gal assays of those yeast transformants. The yeast strains containing pAS2-1 with 26 CAG repeats (L1-1 and L1-2), the strain pAS2-1 with 16 CAG repeats (S7-1 and S7-2) and the beta-galactosidase positive strains (P1 and P2) were grown on plates containing synthetic glucose medium lacking tryptophane (SD-Trp Medium: Synthetic yeast medium with 2% glucose lacking tryptophane). The plate was subjected to the filter lifting X-gal assay. This assay confirmed that these bait plasmids by themselves did not activate the beta-galactosidase reporter gene (**Figure 3**).

Amplification of the cDNA library. A pACT2 cDNA library (human prostate) in *E. coli* was purchased from Clontech. The library was amplified by culturing the bacteria on the solid bacteria medium, and cDNA library plasmids were purified by CsCl gradient centrifugation.

Yeast two-hybrid screening.

1) **Strategy of yeast transformation.** Our hypothesis is that unique proteins bind preferentially to the longer glutamine (CAG) repeats and negatively regulate the transcription activity of the androgen receptor. Therefore to screen a prostate library, we

used the host yeast strain which carries a bait plasmid with 26 CAG repeats, which was designated as pAS2-1L1.

2) **Problems with the yeast transformation.** To set up the screening we faced some difficulties. First, the yeast transformation protocol provided by Clontech did not yield an efficient transformation frequency and was not sufficient to completely screen the library (< $1x10^{2}$ cfu/mg DNA). To obtain sufficient numbers of transformants to cover the number of independent clones in the library ($3.5x10^{6}$), the transformation frequency needed to be >$1x10^{4}$ cfu/mg DNA. This required additional work to establish the transformation protocol. We tried several conditions for culturing the yeast cells, as well as, different transformation procedures. With a protocol we developed, we obtained a transformation frequency of approximately $1x10^{4}$ cfu/mg DNA. The details of this protocol are provided below. Transformation with the cDNA library plasmids was then performed twice in order to obtain a sufficient number of transformants (total 6.6×10^{6}).

Transformation A = $7.2x10^{3}$ cfu/mg
Transformation B = $1.9x10^{4}$ cfu/mg

3) **Description of the yeast transformation protocol developed for the two-hybrid screening.**

-The host yeast CG1954/pAS2-1L1 was cultured in 100ml of the synthetic minimum medium lacking tryptophane (SD-Trp) overnight at 30°C.
-These yeast cells were transferred to 1000ml of fresh SD-Trp medium and cultured overnight at 30°C.
- Yeast cells were collected, reinoculated into 500ml of YPD medium and cultured until the A_{660} reached approximately 1.0.
- Refreshed yeast cells were collected by centrifugation and resuspended in 50 ml of One-Step Transformation buffer, which consists of 0.2N LiAc, 40% PEG, 100mM DTT.
-2ml of the cell suspension was aliquoted into 25 tubes and 1mg of salmon sperm DNA (ssDNA) and 10mg of pACT2cDNA library plasmid were added to each tube.
- Cells were incubated for 30min at 45°C.
- 400ml of the cell suspension was plated onto each of the 100 plates (14cm diameter) containing SD-trp-leu-his medium.
- To determine the transformation efficiency, transformed yeast cells were diluted and plated onto SD-trp-leu plates.
- All the plates were incubated for 7-14 days at 30°C.

4) **Problems with screening of the library.**

The *His*$^{-}$ phenotype of the host yeast strain CG1945, which is a prime selection marker for the two-hybrid interaction is leaky, which results in the growth of false positive clones on the selection medium. To suppress this growth, the histidine analogue 3-aminotriazole (3-AT) can be added to the selection medium. However, this treatment has the possibly of eliminating positive clones, which have a weak interaction between the bait and the binding proteins expressed by library cDNAs. We decided not to add 3-AT into the selection medium. Almost all of the transformants which grew to a 2mm diameter or larger were tested for beta-galactosidase by X-gal assay. (The transformants which stay smaller than 2mm in diameter are false positives according to the instruction

manual.) In the case of the selection plates, which contained a large number of transformants of greater than a 2mm diameter, whole plates were subjected to the X-gal assay. Colonies staining blue indicated the positive clones containing a cDNA that encoded a protein that bound with the N-terminus of hAR.

Identification of proteins expressed in the prostate that interact with the N-terminus of the human androgen receptor. A summary of the yeast two-hybrid screening is shown in **Table 1 (Appendix)**. The four positive clones identified were re-streaked and cultured on SD-trp-leu-his plates and the plate was again subjected to the X-gal assay (**Figure 4**). Again these clones showed growth in the absence of histidine and the blue staining indicated a positive clone.

Task 2: The positive scoring prostate-derived cDNAs will be sequenced to identify the encoded protein.

Recovery of the plasmids carrying prostate cDNAs from the positive yeast clones and restriction enzyme digestion analysis of the recovered plasmid DNAs. To characterize the cloned prostate-derived cDNAs, plasmids DNAs were isolated from the four positive scoring yeast clones, A04, A08, B24, and pACT-B73, and the isolated plasmids were named pACT-A04, pACT-A08, pACT-B24, and pACT-B73, respectively. The positive yeast clones isolated by two-hybrid screening contained two kinds of plasmids: bait plasmid and plasmids derived from the prostate cDNA library. In order to isolate only the library-derived plasmid that has a LEU2 marker for plasmid selection, *E. coli* strain KC8 that has the Leu- phenotype was transformed with yeast extracts prepared from the four positive clones. Plasmid DNAs were purified from the resulting Leu+ bacterial colonies. The recovered plasmids were subjected to restriction enzyme digestion analysis. BglII, whose site flanks the cloning site, and BglII/StuI digestions demonstrated that these four plasmids likely contained identical cDNAs derived from the prostate library (**Figure 5**).

DNA sequence analysis of the prostate-derived cDNAs inserted into the plasmids. These plasmids, pACT-A04, pACT-A08, pACT-B24, and pACT-B73 were subjected to DNA sequencing. DNA sequencing was performed for both directions using MATCHMAKER 5' and 3' AD LD-Insert Screening Amplimers as sequencing primers. However, these primers generated no sequences with the pACT-A04, pACT-A08, pACT-B24, and pACT-B73 plasmids as templates. The second sequencing trial with GAL4 AD Sequencing Primer, whose priming site is located approximately 16 bp upstream of the MATCHMAKER 5' AD LD-Insert Screening Amplimers priming site on the pACT2 vector, generated a weak identical sequence with all the plasmids. DNA database analysis showed that the cloned DNA sequences matched to a portion of the pACT2 plasmid DNA sequence. The result was confusing because the restriction enzyme digestion analysis clearly showed the existence of an insert at the cloning site of the plasmid, which is flanked by the primers used for DNA sequencing. However, we did not find any sequences derived from the prostate cDNA library. These results indicated the following:

a) The sequences corresponding to the MATCHMAKER 5'and 3" AD LD- Insert Screening Amplimers do not exist on the isolated plasmids.

b) The DNA sequence downstream of the GAL4 AD Sequencing Primer site was completely different from the sequence that is described in pACT2 AD vector information of the Clontech manual.

Therefore we made a contact with Clontech technical assistant service and after several correspondences regarding this result, we concluded that a DNA rearrangement could have occurred during propagation of the plasmids in bacterial cells.

PCR analysis of the insert cDNAs recovered directly from the positive scoring yeast clones. To detect prostate-derived cDNAs in the cloning vector, we performed PCR analysis with the plasmids recovered directly from the positive scoring yeast clones. The positive yeast clones, A04, A08, B24, and B73, were patched on the yeast minimal medium lacking leu and trp to ensure the maintenance of the plasmids. Positive and negative control yeast strains were prepared as well. The positive control yeast contained the pACT2 derivative pTD1-1 which has an approximately 2 kb insert between sequences corresponding to the MATCHMAKER 5'and 3" AD LD- Insert Screening Amplimers. The negative control yeast contained the pAS2-1 derivative pVA3-1 which does not have sequences corresponding to the MATCHMAKER 5'and 3" AD LD- Insert Screening Amplimers. The YEASTMAKER Yeast Plasmid Isolation Kit (Clontech) was used to isolate plasmid DNAs from yeast cells.

PCR reactions were performed using the advantage cDNA PCR Kit (Clontech), which includes control template and primers for this template. For pACT2 derivatives, primers provided by LD-Insert Screening Amplimer Sets were used. These primer sequences are the same as the MATCHMAKER 5'and 3" AD LD-Insert Screening Amplimers. The PCR products were analyzed on a 0.8 % agarose gel.

As shown in **Figure 6**, a 3 kb PCR product was obtained with the control template provided by the kit, showing that the PCR reaction worked. As expected the pACT2 derivative pTD1-1 (positive control) gave rise to an approximately 2 kb product and the pAS2-1 derivative pVA3-1 (negative control) did not produce any product, demonstrating that 5'and 3"AD LD-Insert Screening Amplimers specifically amplified the DNA sequence located between these primer sites. However, no PCR products were obtained from the plasmids pACT-A04, pACT-A08, pACT-B24, and pACT-B73, although they are the pACT2 derivatives. This result indicated that the plasmids directly recovered from yeast already contained a rearranged DNA configuration.

Retransformation of the recovered plasmids into the two-hybrid host yeast without the bait plasmid. In parallel with the above experiments, we carried out the retransformation experiment with the plasmids pACT-A04, pACT-A08, pACT-B24, and pACT-B73 recovered from positive yeast clones for further verification. The recipient yeast strain used was CG1945 that was constructed for the yeast two-hybrid screening. This strain has the His- phenotype and contains the B-galactosidase reporter gene construct on its chromosome, but does not contain the bait plasmid pAS2-1L1. The plasmids pACT-A04, pACT-A08, pACT-B24, and pACT-B73 were introduced individually into this host yeast and selected for the plasmid marker LEU2. We prepared the control yeast strains by transforming CG1945 with the plasmids pACT2-1, pTD1-1, pVA3-1 and pTD1-1/pVA3-1 together. The plasmids TD1-1 and pVA3-1 carry the SV40 large T-antigen and p53 cDNA, respectively in their cloning sites and these two

gene products physically interact. The CG1945 cells containing each of these plasmids were tested for the growth on the synthetic medium lacking histidine and for B-galactosidase activity by X-gal assay. The negative control yeast strains containing each of the plasmids pACT2-1, pTD1-1 and pVA3-1 activated neither the His3 nor the B-galactosidase genes. In contrast the positive control yeast containing plasmids pTD1-1 and pVA3-1 together activated these reporter genes resulting in the growth on the his-medium. These showed blue colored colonies on the X-gal assay filter due to the physical interaction of the SV40 large T-antigen and p53 protein, as expected. The yeast strains containing the plasmids pACT-A04, pACT-A08, pACT-B24, and pACT-B73 each activated these reporter genes by themselves. This occurred without any interaction with the protein encoded by the bait plasmid that contains the N-terminus region of hAR with that encoded by the 26 CAG repeats fused to yeast Gal 4 DNA binding domain. These results are summarized in **Table 2**.

Unfortunately, these results indicated that the isolated yeast clones were false positives. In other words, no true prostate cDNAs were identified that encode proteins that interact with the N-terminal domain of the androgen receptor. This negative result made us re-tool the project to examine the biology of the androgen receptor binding proteins in prostate cancer.

Task 3: The protein-receptor interactions will be confirmed in a mammalian two-hybrid system.

Immunoprecipitation of the human androgen receptor to identify interacting proteins. We established an immunoprecipitation assay for the human androgen receptor with the goal of identifying interacting proteins that co-precipitate with hAR. We obtained antibodies that recognize hAR and examined their utility for the immunoprecipitations and Western blot detection of the receptor. This approach was to be used to confirm interactions of the candidate hAR-binding proteins with the androgen receptor in the mammalian two-hybrid system experiments, and was used for the experiments on hAR binding proteins described below.

Protein Co-immunoprecipitations: Proteins of interest were immunoprecipitated (IP) from 2mg total protein using 30µl anti-mouse IgG (SIGMA Cat. # A6531) or anti-rabbit (SIGMA Cat. # A8914) agarose beads plus 4µg of the immunoprecipitating antibody in PBSTDS (PBS, Triton-X 100, sodium deoxycholate, SDS, leupeptin, EDTA and PMSF) to a final volume of 0.5ml. The IP mixture was incubated and rocked overnight at 4°C. The protein-bead complexes were pelleted, washed 4 times with PBSTDS and resuspended in 40µl 2X electrophoresis sample buffer. They were then analyzed by our previously established Western blotting approach (**2**).

Task 4: The expression level data from the CAT assays will be submitted for statistical analyses.

This task was not done because no proteins were identified, and thus, expression levels were not examined.

Objective 2: Identify a protein from the set identified in Objective 1 that differentially is reduced in its ability to bind, or fails to bind, the androgen receptor exon 1 encoded polypeptide with a glutamine repeat length of 10.

As described above, since no hAR N-terminal binding proteins could be identified, this Objective and the following Tasks 5-7 could not be done.

Task 5: Comparative experiments will be done to evaluate the proteins identified in objective 1 for differential binding to androgen receptors with either 10 or 25 glutamine repeats using the mammalian two-hybrid system.

Task 6: Levels of activation of gene expression will be scored using a CAT assay.

Task 7: The expression level data from the CAT assays will be submitted for statistical analyses.

hAR Binding Proteins and the biology of androgen-independence

The negative results described above led to the focus on hAR binding proteins involved in cell cycle responses and the potential roles of RB, Bag1L and *p53* gain of function mutants in androgen-independent growth of prostate cancer cells.

1. Role of the *RB* pathway in the response of androgen-independent prostate cancer cells with reconstituted androgen receptor expression to androgen.

AR is a ligand activated, intracellular transcription factor that belongs to the steroid/nuclear receptor superfamily. When the AR is activated by androgens it translocates to the nucleus and binds androgen response elements located in the promoter of certain genes, thus activating or repressing the expression of androgen-regulated proteins. Excessive androgen action can overstimulate prostatic glandular growth resulting in prostate cancer. Current treatment for metastatic adenocarcinoma of the prostate is hormone ablation, which reduces the endogenous production of androgens. However, nearly all carcinomas of the prostate (CaP) progress to an incurable state of androgen-independence (AI) following an initial response to androgen ablation therapy.

In the normal prostate, androgen withdrawal results in apoptosis of prostate epithelial cells. We have previously shown that in primary CaP the loss of expression of CDKN2/p16 is 43% and loss of retinoblastoma (*RB*) gene expression is 13%, however, in AI tumors the rate of RB loss increases significantly to 36% (p=0.015)(**3-5**). Our *hypothesis* was that the loss of *RB*, an AR co-activator, might contribute to the establishment of hormone-refractory CaP.

The human CaP cell line PC3 was established from a *p53*-null AI cancer that expresses wild-type (wt) *RB* but does not express AR. PC3 cells transfected with wt AR (PC3-AR2) provided a unique model to study responses to androgens. Recently many co-factors for the androgen receptor have been identified. One of these co-factors is RB, which has been shown to increase the transcriptional activity of AR when overexpressed. We show here that RB forms a physical complex with AR in prostate cancer cell lines (**Figure 7**).

Treatment of PC3-AR2 with the synthetic androgen R1881 resulted in cell cycle arrest and eventual cell death. While this was unusual, it is similar to what has been seen in breast cancer models where estrogen receptor (ER) negative cells with an introduced ER gene growth arrest and die in response to estrogen treatment. Thus, this suggests that cancer cells of hormone responsive tissues that have evolved to be hormone-independent through loss of the hormone receptor have a common response to re-establishment of the receptor, and may be particularly sensitive to this type of modulation. We examined the molecular responses at 24-hr intervals over a period of six days. Expression of cell cycle-related genes *RB*, *CCNA* (Cyclin A), *CDKN1/p21*, and *p27/Kip1* were assessed by Western blotting at each time point. The results are shown in **Figures 8 and 9**. Our data showed that, when PC3-AR2 cells are grown in the presence of androgen, they rapidly arrest in the G1 phase of the cell cycle. The results also showed that this response occurred through a mechanism of *p53*-independent induction of p21 (PC3-AR2 cells are effectively *p53*-null) which leads to hypophosphorylation of Rb. Further study of this biology in prostate cells may identify targets for therapeutic intervention.

Cell culturing: Cells were cultured in RPMI or DMEM media (BioWhittaker, Walkersville, MD) with 7-10% heat-inactivated fetal bovine serum, supplemented with penicillin and streptomycin. Cells were maintained at 37°C in 5% CO_2 in air. For the androgen stimulation experiments, one day after plating cells, the dishes were washed and one set of dishes received medium containing R1881 (0.2nM) or DHT (1nM) and the second set of dishes were not supplemented with androgen. Cells were then harvested a defined timepoints.

Western blotting. Cells were harvested and whole cell soluble proteins extracted by lysing the cells in Lysis Buffer supplemented with Leupeptin (10mg/ml), Aprotinin (0.1M), $NaVO_4$ (0.1 M), PMSF (0.1M) and DNase. Non-soluble components were removed by high-speed centrifugation at 4°C. Samples were quantitated using a modified Bradford assay and diluted to equivalent protein concentrations. Samples were then fractionated by SDS-PAGE and transferred onto nitrocellulose membranes. The AR primary antibody was a purified mouse anti-human antibody from NeoMarkers. β-actin was detected with a monoclonal mouse IgG antibody from Sigma and the RB antibody was a mouse anti-human antibody from Pharmingen.

2. Disrupting RB reverses the androgen-induced cell death in PC3-AR2 cells.

To further investigate this *RB*-associated biology in response to androgens, PC3-AR2 cells were transfected with the human papilloma virus (HPV) E7 gene, whose protein product inhibits and degrades the RB protein. As shown in **Figure 10**, a survival advantage is conferred by the disruption of RB by the HPV-E7 gene. Because survival was not fully restored, the data suggested that the androgen-induced response is more complex than through RB alone.

3. Construction of DU-145 cells expressing a functional androgen receptor.

To extend this model, DU-145 cells were chosen for reconstitution of hAR expression because they have mutant *RB*. To accomplish this, an appropriate mammalian expression vector for hAR needed to be constructed. Yeast hAR expression vectors were

available from Dr. Xu-Bao Shi, and diagrams of these are shown in **Figure 11**: pXB-6: hAR cDNA containing 17 CAG repeats and a long 5' untranslated sequence, and pXB-10: hAR cDNA containing 23 CAG repeats and a short 5' untranslated sequence.

To express the hAR cDNAs in mammalian cells, we subcloned these hAR cDNAs into the mammalian expression vector pREP 9 (Invitrogen) using NheI and BamHI as cloning sites. pREP 9 is a low copy episomal plasmid in mammalian cells. We named these plasmids pREP-6 and pREP-10 derived from pXB-6 and pXB-10, respectively (**Figure 11**).

These plasmids were used to transfect DU145 cells to obtain hAR expressing stable sublines. We have successfully isolated a clone, DU145-AR4, and this was examined for the transcriptional activation function of the transfected hAR gene. These cells were either treated with R1181 or not, and mRNA was extracted after 24 hours. RT-PCR was performed to detect expression of prostate specific antigen (PSA) mRNA, a gene known to be induced by androgens through activation of hAR. As shown in **Figure 12**, PSA expression was induced by androgens, confirming the expression of a functional androgen receptor in this DU-145-AR4 subline. This subline will be useful for further studies of the mechanisms of androgen independence. This has led us to catalogue the potential mechanisms of androgen-independence and propose a model based on our previous work, our work under this project, and literature review (**Figure 13**).

3. Bag1L binds to and may be a co-activator of hAR.

hAR is a ligand activated, intracellular transcription factor that belongs to the steroid/nuclear receptor superfamily. When the AR is activated by androgens it translocates to the nucleus and binds androgen response elements located in the promoter of certain genes, thus activating or repressing the expression of androgen-regulated proteins. Bag1L, an isoform of the human Bag1 protein, binds several steroid receptors including AR. Bag1 is a protein with anti-apoptotic function and is comprised of three isoforms (Bag1, Bag1M, and Bag1L). Bag1L is preferentially found in steroid hormone-dependent tissues such as testis, ovary, breast, and prostate. It has been suggested that the Bag1L protein may play a role in the AR signaling pathway by binding AR and enhancing the androgen-dependent transactivation function of the steroid hormone receptor. To begin this study, we surveyed the expression of Bag1 isoforms in human cancer cell lines of various origins. As shown in **Figure 14**, expression patterns for the three Bag1 isoforms varied, with all three forms found in all prostate cancer cells (PC3-AR2, CWR22, and LNCaP), though at different levels. Of note is also the lack of Bag1L expression in the HL60 promyelocytic leukemia cell line.

Using human prostate cancer PC3 cells transfected with a wild-type androgen receptor (PC3-AR2), we immunoprecipitated the AR and performed Western blotting using an anti-Bag1 monoclonal antibody. We found that of the three isoforms of Bag1 (Bag1, Bag1M, and Bag1L) only the Bag1L protein co-immunoprecipitated with AR (**Figure 15**). Here our work has confirmed that Bag1L does bind the androgen receptor in prostate cancer cells. These results, combined with the findings of others, suggest that Bag1L may be a co-activator for hAR.

4. Bag1L is induced by *p53* gain-of-function mutants, which contributes to androgen-independent growth of human prostate cancer cells.

We have shown that mutations in the *p53* gene are common in CaP, with a high frequency (approximately 70%) found in hormone-refractory disease (**6-8**). To date there has been no evidence to link *p53* mutations to androgen-independence. Recently the Bag1L protein was shown to sensitize the androgen receptor (AR) to low levels of androgen. In addition some gain-of-function *p53* mutants were shown to upregulate transcription from the *BAG1* promoter. Our *hypothesis* was that certain gain-of-function *p53* mutations would lead to androgen-independence through Bag1L induction. PC3 sub-lines containing 5 different mutant *p53* alleles were constructed by transfection. Levels of the Bag1L protein were assessed by Western blotting (**Figure 16**). For quantitation of expression, levels of Bag1L were normalized to B-actin, and the results are shown in **Figure 17**. The expression of Bag1L was induced in 4 of these sub-lines compared to the untransfected control. These results supported our hypothesis that certain gain-of-function mutant *p53* alleles may contribute to androgen-independence by the induction of Bag1L.

These studies were extended to examine the Bag1L induction under androgen-depleted conditions. Here it was necessary to use a different model, the androgen sensitive prostate cancer cell line LNCaP. The mutant *p53* alleles were transfected into LNCaP and sublines established. The induction of Bag1L by mutant *p53* alleles was also observed in the LNCaP cells, and the LNCaP sublines with the gain-of-function *p53* mutant alleles continued to proliferate in androgen-depleted media, demonstrating a new gain-of-function for these alleles: they contribute to androgen-independent growth of human prostate cancer cells. These exciting findings are being further pursued in continuing collaboration with Dr. Ralph deVere White.

III. KEY RESEARCH ACCOMPLISHMENTS

- Failure to identify any binding proteins for the Gln repeat region of the human androgen receptor.
- Role of the *RB* pathway growth arrest in the androgen response in prostate cancer (**9, 10**).
- Bag1L binds the androgen receptor, is induced by gain-of-function *p53* mutants, and contributes to androgen-independent growth of prostate cancer cells (**11, 12**).

IV. REPORTABLE OUTCOMES

A. Manuscripts and abstracts:

Publications

1. Lara, P.N. Jr., H-J. Kung, P. H. Gumerlock, F. J. Meyers. Molecular biology of prostate carcinogenesis. *Critical Reviews in Oncology/Hematology* 32:197-208, 1999.

Abstracts: National Meetings

1. Gumerlock, P. H., M. H. Gustafsson, J. B. Schnier, K. Nishi-Schnier. Role of the RB pathway in the androgen response of prostate cancer (CaP) cells with reconstituted androgen receptor (AR). *Proceedings of the American Association for Cancer Research* 42:547 (Abstract # 2938), 2001.

2. Nesslinger, N. J., X-B. Shi, M. H. Gustafsson, P. H. Gumerlock, R. W. deVere White. Mutant p53 induction of Bag1L as a potential mechanism for androgen-independence in human prostate cancer. *Proceedings of the American Association for Cancer Research* 42:218 (Abstract # 1171), 2001.

3. Gumerlock, P. H., M. H. Gustafsson, K. Nishi-Schnier, J. B. Schnier. Role of RB in the response of androgen-independent prostate cancer cells to androgen. *Proceedings of the American Association for Cancer Research* 43:806 (Abstract # 3998), 2002.

4. Nesslinger, N. J., X-B. Shi, P. H. Gumerlock, R. W. deVere White. Gain-of-function mutant p53 mediates androgen-independent growth of human LNCaP prostate cancer cells. *Proceedings of the American Association for Cancer Research* 43:1137-1138 (Abstract # 5636), 2002.

Abstracts: Local Meetings

1. Gumerlock, P. H., M. H. Gustafsson, J. B. Schnier, K. Nishi-Schnier. Role of the *RB* pathway in the androgen response of prostate cancer (CaP) cells with reconstituted androgen receptor (AR). University of California, Davis Cancer Center Symposium, 2000.

2. Gustafsson, M. H., N. J. Nesslinger, J. B. Schnier, X-B. Shi, R.W. deVere White, P.H. Gumerlock. Induction of Bag1L by mutant *p53* as a potential mechanism for androgen-independence in human prostate cancer. University of California, Davis Cancer Center Symposium, 2000.

3. Gustafsson M.H., J. B. Schnier, K. Nishi-Schnier, P.H. Gumerlock. Role of *RB* in the response of androgen-independent prostate cancer cells to androgen as revealed by transfection of the E7 gene. University of California, Davis Cancer Center Symposium, 2001.

B. Patents and licenses applied for and/or issued

None

C. Degrees obtained that are supported by this award:

None

D. Development of cell lines, tissue or serum repositories

1. Expression plasmids containing the hAR N-terminal region with either 26 CAG repeats or 16 CAG repeats were created and confirmed by DNA sequencing.
2. Mammalian hAR expression vectors pREP-6 and pREP-10.

3. DU-145-AR4 subline expressing a functional androgen receptor.

E. Informatics such as databases and animal models, etc.
None

F. Funding applied for based on work supported by this award
NIH-NCI grant application on abnormal androgen receptors in prostate cancer using primarily the Bag-1L data described above.

G. Employment or research opportunities applied for and/or received based on experience/training supported by this award
None.

V. CONCLUSIONS

This project, which began with the goal of identifying proteins that bind to the androgen receptor differentially based on the length of the CAG repeat sequence, has been instrumental in revealing new biological findings of the role of the androgen receptor in prostate cancer progression to androgen-independence.

1. Failure to identify any binding proteins for the Gln repeat region of the human androgen receptor.

Since the start of this project, there were two reports on identification of proteins that bind to the N-terminus of hAR. These were ARA 24 and ARA160 (**13, 14**). ARA160 binds to the N-terminal amino acids 38-566 region of hAR and coactivates this receptor transcription factor by cooperating with the other coregulator ARA70 that binds to the ligand-binding domain of hAR. ARA24 was isolated by yeast two-hybrid screening (the same technique we employed) using the N-terminal 11-208 amino acids of hAR that included poly-glutamine (poly-Q)(CAG repeats). Utilizing the mutant hAR proteins that have different lengths of poly-Q (Q0, Q1, Q25 and Q49), the authors found that an expanding poly-Q length decreases ARA24-binding to hAR. The coactivation of ARA24 also diminishes with the poly-Q expansion within hAR. These results indicate that ARA24 may bind to the N-terminus regions that flank the poly-Q stretch and that the longer poly-Q stretch may interfere with the ARA24-binding. The authors correlated these findings to the partial androgen insensitivity observed in the development of Kennedy's disease in which the mutant hAR proteins contain 40-72 Q expansion (17-29Q in normal hAR).

Our extensive attempts to identify proteins that specifically bind the poly-Gln (Q) encoded by the CAG repeats in the hAR gene failed to identify any such proteins. Thus, our negative results provide some support for their hypothesis that expansion of the repeat negatively influences ARA24 binding in a region near the poly-Gln stretch. Our conclusion was that the continued search for such binding proteins is not warranted at this time, however further study of ARA24 is warranted.

2. Role of the *RB* pathway growth arrest in the androgen response in prostate cancer.

We have shown that hAR does bind the tumor suppressor protein RB, and that RB is involved in androgen-induced growth arrest. Indeed, restoring RB to advanced prostate cancer cells that have evolved to androgen-independence by deleting expression of hAR, may be restoring the natural response to androgens seen in fully differentiated prostate epithelial cells where androgen is considered a survival factor. The unexpected occurrence of prostate cancer cell death several days following the growth arrest, may reflect an normal response to conflicting signals that has often been observed in cancer cells therapeutically induced to growth arrest. We have reported these results (**9**).

We extended these results by showing that disruption of RB in those same cells using the HPV-E7 gene partially restored cell survival after androgen treatment. These results were also reported (**10**). A manuscript detailing this work is being organized. Our conclusions from this work are that this unique biology could possibly be exploited in treating the subset of hormone-refractory prostate cancer that has become so by deletion of the androgen receptor. Although this will require additional work, the aspects revealed under this project are sufficiently compelling to propose additional projects to further dissect this.

3. Bag1L binds the androgen receptor, is induced by gain-of-function *p53* mutants, and contributes to androgen-independent growth of prostate cancer cells.

We have demonstrated that of the three protein isoforms encoded by the anti-apoptotic gene *BAG1*, only the Bag1L form binds the androgen receptor. Further, Bag1L levels are increased following transfection of gain-of-function *p53* alleles into *p53*-null PC3 prostate cancer cells. Because Bag1L has been suggested to be a co-activator of hAR that sensitizes the receptor to low levels of androgen, this association of mutant *p53* with Bag1L induction brings together several lines of our continuing investigations into androgen independence. As mentioned above, we have established that mutations in *p53* are frequently found in AI metastatic prostate cancer. We are excited about the merging of these research areas, and continued studies on this linkage are clearly warranted. These results were also reported (**11**).

These studies were extended to examine the Bag1L induction under androgen-depleted conditions. The induction of Bag1L by mutant *p53* alleles was also observed in the LNCaP cells, and the LNCaP sublines with the gain-of-function *p53* mutant alleles continued to proliferate in androgen-depleted media, demonstrating a new gain-of-function for these alleles: they contribute to androgen-independent growth of human prostate cancer cells. These results have also been reported, and manuscripts detailing this biology are being prepared (**12**).

From these studies, we have now found a link between mutant *p53* and androgen independence that involves the androgen receptor. This concerns the other subset of hormone refractory prostate cancer that has become so while retaining expression of the receptor, even through hormone ablation. Our previous work established that abnormally high levels of androgen receptor expression are found in many hormone-refractory prostate cancers (**5**). Our conclusions are that this is a key area for further investigation, and one likely to yield the identification of genes downstream of mutant *p53* that are involved in this androgen-independent growth. Further study is also warranted on whether there is a link between the high AR expressors and the ones with gain-of-function *p53* alleles.

So What? We believe these findings make important and extremely valuable contributions toward understanding the role of the androgen receptor in hormone-refractory prostate cancer. Molecular biological hypotheses have been generated from our work under this project for both the role of *RB* in hormone refractory prostate cancer that is characterized by the loss of AR expression and for the role of mutant *p53* inducing Bag1L, which in turn aids in activating AR in hormone refractory prostate cancer that has retained expression of AR. These multiple findings provide clearer foundations on which to build and propose new projects for study of this important aspect of human prostate cancer.

VI. REFERENCES

1. McEwan, I.J., and J. Gustafsson. Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. *Proceedings of the National Academy of Sciences, USA* 94:8485-8490, 1997.

2. Gumerlock, P.H., F.J. Meyers, S.P. Kokoris, G. Wong, F.P. McCormick, and R.W. deVere White. *ras* enzyme-linked immunoblot assay discriminates p21 species: a technique to dissect gene family expression. *Analytical Biochemistry* 180:158-168, 1989.

3. Chi, S-G., R.W. deVere White, J.T. Muenzer, and P.H. Gumerlock. Frequent alteration of *CDKN2(p16^{INK4A}/MTS1)* expression in human primary prostate carcinomas. *Clinical Cancer Research* 3:1889-1897, 1997.

4. Mack, P.C., S-G. Chi, F.J. Meyers, S.L. Stewart, R.W. deVere White, and P.H. Gumerlock. Increased *RB1* abnormalities in human primary prostate cancer following combined androgen blockade. *The Prostate* 34:145-151, 1998.

5. deVere White, R., F. Meyers, S-G. Chi, S. Chamberlain, D. Siders, F. Lee, S. Stewart, and P.H. Gumerlock. Human androgen receptor expression in prostate cancer following androgen ablation. *European Urology* 31:1-6, 1997.

6. Chi, S-G., R.W. deVere White, F.J. Meyers, D.B. Siders, F. Lee, and P.H. Gumerlock. p53 in prostate Cancer: frequent expressed transition mutations. *Journal of the National Cancer Institute* 86:926-933, 1994.

7. Gumerlock, P.H., S-G. Chi, X-B Shi, H.J. Voeller, J.W. Jacobson, E.P. Gelmann, R.W. deVere White, and The Cooperative Prostate Network. *p53* abnormalities in primary prostate cancer: single-strand conformation polymorphism analysis of complementary DNA in comparison with genomic DNA. *Journal of the National Cancer Institute* 89:66-71, 1997.

8. Meyers, F.J., P.H. Gumerlock, S.G. Chi, H. Borchers, A.D. Deitch, and R.W. deVere White. Very frequent p53 mutations in metastatic prostate carcinoma and in matched primary tumors. *Cancer* 83:2534-2539, 1998.

9. Gumerlock, P. H., M. H. Gustafsson, J. B. Schnier, and K. Nishi-Schnier. Role of the RB pathway in the androgen response of prostate cancer (CaP) cells with reconstituted androgen receptor (AR). *Proceedings of the American Association for Cancer Research* 42:547 (Abstract # 2938), 2001.

10. Gumerlock, P. H., M. H. Gustafsson, K. Nishi-Schnier, and J. B. Schnier. Role of RB in the response of androgen-independent prostate cancer cells to androgen. *Proceedings of the American Association for Cancer Research* 43:806 (Abstract # 3998), 2002.

11. Nesslinger, N. J., X-B. Shi, M. H. Gustafsson, P. H. Gumerlock, and R. W. deVere White. Mutant p53 induction of Bag1L as a potential mechanism for androgen-independence in human prostate cancer. *Proceedings of the American Association for Cancer Research* 42:218 (Abstract # 1171), 2001.

12. Nesslinger, N. J., X-B. Shi, P. H. Gumerlock, R. W. deVere White. Gain-of-function mutant p53 mediates androgen-independent growth of human LNCaP prostate cancer cells. *Proceedings of the American Association for Cancer Research* 43:1137-1138 (Abstract # 5636), 2002.

13. Hsiao P.W., D.L. Lin, R. Nakao, and C. Chang. The linkage of Kennedy's neuron disease to ARA24, the first identified androgen receptor polyglutamine region-associated coactivator. *Journal of Biological Chemistry* 274:20229-20234, 1999.

14. Hsiao, P.W., and C. Chang. Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells. *Journal of Biological Chemistry* 274:22373-22379, 1999.

VII. APPENDIX

Attached in the Appendix are the following:

Appendix
Figures and Tables

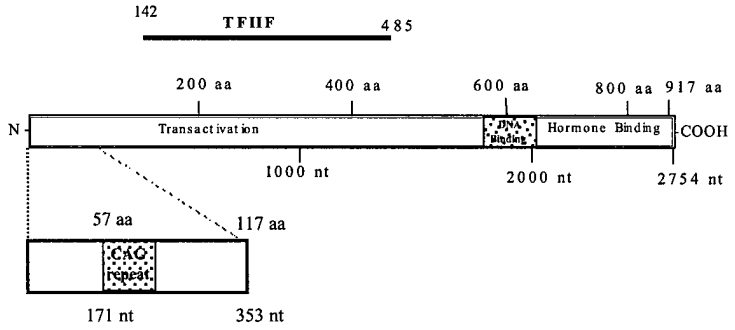


Figure 1. Schematic representation of *hAR*.

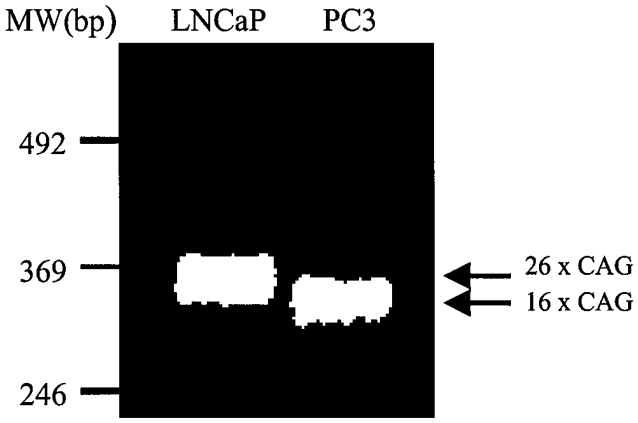


Figure 2. Agarrose gel electrophoresis of the PCR products.

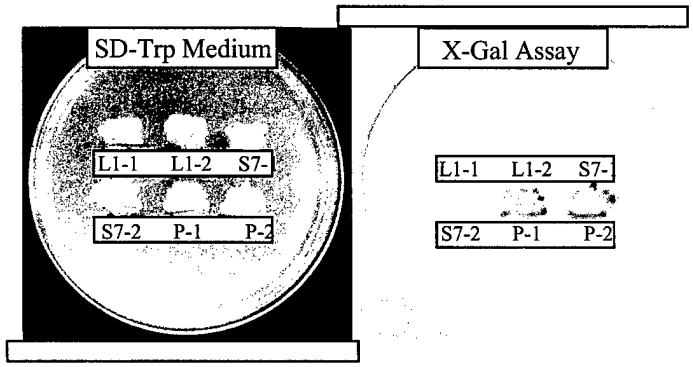


Figure 3. X-gal assay of the yeast cells containing the two-hybrid plasmids with the DNA fragments, which encode N-terminus of hAR. SD-Trp Medium: Synthetic yeast medium with 2% glucose lacking tryptophane.L1-1and L1-2: yeast strains containing pAS2-1 with 26 CAG repeats. S7-1 and S7-2: yeast strains containing pAS2-1 with 16 CAG repeats. P1 and P2: β galactosidase positive yeast strains.

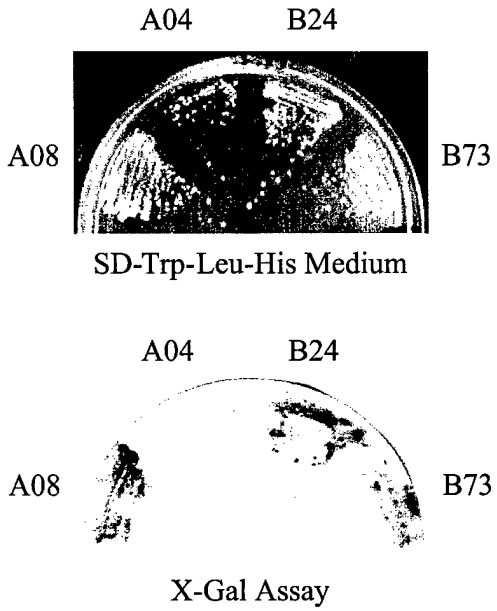


Figure 4. His+ growth and X-gal assay of the positive clones.

Table 1. Summary of the screening

Transformation	Number oftransformants screened	Number of His+ transformantsisolated	Number of β-galactosidase positive transformants
1	1.8×10^6	24	2
2	4.8×10^6	73	2
total	6.6×10^6	97	4

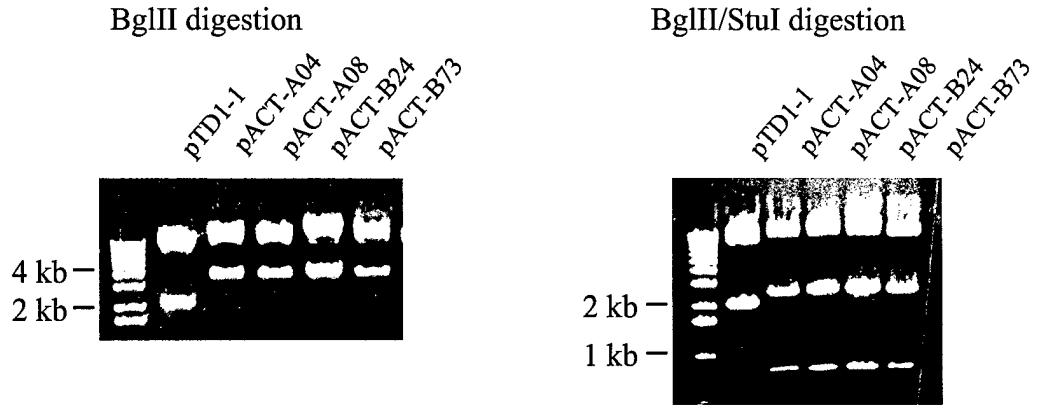


Figure 5. Restriction digestion analysis of the plasmids isolated from positive scoring clones

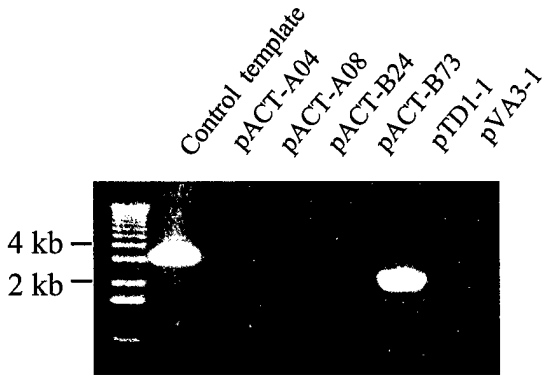


Figure 6. PCR analysis of the insert DNA on the plamids directly isolated from yeast clones

Table 2. Reporter gene activation by the isolated plasmids in the absence of the bait plasmid

Plasmid	Insert	growth on the his- medium	X-gal assay
pACT2-1	none	-	-
pACT-A04	?	+	+
pACT-A08	?	+	+
pACT-B24	?	+	+
pACT-B73	?	+	+
pTD1-1	T-antigen	-	-
pVA3-1	p53 cDNA	-	-
pTD1-1/pVA3-1	T-antigen/p53 cDNA	+	+

+: blue color on filter lifting X-gal assay

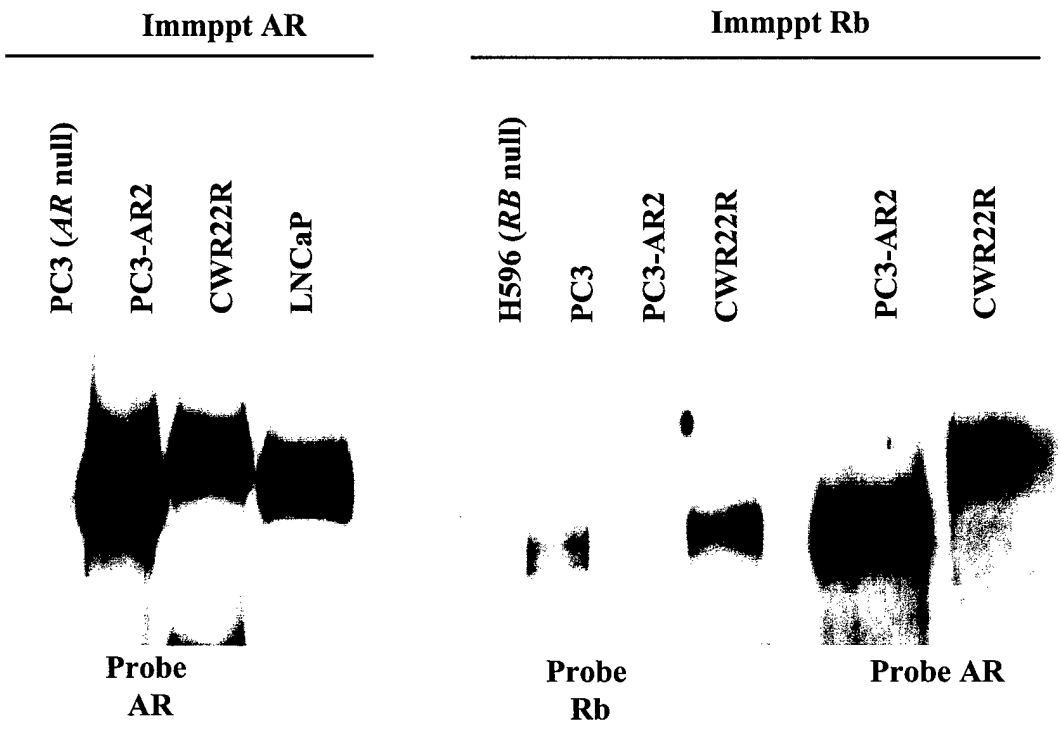


Figure 7. Rb binds AR

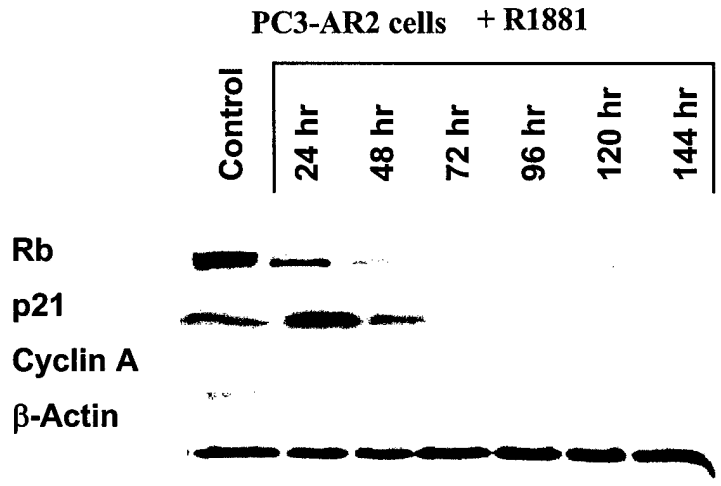


Figure 8. G1 Arrest Response to Androgens in PC3 Reconstituted with AR

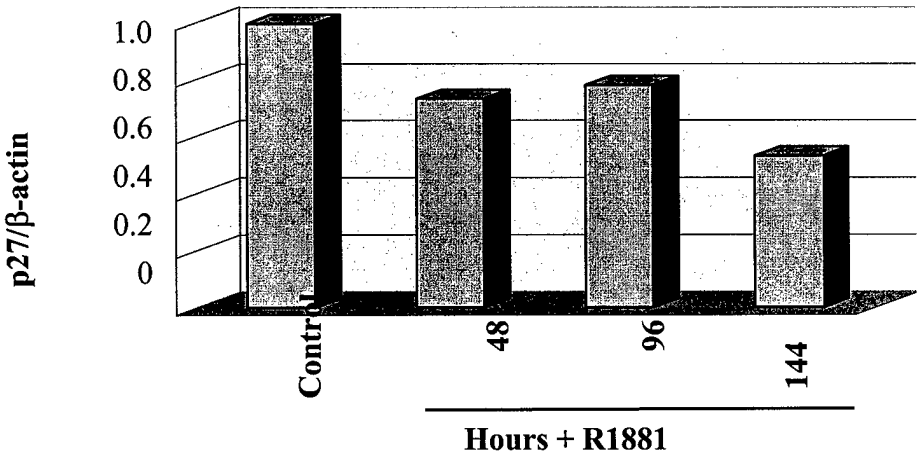


Figure 9. Decreased p27 in Response to Androgen in PC3-AR2 Cells

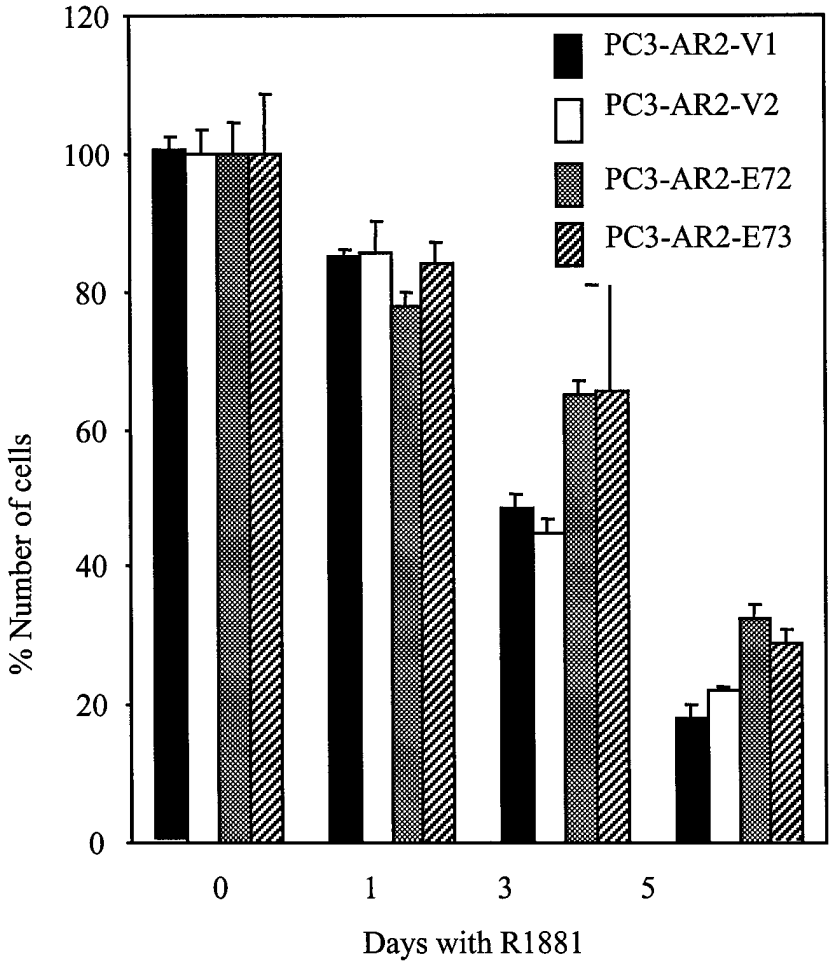


Figure 10. Survival of PC3-AR-V and PC3-AR-E7 cells in response to androgen

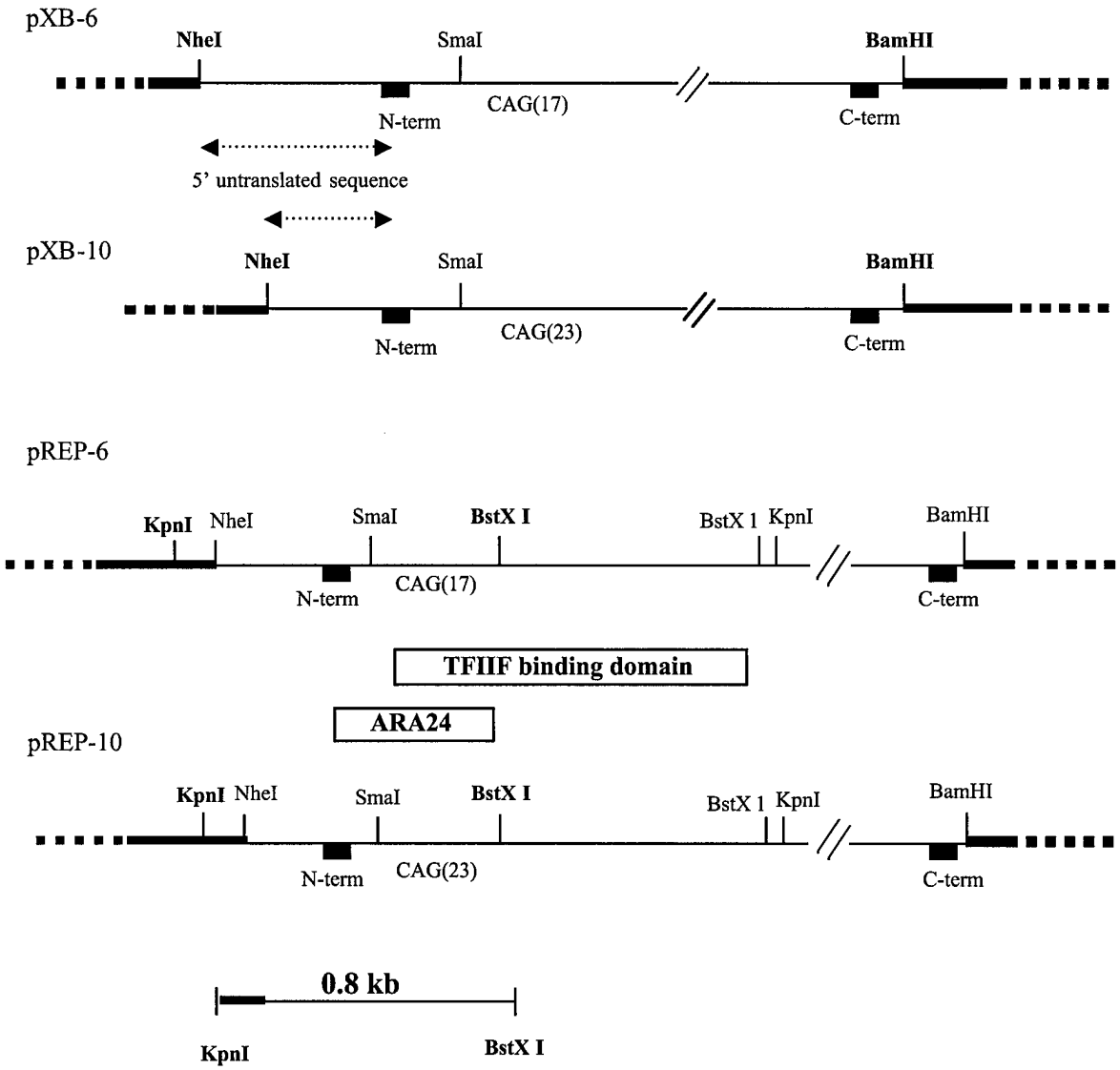


Figure 11. Diagrams of yeast and mammalian expression vectors with *hAR* for transfections.

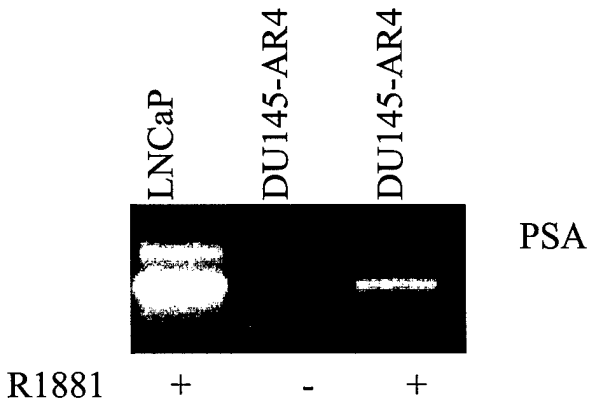


Figure 12. PSA Expression in DU145-AR4 Cells.

PSA expression in LNCaP and DU145-AR4. Cells were grown in the absence or presence of 4nM R1881. RNA was isolated and cDNA prepared from the 2 mg RNA using random primers. cDNA was amplified using PCR. The PCR products were separated by 2% agarose electrophoresis.

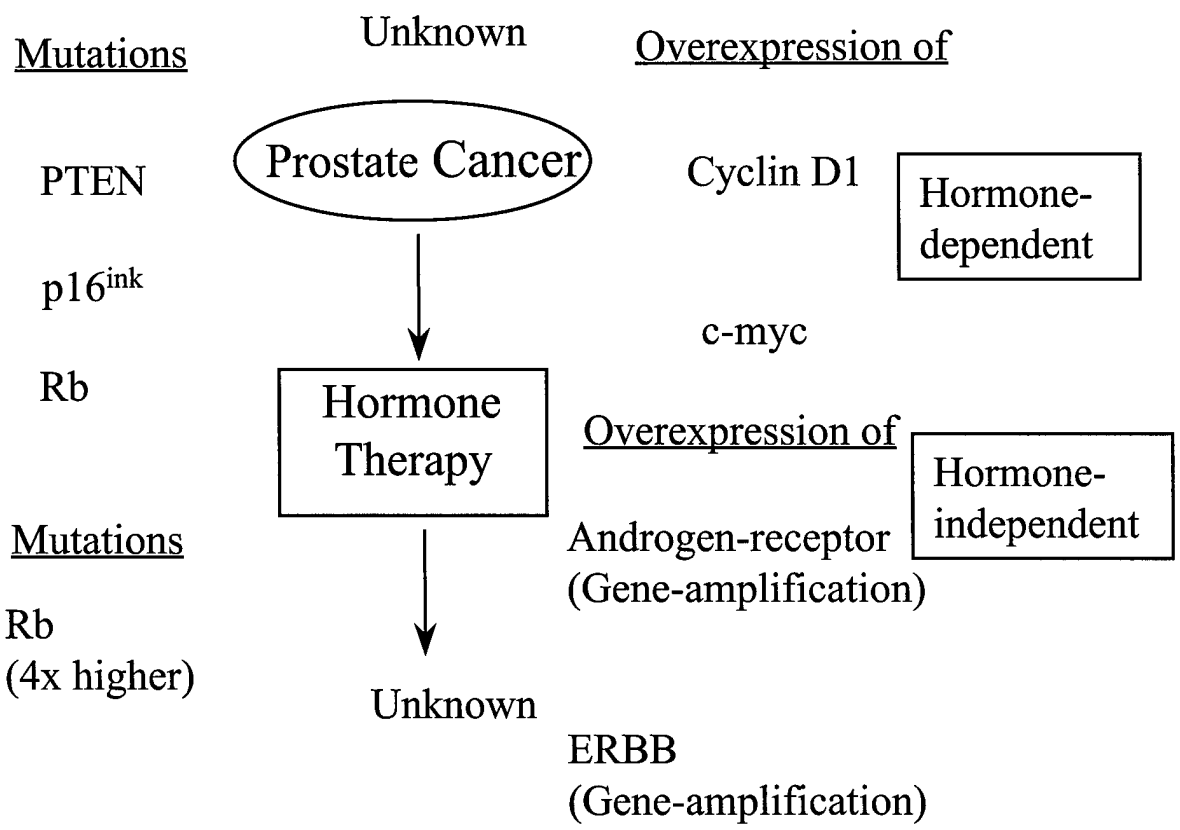


Figure 13. The Involvement of Rb in the Development of Hormone-Independent Prostate Cancer

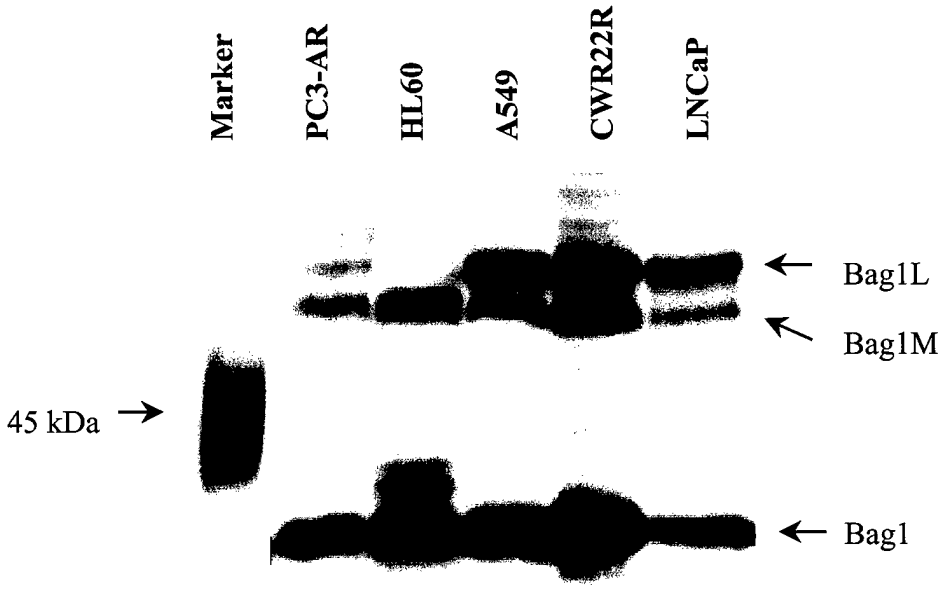
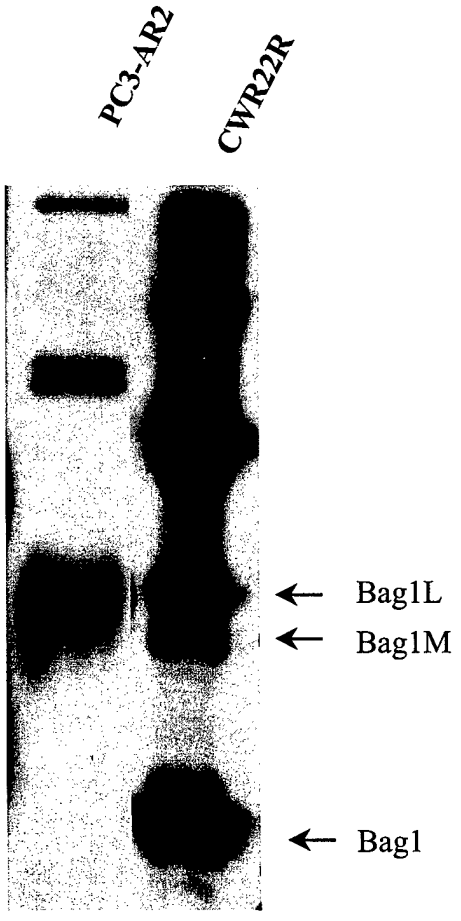


Figure 14. Bag1L Expression in Various Human Cancer Cell Lines



PC3-AR2: Immunoprecipitatied with an anti-AR antibody and probed with an anti-Bag1 antibody.

CWR22R: Whole cell extracts probed with an anti-Bag1 antibody.

Figure 15. Bag1L Binds the Androgen Receptor

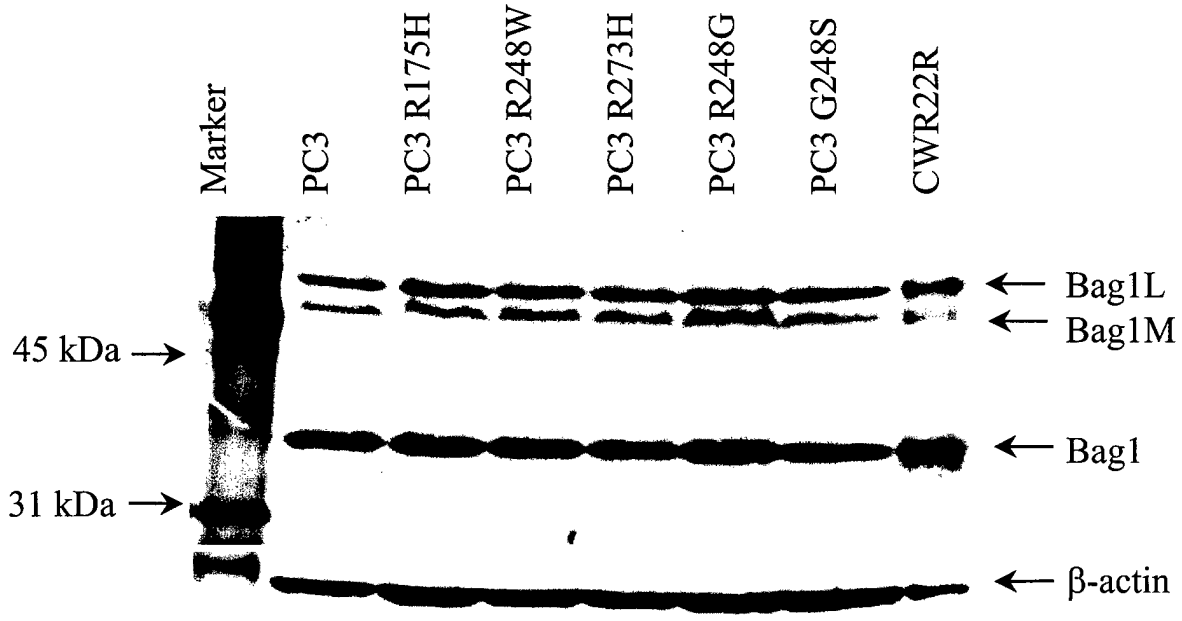


Figure 16. Bag1L Expression is induced in PC3 Cells Transfected with Mutant *p53* Alleles

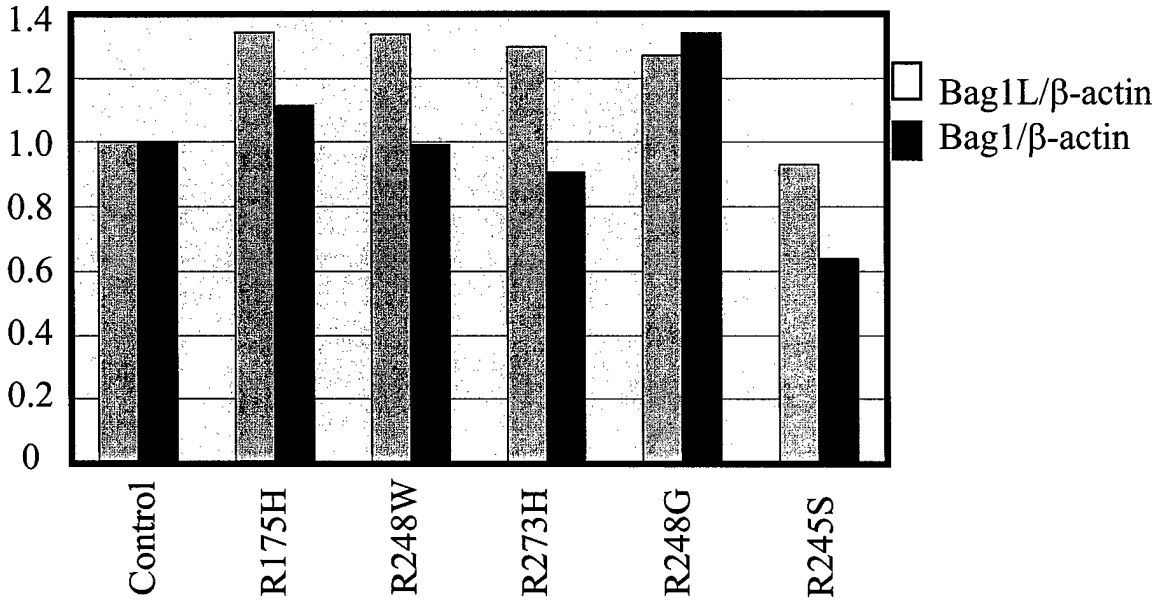


Figure 17. Induction of Bag1L by p53 Mutant Alleles (PC3 Cells)

DAMD17-98-1-8565
PI: Paul H. Gumerlock, Ph.D.

FINAL REPORT: BIBLIOGRAPHY

PUBLICATION

1. Lara, P.N. Jr., H-J. Kung, P. H. Gumerlock, F. J. Meyers. Molecular biology of prostate carcinogenesis. *Critical Reviews in Oncology/Hematology* 32:197-208, 1999.

ABSTRACTS

National Meetings

1. Gumerlock, P. H., M. H. Gustafsson, J. B. Schnier, K. Nishi-Schnier. Role of the RB pathway in the androgen response of prostate cancer (CaP) cells with reconstituted androgen receptor (AR). *Proceedings of the American Association for Cancer Research* 42:547 (Abstract # 2938), 2001.

2. Nesslinger, N. J., X-B. Shi, M. H. Gustafsson, P. H. Gumerlock, R. W. DeVere White. Mutant p53 induction of Bag1L as a potential mechanism for androgen-independence in human prostate cancer. *Proceedings of the American Association for Cancer Research* 42:218 (Abstract # 1171), 2001.

3. Gumerlock, P. H., M. H. Gustafsson, K. Nishi-Schnier, J. B. Schnier. Role of RB in the response of androgen-independent prostate cancer cells to androgen. *Proceedings of the American Association for Cancer Research* 43:806 (Abstract # 3998), 2002.

4. Nesslinger, N. J., X-B. Shi, P. H. Gumerlock, R. W. deVere White. Gain-of-function mutant p53 mediates androgen-independent growth of human LNCaP prostate cancer cells. *Proceedings of the American Association for Cancer Research* 43:1137-1138 (Abstract # 5636), 2002.

Local Meetings

1. Gumerlock, P. H., M. H. Gustafsson, J. B. Schnier, K. Nishi-Schnier. Role of the *RB* pathway in the androgen response of prostate cancer (CaP) cells with reconstituted androgen receptor (AR). University of California, Davis Cancer Center Symposium, 2000.

2. Gustafsson, M. H., N. J. Nesslinger, J. B. Schnier, X-B. Shi, R.W. deVere White, P.H. Gumerlock. Induction of Bag1L by mutant *p53* as a potential mechanism for androgen-independence in human prostate cancer. University of California, Davis Cancer Center Symposium, 2000.

3. Gustafsson M.H., J. B. Schnier, K. Nishi-Schnier, P.H. Gumerlock. Role of *RB* in the response of androgen-independent prostate cancer cells to androgen as revealed by transfection of the E7 gene. University of California, Davis Cancer Center Symposium, 2001.

ELSEVIER

Critical Reviews in Oncology/Hematology 32 (1999) 197–208

Critical Reviews in
Oncology
Hematology

www.elsevier.com/locate/critrevonc

Molecular biology of prostate carcinogenesis

Primo N. Lara Jr*, Hsing-Jien Kung, Paul H. Gumerlock, Frederick J. Meyers

Division of Hematology-Oncology, Department of Internal Medicine, and the Department of Biological Chemistry, University of California Davis Cancer Center, 4501 X Street, Sacramento, CA 95817, USA

Accepted 6 August 1999

Contents

Prostate cancer is a leading cause of cancer-related deaths in men. Investigations into the genetic and molecular events involved in the development of prostate cancer have intensified as a result of evolving technology and the promise of novel therapeutic targets. It is now apparent that a series of genetic alterations transform normal glandular epithelium from a putative pre-neoplastic state to prostate intraepithelial neoplasia and invasive carcinoma. Further molecular insults in hormone-sensitive disease promote the development of androgen independence. This article reviews the current literature on specific genetic alterations reported to be operative in prostate cancer, including changes in the status of tumor suppressor genes, oncogenesis, signal transduction pathways, growth factors, telomerase, angiogenesis, and cytogenetics. In addition, there is an attempt to frame these alterations into a potential sequence that delineates the multistep process of prostate carcinogenesis. Although the steps in this process do not universally apply to all prostate cancers, it serves as a model on which to base further studies and identify potential molecular targets for diagnosis, prognosis and therapy.

* Corresponding author. Tel.: +1-916-7343771; fax: +1-916-7344879.

1040-8428/99/$ - see front matter
PII: S1040-8428(99)00041-4

1. Background

Prostate cancer is the most common malignancy in American males, with an estimated incidence of 184 500 new cases diagnosed in 1998 [1]. It is also a leading cause of cancer-related death in men, second only to lung cancer. In 1999, an estimated 37 000 men are expected to die from prostate cancer. Nearly all these deaths are due to the emergence of androgen-independent clones that are refractory to known therapeutic modalities.

The incidence of prostate cancer increases sharply with advancing age, with an estimated rate of 82 per 100 000 in men aged 50–54 compared to 1326 per 100 000 in the 70–74-year-old age group [2]. Mortality rates vary widely, especially across ethnic groups. For instance, the 5-year overall survival rate of African Americans is 75% as compared to 90% for Caucasians [2]. Even within ethnic groups, prostate cancer does not follow a predictable natural history. This mirrors the marked heterogeneity in the clinical behavior and molecular characteristics of this disease.

The heterogeneity of prostate cancer defines the current clinical challenges. One challenge is to identify molecular markers in early stage prostate cancer (i.e. potentially curable) that predict aggressive or metastatic behavior. Another is to develop a more precise diagnosis of preneoplastic states that would respond to chemoprevention or lifestyle changes. A third, albeit more difficult challenge, is to select appropriate molecular or genetic targets in both preneoplasia and invasive malignancies for novel therapies.

To confront these challenges, increasing efforts are being undertaken to characterize the molecular events involved in the transformation of normal prostate tissue to that of a malignant phenotype with a propensity towards metastasis and treatment resistance. One model that has been proposed is the multistep process of carcinogenesis. This model, best characterized in colorectal cancer, indicates that specific genetic perturbations at certain timepoints in turn initiate and promote neoplastic transformation in normal tissue [3,4]. Later mutations or 'hits' would increase the likelihood of a more biologically aggressive behavior, including metastasis and androgen-independence. The presence of a predisposing genetic alteration, the first of Knudson's two hits [5], appears to explain some instances of familial clustering of prostate cancer.

At the present time, the carcinogenesis model for prostate cancer is difficult to construct with confidence, principally because of the recognized heterogeneity of the disease. Indeed, how is it that one man may live for decades with an indolent tumor while another succumbs to a virulent variety within months of diagnosis? Certainly, the pathways that lead to malignant transformation are not uniform across all prostate cancers. Nevertheless, recent advances in the molecular characterization of prostate cancer suggest that certain genetic alterations may be common in the evolution to malignancy. This review will concentrate on the current understanding of the molecular changes associated with prostate cancer development. We will describe the morphologic evolution of prostate cancer from normal epithelia to metastatic disease. We will then discuss in detail the various cytogenetic and molecular changes that have been described in prostate cancer. Finally, we will attempt to construct a model for prostate carcinogenesis with proposed molecular changes that accompany the change from a benign to a malignant phenotype.

2. In-vitro molecular studies

Prostate cancer, like all other cancers and unlike normal tissues, is characterized by molecular and genetic aberrations that confer either a proliferative advantage or resistance to programmed cell death (apoptosis). Recent advances in molecular biologic techniques have allowed investigators to pursue these molecular aberrations which provide not only prognostic information but also potential therapeutic targets. These modern techniques, which are summarized in Table 1 and are extensively reviewed elsewhere [6], generally utilize two types of material: human tumor tissue or cell lines. Nearly all human prostate tissue specimens are acquired from either radical prostatectomy or transurethral resection of prostate (TURP) procedures. Occasionally, nodal and bone marrow metastatic deposits are obtained for analysis. These tissue sources allow investigators to identify molecular differences between the primary tumor, the surrounding normal prostate tissue, and metastatic foci, providing information to delineate prostate carcinogenesis.

Much of our current knowledge on prostate cancer development has been derived from prostate cancer cells lines and xenografts. The three human cell lines most commonly utilized are LNCaP, PC3, and DU145. These cells lines (as well as xenografts) possess different molecular profiles of abnormalities in cell cycle- and apoptosis-associated genes. Table 2 illustrates the molecular characterization of the cell line panel and xenografts currently in use at the UC Davis Cancer Center. Translational research has focused on exploiting the known differences between the cell lines to evaluate the molecular basis of response or resistance to systemic or radiation therapy.

Table 1
Summary of some molecular biologic techniques used in prostate cancer research (adapted from Lalani et al. [6])

I. Detection of point mutations
1. Allele-specific oligonucleotide hybridization
2. Direct sequencing of DNA amplified by polymerase chain reaction (PCR)
3. Single strand conformational polymorphism (SSCP)

II. Detection of gene amplification
1. Dot-blot analysis
2. Southern/Northern/gel electrophoresis blot

III. Detection of loss of heterozygosity (LOH)
1. Restriction fragment length polymorphism (RFLP) analysis
2. Blotting techniques
3. PCR of repeat sequences

IV. Hybridization techniques
1. In-situ (ISH) hybridization
2. Fluorescent in situ hybridization (FISH)
3. Comparative genomic hybridization (CGH)

V. PCR and reverse transcriptase PCR (RT PCR)

VI. Protein analysis
1. Immunohistochemistry (IHC)
2. Enzyme linked immunoassay (ELISA)
3. Western blot

VII. Cytogenetic analysis

VIII. Flow cytometry (DNA content and cell surface markers)

IX. Apoptosis analysis
1. PARP cleavage
2. TUNEL assay
3. Electron microscopy
4. DAPI staining

3. Morphologic evolution of prostate neoplasia

The diagnosis of prostate cancer relies on the morphologic assessment of a tissue specimen by an experienced histopathologist. The Gleason's scoring system [7], based on histologic atypia, has been widely used and is the most important single prognostic factor in established prostate cancer [8,9]. However, the identification of preneoplastic lesions is less clear because of the lack of reproducible objective criteria that identify a preneoplastic lesion. Like cervix cancer, it is thought that invasive prostate cancer evolves through certain histopathologic stages such as hyperplasia, metaplasia, dysplasia, and in-situ carcinoma [10]. Candidates for putative preneoplastic lesions include intraductal dysplasia [11], prostatic intraepithelial neoplasia [12] (PIN) and atypical adenomatous hyperplasia. PIN is considered by a number of investigators as the most likely precursor lesion. Its presence has been shown to increase the risk of developing overt prostate cancer [13], but the natural history of PIN has not been established [14].

4. Etiology

The etiology of prostate cancer remains speculative. In mouse models, mutagens that have been shown to cause prostate dysplasia and cancer include diethylstylbestrol [15], *N*-methyl-*N*-nitrosourea (MNU) [16], 3,2,-dimethyl-4-aminobiphenyl (DMAB), and estradiol-17-beta. Most morphologic changes observed in these models occurred following chronic mutagen exposure and testosterone promotion. Cadmium exposure has also been associated with increased risk of prostate cancer [17].

A small proportion of patients have an inherited predisposition to prostate cancer, as seen in several epidemiologic studies [18–20]. Familial clustering has been attributed to the dominant inheritance of a rare high risk allele [21]. This appears to be analogous to predisposing alleles associated with inherited cancers of the colon and breast. Family studies indicate that a region on the long arm of chromosome 1 (specifically 1q24-25) likely contains a prostate cancer susceptibility gene [22].

5. Chromosomal changes

Karyotypic analysis of primary prostate cancers usually yields a normal male karyotype [23]. However, various cytogenetic changes involving several chromo-

Table 2
Molecular characterization of prostate cancer cell lines and xenografts[a]

Cell line	*p53*	*p21*	*Rb*	*p16*	*Cyclin D1*	*bcl2*	Hormonal sensitivity	*HER2/neu*
LNCaP	Norm	Norm	Norm	Ab low	Norm	OvEx	Yes	OvEx
DU145	Mt	Ab low	Mt	OvEx	Ab low	Norm	No	OvEx
PC3	Mt	Ab low	Norm	Mt	Norm	OvEx	No	OvEx
Xenograft								
CWR22	Norm	Norm	Norm	Ab low	Norm	OvEx	Yes	ND
CWR22R	Norm	Ab low	ND	ND	ND	ND	No	ND
CWR31	Null	Ab low	Norm	OvEx	Norm	OvEx	No	ND

[a] Ab low, abnormally low; Mt, mutant; ND, not determined; Norm, normal expression; OvEx, overexpressed.

somes have been reported. Improved techniques such as comparative genomic hybridization and allelotyping have identified previously undetected regions of chromosomal loss or gain [24]. Generally, frank chromosomal abnormalities in prostate cancer specimens have been associated with poor clinical outcome [25]. Unlike acute and chronic myeloid leukemia, prostate cancer cytogenetics, if abnormal, consist mainly of deletions rather than translocations. Loss of heterozygosity (LOH) studies on prostate cancer have confirmed the karyotypic observation of chromosomal loss [26]. It is thought that loss of a putative tumor suppressor gene in the involved chromosomal segment initiates and promotes prostate cancer. Loss of chromosome Y has also been reported [27].

Abnormalities in chromosome 7 have been observed in several studies. One such alteration involves the long arm of chromosome 7 (7q31.1). In one study, loss of this segment correlated with higher grade and metastatic potential [28]. Another study reported trisomy 7 as being seen in higher stage (T3) compared to lower stage (T2) prostate cancer [29]. Trisomy 7 can be detected using dual-color fluorescence in situ hybridization and has been proposed to be a potential biological marker for prostate cancer progression [30].

Chromosomes 8p, 10q, and 16q have also been shown to be frequently deleted [31–33]. 8p is of particular interest because of its frequent LOH in other cancers such as hepatocellular, colorectal, and lung carcinoma [34]. It has also been suggested that loss of 8p may be related to the development of androgen independence [35]. Deletion of portions of both 10p and 10q has been associated with advanced stage and rapidly progressive disease [36]. Among the genes located in 10q, the loss of the *PTEN* tumor suppressor gene (which modulates signal transduction through its protein phosphatase and lipid phosphatase [37] gene product) is felt to be involved in the development of the malignant phenotype [38]. The gene for E-cadherin, a cell adhesion molecule crucial for normal differentiation, has been mapped to 16q [39]. Loss or methylation of the promoter leading to lack of E-cadherin expression has been described in both prostate cancer cell lines and tumor tissue [40–42].

Other chromosomal losses have been reported in 3p, 5q, 9q, 11p, 13q, 16q, 17p and 18q, among others [8,43]. Allelic loss of 6q14-21 has also been recently described [44]. Interestingly, the *p53* gene maps to 17p, the retinoblastoma gene (*Rb*) to 13q, and *DCC* (deleted in colon cancer) to 18q. Loss of these three genes in various tumor types has clearly been shown to initiate and promote cancer growth and progression.

6. Oncogenes and proto-oncogenes

The role of the expression or amplification of oncogenes in prostate cancer remains unclear, although there is some evidence to suggest that it contributes to the transformation of preneoplastic lesions to overt carcinoma.

One of the first protein families identified as a product of proto-oncogene expression is *MYC*. The *MYC* gene has been extensively evaluated in prostate cancer because of its known amplification in a variety of other tumors. Fluorescence in-situ hybridization (FISH) with a region-specific probe for *C-MYC* was performed in PIN, localized disease, and metastatic carcinoma [45]. The results suggest that gain of chromosome 8 and amplification of *MYC* may be potential markers for prostate cancer progression. However, earlier studies have yielded conflicting results [46]. Overall, the contribution of *MYC* to prostate cancer development and progression is believed to be infrequent.

The frequency of *RAS* mutations in prostate cancer has been highly variable. Mutations of *K-RAS*, *H-RAS*, and *N-RAS* are found at a frequency of less than 5% in North Americans [47,48], but at an increased frequency (25%) in Japanese men [49]. This molecular difference may reflect the dissimilar epidemiologies for this disease in the two populations.

The *HER2/neu* oncogene encodes the $p185^{neu}$ transmembrane glycoprotein which belongs to the epidermal growth factor-receptor family of tyrosine kinases [50]. *HER2/neu* over-expression is well described in breast cancer, and has been reported in 20% of primary invasive breast carcinomas [51]. Furthermore, amplification of this oncogene in breast cancer has been shown to be a significant predictor of time to first relapse and inferior overall survival [52]. Similarly, *HER2/neu* has been shown to be amplified in 30–70% of prostate carcinomas [53,54], is associated with androgen-independent prostate cancer growth [55], and correlates with increased probability of disease recurrence [56]. The activation of *HER-2/neu* requires dimerization with itself or with other members of the EGF receptor family, *HER1/EGF* receptor or *HER3/erbB3*. (The other potential partner, *HER4/erbB4*, is not expressed in most prostate cancer cells [57].) Overexpression of *HER1* or *HER3* can diversify and increase the potency of *HER2/neu* signals. In prostate cancer cells, overexpression of *HER3* has also been reported [58,59]. This underscores the importance of *HER2* signaling in the transformation and progression of prostate cancer. As described above, deregulated *HER2* signals may be relevant to androgen independence in advanced prostate cancer. In a recent study, introduction of a prostatic acid phosphatase gene (which inactivates *HER2* kinase activity) into an androgen-independent prostate cell line resulted in the

restoration of androgen-dependent growth [60]. In another in vitro study, forced expression of *HER2* in androgen-dependent prostate cancer cells allowed activation of the androgen receptor pathway in the absence of ligand [55]. Furthermore, increased PSA levels were observed in prostate cancer cells expressing *HER2*. It thus appears that the *HER2* family of receptors, which play a significant role in the progression of breast carcinoma, may also participate in prostate carcinogenesis.

The proto-oncogenes *MET* and *RET* have tyrosine kinase gene products that have been implicated in renal and prostatic development. Furthermore, both oncogenes have similar activating mutations. The expression of the *MET* proto-oncogene, which encodes for the membrane growth factor receptor for hepatocyte growth factor/scatter factor (HGF/SF), has been described in PIN and prostate cancer [61]. It has been proposed that *MET* could be a tumor marker for early progression of human prostate cancer [62]. The more recently described *RET* proto-oncogene has already been associated with patients diagnosed with multiple endocrine neoplasia (MEN) syndrome. One study demonstrated that *RET* was over-expressed in high-grade PIN and prostate cancer as compared to normal prostate epithelium [63].

The *BCL2* oncoprotein is a blocker of programmed cell death (apoptosis) [64]. It was first identified in studies of the t(14;18) in follicular lymphomas [65]. *BCL2* overexpression has been associated with shortened survival in prostate cancer [66]. It is normally expressed in prostatic basal epithelial cells that are typically unaffected by androgen withdrawal [67,68]. It is frequently detected in hormone-refractory prostate cancers, suggesting that *BCL2* overexpression is a relatively late event in prostate carcinogenesis [69,70]. Furthermore, data suggest that androgen ablation therapy can result in either enhanced expression of *BCL2* in prostate cancer cells or clonal selection [71]. Phosphorylation of *BCL2* inhibits its binding to *BAX*, a related pro-apoptotic protein [72]. Paclitaxel induces *BCL2* phosphorylation and death of prostate cancer cells, indicating potential therapeutic implications for paclitaxel in hormone-refractory prostate cancer [73].

7. Tumor suppressor genes

Tumor suppressor gene alterations are the most frequent type of change so far noted in prostate carcinoma [74]. Indeed, the concept of prostate cancer as a disease of tumor suppressor abnormalities in contrast to an oncogene-driven cancer fits the observed biology of a chronic, slow growing disease. The frequency of tumor suppressor alterations is greater in advanced than in localized disease. Thus, the molecular profile mirrors the clinical profile that metastatic prostate cancer is a product of the 'survival of the fittest' malignant cells [75]. Two of the most extensively studied tumor suppressor genes are *p53* and *RB*. Loss of *p53* or *RB* function has been unquestionably linked to cancer development [76–78].

The *p53* gene is located at 17p13 and yields a 53-kDa protein. Many mutant type *p53* proteins have an extended half-life that permits their detection using standard IHC techniques. IHC detection of *p53* is markedly enhanced by using heat-induced antigen unmasking (or retrieval) and a combination of antibodies to the protein product [79]. More recent techniques, including single-strand conformation polymorphism analysis of PCR products from both DNA and cDNA, have yielded increased sensitivity of *p53* mutation detection [80]. In addition, while initial studies focused on codons 5–8, expanded studies have examined codons 4–11. The aggregate of studies indicates that ~20–30% of patients will have detectable *p53* mutations and that this is associated with poor outcome [81,82]. Our initial studies reported the frequency of *p53* mutations to be 42% [83]. This initial report emphasized the relatively high frequency of nucleotide base pair transitions, consistent with or suggestive of an environmental or carcinogenic etiology rather than spontaneous conversion to the malignant phenotype. Our recent studies indicate that *p53* abnormalities occur in 30–35% of primary prostate cancers and in 70% of metastatic deposits in the bone marrow [84]. The accumulation of *p53* has been observed more commonly in metastatic as opposed to localized disease [85], suggesting that *p53* mutations are late events in prostate carcinogenesis or become manifest due to selection pressures. Therefore, the initial reports that *p53* mutations are rare and may not be of prognostic value to predict for dissemination need to be re-evaluated.

Similar observations have been reported with the retinoblastoma (*RB*) gene which is located on chromosome 13q14. *RB* is a key determinant of cell cycle progression through its interactions with transcription factors such as the E2F family [86]. It has been shown through RNA expression analysis that *RB* abnormalities are significantly more frequent in tumors from prostate cancer patients who have received androgen blockade therapy (36%) compared to those who have had no treatment (13%), thus implicating abnormal *RB* in survival from androgen blockade [87].

The tumor suppressor gene *DCC* is frequently deleted or is expressed at low levels in esophageal, gastric, pancreatic, and colorectal carcinomas. It has been cloned and mapped to chromosome 18q21.3 [88]. *DCC* encodes a protein that is homologous to the neural cell adhesion molecule and is involved in diverse cellular processes such as thrombosis, wound healing, embryogenesis, immunoreaction, and tumor progres-

sion or metastasis. The *DCC* gene has been assessed through mRNA expression and loss of heterozygosity (LOH) studies and was found to have decreased expression in both human prostate cancer cell lines and patient tissues. Data suggest that LOH at the *DCC* locus may partly explain this observation [89].

The tumor suppressor gene *CDKN2* (formerly *MTS1*) which encodes the cyclin dependent kinase inhibitor $p16^{INK4A}$ regulates the phosphorylation state of the *Rb* protein [90]. $p16^{INK4A}$ ultimately decreases phosphorylation of the *RB* protein and leads to arrest at the G1 checkpoint, leading to cell growth inhibition. Markedly reduced expression of *CDKN2* mRNA has been detected in 43% of untreated prostate carcinomas, while no alterations were seen in benign prostatic hyperplasia [91]. Thus, *CDKN2* is one of the most frequently altered genes thus far identified in prostate cancer.

The more recently described tumor suppressor gene *PTEN/MMAC1*, located on chromosome 10q23, has a high frequency of mutations (60%) in prostate cancer cell lines and xenografts [92]. In another study, *PTEN* mutations were observed in 23 of 80 prostate tumors [93]. Sequence analysis revealed that a second mutational event at the *PTEN/MMAC1* locus occurred in 43% of the tumors with loss of heterozygosity of this gene. In addition, inactivation of *PTEN* in localized prostate cancer has been associated with increased angiogenesis independent of thrombospondin, a negative regulator of angiogenesis [94].

8. Metastasis suppressor genes

Certain suppressor genes regulate metastatic tumor behavior. One of the first metastasis suppressor genes described is *KAI1*, located on chromosome 11p11.2. Down-regulation of *KAI1* has been observed in patients with androgen-independent disease, although no allelic loss or mutation of the gene was seen [95]. Another candidate metastasis suppressor is the *CD44* gene located on 11p13. It has also been shown that decreased expression of *CD44* is involved in prostate cancer progression [96]. A putative metastasis suppressor region has also been identified on chromosome 12 [97].

9. Androgen receptor

The human androgen receptor (hAR) is a cytoplasmic, 98-kDa protein encoded by a gene localized at Xq11-12. After binding to its androgen ligand, hAR translocates to the nucleus and binds to androgen response elements (ARE) in the promoters of specific genes in DNA. Recent studies have implicated the hAR N-terminus in the biology of prostate cancer. Two regions of triplet nucleotide repeats are found in exon 1 of hAR, the first a CAG repeat encoding a string of glutamine residues (approximately amino acids 58–78) and the second, a GGT repeat encoding a run of glycines (approximately amino acids 448–471) [98]. It is the CAG repeat that is of interest here, because of the association of CAG triplet repeats in several genes with human diseases [99]. In different individuals, the CAG repeat length in the hAR varies from about eight to 31 repeats. Caskey's group was the first to show an association of the length of first triplet nucleotide repeat with different racial groups [86]. The shortest average CAG repeat length was found in African Americans, Caucasians had an intermediate length and Asians had the longest average repeat length. This ranking of the racial groups parallels the incidence of and mortality from prostate cancer found in these groups. hAR transactivation activity was shown to be inversely correlated with the length of the CAG repeat [100]. Elimination of the repeats increased transcriptional activity of hAR. Expansion of the repeat reduced hAR activity. These data suggest that the function of the CAG repeat sequences is to inhibit transcription. Giovannucci et al., recently showed that an association existed between fewer CAG repeats and higher risk of prostate cancer for men with 18 repeats or less compared to those with 26 or more [101]. Those with shorter repeats were at a particularly high risk for metastases and death from prostate cancer. Further support for the role of short repeats in this disease comes from our finding a shorter CAG repeat length (ten repeats) in the tumor of a prostate cancer patient compared to the patient's matched peripheral blood cell DNA (25 repeats). This finding of a somatic abnormality in the tumor suggests a role of selection for shorter CAG repeat lengths, and by extrapolation increased hAR activity, during the genesis or progression of prostate cancer.

Proposed mechanisms for the development of androgen independence following therapy with complete androgen blockade (CAB) include hAR mutations [102], downregulation of hAR expression [103], and hAR gene amplification [104]. The most extensively studied mechanism involves androgen receptor mutations that have been associated with tumor progression [105,106]. In one study of hormone refractory prostate cancer, hAR mutations at codon 877 (which corresponds in the ligand-binding domain of protein) were found to be frequent [107]. Analysis of the entire AR coding region and of the X-linked hypoxanthine guanine phosphoribosyl transferease (*HGPRT*) gene in the same prostate cancer samples indicate that the high frequency of hAR mutations are not a consequence of generalized genetic instability [106]. Previous studies by our group have demonstrated that hAR expression following CAB is preferentially noted in high-grade, high-stage tumors and that hAR-positive tumors in clinical remission pose

a high risk of relapse [108]. It remains to be clarified whether CAB induces the alterations in hAR structure and/or function or whether it merely selects out the already-mutated forms. Furthermore, androgen independent tumors have been shown to continually express high levels of hAR gene transcripts [109]. In fact, hAR has been detected in 100% of distant metastases by immunohistochemistry [110]. It is presently believed that androgen independent prostate cancer may continue to have in-vivo hormone responsiveness or that an agonist binds to hAR to promote progression. Alternatively, deregulated growth signals such as *HER2/neu* overexpression may override androgen independence, resulting in uncontrolled proliferation.

10. Telomerase

Telomeres, repeat DNA sequences at the ends of chromosome, have been described as the biological intracellular clock, regulating the number of divisions a cell can undergo [111]. After a telomere reaches a critically short length, the cell cannot undergo further mitoses. Telomerase is a ribonucleoprotein that stabilizes telomere length and is highly active in many cancers and immortalized cell lines [112]. Little data is presently available for its role in prostate cancer. In one study, telomerase activity was found in 92% of prostate cancers, 73% of PINs, 50% of BPHs, and 36% of normal tissues [113]. There appeared to be no correlation with Gleason's score or PSA level. The high frequency of telomerase activity in prostate cancer makes it a potential therapeutic target.

11. Growth factors

Peptide growth factors influence cellular proliferation through their paracrine or autocrine actions. As prostate cancer achieves androgen independence, these growth factors may assume critical roles in tumor progression [114]. Among the most widely investigated of these growth factors are the transforming growth factor (TGF), epidermal growth factor (EGF), and insulin-like growth factor (IGF) families.

TGF-β inhibits epithelial cell growth through a heteromeric complex of two kinases (called TGF-β receptor I and II) which, in turn, are modulated by the extracellular matrix, androgens, and other growth factors [115]. Either receptor may be inactivated or downregulated, which may lead to cancer development [116]. It has been hypothesized that downregulation of type I and II receptors correlates with histologic progression of prostate cancer [117]. Paradoxically, high levels of TGF-β have been seen in malignant epithelial cells compared to BPH specimens [118]. In mouse models, TGF-β mRNA is markedly elevated in poorly differentiated prostate adenocarcinomas and is thought to be induced by *RAS* and *MYC* oncogene overexpression [119]. In-vitro studies with prostate cancer cell lines have shown highly variable effects of TGF-β on growth and proliferation, partially explaining the variance in observations on TGF-β's role in prostate carcinogenesis [101].

TGF-α and EGF are homologous growth factors that bind to EGF receptors and have roles in normal prostatic development and differentiation. EGF is known to be mitogenic in normal and malignant prostate cancer cells [103]. High affinity EGF receptors have also been identified in both normal human prostate specimens [120,121]. TGF-α mRNA has likewise been demonstrated to be overexpressed in prostate cancer cell lines [122] as well as in samples of human prostate cancer compared to BPH tissues [123]. It is believed that the efficacy of the experimental agent suramin in prostate cancer is through this drug's ability to inhibit the binding of TGF-α to its receptor.

The members of the IGF family have also been shown to have mitogenic and anti-apoptotic effects on normal and transformed prostate epithelial cells. The two major ligands, IGF-1 and -2, have many potential receptors and include the hAR in the case of IGF-1 [124]. The PC3 and DU145 cell lines have increased proliferation in the presence of IGF while the LNCaP cell line will only respond to IGF in the presence of androgen [125]. Increased levels of plasma IGF-1 have already been shown to be a predictor of prostate cancer risk [126]. Likewise, increased regional loss of IGF-2 gene imprinting (i.e. biallelic expression) has been demonstrated in human prostate cancers compared with BPH specimens [127].

Recently, basic fibroblast growth factor (FGF-2) was found to be significantly increased in localized prostate cancers when compared to uninvolved prostate tissue [128]. Furthermore, there was an increase in the expression of the receptor (FGFR) that possesses the capacity to respond to FGF-2. This receptor overexpression was also shown to correlate with poorer tumor differentiation.

12. Adhesion molecules and proteases

The cellular basement membrane and the extracellular matrix serve as crucial barriers to tumor invasion [129]. Once these barriers are breached, cancer cells can find access to blood and lymphatic channels and metastasize. The key components of the extracellular matrix include integrins and cadherins while the most important proteases are those that belong to the matrix metalloproteinase family.

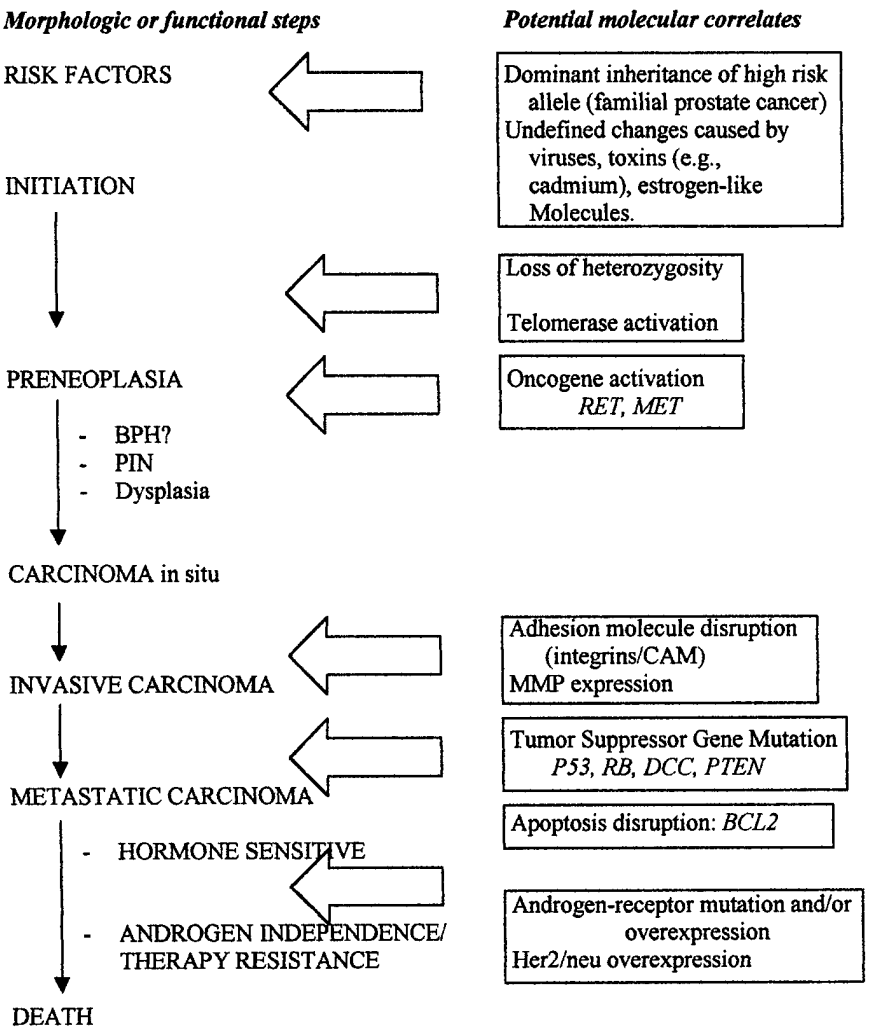


Fig. 1. Multistep carcinogenesis model for prostate cancer.

Integrins are cell surface adhesion proteins (or extracellular matrix receptors) that are important in cell-cell interaction. Normal prostate basal cells have been reported to express several integrins such as α-2, α-3, α-4, α-5, α-6, α-v, β-1, and β-4. Although the dimers α-3, β-1 and α-6, β-1 are retained in the early PIN lesions, the expression of α-2, α-4, α-5, α-v, and β-4 are lost in overt carcinoma. The α-3, β-1 and α-6, β-1 integrins have been shown to be continually expressed in invasive prostate cancer. The α-6, β-1 integrin is now suspected to be a prime candidate for conferring the invasive phenotype in prostate cancer [130].

Cadherins are a family of calcium-dependent cell surface proteins that are critical for normal cellular differentiation and adhesion. Among the most widely evaluated is E-cadherin which has been mapped to 16q22, a locus that is deleted in some prostate cancers [131]. It has been shown that E-cadherin expression is reduced or absent in high-grade prostate cancer [132]. Furthermore, E-cadherin expression decreases as the Gleason score increases [133]. Likewise, less E-cadherin has been found in lymph node metastases compared to primary tumors [134]. In a recent study, aberrant E-cadherin expression was found in 56% of prostate cancer specimens and was associated with disease extent and decreased survival [135].

Metalloproteinases (MMPs) are a family of proteases that dissolve the extracellular matrix and include such enzymes as collegenase and gellatinase. It is thought that these proteins are required by cancer cells to achieve an invasive phenotype. In vitro studies revealed higher MMP levels in prostate cancer cell lines metastatic to bone compared to the non-metastatic cells [136]. MMP-7 and collagenase transcripts have been detected in most prostate carcinomas [137] while collagenase levels were higher in prostate cancer patients with known metastasis compared to those without [138].

13. Proposed carcinogenesis model

Although our understanding of the molecular biology of prostate cancer is far from complete, it is becoming apparent that a molecular carcinogenesis model akin to colorectal cancer is appropriate for prostate cancer. A carcinogenesis pathway was proposed by Sandberg in 1992 based on chromosomal (or cytogenetic) changes [139]. Because of the marked heterogeneity of the disease both clinically and molecularly, there are likely to be multiple intersecting pathways that lead from benign epithelia to therapy-resistant disease. However, a general model is proposed in Fig. 1, with presumed accompanying molecular changes as reported in the literature. This is by no means definitive, but it serves as a potential model for the development of preventive and therapeutic trials aimed at the likely molecular targets operating at each step.

14. Conclusion

Prostate cancer is characterized by a multitude of genetic and molecular changes during its stepwise transformation from benign tissue to its malignant metastatic form. Efforts should be directed towards increasing research activity to delineate the still unclear mechanisms relevant to cancer progression. This must involve patient tumor tissue both from primary and metastatic sites as well as from hormone-responsive and therapy-resistant patients. Only after the molecular alterations that lead to cancer development or invasiveness are delineated can we begin to address appropriate therapeutic solutions to this public health problem.

15. Reviewers

This paper was reviewed by Professor Christophe Iselin, Clinique d'Urologie, Département de Chirurgie, Hôpitaux Universitaires de Genève, CH-1211 Genève, and Dr Ian M. Thompson, Brook Army Medical Center, Department of Surgery, Urology Service, San Antonio, TX 78234-6200, USA, and Professor Nicholas J. Vogelzang, M.D., Director of Genitourinary Oncology, Section of Hematology/Oncology, The University of Chicago Medical Center, 5841 S. Maryland Avenue, MC 2115, Chicago, IL 60637-1470, USA.

Acknowledgements

P.N.L. was supported in part by the Veterans Administration of Northern California Health Care System. P.H.G. was supported in part by the National Institute of Aging (AG15404) and the Department of Defense (DAMD 17-98-8565).

References

[1] American Cancer Society. Cancer facts and figures 1998. Atlanta, GA: American Cancer Society, 1998:20–4.
[2] Ries L, Kosary C, Hankey BF, et al. SEER cancer statistics review, 1973–1994: tables and graphs. Bethesda, MD: National Cancer Institute, 1997 NIH Publication No. 97-2789.
[3] Vogelstein B, Fearon E, Kern S, et al. Allelotype of colorectal carcinomas. Science 1989;244:207–11.
[4] Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759–67.
[5] Knudson AG. Hereditary cancer, oncogenes, and anti-oncogenes. Cancer Res 1985;45:1437.
[6] Lalani E, Laniado ME, Abel PD. Molecular and cellular biology of prostate cancer. Cancer Metastasis Rev 1997;16:29–66.
[7] Gleason DF. Histologic grading and clinical staging of prostatic carcinoma. In: Tannenbaum M, editor. Urologic pathology: the prostate. Philadephia: Lea and Febringer, 1997:171–97.
[8] Lerner SE, Blute ML, Zincke H. Risk factors for progression in patients with prostate cancer treated with radical prostatectomy. Semin Urol Oncol 1996;14(suppl 2):12–21.
[9] Iselin CE, Vollmer RE, Box J, et al. Surgical control of clinically localized prostate cancer is equivalent in African American and white males. Cancer 1998;83:2353–60.
[10] Pretlow TG, Nagabhusahn M, Sy M, et al. Putative preneoplastic foci in the human prostate. J Cell Biochem 1994;19(suppl):224–31.
[11] McNeal JE, Bostwick DG. Intraductal dysplasia: a premalignant lesion of the prostate. Hum Pathol 1986;17:64–71.
[12] Mostofi FK, Sesterhenn IA, Davis CJ. Prostatic intraepithelial neoplasia (PIN): morphological clinical significance. Prostate 1992;4(suppl):71–7.
[13] Bostwick DG, Brawer MK. Prostatic intraepithelial neoplasia and early invasion in prostate cancer. Cancer 1988;50:788–94.
[14] Bostwick DG. Progression of prostatic intraepithelial neoplasia to early invasive adenocarcinoma. Eur Urol 1996;30:145–52.
[15] Pylkkanen L, Makela S, Santti R. Animal models for the preneoplastic lesions of the prostate. Eur Urol 1996;30(2):243–8.
[16] Bosland MC. Animal models for the study of prostate carcinogenesis. J Cell Biochem 1992;16H(suppl):89–98.
[17] Elghany NA, Schumacher MC, Slattery ML, West DW, Lee JS. Occupation, cadmium exposure, and prostate cancer. Epidemiology 1990;1(2):107–15.
[18] Spitz MR, Currier RD, Fuerger JJ, et al. Familial patterns of prostate cancer: a case control analysis. J Urol 1991;146:1305–7.
[19] Steinberg GD, Carter BS, Beaty TH, et al. Family history and the risk of prostate cancer. Prostate 1990;17:337–47.
[20] Cannon L, Bishop DT, Skolnick M, et al. Genetic epidemiology of prostate cancer in the Utah Mormon genealogy. Cancer Surv 1982;1:47–9.
[21] Carter BS, Beaty TH, Steinberg GD, et al. Mendelian inheritance of familial prostate cancer. Proc Nat Acad Sci USA 1992;89:3367–71.
[22] Cooney KA, McCarthy JD, Lange E, et al. Prostate cancer susceptibility locus on chromosome 1q: a confirmatory study. J Natl Cancer Inst 1997;89:1893–4.
[23] Micale MA, Mohammed A, Sakr W, et al. Cytogenetics of primary prostatic adenocarcinoma: clonality and chromosome instability. Cancer Genet Cytogenet 1992;61:165–73.

[24] Cher ML, Bova GS, Moore DS, et al. Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. Cancer Res 1996;56:3091–102.
[25] Lundgren R, Heim S, Mandahl N, et al. Chromosomal abnormalities are associated with unfavorable outcome in prostatic cancer patients. J Urol 1992;147:784–8.
[26] Carter BS, Ewing CM, Ward WS, et al. Allelic loss of chromosomes 16q and 10q in human prostate cancer. Proc Nat Acad Sci USA 1990;87:8751–5.
[27] Lundgren R, Mandahl N, Heim S, et al. Cytogenetic analysis of 57 primary prostatic adenocarcinomas. Genes Chromosomes Cancer 1992;4:16–24.
[28] Takahashi S, Shan A, Ritland SR, et al. Frequent loss of heterozygosity at 7q31.1 in primary prostate cancer is associated with tumor aggressiveness and progression. Cancer Res 1995;55:4114–9.
[29] Bandyk MG, Zhao L, Troncoso P, et al. Trisomy 7: a potential cytogenetic marker of human prostate cancer progression. Genes Chromosomes Cancer 1994;9:19–27.
[30] Wang RY, Troncoso P, Palmer JL, et al. Trisomy 7 by dual-color fluorescence in situ hybridization: a potential biological marker for prostate cancer progression. Clin Cancer Res 1996;2:1553–8.
[31] Kunimi K, Bergenheim US, Larsson IL, et al. Allelotyping of human prostatic adenocarcinoma. Genomics 1991;11:530–6.
[32] Bergenheim US, Kunimi K, Collins VP, Ekman P. Deletion mapping of chromosomes 8, 10, and 16 in human prostatic carcinoma. Genes Chromosomes Cancer 1991;3:215–20.
[33] Macoska J, Trybus T, Sakr W, et al. Fluorescence in situ hybridization analysis of 8p allelic loss and chromosome 8 instability in human prostate cancer. Cancer Res 1994;54:3824–30.
[34] Emi M, Fujiwara Y, Nakajima T, et al. Frequent loss of heterozygozity for loci on chromosome 8p in hepatocellular carcinoma, colorectal cancer, and lung cancer. Cancer Res 1992;5:5368–72.
[35] Konig JJ, Kamst E, Hagemeijer A, et al. Cytogenetic characterization of several androgen responsive and unresponsive sublines of the human prostate carcinoma cell line LNCaP. Urol Res 1989;17:79–86.
[36] Ittman M. Allelic loss of chromosome 10 in prostate adenocarcinoma. Cancer Res 1996;56:2143–7.
[37] Maehama T, Dixon JE. The tumor suppressor PTEN/MMAC1 dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-triphosphate. J Biol Chem 1998;273:13375–8.
[38] Ittmann MM. Chromosome 10 alterations in prostate adenocarcinoma. Oncol Rep 1998;5(6):1329–35.
[39] Natt E, Magenis RE, Zimmer J, et al. Regional assignment of the human loci for uvomorulin (UVO) and chymotrypsinogen B (CTRB) with the help of two overlapping deletions on the long arm of chromosome 16. Cytogenet Cell Genet 1989;50:145–8.
[40] Graff JR, Herman JG, Lapidus RG, et al. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostatic carcinomas. Cancer Res 1995;55:5195–9.
[41] Schalken JA, Smit F, Bringuier PP, et al. The role of E-cadherin mutations in bladder and prostate cancer: Is E-cadherin a classical suppressor gene? J Urol 1996;155:339A.
[42] Giroldi LA, Schalken JA. Decreased expression of the intracellular adhesion molecule E-cadherin in prostate cancer: biological significance and clinical implications. Cancer Metastasis Rev 1993;12:29–37.
[43] Isaacs WB. Molecular genetics of prostate cancer. Cancer Surv 1995;25:357–79.
[44] Cooney KA, Wetzel JC, Consolino CM, Wojno KJ. Identification and characterization of proximal 6q deletions in prostate cancer. Cancer Res 1996;56:4150–3.
[45] Jenkins RB, Qian J, Lieber MM, Bostwick DG. Detection of c-myc oncogene amplification and chromosomal anomalies in metastatic prostatic carcinoma by fluorescence in-situ hybridization. Cancer Res 1997;57:524–31.
[46] Matusik RJ, Fleming WH, Hamel A, et al. Expression of the c-myc proto-oncogene in prostatic tissue. Prog Clin Biol Res 1987;239:91–112.
[47] Gumerlock PH, Poonamallee UR, Meyers FJ, DeVere White R. Activated ras alleles in human carcinoma are rare. Cancer Res 1991;51:1632–7.
[48] Moul JW, Friedrichs PA, Lance RS, et al. Infrequent ras oncogene mutations in prostate cancer. Prostate 1992;20:327–38.
[49] Konishi N, Enomoto T, Buzard G, et al. K ras activation and ras p21 expression in latent prostatic carcinoma in Japanese men. Cancer 1992;69:2293–9.
[50] Coussens L, Yang Feng T, Liao YC, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 1985;230:1132–9.
[51] Gullick WJ. The role of epidermal growth factor and the c-erbB-2 protein in breast cancer. Int J Cancer 1990;5(suppl):55–61.
[52] Slamon DJ, Godolphin W, Jones L, et al. Studies of HER-2/neu protooncogene in human breast and ovarian cancer. Science 1989;244:707–12.
[53] Zhau HE, Wan DS, Zhou J, et al. Expression of c-erb b-2/neu proto-oncogene in human prostatic cancer tissues and cell lines. Mol Carcinog 1992;5(4):320–7.
[54] Kuhn EJ, Kurnot RA, Sesterhem I, et al. Expression of cerbB2 (HER-2/neu) oncoprotein in human prostatic carcinoma. J Urol 1993;150(5):1427–33.
[55] Craft N, Shostak Y, Carey M, Sawyers C. A mechanism of hormone independent prostate cancer through modulation of androgen receptor signaling by the Her2/neu tyrosine kinase. Nat Med 1999;5:280–5.
[56] Kallakury BV, Sheehan C, Ambros R, et al. Correlation of p34-cdc2 cyclin dependent kinase overexpression, cd44 down-regulation, and Her2/neu oncogene amplification with recurrence in prostatic adenocarcinoma. J Clin Oncol 1998;16(4):1302–9.
[57] Robinson D, He F, Pretlow T, Kung HJ. A tyrosine kinase profile of prostate carcinoma. Proc Natl Acad Sci USA 1996;93(12):5958–62.
[58] Meyers RB, Srivastava S, Oelschlager DK, Grizzle WE. Expression of p160 erbB-3 and p185 erbB-2 in prostate intraepithelial neoplasia and prostatic adenocarcinoma. J Natl Cancer Inst 1994;86:1140–5.
[59] Leung HY, Weston J, Gullick WJ, Williams G. A potential autocrine loop between heregulin-α and erbB3 receptor in human prostatic adenocarcinoma. Br J Urol 1997;79(2):212–6.
[60] Lin MF, Meng TC, Rao PS, et al. Expression of human prostatic acid phosphatase correlates with androgen-stimulated cell proliferation in prostate cancer cell lines. J Biochem 1998;6:5939–47.
[61] Pisters LL, Troncoso P, Zhau HYE, et al. The role of hepatocyte growth factor/scatter factor and its receptor, the c-met proto-oncogene, in human prostatic tumor growth. J Urol 1993;149:482A.
[62] Zhau HE, Pisters L, Hall MC, et al. Biomarkers associated with prostate cancer progression. J Cell Biochem 1994;19(suppl):208–16.
[63] Dawson DM, Lawrence EG, MacLennan GT, et al. Altered expression of RET proto-oncogene in prostatic intraepithelial neoplasia and prostate cancer. J Natl Cancer Inst 1998;90(7):519–23.

[64] Miyashita T, Reed J. Bcl2 oncoprotein blocks chemotherapy induced apoptosis in a human leukemia cell line. Blood 1993;11:151–7.
[65] Reed JC. Dysregulation of apoptosis in cancer. Cancer J Sci Am 1998;4(suppl 1):S8–S14.
[66] Reed JC. Regulation of apoptosis by bcl-2 family proteins and its role in cancer and chemoresistance. Curr Opin Oncol 1995;7:541–6.
[67] Kyprianou N, English HF, Isaacs JT. Programmed cell death during regression of PC-82 human prostate cancer following androgen ablation. Cancer Res 1990;50:3748–53.
[68] Hockenberry DM, Zutter M, Hickey W, et al. Bcl-2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci USA 1991;88:6961–5.
[69] Raffo AJ, Perlman H, Chen MW, et al. Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. Cancer Res 1995;55:4438–45.
[70] Colombel M, Symmans F, Gil S, et al. Detection of the apoptosis-suppressing oncoprotein bcl-2 in hormone-refractory human prostate cancers. Am J Pathol 1993;143:390–400.
[71] McDonnell TJ, Troncoso P, Brisbay SM, et al. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 1992;52:6940–4.
[72] Krajewski S, Krajewska M, Shabaik A, et al. Immunohistochemical determination of in-vivo distribution of bax, a dominant inhibitor of bcl-2. Am J Pathol 1994;145:1323–36.
[73] Haldar S, Chintapalli J, Croce CM. Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. Cancer Res 1996;56:1253–5.
[74] Gao X, Porter AT, Honn KV. Involvement of multiple tumor suppressor genes and 12-lipoxygenase in human prostate cancer: therapeutic implications. Adv Exp Med Biol 1997;407:41–53.
[75] Hopkin K. Survival of the fittest cells. J Natl Inst Health Res 1996;8:37–41.
[76] Weinberg RA. Tumor suppressor genes. Science 1991;254:249–70.
[77] Hollstein M, Sidransky D, Vogelstein B, Harris CC. P53 mutations in human cancers. Science 1991;253:49–53.
[78] Bookstein R. Tumor suppressor genes in prostatic oncogenesis. J Cell Biochem 1994;19(suppl):217–23.
[79] Wertz IE, Deitch AD, Gumerlock PH, et al. Correlation of genetic and immunodetection of TP53 mutations in malignant and benign prostate tissues. Hum Pathol 1996;27(6):573–80.
[80] Gumerlock PH, Chi SG, Shi XB, et al. P53 abnormalities in primary prostate cancer: single strand conformation polymorphism analysis of complementary DNA in comparison with genomic DNA. The Cooperative Prostate Network. J Natl Cancer Inst 1997;89(1):66–71.
[81] Navone NM, Troncoso P, Pisters LL, et al. P53 accumulation and gene mutation in the progression of human prostate carcinoma. J Natl Cancer Inst 1993;85:1657–69.
[82] Effert PJ, McCoy RH, Walther PJ, et al. P53 gene alterations in human prostate carcinoma. J Urol 1993;150:257–61.
[83] Chi SG, et al. P53 in prostate cancer: frequent expressed transition mutations. J Natl Cancer Inst 1994;12:926–33.
[84] Meyers FJ, Gumerlock PH, Chi S-G, et al. Very frequent p53 mutations in metastatic prostate carcinoma and in matched primary tumors. Cancer 1998;83(12):2534–9.
[85] Myers RB, Oelschlager D, Srivastava S, Grizzle WE. Accumulation of the p53 protein occurs more frequently in metastatic than in localized prostatic adenocarcinomas. Prostate 1994;25:243–8.
[86] Tricoli JV, Gumerlock PH, Yao JL, et al. Alterations of the retinoblastoma gene in human prostate adenocarcinoma. Genes Chromosomes Cancer 1996;15:108–14.
[87] Mack PC, Chi SG, Meyers FJ, et al. Increased RB1 abnormalities in human primary prostate cancer following combined androgen blockade. Prostate 1998;34(2):145–51.
[88] Fearon ER, Cho KR, Nigro JM, et al. Identification of a chromosome 18q gene that is altered in colorectal carcinoma. Science 1990;247:49–56.
[89] Gao X, Honn KV, Grignon D, et al. Frequent loss of expression and loss of heterozygosity of the putative tumor suppressor gene DCC in prostatic carcinomas. Cancer Res 1993;53:2723–7.
[90] Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK. Nature 1993;366:704–7.
[91] Chi SG, deVere White RW, Muenzer JT, Gumerlock PH. Frequent alteration of CDKN2 (p16/MTS1) expression in human primary prostate carcinomas. Clin Cancer Res 1997;3:1887–9.
[92] Vlietstra RJ, van Alewijk DC, Hermans KG, et al. Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. Cancer Res 1998;58:2720–3.
[93] Cairns P, Okami K, Halchmi S, et al. Frequent inactivation of the PTEN/MMAC1 in primary prostate cancer. Cancer Res 1997;57:4997–5000.
[94] Giri D, Ittmann M. Inactivation of the PTEN tumor suppressor gene is associated with increased angiogenesis in clinically localized prostate cancer. Hum Pathol 1999;30:419–24.
[95] Dong JT, Suzuki H, Pin SS, et al. Down regulation of KAI1 metastasis suppressor gene during progression of human prostatic cancer infrequently involves gene mutation or loss. Cancer Res 1996;56:4387–90.
[96] Gao AC, Lou W, Dong JT, Isaacs JT. CD44 is a metastasis suppressor gene for prostate cancer located on human chromosome 11p13. Cancer Res 1997;57:846–9.
[97] Luu HH, Zagaja GP, Dubauskas Z, et al. Identification of a novel metastasis suppressor gene on human chromosome 12. Cancer Res 1998;58:3561–5.
[98] Tilley WD, Marcelli M, Wilson JD, McPaul MJ. Characterization and expression of a cDNA encoding the human androgen receptor. Proc Natl Acad Sci USA 1989;86:327–31.
[99] Caskey CT, Pizzuti A, Fu YH, Fenwick RG, Nelson DL. Triplet repeat mutations in human disease. Science 1992;256:784–8.
[100] Chamberlain NL, Driver ED, Miesfeld RL. The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. Nucleic Acids Res 1994;22:3181–6.
[101] Giovannucci E, Stampfer MJ, Krithivas K, Brown M, Brufsky A, Talcott J, et al. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. Proc Natl Acad Sci USA 1997;94:3320–3.
[102] Newmark JR, Hardy DO, et al. Androgen receptor gene mutations in human prostate cancer. Proc Natl Acad Sci USA 1992;89:6319–23.
[103] Tilley WD, Wilson CM, Marcelli M, McPhaul MH. Androgen receptor gene expression in human prostate carcinoma cell lines. Cancer Res 1990;50:5382–6.
[104] 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.
[105] Klocker H, Culig Z, Hobisch A, et al. Androgen receptor alterations in prostatic carcinoma. Prostate 1994;25:266–73.
[106] Tilley WD, Buchanan G, Hickey TE, Bentel JM. Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin Cancer Res 1996;2:277–85.
[107] Gaddipati JP, McLeod DG, Heidenberg HB, et al. Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. Cancer Res 1994;54:2861–4.

[108] De Vere White R, Meyers F, Chi SG, et al. Human androgen receptor expression in prostate cancer following androgen ablation. Eur Urol 1997;31(1):1–6.
[109] Taplin M, Bubley G, Shuster T, et al. Mutation of the androgen receptor gene in metastatic androgen independent prostate cancer. N Engl J Med 1995;332:1393–8.
[110] Hobisch A, Culig Z, Radmayr C, et al. Distant metastases from prostate carcinoma express androgen receptor protein. Cancer Res 1995;55:3068–72.
[111] Allsopp RC, Vaziri H, Patterson J, et al. Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci USA 1992;89:10114–8.
[112] Bacchetti S, Counter CM. Telomerase: a key to cell immortality. Int J Oncol 1995;7:423–32.
[113] Zhang W, Kapusta LR, Slingerland JM, Klotz LH. Telomerase activity in prostate cancer, prostatic intraepithelial neoplasia, and benign prostatic epithelium. Cancer Res 1998;58:619–21.
[114] Byrne R, Leung H, Neal DE. Peptide growth factors in the prostate as mediators of stromal epithelial interactions. Br J Urol 1996;77:627–33.
[115] Dong JT, Isaacs WB, Isaacs JT. Molecular advances in prostate cancer. Curr Opin Oncol 1997;9:101–7.
[116] Polyak K. Negative regulation of cell growth by TGF-B. Biochem Biophys Acta 1996;1242:185–99.
[117] Kim IY, Ahn HJ, Zelner DJ, et al. Loss of expression of transforming growth factor beta type I and type II receptors correlates with tumor grade in human prostate cancer tissues. Clin Cancer Res 1996;2:1255–61.
[118] Truong LD, Kadmon D, McCune BK, et al. Association of transforming growth factor beta 1 with prostate cancer: an immunohistochemical study. Hum Pathol 1993;24:4–9.
[119] Thompson TC. Growth factors and oncogenes in prostate cancer. Cancer Cells 1990;2:345–54.
[120] Davies P, Eaton C. Binding of epidermal growth factor by human normal, hypertrophic, and carcinomatous prostate. Prostate 1989;14:123–32.
[121] Traish AM, Wortiz HH. Prostatic epidermal growth factor receptors and their regulation by androgens. Endocrinology 1987;121:1461–7.
[122] Wilding G, Valverius E, Knabbe C, Gelmann EP. Role of transforming growth factor alpha in human prostate cancer cell growth. Prostate 1989;15:1–12.
[123] Lloyd SN, Brown LL, Leake RE. Transforming growth factor alpha expression in benign and malignant human prostatic disease. Int J Biol Markers 1992;7:27–34.
[124] Culig Z, Hobich A, Cronauer MV, et al. Androgen receptor activation in prostatic tumor cell lines by insulin like growth factor I, keratinocyte growth factor, and epidermal growth factor. Cancer Res 1994;54:5474–8.
[125] Iwamura M, Sluss PM, Casamento JB, et al. Insulin like growth factor I: action and receptor characterization in human prostate cancer cell lines. Prostate 1993;22:243–52.
[126] Chan JM, Stampfer MJ, Giovannucci E, et al. Plasma insulin like growth factor I and prostate cancer risk: a prospective study. Science 1998;279:556–63.
[127] Jarrard DF, Bussemakers MJG, Bova GS, Isaacs WB. Regional loss of imprinting of the insulin like growth factor II occurs in human prostate tissues. Clin Cancer Res 1995;1:1471–8.
[128] Giri D, Ropiquet F, Ittmanm M. Alterations in expression of basic fibroblast growth factor (FGF)2 and its receptor FGFR-1 in human prostate cancer. Clin Cancer Res 1999;5:1063–71.
[129] Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer angiogenesis and metastasis: an imbalance of positive and negative regulation. Cell 1991;64:327–36.
[130] Cress AE, Rabinovitz I, Zhu W, Nagle RB. The alpha 6 beta 1 and alpha 6 beta 4 integrins in human prostate cancer progression. Cancer Metastasis Rev 1995;14(3):219–28.
[131] Graff JR, Herman JG, Lapidus RG, et al. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res 1995;55:5195–9.
[132] Umbas R, Schalken JA, Aalders TW, et al. Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. Cancer Res 1992;52:5104–9.
[133] Ross JS, Figge HL, Bui HX, et al. E-cadherin expression in prostatic carcinoma biopsies: correlation with tumor grade, DNA content, pathologic stage, and clinical outcome. Mod Pathol 1994;7:835–41.
[134] Giroldi LA, Schalken JA. Decreased expression of the intracellular adhesion molecule E-cadherin in prostate cancer: biological significance and clinical implications. Cancer Metastasis Rev 1993;12:29–37.
[135] Richmond PJ, Karayiannakis AJ, Nagafuchi A, et al. Aberrant E-cadherin and alpha-catenin expression in prostate cancer: correlation with patient survival. Cancer Res 1997;57:3189–93.
[136] Stearns ME, Wang M. Immunoassays of the metalloproteinase (MMP-2) and tissue inhibitor of metalloproteinase (TIMP1 and 2) levels in non-invasive and metastatic PC-3 clones: effects of taxol. Oncol Res 1994;6:195–201.
[137] Pajouh MS, Nagle RB, Breathnach R, et al. Expression of metalloproteinase genes in human prostate cancer. J Cancer Res Clin Oncol 1991;117:144–50.
[138] Baker T, Tickle S, Wasan H, et al. Serum metalloproteinases and their inhibitors: markers for malignant potential. Br J Cancer 1994;70:506–12.
[139] Sandberg AA. Chromosomal abnormalities and related events in prostate cancer. Hum Pathol 1992;23:368–80.

Biography

Dr Lara is an Assistant Professor of Medicine at the Division of Hematology-Oncology, Department of Internal Medicine, University of California Davis School of Medicine, UCD Cancer Center in Sacramento, California, USA. His research activities revolve around developmental therapeutics and genitourinary malignancies.

American Association for **Cancer Research**

92nd Annual Meeting

March 24-28, 2001 • New Orleans, LA

Volume 42 • March 2001

Proceedings

#2938 **Role of the *RB* Pathway in the Androgen Response of Prostate Cancer (CaP) Cells with Reconstituted Androgen Receptor (*AR*).** Paul H. Gumerlock, Matthew H. Gustafsson, Joachim B. Schnier, and Kayoko Nishi-Schnier. *University of California, Davis Cancer Center, Sacramento, CA.*

Virtually all CaPs progress to an incurable state of androgen-independence (AI) that will be the cause of nearly 32,000 deaths of men in the US in 2000. We previously showed that *RB* pathway abnormalities in primary CaP include loss of expression of the tumor suppressors *CDKN2/p16* in 43% and *RB* in 13%. Loss of *RB* expression was found significantly increased to 36% in AI tumors ($p=0.015$). Rb has also been found to be a nuclear co-factor for the AR. Our hypothesis is that loss of Rb contributes to AI CaP. The PC3 cell line was established from a wild-type (wt) *RB*, *p53*-null, AI CaP. PC3 cells transfected with wt *AR* (PC3-AR2) provide a unique model to study the role of RB in responses to androgens. Treatment of PC3-AR2 with the synthetic androgen R1881 resulted in cell cycle arrest and eventual cell death. Molecular responses were examined at 24-hr intervals over a period of six days. Cell cycle-related proteins [Rb, p21, p27, Cyclin A] and AR were assessed by Western blotting at each time point and normalized to -actin. Rb co-immunoprecipitated with AR, demonstrating interaction of the two proteins in this model system. Rb was completely hypophosphorylated after 24-hrs of R1881, and levels declined markedly up to six days. p21 was induced within 24-hrs, but decreased by 48-hrs and was undetectable at 72-hrs. After 24-hrs, no expression of Cyclin A was seen, in contrast to moderate levels in control cells, providing evidence of cell arrest at the R point in G1. Levels of p27 decreased over the entire period. Our data suggest that this androgen-induced G1 arrest occurs by the mechanism of *p53*-independent induction of *p21* and hypophosphorylation of Rb, and demonstrate a role for *RB* in the androgen-response of CaP cells. (Support: DAMD17-98-1-8565.)

#1171 **Mutant p53 Induction of Bag1L as a Potential Mechanism for Androgen-Independence in Human Prostate Cancer.** Nancy Jane Nesslinger, Xu-Bao Shi, Matthew H. Gustafsson, Paul H. Gumerlock, and Ralph W. deVere White. *UC Davis, Sacramento, CA.*

Prostate cancer (CaP) is the most common tumor found in American men and is the second leading cause of cancer death. Since proliferation and maintenance of function in malignant prostate epithelial cells is initially androgen-dependent, androgen ablation has been the first line of treatment for patients with metastatic prostate cancer. However after an initial response, both the primary tumors and their metastases eventually become androgen-independent. Mutations of the *p53* gene are common in CaP, with higher rates in hormone-refractory disease. To date there has been no evidence to link *p53* mutations to androgen-independence. Recently the Bag1L protein was shown to sensitize the androgen receptor (AR) to low levels of androgen. In addition, some gain-of-function *p53* mutants were shown to upregulate transcription of the *BAG1* promoter. Our hypothesis is that certain gain-of-function *p53* mutations will lead to androgen-independence through Bag1L induction. Using human prostate cancer PC3 cells transfected with a wild-type androgen receptor (PC3-AR2), we immunoprecipitated the AR and performed Western blotting using an anti-Bag1 monoclonal antibody. We found that of the 3 isoforms of Bag1 (Bag1, Bag1M and Bag1L), only the Bag1L protein co-immunoprecipitated with AR. PC3 sub-lines containing 5 different mutant *p53* alleles were constructed by stable transfection. Levels of Bag1L protein were measured by Western blotting and normalized to β-actin. The expression of Bag1L was higher in 4 of these sub-lines compared to the untransfected control. In addition, 2 of the PC3 sub-lines containing gain-of-function, dominant negative *p53* alleles were grown in medium containing 5% charcoal-stripped serum before determining cell numbers. The parental cells did not grow whereas the mutant *p53* transfectants were able to proliferate in the androgen-deprived medium. This provides the first evidence that *p53* mutations may act through Bag1L to promote androgen-independence.

AACR American Association for Cancer Research

93rd Annual Meeting

April 6-10, 2002 • San Francisco, California

Volume 43 • March 2002

In joint sponsorship with the Keck School of Medicine of the University of Southern California

The premier meeting for cancer research in the post-genomic era

Featuring the latest developments in basic, translational, and clinical cancer research

Proceedings

#3998 **Role of *RB* in the response of androgen-independent prostate cancer cells to androgen.** Paul H. Gumerlock, Matthew H. Gustafsson, Kayoko Nishi-Schnier, and Joachim B. Schnier. *University of California, Davis Cancer Center, Sacramento, CA, and University of California, Davis, Davis, CA.*

The vast majority of prostate cancers (CaP) progresses to an incurable state of androgen-independence (AI) and will be the cause of nearly 30,000 deaths of men in the US in 2001. We have shown that abnormalities in the *RB* pathway in primary CaP include loss of expression of the tumor suppressors *CDKN2/p16* in 43% and *RB* in 13%. However, in AI tumors the loss of *RB* expression significantly increased to 36% ($p=0.015$). Further, in the PC3-AR2 model system [wild-type (wt) [i]*RB*, *p53*-null, AI CaP, transfected with wt androgen receptor(*AR*)], treatment with the synthetic androgen R1881 resulted in cell cycle arrest and eventual cell death. Molecular analyses showed that Rb co-immunoprecipitated with AR and that there was *p53*-independent induction of p21, hypophosphorylation of Rb, loss of Cyclin A, and gradual decreases of p27, implicating the *RB* gene in this response of AI prostate cancer cells. To further clarify the role of *RB*, the PC3-AR2 cells were secondarily transfected with the human papilloma virus (HPV) *E7* gene, whose protein binds and inactivates Rb. We hypothesized that disruption of *RB* in the PC3-AR2-E7 model would attenuate the G1 arrest response to androgens. Cells were treated with R1881 and molecular responses were examined at 24-hr intervals over a period of four to six days. Cell cycle-related proteins [Rb, p21, p27] were assessed by Western blotting and normalized to β-actin. The levels of p21 in the PC3-AR2-E7 cells closely resembled those previously in the PC3-AR2 cells. Levels were induced at 24 hr, but declined to basal levels by 48 hr. After five days, PC3-AR2-E7 cells survived approximately 2-fold greater than the vector alone transfected control cells. Conclusions: *RB* functions in the androgen-response of CaP cells and its disruption leads to increased survival of AI cells in the presence of androgens. (Support: DAMD17-98-1-8565)

AACR American Association for Cancer Research

93rd Annual Meeting

April 6-10, 2002 · San Francisco, California

Volume 43 · March 2002

In joint sponsorship with the Keck School of Medicine of the University of Southern California

The premier meeting for cancer research in the post-genomic era

Featuring the latest developments in basic, translational, and clinical cancer research

Proceedings

#5636 Gain-of-function mutant *p53* mediates androgen-independent growth of human LNCaP prostate cancer cells. Nancy J. Nesslinger, Xu-Bao Shi, Paul H. Gumerlock, and Ralph W. Devere White. *University of California, Davis, Sacramento, CA.*

Androgen ablation has traditionally been the first line of treatment for patients with advanced prostate cancer (CaP). However, after an initial response these

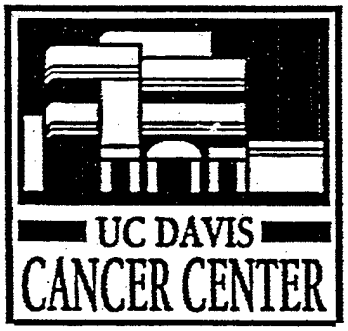
tumors eventually become androgen-independent (AI) with no effective therapy currently available. Our previous studies have shown that mutations of *p53* occur at a rate of 70% in hormone-refractory CaP, suggesting that these mutations may be involved in the progression of CaP to androgen-independent growth. However, to date there has been no direct evidence linking *p53* mutations with AI growth of CaP cells. The LNCaP cell line expresses wild-type *p53* and is a useful *in vitro* model for CaP study because it is sensitive to androgen stimulation and its growth is inhibited in the absence of androgens. We established 5 stably-transfected LNCaP cell lines: 4 containing gain-of function mutant *p53* alleles (G245S, R248W, R273H, R273C) and one containing a mutant *p53* allele (P151S) with no gain-of-function characteristics. The 4 LNCaP sub-lines containing gain-of-function mutant *p53* alleles were found to grow under androgen-depleted conditions, but the LNCaP parental line and the remaining sub-line with no gain-of-function abilities did not grow. To investigate the mechanism of the androgen-independent growth displayed by the gain-of-function mutant *p53* alleles, Western blotting or ELISA were used to examine the expression of the androgen receptor (AR), AR-regulated prostate-specific antigen (PSA), Bag1 and Akt under androgen-depleted conditions. Upon androgen ablation, the levels of AR decreased in the 4 gain-of-function mutant *p53* sub-lines compared to the parental cells. This decreased AR expression was accompanied by attenuated receptor activity, since a decrease in PSA levels compared to parental LNCaP cells was also observed. However, Bag1L levels were increased under androgen-depleted conditions in 3 of the 4 gain-of-function mutant *p53* sub-lines as compared to the parental line. In contrast, levels of phosphorylated Akt increased in both the mutant *p53*-transfected sub-lines and the parental line. These observations suggest that only gain-of-function mutant *p53* alleles mediate the androgen-independent growth of CaP cells and that Bag1, but not Akt, may be involved in this process. (Support: NCI RO1 CA 77662, NCI RO1 92069, DOD DAMD 17-98-8565)

UC Davis Cancer Center

Sixth Annual

Cancer Research Symposium

Program & Abstracts
October 6 –7, 2000



Poster Abstracts

Session II – Saturday, October 7

Role of the *RB* pathway in the androgen response of prostate cancer (CaP) cells with reconstituted androgen receptor (AR). PH Gumerlock, JB Schnier, K Nishi-Schnier, MH Gustafsson. Departments of Biochemistry and Internal Medicine: Hematology/Oncology, UCD School of Medicine.

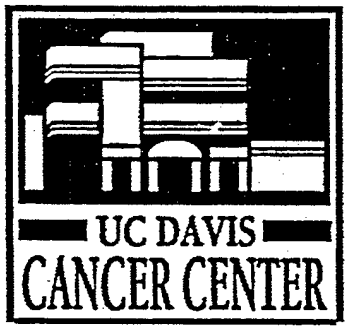
Virtually all CaPs progress to an incurable state of androgen-independence (AI) that is the cause of nearly 40,000 deaths of men in the US each year. We previously showed that *RB* pathway abnormalities in primary CaP include loss of expression of the tumor suppressors *CDKN2/p16* in 43% and *RB* in 13%. Loss of *RB* expression was found significantly increased to 36% in AI tumors (p=0.015). Rb has also been found to be a nuclear co-factor for the AR. Our **hypothesis** is that loss of *RB* contributes to AI CaP. The PC3 cell line was established from a wild-type *RB*, *p53*-null, AI CaP. PC3 cells transfected with wt *AR* (PC3-AR2) provide a unique model to study the role of *RB* in responses to androgens. Treatment of PC3-AR2 with the synthetic androgen R1881 resulted in cell cycle arrest and eventual cell death. Molecular responses were examined at 24-hr intervals over a period of six days. Cell cycle-related proteins [Rb, p21, p27, Cyclin A] and AR were assessed by Western blotting at each time point and normalized to β-actin. Rb co-immunoprecipitated with AR, demonstrating interaction of the two proteins in this model system. Rb was completely hypophosphorylated after 24-hrs of R1881. p21 was induced within 24-hrs, but decreased by 48-hrs and was undetectable at 72-hrs. After 24-hrs, no expression of Cyclin A was seen, in contrast to moderate levels in control cells, providing evidence of cell arrest at the R point in G1. Levels of p27 decreased until 96-hrs where the levels dropped to lower than the control. Our data suggest that this androgen-induced G1 arrest occurs by the mechanism of *p53*-independent induction of p21 and hypophosphorylation of Rb, and demonstrate a role for *RB* in the androgen-response of CaP cells. (Support: DAMD17-98-1-8565.)

UC Davis Cancer Center

Sixth Annual

Cancer Research Symposium

Program & Abstracts
October 6 –7, 2000



Poster Abstracts

Session II – Saturday, October 7

Induction of Bag1L by mutant *p53* as a potential mechanism for androgen-independence in human prostate cancer. MH Gustafsson, NJ Nesslinger, JB Schnier, X-B Shi, RW deVere White, PH Gumerlock. Departments of Internal Medicine (Hematology/Oncology) and Urology, UC Davis Cancer Center.

Despite the Nobel-winning observation by Charles Huggins in the 1940's that castration causes regression of metastatic carcinoma of the prostate (CaP) in dogs, CaP remains the second leading cause of cancer-related deaths among men in the United States. Virtually all CaPs progress to an incurable state of androgen-independence after initial response to androgen-ablation. Mutations in the *p53* gene are common in CaP, with higher rates in hormone-refractory disease. To date there has been no evidence to link *p53* mutations to androgen-independence. Recently the Bag1L protein was shown to sensitize the androgen receptor (AR) to low levels of androgen. In addition some gain-of-function *p53* mutants were shown to upregulate transcription of the *BAG1* promotor. Our hypothesis is that certain gain-of-function *p53* mutations will lead to androgen-independence through Bag1L induction. Using human prostate cancer PC3 cells transfected with a wild-type androgen receptor (PC3-AR2), we immunoprecipitated the AR and performed Western blotting using an anti-Bag1 monoclonal antibody. We found that of the three isoforms of Bag1 (Bag1, Bag1M, and Bag1L) only the Bag1L protein co-immunoprecipitated with AR. PC3 sub-lines containing 5 different mutant *p53* alleles were constructed by transfection. Levels of Bag1L protein were measured by Western blotting and normalized to β-actin. The expression of Bag1L was induced in 4 of these sub-lines compared to the untransfected control. These results support our hypothesis that certain gain-of-function mutant *p53* alleles may contribute to androgen-independence by the induction of Bag1L. (Supported by DAMD17-98-1-8565 and CA77662.)

UC Davis Cancer Center
Seventh Annual Cancer Research Symposium
2001 Abstract Form

1. Abstract Presented By: Matthew H. Gustafsson

2. Position/Title: Post Graduate Researcher

3. Department/Affiliation: Department of Radiation Oncology

4. Mailing Address: UCD Cancer Center, 4501 X Street, Sacramernto, CA 95817

5. Telephone: 916-734-3734 E-mail: mhgustafsson@ucdavis.edu

6. Abstract Title: **Role of *RB* in the response of androgen-independent prostate cancer cells to androgen as revealed by transfection of the E7 gene.**

Paper
X Poster

7. To submit an abstract in hard copy, **photocopy this form**, use only the space within the square below (or within a 5 inch square on your word processor) for the abstract, and mail the completed form **unfolded and without creases** to the UC Davis Cancer Center. Submission deadline is **September 7, 2001**. You may fax a version to (916) 451-4464; hard copy or e-mail **must** follow.

Mail to:
7th Annual Symposium
UC Davis Cancer Center
4501 X Street, Rm 3009A
Sacramento, CA 95817

(916) 734-5818

e-mail submission:
cancer.center
@ucdmc.ucdavis.edu

Role of *RB* in the response of androgen-independent prostate cancer cells to androgen as revealed by transfection of the E7 gene. MH Gustafsson, JB Schnier, K Nishi-Schnier, PH Gumerlock. Departments of Internal Medicine and Medical Biochemistry, UC Davis School of Medicine, Sacramento & Davis, CA.

The vast majority of prostate cancers (CaP) progresses to an incurable state of androgen-independence (AI) and will be the cause of nearly 30,000 deaths of men in the US in 2001. We have shown that abnormalities in the *RB* pathway include loss of expression of the tumor suppressors *CDKN2/p16* in 43% and *RB* in 13% in primary CaP, however, in AI tumors the loss of *RB* expression significantly increased to 36% (p=0.015). Further, in the PC3-AR2 model system (wt *RB*, *p53*-null, AI CaP, transfected with wt *AR*), treatment with the synthetic androgen R1881 resulted in cell cycle arrest and eventual cell death. Molecular analyses showed that Rb co-immunoprecipitated with AR and that there was *p53*-independent induction of p21, hypophosphorylation of Rb, loss of Cyclin A, and gradual decreases of p27, implicating the *RB* gene in this response of AI prostate cancer cells. To further clarify the role of *RB*, the PC3-AR2 cells were secondarily transfected with the human papilloma virus (HPV) E7 gene, whose protein binds and inactivates Rb. We hypothesized that disruption of *RB* in the PC3-AR2-E7 model would attenuate the G1 arrest response to androgens. Cells were treated with R1881 and molecular responses were examined at 24-hr intervals over a period of four to six days. Cell cycle-related proteins [Rb, p21, p27] were assessed by Western blotting and normalized to β-actin. The levels of p21 in the PC3-AR2-E7 cells closely resembled those previously in the PC3-AR2 cells. Levels were induced at 24 hr, but declined to basal levels by 48 hr. After five days, PC3-AR2-E7 cells survived approximately 2-fold greater than the vector alone transfected control cells. Conclusions: *RB* functions in the androgen-response of CaP cells and its disruption leads to increased survival of AI cells in the presence of androgens. (Support: DAMD17-98-1-8565)

DAMD17-98-1-8565
PI: Paul H. Gumerlock, Ph.D.

FINAL REPORT:
PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Paul H. Gumerlock, Ph.D.
Joachin B. Schnier, Ph.D.
Kayoko Nishi-Schnier, Ph.D.
Dominika Maglasang, B.S.
Matthew Gustaffsson, B.S.