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Project B - Androgen Receptor (AR) Signaling in Prostate Cancer Progression
Project C - Cellular and Molecular Markers of Prostate Cancer Progression
Core - Epidemiology Core

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Project B - Gerhard A. Coetzee, Ph.D.
Project C - Richard Cote, M.D.
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13. ABSTRACT ***(Maximum 200 Words)***

This Prostate Cancer Center Initiation grant has been designed to identify genetic and molecular markers of prostate cancer progression within and between racial ethnic groups (African-Americans, Latinos, Whites, Japanese) at substantially distinct underlying risk of prostate cancer. Our Epidemiology Core has obtained signed tissue releases from prostate cancer patients to date identified during follow-up of the Hawaii/Los Angeles Multiethnic Cohort study. Two hundred thirty-nine tissue samples have been received and processed histopathologically by Project C, which has begun immunohistochemical staining for COX-2, p27, p21, p16 and Caveolin-1 markers with additional markers to follow. Project B, studying the androgen receptor (AR) gene in detail, has identified 54 sequence variants in 90 samples analyzed to date. Two functional assays were developed this year to better assess these sequence variants. In Project A, studying the SRD5A2 gene in detail, in 87 tumors, the 13 mutations detected to date have been reconstructed by site-directed mutagenesis.

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Overall Introduction

We report below our second year progress in our Prostate Cancer Center Initiation grant. The overall goal of our Center is to understand molecular and genetic factors associated with progression of occult prostate cancer to invasive disease, as only a small subset of patients progress, but for which no mechanism currently exist to identify which ones. Moreover, the rate of progression appears to differ substantially among racial-ethnic groups as the prevalence of occult cancer is similar among African-Americans, Latinos, Whites and Japanese-Americans but the incidence of invasive disease varies several-fold across these same groups.

The Center consists of three Projects and one Core and we organize our progress report along these lines. The Core is designed to identify prostate cancer patients from a large prospective study in Los Angeles and Hawaii, obtain signed tissue release forms from these patients, secure tissue samples from hospitals and distribute these to the three Project laboratories. We had substantial delay in starting this critical aspect of the project because of IRB issues locally and at the Army Medical Research Center; these have all been resolved and all aspects of this project are proceeding in Hawaii. We have signed releases from subjects and have secured tissue. We have had substantial success (see below) in identifying and characterizing sequence variants in the androgen receptor gene and the steroid 5-alpha reductase type II genes, the major goals of Projects B and A, respectively. Although the budget for Project C was reduced by more than 50% and much of the activity of this Project to date has been to process and characterize the tissue samples as they are sent by hospitals, we have nonetheless been conducting a number of immunohistochemical studies of molecular markers of progression as planned in this Project including p27, p21, p16, COX-2 and Caveolin-1 (see below).

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Project A: The Human 5RD5A2 Gene and Prostate Cancer Progression
Principal Investigator: Juergen Reichardt, Ph.D.

INTRODUCTION

There is a large variation in prostate cancer rates between racial-ethnic groups in the US. We have taken a "candidate gene" approach to prostate cancer. We have focused on androgen-metabolic genes since they can regulate prostatic growth. Specifically, we proposed to examine the hypothesis that *de novo* DNA sequence variations (i.e. somatic mutations) in the type II (or prostatic) steroid 5α-reductase (SRD5A2) gene contribute substantially to the progression of prostate cancer particularly across racial/ethnic lines.

BODY

In our application we had proposed to investigate the following three interrelated specific aims:

1. To identify somatic mutations in prostatic tumors of men from four racial/ethnic groups [African-Americans, Asian-Americans, Caucasians and Latinos] in the regulatory elements of the SRD5A2 gene, specifically its promoter and the 5' and 3' untranslated regions (UTR);

2. To determine the frequency of somatic SRD5A2 mutations in prostate cancers in four racial-ethnic groups [African-Americans, Asian-Americans, Caucasians and Latinos];

3. To determine the contribution of the SRD5A2 somatic mutations screened for in specific aim 2 to prostate cancer grade and stage of disease as surrogates for outcome.

KEY RESEARCH ACCOMPLISHMENTS

Substantial progress was made toward specific aim 1 last year as reported. Furthermore, we have now reconstructed all *de novo* somatic amino acid substitutions in the SRD5A2 cDNA. Therefore, we are poised to express them in *cos* cells to determine their biochemical properties. Specifically, we have reconstructed by site-directed mutagenesis eleven single amino acid substitutions and two double mutants for a total of 13 SRD5A2 somatic mutants to be assayed. We have also made significant progress toward specific aim 2: we have genotyped 87 DNA samples for the A49T (alanine at codon 49 replaced by threonine) and V63M (valine-63 to methionine) *de novo* somatic mutations. Both mutations were identified as detailed in Table 1 along with five additional potential new mutations.

Table 1
Somatic Mutations in the SRD5A2 Gene

Mutation	Number	Comments
A49T	2	
V63M	4	
Other	5	To be sequenced

REPORTABLE OUTCOMES

None thus far besides Table 1.

CONCLUSIONS

This laboratory has completed the sequencing component of specific aim 1. We are, therefore, poised to begin with the investigation of the biochemical properties of the somatic mutations we identified in the SRD5A2 locus. In specific aim 2, we have already begun screening for the A49T and V63M recurrent protein-coding mutations in tumor and "normal" samples obtained through this grant. Both were found repeatedly as outlined in Table 1. Genotyping will, therefore, continue in the next year and novel mutations will be identified by DNA sequencing (cf. Table 1). This strategy will allow us to determine their contributions to tumor progression in specific aim 3 in the coming year.

REFERENCES

None

APPENDICES

None

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Project B: Androgen Receptor (AR) Signaling in Prostate Cancer Progression
Principal Investigator: Gerhard A. Coetzee, Ph.D.

INTRODUCTION

Prostate tumorigenesis requires a functional androgen-signaling axis, the components of which form the principal targets of androgen ablation therapies that inhibit the growth of prostate cancer. For patients who are either diagnosed with or subsequently develop metastatic disease, the only treatment option is androgen ablation [i.e. orchidectomy, treatment with LHRH agonists/antagonists and/or AR antagonists]. Despite an initial good response in 80-90% of patients with metastatic disease, androgen ablation is essentially palliative and disease progression eventually ensues. Resistance to androgen ablation is not necessarily due to loss of androgen sensitivity, but may develop as a consequence of a deregulated androgen signaling axis resulting from amplification or mutation of the AR gene, altered interaction of co-regulatory molecules, or ligand independent activation of the AR by growth factors and cytokines. Thus the AR is involved in most phases of prostate cancer biology, from genetic predisposition (due to the existence of polymorphic variants), through to disease progression that includes resistance to androgen-ablation therapies.

Recent studies in clinical prostate cancer have identified several mechanisms that potentially explain how prostate tumors progress following initiation of androgen ablation. These include amplification or mutation of the AR gene, altered interaction with co-regulatory molecules and ligand-independent activation of the AR. These studies suggest that resistance to conventional androgen ablation therapy is not due to a loss of androgen sensitivity but rather may be a consequence of a deregulated androgen-signaling axis. Gain-of-function mutations at the AR locus seem to be selected in advanced prostate cancer, especially as a consequence of androgen ablation treatments. Somatic missense mutations have been detected throughout the AR coding sequence at frequencies of up to 50% in metastatic deposits from hormone refractory patients. These mutations consistently result in receptors that exhibit decreased specificity of ligand-binding and/or enhanced receptor activation by androgens and non-classical ligands compared to wild type AR (wtAR). A survey of AR mutations in prostate tumors revealed that nearly 80% cluster to discrete regions of the receptor that collectively span less than 15% of the coding sequence. It is for this reason that we have targeted these regions (hotspots) in our mutation detection strategy in the present study.

BODY

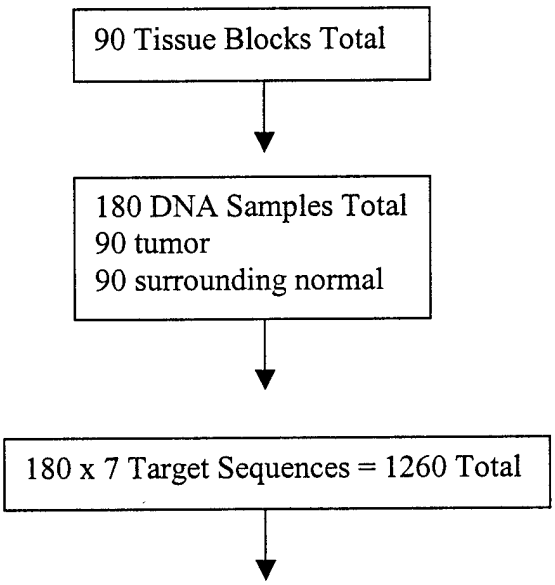
Our initial plan was to analyze 160 prostate cancer tumor blocks per year by two main tasks:

Task 1. To measure the frequency of somatic AR mutations in tumors from prostate cancer patients with 'localized' and 'advanced' disease, and to determine if the frequency differs between the two groups among different racial-ethnic groups (weeks 1-24). Microdissect samples (week 1). Extract DNA (week 2). PCR amplify AR exons and prepare for sequencing (weeks 3-24). Sequence AR exons and analyze results (weeks 4-24).

Task 2. To determine the functional significance of somatic AR mutations (weeks 10-50). Reconstruct 15 mutations by site-directed mutagenesis (weeks 10-50). Transfect PC3 cells and measure AR activity under various conditions (weeks 10-50). Transfect PrEC cells and measure AR activity under various conditions (weeks 10-50).

Task 1: We are behind schedule in our tissue block accrual, but hopefully will be able to complete the tissue accrual (160 blocks) during the next year.

Summary of tissues block analyses



Hotspot	Amino Acids	# PCR Performed	# Sequences Obtained	Percent of PCR Success	Potential Mutations	Percent Mutations in Sequences
HS I	Glu-Arg (43-99)	152	146	96%	19	13%
HS II	Leu-Cys (237-282)	78	55	71%	3	5%
HS III	Pro-Intron 2 (485-537)	78	67	86%	3	4%
HS IV	Gly-Asp (666-690)	180	134	74%	0	0%
HS V exon 4	Phe-Intron 4 (696-723)	180	90	50%	3	3%
HS V exon 5	Arg-Gly (726-743)	180	171	95%	12	7%
HS VI	Ile-Intron 8 (868-919)	180	166	92%	14	8%

Task 2: To enable a better functional analysis of mutant ARs, we have developed two new functional assays in addition to our classic co-transfection transient reporter system of AR transactivation activity. They are:

(i) The mammalian two-hybrid system in which we assay protein-protein interactions. For this pupose, we have established the Clontech mammalian MATCHMAKER two-hybrid system. The pM vector was engineered to express a chimeric protein consisting of a protein of interest (or protein fragment e.g. AR-LBD) fused to the DNA binding domain of the yeast GAL4 gene. The chimeric protein contains a nuclear localization signal and can bind constitutively to the 5 consensus GAL4 response elements of the cotransfected pGK1 reporter construct (expressing luciferase). As long as the protein (or fragment) does not contain an intrinsic activation function, the construct is unable to activate transcription of the luciferase reporter gene. The pVP16 vector is engineered to express a chimeric protein consisting of the other binding protein of interest (e.g. AR-NTD) or fragment (e.g. subdomains of the AR-NTD) fused to the intrinsic activation domain (Ta) of the VP16 protein. The VP16 fusion protein contains a nuclear localization signal but is unable to interact with the consensus GAL4 response elements in the luciferase reporter construct. Mammalian cells are transfected with the pM chimer, the pVP16 chimer and pGK1 reporter at a molar ratio of approximately 1:1:2.5. If the one protein is able to interact with the other, the tethering of the VP16-Ta and GAL4-DBD via the interaction is sufficient to activate transcription of the luciferase reporter gene. A semi-quantitative evaluation of the strength of interaction is provided by the relative luciferase activity compared to cells transfected with pM and pVP16 vectors, either expressing fusions of proteins known to interact [i.e. pM-53 (p53) and pVP16-T (SV40 large T antigen)] or known not to interact [i.e. pM-53 (p53) and pVP16-CP (SV40 coat protein). additional controls include the parent pM and pVP16 vectors.

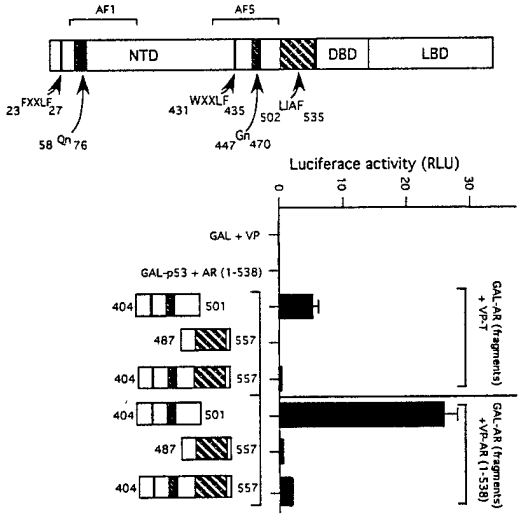


Figure 1: *Mammalian two-hybrid analysis of AR N-N interactions: Cos-7 cells were tranfected as indicated with GAL4 DBD (10ng) and VP16Ta (10ng) fusion proteins and a GAL4 promoter driven Luciferase reporter in 96 well cluster dishes. Exposure to DHT (10nM) to indicated wells for 24h was followed by Luciferase activity determinations. Values are mean +/- SE of at least 4 independent wells. Structures within the AR-NTD are schematically represented. Intrinsic transactivation activity of AR fragments is measured as 'background' in the controls containing large T antigen as a fusion protein with VP16Ta.*

Using this method we have discovered a novel N-terminal/N-terminal interaction in the AR probly being manifested during AR dimerization. Three AR-NTD fragments were constructed to either span the C-terminal part of the NTD including AF-1, or contain most aspects of the AF-5 and LIAF subdomains (***Figure 1***). These fragments were tested in the mammalian two-hybrid system, described above, for interaction with the entire AR-NTD. Because of high autologous transactivation activity of the AR-NTD, the orientation of the two-hybrid system was such that the AR-NTD was fused with the VP16Ta and different NTD fragments fused with the GAL4-DBD. Even in this orientation, some of the fragments displayed significant transactivation activity without interaction. Positive interaction was scored as increased activity over this background, as was the case for fragment (aa 404-501), indicating that this region of the

AR-NTD can interact with the entire NTD in an apparent N-N interaction. Of considerable interest was the observation that a fragment, which included the LIAF domain (aa 502-535), completely abolished this interaction/activity. While these results support an N-N interaction, we do not know yet which subdomain in this region forms the interaction interface, although the two LXXLL-like motifs in the AR-NTD and the Q_n and G_n stretches are good candidates. Of interest was the added observation that the LIAF also apparently functions as a strong inhibitory domain in this setting. It should be noted that in other assays, in the context of the entire NTD, the LIAF possesses strong constitutive transactivation activity. At this stage we do not understand the different functions of the AR-NTD subdomains but it is likely that structural alterations within them might influence inter- and intra molecular AR interactions as well as interactions with transcriptional coregulators and thus effect AR transactivation activity.

(ii) ChIP assays: These assays are being employed to determine how alterations in AR-NTD influence the composition and kinetics of occupancy of the AR transcription complex on a natural promoter. Since most of the preceding work is based on AR analyses that depend on functional and interaction assays performed in artificial transfection systems, a more physiological system was sought. Chromatin immunoprecipitation (ChIP) assays provide a method to interrogate the molecular composition of complexes on natural promoters *in vivo*. In the case of steroid receptors, promoter occupancy can be synchronized by the addition of ligand to the cultures. As a result occupancy can be assessed as a function of time (i.e. kinetically) and composition by using appropriate antibodies. Negative controls include the use of non-specific antibodies, absence of ligand and PCR amplification of irrelevant DNA fragments. Steroid receptor mediated transactivation on chromatin integrated promoters is thought to occur in at least two distinct steps, namely chromatin remodeling followed by transcription initiation. In the case of glucocorticoid receptor (GR) action on chromosomal integrated MMTV LTR, both these steps seem to be hormone dependent. It is likely that either of these steps is affected by genetic variations of the AR. By coupling natural promoter occupancy (ChIP assays) with expression of the associated androgen regulated gene (e.g. PSA), the two steps can be evaluated separately. Multiple protein-protein interactions are likely to be

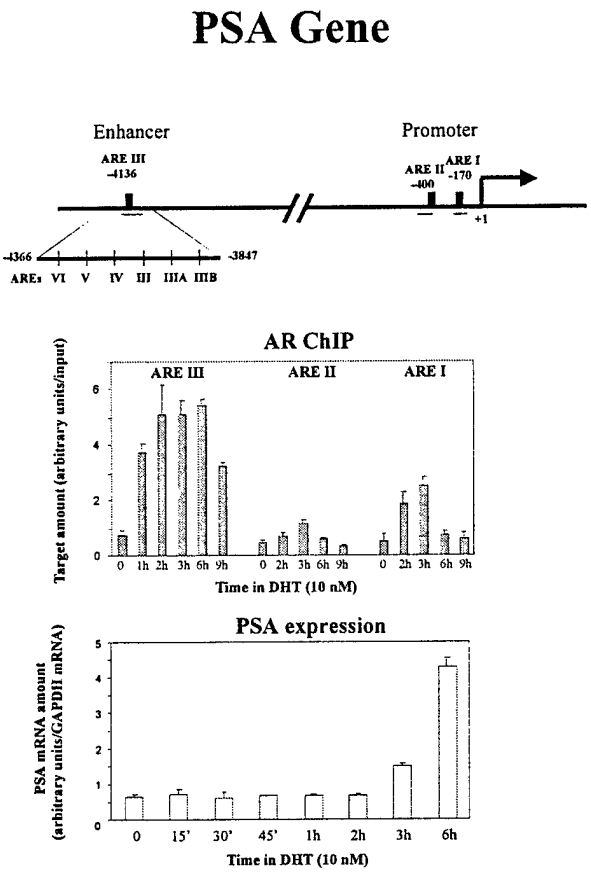


Figure 2: *PSA promoter promoter/enhancer occupancy by AR. LNCaP cells were grown in Medium containing 10% charcoal-stripped FBS. At time 0, 10nM DHT were added to the medium and at the indicated times cells were fixed in formalin (ChIP) or RNA extracted (PSA expression). For the ChIP assay isolated cross-linked chromatin was somicated, IPed with AR antibody, the cross-link reversed and target DNA quantitated in a real-time PCR reaction (BioRad). Input DNA was treated similarly, except that samples were taken before the IP step. RT-PCR of PSA quantitation was conducted on total RNA isolated and normalized toGAPDH mRNA. Values are means (±SD) of triplicated PCR determinations.*

required to bring AR and its associate proteins into the appropriate vicinity/conformation to initiate transcription via the basal transcription machinery. If a particular mutant AR ineffectively interacts with one protein, it could affect a cascade of downstream interactions and severely alter the final transcriptional output. AR mutants that can transactivate transiently expressed reporter genes, might occupy or recruit a different complement of protein partners to the basal transcription complex on natural promoters to affect transcription initiation in a manner different from wild type AR. For example, a mutation (C644G) in the mouse GR activates transcription from a transiently transfected promoter, but with an integrated promoter the mutant GR does not activate transcription due to its failure to induce chromatin remodeling. The importance of chromatin-integrated systems is highlighted by such observations and it is for this reason that we are compelled to extend our analysis of AR function to include natural promoters. Our approaches using ChIP assays and permanently transfected reporters go a long way to evaluate the proper physiological functions of the AR in healthy and diseased cells.

In preliminary experiments we have used Chromatin IP (ChIP) assays to analyze the molecular components of the endogenous PSA promoter and enhancer. The promoter has two putative AREs and the enhancer has six as indicated in ***Figure 2***. The enhancer is located some 4kb upstream from the transcription start site. No antibody, absence of DHT and irrelevant DNA sequences serve as negative controls. Interestingly it seems that the AR occupies the enhancer quicker and more extensively than the promoter AREs. Furthermore PSA expression seems to be delayed some hours later than enhancer AR occupancy possibly due to chromatin remodeling having to occur prior to transcription initiation.

In the present project, we will utilize ChIP assays of natural promoters (like the PSA) to better define the functional roles of AR-NTD subdomains *in vivo*, to examine the recruitment of cofactors known to interact with the AR-NTD *in vitro*, and to determine the relative contribution of these cofactors to both ligand and ligand independent activation of the AR *in vivo*.

Thereoretical Considerations

The single most important reason men die of prostate cancer is failure of hormone ablation therapy. The molecular reason for this failure is poorly understood. In the present application we pursue the hypothesis that the AR signaling axis provides a unifying mechanism to understand ablation therapy failure. The molecular consequences of sustained ablation therapy are the selection of cell clones showing classic drug (ablation therapy modalities) resistance with the aberrant AR being the main target of functional selection. To enable future work to possibly target the AR (as appose to the ligand) in treatment, a more thorough understanding is absolutely required of the molecular changes and subsequent function of the AR. Without this knowledge novel treatment designs are largely based on empirical considerations. Our work will provide the necessary molecular knowledge for future rational design of treatment strategies.

KEY RESEARCH ACCOMPLISHMENTS

The realization and formulation of the central role of the AR signaling axis in all phases of prostate cancer development.

REPORTABLE OUTCOMES

Manuscripts:

Buchanan G, Irvine RA, Coetzee GA & Tilley WD: Contribution of the androgen receptor to prostate cancer predisposition and progression. Cancer and Metastasis Reviews (in press) 2002. Galley proofs included.

Funding applied for based on work supported by this award:

1) An application to the NCI for a specialized program of research excellence (SPORE) in prostate cancer is currently being prepared by our group. Some of the data and experience from the present work will form the basis of this application.
2) An R01 application to the NIH/NCI is currently pending and entitled: "Androgen Receptor NTD and Prostate Cancer." The major goals of this project are to functionally characterize sub-domains in the AR-NTD, to assess functional consequences of polymorphic variations and somatic mutations in the AR-NTD and to determine how such alterations influence the composition of the AR transcriptional complex.

CONCLUSIONS

The single main finding from the first months of work (supported by the present grant) is that the AR plays a vital role in prostate cancer progression in cases where it is the target for somatic change.

REFERENCES

None

APPENDICES

Appendix A: Buchanan et al, Manuscript Cancer and Metastasis Reviews, in press (2002).

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Project C: Cellular and Molecular Markers of Prostate Cancer Progression
Principal Investigator: Richard Cote, M.D.

INTRODUCTION

Prostate cancer is a highly heterogeneous disease with an unpredictable course. Although the epidemiology and etiology of prostate cancer is largely unknown, it is a disease with extraordinary racial-ethnic variation in incidence, mortality, and survival. African-American men have by far the highest rates of prostate cancer in the world, whereas Asian men native to China, Japan and Korea have the lowest. Even for prostate cancers presenting at a specific stage, African-Americans have substantially worse survival, whereas Asian-Americans appear to have substantially better survival than whites including Hispanics. Indeed, a recent report shows that even in an equal-access medical care setting, prostate cancer survival for black men is poorer compared to white men, suggesting that the disease is particularly aggressive in black men.

The steps that a tumor must undergo to be invasive and metastatic (i.e. the critical factors leading to patient death) are becoming increasingly well characterized. These include:

- Loss of hormonal regulation that can also have important implications in the control of metastatic disease.
- Loss of cell cycle control: Loss of tumor suppressor function (e.g. p53, Rb, PTEN) that can have multiple effects on regulation of cell growth, angiogenesis, and the ability of a tumor to enter the cell death (apoptotic) pathway. Similarly, inactivation of cdk-inhibitors (p27, p21, p16) is expected to result in increased proliferation rates of tumor cells (as detected by PCNA, Ki67 and Topoisomerase II (expression).
- Loss of growth control: In the last year a number of groups have identified loss of function of the PTEN phosphatase as a common event, particularly in advanced prostate cancer. The primary consequence of loss of PTEN function is deregulation of the PI3-kinaseAkt pathway, which is oncogenic in many tumor models. By measuring the status of this pathway at multiple levels, we will define the frequency of this change in multiple ethnic groups.
- The ability to form a new blood supply (angiogenesis), which is important in delivering nutrients and removing waste from a tumor, and also in providing a route for tumor metastasis. Loss of normal inhibitors of angiogenesis (thrombospondin-1) can lead to increased neovascularization (detected by microvessel density).
- Loss of normal cell matrix adhesion properties and cell-cell interactions (including contact inhibition), which allow tumor cells to grow past normal cell density and to break away from their primary site and form occult metastases, or overt metastases.

BODY

We are constantly searching for new markers that will help us address the biovariability of tumors among members of different racial-ethnic groups. To this end in the past year we have identified two new markers to add to our test battery. These are Cox-2 and Caveolin-1. COX-2 is an isoform of cyclooxygenase and is an enzyme that metabolizes arachidonate to prostaglandin G2 and then to prostaglandin H2. COX-2 activity has been implicated as an important factor in

tumorigenesis. The model most studies is that of colorectal cancer. More than 80% of human colorectal cancers have increased levels of COX-2 mRNA, as do about 40% of colorectal adenomas (Eberhart et al 1994). COX-2 inhibitors (such as NSAIDS) exhibit dramatic anti-neoplastic activity in experimental models of colorectal cancer. These include colorectal cancer cells implanted into nude mice, colon tumor production in APC (adenomatous polyposis coli) mutant mice, and carcinogen-induced colon tumors in rats (Oshima et al 1996; Sheng et al, 1997; Kawamori et al 1998). Transfection of COX-2 into human colon cancer cells have shown that COX-2 is involved in a number of processes fundamental to tumor development-apoptosis, tumor invasion and metastasis, and angiogenesis (Tsujii and DuBois, 1995; Tsujii et al, 1997, 1998). COX-2 appears to regulate the expression of a large number of genes associated with these processes. We are testing the hypothesis that COX-2 expression tumors are an important molecular pathway of carcinogenesis in human prostate cancer. We are using a modified protocol of Masferrer et al (2000) to measure COX-2 expression in prostate cancer. This protocol has been tested and optimized by our laboratory and applied to patient samples to date. The second new marker we have added is Caveolin-1. Caveolins are major structural proteins of caveolae-specialized plasma membrane invaginations that are abundant in smooth muscle cells, adipocytes, and endothelium, and mediate signal transduction activities and molecular transport (Harder et al, 1997). Initial studies based on the work by Thompson and Yang et al on Caveolin-1 expression in a large number of primary and metastatic pairs of cell lines derived from the MPR model system. Their results indicated that cavelin-1 protein was elevated in metastasis-derived cells relative to their matched primary tumor counterparts (Yang et al 1998). A further study by this group indicated that Caveolin-1 levels as measured by IHC were different in African-American versus Non-Hispanic whites with prostate cancer (Yang et al 2002). We have obtained this antibody and are subjecting it to our validation protocol. When this is completed, we will begin analyzing our multi-racial cohort for differences in Caveolin-1 expression. Since we have numerous markers to analyze, we have developed, in collaboration with George McNamara, a multi-marker technique by which we can look at up to 4 or more different markers of biologic status on a single tissue section using spectral imaging techniques. Using this technique, we will be able assess three to four different tumor markers on a single tissue section, thus multiplying our resources significantly. Until this technique was developed and optimized for use in our laboratory we were unable to test all of the markers proposed on the limited number of slides available to us.

These new markers and novel techniques to maximize available tissue will better enable us to determine the relationship between the changes in these key biological pathways and a) race/ethnicity, b) age, and c) intermediate markers of tumor progression (tumor stage and grade). We will eventually be in a position to eventually relate these changes to clinical outcome (survival and mortality across racial-ethnic groups).

It is our hypothesis that the difference in tumor behavior observed in prostate cancer arising in men of different racial groups has a molecular and cellular basis. We are obtaining patient specimens from highly multiethnic population of Los Angeles, California in planning and executing this project. These studies are expected to provide information leading to a better understanding of prostate cancer progression in men of different racial/ethnic groups. While our study will have emphasis on racial/ethnic variability, it will also address important issues concerning prostate cancer outcome for all men. Facts that predispose one group of men to have

more aggressive tumor, may be predictive of behavior of prostate cancer in all men. Our initial focus will be on known pathways of tumor progression, studying factors that have been shown to be important (or potentially important) predictors of prostate cancer behavior. The registry maintains the main patient database and provides us with glass slides from patients with prostate cancer, stripped of patient names. We are blinded to the clinical stage and grade of the tumor as well as the patient's race.

KEY RESEARCH ACCOMPLISHMENTS

To date we have received formalin-fixed, paraffin-embedded tissue from 239 cases of prostate cancer and entered these into the laboratory database providing them with a laboratory number. This number is linked in our database to the patient's study identification number. We have assessed 219 of these for the presence of tumor, for the percentage of tumor to normal prostate tissue and recorded the Gleason grade of the tumor in the slides provided. In most cases (>90%), sufficient tissue is available for immunohistochemical analysis. In addition, we have examined the tissue and assessed its suitability for DNA extraction. We have identified 103 of the 219 specimens that most likely contain sufficient tumor for successful extraction. Tissue from these cases has been supplied to Dr. Coetzee (Project B) and Dr. Reichardt (Project A) for analysis. To date we have stained, reviewed and recorded the results on 173 of the 219 cases received with antibody against p27. We have stained the same 173 cases with antibody against COX-2 and have analyzed and recorded the data on 77 of these. The remaining will be analyzed within the next few weeks. The same 173 cases are also currently being analyzed for Caveolin-1. We will also examine other factors known to be involved in tumor progression in various cancers, including prostate cancer, and other factors that play a potential role in prostate cancer progression. These include bcl-2, E-cadherin p53, Rb, CD34, p21, p16, Ki67, PCNA, Topoisomerase-II and thrombospondin-1. The development of the multiple marker analysis by Spectral Imaging will allow us to do most, if not all, markers listed on virtually every tissue with sufficient tumor present and will allow us to determine the molecular basis for the racial/ethnic differences in prostate cancer progression and morality.

REPORTABLE OUTCOMES

None

CONCLUSIONS

This study is a molecular epidemiologic study designed to study prostate cancer progression. It will specifically elucidate multi-ethnic differences in prostate cancer risk and progression. It takes an innovative approach to develop and apply novel biologic markers of prostate cancer progression. It is investigating understudied populations of contrasting risk (African-American, Asian, Latino and white men). We have made significant progress in our goals to assess multiple, significant markers of disease progression and will continue to make even further progress over the next twelve months.

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APPENDICES

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Core: Epidemiology Core
Director: Brian E. Henderson

INTRODUCTION

This is a study looking at the differences in tumor behavior (molecular and cellular behavior) observed in prostate cancer arising in men of different racial groups. The specific aims of the project are to: (1) identify and contact incident prostate cancer patients diagnosed among participants in the Hawaii-Los Angeles multiethnic cohort study to obtain signed tissue release forms, (2) secure formalin-fixed tissues on these individuals; to process these samples; and to distribute these samples to laboratories involved, (3) develop and implement data forms to record laboratory results and histologic reviews; to conduct data management activities including data entry and editing, (4) Project A will try to determine the frequency of somatic mutations in the SRD5A2 gene, (5) Project B will try to determine the frequency of somatic androgen receptor gene mutations in prostate cancer and the functional significance of these, and (6) Project C will use immunohistochemistry to look at a panel of molecular markers thought to possibly be indices of progression.

Our data may partly explain the differing rates of progression from occult to clinically meaningful disease across racial-ethnic groups. These data may be useful in identifying prostate cancer cases who would benefit from improved treatment modalities based in part on somatic alterations in the SRD5A2 or AR genes in their tumors or the presence of other molecular markers of progression, and in identifying those occult lesions requiring the most (or least) aggressive therapy.

BODY

We have identified all of the African-American and Latino-American prostate cancer cases in the multi-ethnic cohort. 1307 men have been identified and contacted by mail, and in some cases, by phone and asked to sign the tissue release forms. These consents were approved by the University of Southern California IRB office and sent to the men identified as having prostate cancer through follow-up linkages with our SEER cancer registry. 600 men (320 African-American and 280 Latinos) have signed the forms and returned them to us by mail. We are in the process of calling the other respondents to encourage them to sign and return the consent forms. We are also tracking cases through our cancer registry follow-up department, whose letter has been returned to us as undeliverable. Seventy-seven subjects have died have and we are trying to secure tissue release forms signed by next-of-kin. Sixty-three subjects have refused participation.

We have given 468 (230 African-American and 238 Latinos) tissue release request forms to date to the Tissue Procurement Core Resource at USC/Norris Comprehensive Cancer Center. We have received tissue on 120 African-Americans and 103 Latinos. Two hundred and twenty-one samples have been forwarded to Dr. Richard Cote's lab. Ninety tissue samples have been forwarded to Dr. Gerhard Coetzee's lab and 87 tissue samples have been forwarded to Dr. Juergen Reichardt's lab.

We have received 70 tissues samples from the University of Hawaii; 37 from Caucasians and 33 from Japanese-Americans. Eighteen samples have been forwarded to Dr. Richard Cote's lab and then will be distributed to Dr. Coetzee and Dr. Reichardt.

KEY RESEARCH ACCOMPLISHMENTS

None: This is a Core resource to support the three Projects.

REPORTABLE OUTCOMES

None

CONCLUSIONS

This Core is now functioning very effectively. Prostate cancer patients are being routinely collected among all four racial ethnic groups in this study and signed tissue releases are being routinely obtained. Tissue procurement and processing is going along well in African-Americans and Latinos and, after delays due to IRB issues, we are now poised to begin tissue procurement of Japanese and Whites.

REFERENCES

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Appendix 1

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Contribution of the androgen receptor to prostate cancer predisposition and progression

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Key words: androgen-signaling axis, CAG, GGC, mutation, androgen-ablation therapy

Abstract

Although prostate cancer is heterogeneous in its etiology and progression, androgen signaling through the androgen receptor (AR) appears to be involved in all aspects of the disease, from initiation to development of treatment resistance. Lifetime exposure to a constitutively more active AR, encoded by AR alleles as defined by two translated polymorphic microsatellites (CAG and GGC), results in a significant increase in prostate cancer risk. The AR gene is amplified or a target for somatic gain-of-function mutations in metastatic prostate cancer. Gain-of-function AR gene mutations may result in inappropriate activation of the AR, thereby contributing to the failure of conventional androgen-ablation treatments. In cases where no genetically altered receptors are observed, altered signaling through the AR, achieved by cross-talk with other signaling pathways (e.g. kinase-mediated pathways) and/or inappropriate expression of coregulatory proteins, may contribute to disease progression. Thus, the AR-signaling axis contributes to many aspects of prostate cancer, including initiation, progression and resistance to current forms of therapy. This recognition represents a paradigm shift in our understanding of the molecular mechanisms involved in progression of prostate cancer, and provides insight into novel AR-targeted therapies which ultimately may be more effective than current forms of androgen ablation.

Introduction

The development and maintenance of the normal prostate gland requires a functional androgen-signaling axis [1,2]. The primary components of this axis include testicular biosynthesis and transport of testosterone to target tissues, conversion of testosterone to its more active metabolite 5α-dihydrotestosterone (DHT), maturation of the androgen receptor (AR) to its ligand-binding competent form, and the subsequent transcriptional regulation of AR target genes. Through the AR, the androgen-signaling axis mediates diverse cellular functions in the prostate including differentiation, morphogenesis, angiogenesis, proliferation and apoptosis [1–5].

Prostate tumorigenesis also requires a functional androgen-signaling axis, the components of which form the principal targets of androgen-ablation therapies that inhibit the growth of prostate cancer. For patients who are either diagnosed with or subsequently develop metastatic disease, the only treatment option is androgen ablation (i.e., orchidectomy, treatment with LHRH agonists/antagonists and/or AR antagonists [6,7]). Despite an initial good response in 80–90% of patients with metastatic disease, androgen ablation is essentially palliative and disease progression eventually ensues [7,8]. Resistance to androgen ablation is not necessarily due to loss of androgen sensitivity, but may develop as a consequence of a deregulated androgen-signaling axis resulting from amplification or mutation of the AR gene, or ligand-independent activation (LIA) of the AR by growth factors and cytokines ([9–11]; reviewed in [12–14]).

Recent evidence suggests that the AR is involved in many phases of prostate cancer biology, including genetic predisposition (due to the existence of polymorphic variants), disease progression, and the development of resistance to androgen-ablation therapies. In this review, we document the contribution of the AR to each of these phases of prostate cancer.

Androgen receptor structure and function

The AR gene is located on the long arm of the X chromosome at Xq11–12, and comprises 8 exons that encode a protein of ~~about 910 amino acids~~ (Figure 1). approximately 110kDa The AR can be broadly defined in terms of three distinct functional domains: a large amino-terminal transactivation domain (NTD) containing at least two strong constitutive transactivation functions; a DNA-binding domain (DBD); and a carboxy-terminal ligand-binding domain (LBD) that contains a highly conserved ligand-dependent transactivation function (AF-2) (Figure 1; [15]). The large NTD is encoded in its entirety by exon 1 of the gene and contains two polymorphic trinucleotide microsatellites, CAG and GGC, which encode variable-length polyglutamine (poly-Q) and polyglycine (poly-G) tracts, respectively, in the receptor (Figure 1). The CAG and GGC microsatellites have a normal size distribution of 6–39 and 7–20 repeats respectively ~~with an average of 21 CAG and 16 GGC repeats~~ [16,17]. The CAG and GGC microsatellites have expanded during primate evolution [18,19]. The Old World marmoset, drill and macaque monkeys, for example, possess only 3, 8 and 7 uninterrupted AR-CAG repeats, respectively [18], and the macaque and the prosimian lemur possess only 6 and 2 uninterrupted AR-GGC repeats, respectively [19]. Rubinsztein et al. [20] have shown that human microsatellite repeats statistically are more likely to be longer than their primate counterparts, suggesting that phylogenetic microsatellite expansion may be reflective of a mutational bias in favor of longer repeat lengths specifically in humans. A directional expansion of coding microsatellite repeats could be tolerated evolutionarily until it significantly alters function of the receptor such that reproduction is compromised.

Expansion of the CAG microsatellite to 40 or more repeats causes a rare, X-linked, adult onset,

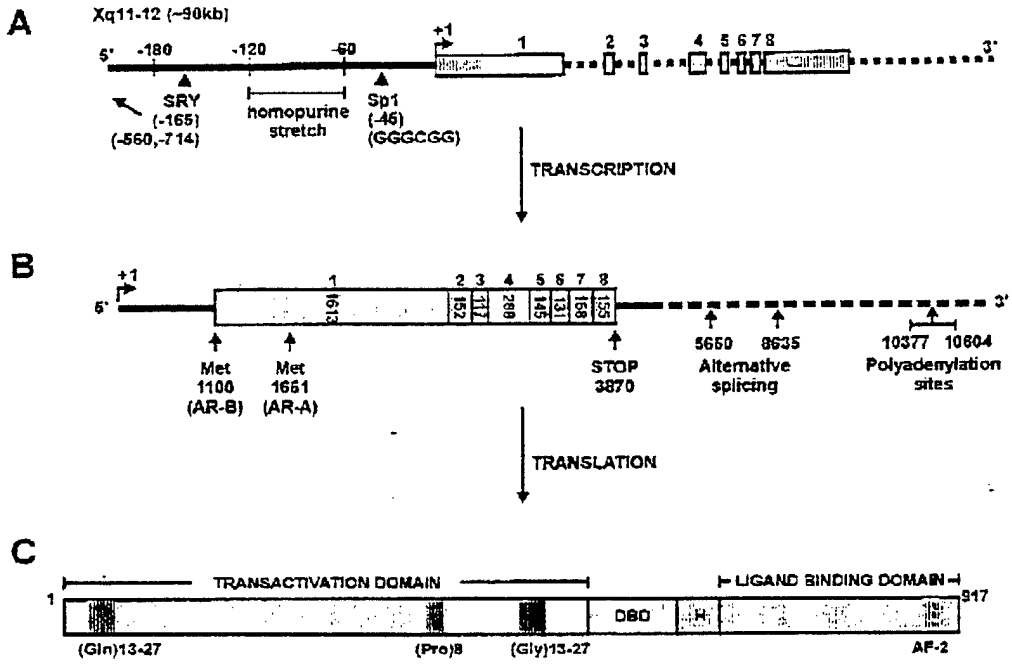

Figure 1. Transcription and translation of the AR. (A) Schematic representation of the AR gene structure on chromosome Xq11–12 showing important binding sites for SRY and SP1 transcription factors. Individual exons are separated by up to 16 kb of intronic sequence. (B) AR mRNA transcript showing alternative splice and polyadenylation sites. Translation is primarily directed from the first of two initiating methionine residues. (C) Structure of the predominant (AR-B) form of the AR. Indicated are the NTD, the DBD, the hinge region (H) and LBD. The positions of the three polymeric amino acid stretches in the NTD and the activation function, AF-2 in the LBD are shown (shaded).

neurodegenerative disorder called spinal and bulbar muscular atrophy (SBMA) or Kennedy's disease [21,22]. In addition to progressive muscle weakness and atrophy due to loss of brain stem and spinal cord motor neurons, men with this disorder frequently present with symptoms of partial androgen insensitivity (i.e., gynecomastia and testicular atrophy), indicative of aberrant AR function [23,24]. Receptor proteins encoded by SBMA AR alleles have normal androgen-binding affinities but reduced transactivation capacity compared to wild-type AR [25,26]. Indeed, an inverse relationship between AR transactivation activity and CAG repeat length has been well-established over a CAG size range encompassing normal AR alleles [27–30].

Following translation of the AR, conformational maturation of steroid receptors by a multi-protein chaperone heterocomplex is essential for the acquisition of ligand-binding competence [31]. The specific details of this process for the AR are poorly defined, but in general, it requires at least three heat shock proteins, Hsp40, Hsp70 and Hsp90, and the co-chaperones p23 and Hop [31]. In the final stages of receptor maturation, Hsp90 becomes directly associated with the receptor LBD in a process stabilized by p23, and with one of the tetratricopeptide repeat (TPR) containing proteins which include the immunophilins FKBP51/52 and CyP40, and the protein–serine phosphatase, PP5 [31]. The interaction of the co-chaperone p23 with Hsp90 is an absolute requirement for heterocomplex stabilization of unliganded nuclear receptors [31]. Although many TPR containing proteins appear to have a similar affinity for Hsp90, they exhibit specific preferences for different steroid receptors, and may play a role in hormone action by altering the affinity and specificity for ligand. It is thought that the Hsp90-containing heterocomplex dynamically associates with steroid receptors to maintain them in a conformation that, although unstable, has a high affinity for ligand binding [31]. Following hormone binding in the cytoplasm, the Hsp90-containing heterocomplex is dissociated and the steroid receptor is rapidly translocated into the nucleus.

In the nucleus, the AR dimerizes and binds in the major groove of the DNA double-helix at specific DNA sequences called androgen response elements (AREs). The DNA bound AR dimer recruits a multi-protein complex containing members of the basal transcription machinery (e.g. TFIIF) and additional essential proteins termed cofactors, which act to up-regulate (coactivators) or inhibit (corepressors) target gene expression [32]. Chromatin remodeling occurs via targeted histone acetylation by recruited coactivators, resulting in the stable assembly of the pre-initiation transcriptional complex and enhanced rates of transcription initiation by RNA polymerase II [32]. Following ligand dissociation, the AR is shuttled back to the cytoplasm where it can re-associate with Hsp90 and ligand, subsequently undergoing multiple rounds of nucleocytoplasmic recycling and gene activation [33].

The DBD of steroid receptors contains two zinc finger motifs and a short C-terminal extension that form part of the hinge region [34,35]. Conserved amino acids in the second zinc finger of the DBD ('D' box) form a dimerization interface between steroid receptor monomers. The specificity of steroid receptors is determined at the level of DNA binding by conserved amino acids in the α-helix of the first zinc finger ('P' box) which contact specific base pairs in steroid receptor response elements [34]. Despite their diverse biological effects, the 'P' boxes of class I steroid receptors (i.e., AR and the corresponding receptors for glucocorticoids (GR), mineralocorticoids and progestins) are almost identical and the core DBD, which includes both zinc fingers, shares up to 75% identity [35]. However, sequence differences in the C-terminal extensions, which diverge considerably between steroid receptors (approximately 30% identity), may confer receptor selectivity for particular target sequences by allowing alternative modes of DNA binding [36]. Response elements for steroid receptors generally consist of hexameric half-sites arranged as either inverted repeats (symmetrically arranged palindromes) or direct repeats separated by three nucleotides. A recent analysis of AR-regulated genes has shown that the AR-responsive half-sites arranged as inverted repeats may induce head-to-head dimerization of the receptor, while the polarity of direct repeats may lead to head-to-tail dimerization [37–39]. These two distinct classes of AREs mediate cooperativity of AR binding and the unique regulation of target genes [40].

The contribution of AR-CAG size variation to prostate cancer risk

In 1992, Edwards et al. [16] reported the allelic frequency distribution of AR-CAG repeat size in different US racial–ethnic populations as part of a larger survey of genetic variation in a series of different trimeric and tetrameric tandem repeats. Among

African-Americans, the frequency of AR alleles with less than 22 CAG repeats was 65%, as compared to 53% in Caucasians and 34% in Asian-Americans. On the basis of these observations, we hypothesized that AR-CAG repeat length might be associated with the higher risk of prostate cancer in African-Americans, and the intermediate and low risk in Caucasions and Asian-Americans respectively, and that enhanced transcriptional activity of receptors with a shorter AR-CAG allele could promote tumorigenesis by enhancing prostatic epithelial cell turnover [41].

In 1995 we directly tested this hypothesis in a pilot case-control study comprising 68 prostate cancer patients and 123 control subjects [42]. In agreement with Edwards et al. [16] there was a prevalence of short AR-CAG alleles in African-American vs. Caucasian and Asian controls. In addition, modest though not statistically significant enrichment of short AR-CAG alleles was observed in the Caucasian prostate cancer patients. These findings were extended in an expanded follow-up study that showed a significantly higher prevalence of short AR-CAG alleles among prostate cancer patients, especially among those with advanced disease (Table 1; [43]). In addition to our studies, Hakimi et al. [44] identified a subgroup of patients diagnosed with advanced prostate cancer who had shorter AR-CAG repeats. Hardy et al. [45] furthermore, demonstrated an association between age of onset and AR-CAG repeat length.

Subsequently, several well-designed matched case-control studies demonstrated an approximate 2-fold increased prostate cancer risk, decreased age of onset and/or increased risk of advanced disease for reduced AR-CAG repeat length (Table 1). Giovannucci et al. [17] used a population selected from the Physicians Health Study that included 587 prostate cancer cases and 588 matched controls. The large sample size of this study allowed the authors to stratify cases by tumor grade and stage. A highly significant inverse correlation between AR-CAG repeat length and risk of developing prostate cancer was observed when repeat size was analyzed as a semi-continuous variable. Short AR-CAG

Table 1. Studies evaluating the roles of the AR CAG and/or GGC microsatellites in prostate cancer risk, progression, and age at onset.

Study	Subjects	AR CAG repeat correlation with PCa			AR GGC repeat correlation with PCa		
		Risk	Stage/grade	Age at onset	Risk	Stage/grade	Age at onset
Pilot studies							
Irvine et al., 1995 [42]	US Caucasian	Yes	N/A	N/A	Yes	N/A	N/A
Hardy et al., 1996 [45]	US Caucasian	N/A	No	Yes	N/A	N/A	N/A
Ingles et al., 1997 [43]	US Caucasian	Yes	Yes	N/A	N/A	N/A	N/A
Hakimi et al., 1997 [44]	US Caucasian	Yes	Yes	No	Yes	No	No
Matched case-control studies							
Giovannucci et al., 1997 [17]	US Caucasian	Yes	Yes	No	N/A	N/A	N/A
Standford et al., 1997 [46]	US Caucasian	Yes	No	Yes	Yes	No	Yes
Platz et al., 1998 [53]	US Caucasian	N/A	N/A	N/A	Yes	N/A	N/A
Hsing et al., 2000 [47]	Chinese	Yes	No	No	Yes	No	No
Beilin et al., 2001 [30]	Australian White	No	No	Yes	N/A	N/A	N/A
Other studies							
Ekman et al., 1999 [49]	Swedish White	Yes	N/A	N/A	N/A	N/A	N/A
Edwards et al., 1999 [50]	British Caucasian	No	No	N/A	Yes	No	N/A
Correu-Cerro et al., 1999 [51]	French/German White	No	No	No	No	No	No
Bratt et al., 1999 [52]	Swedish White	No	Yes	Yes	N/A	N/A	N/A
Lange et al., 2000 [53]	US Caucasian (high risk)	No	No	No	N/A	N/A	N/A
Nam et al., 2000 [54]	Canadian	N/A	Yes	N/A	N/A	N/A	N/A
Latil et al., 2001 [55]	French White	No	No	Yes	N/A	N/A	N/A
Modugno et al., 2001 [56]	US Caucasian	Yes	N/A	N/A	N/A	N/A	N/A
Miller et al., 2001 [57]	US Caucasian	No	N/A	N/A	No	N/A	N/A
Panz et al., 2001 [58]	S. Africans (Black & White)	Yes	Yes	N/A	N/A	N/A	N/A

N/A, not applicable or not assessed; Yes, association between polymorphism and listed parameter; No, no significant association detected between polymorphism and listed parameter.

alleles also correlated with an increased risk of having advanced disease, defined as a high-stage or high-grade tumor at diagnosis [17]. In another study, Stanford et al. [46] analyzed AR-CAG repeat length and prostate cancer risk in 301 prostate cancer cases and 277 matched controls [46]. They noted only a small increase in the frequency of AR-CAG alleles with less than 22 repeats in cancer patients compared with controls. Nevertheless, when AR-CAG repeat size was examined as a continuous variable, an overall age-adjusted relative odds of developing prostate cancer of 0.97 was observed for each additional CAG. More recently, Hsing et al. [47] reported that AR-CAG alleles were significantly shorter in prostate cancer patients compared to controls among Shanghai Chinese. This study is important as it was the first to demonstrate this association in a population group other than Caucasian. In a recent case-control study in an Australian Caucasian population [48], no association was observed between AR-CAG repeat length and prostate cancer risk, but a significant effect on the age of onset was observed. In other studies (Table 1), associations between AR-CAG repeat length and prostate cancer risk were not consistently observed, possibly due to small sample sizes, population differences and/or failure to appropriately match cases and controls [49–58].

While the consistent finding of the epidemiologic studies discussed above has provided evidence for an association between AR-CAG repeat length and prostate cancer risk, those studies did not address the molecular mechanisms underlying changes in receptor activity with length variation of the poly-Q tract (encoded by the polymorphic CAG repeat). As stated above, *in vitro* transient cotransfection studies have shown that ARs with longer poly-Q repeats have normal ligand-binding affinities but lower transactivation activities [25–28,48]. Protein expression levels are unlikely to account for this effect since they have been found to be similar for ARs containing between 9 and 42 poly-Q repeats [29]. However, two studies have reported that AR constructs with longer repeat lengths (CAG-50–52) are unstable and undergo accelerated degradation, potentially in a ligand-dependent manner [29,59]. The poly-Q size effect in AR transactivation activity observed in most *in vitro* studies is thought to be mediated, at least in part, through altered functional interactions with cofactors. In transient cotransfection experiments, the p160 coactivators, GRIP1, AIB1 and SRC-1 exaggerate the relative difference in AR transactivation activity with altered poly-Q length [29]. As the p160 coactivators bind to regions of the AR distinct from the poly-Q tract, this effect may be mediated by steric hindrance of p160–receptor interactions when poly-Q length is increased [29]. The RAS related G-protein, Ran/ARA24, which binds to the AR NTD in the region of the poly-Q, is an AR cofactor that appears to enhance AR activity in a poly-Q size-dependent manner [60]. Given the well-described role for Ran in protein nuclear transport, it is possible that larger poly-Q tracts inhibit the efficiency of Ran-directed AR nuclear import [61]. Clearly, more studies are required to determine whether the effects of other cofactors that act in a cell-, promoter- and/or AR-specific manner can be directly influenced by poly-Q length, and to determine how variation in AR poly-Q length can influence prostate cancer cell growth.

The contribution of AR-GGC size variation to prostate cancer risk

Allelic distributions of the GGC microsatellite are significantly different among racial–ethnic groups [42], with the 16-repeat GGC allele being least prevalent amongst high-risk African-Americans (i.e., 20%) and most prevalent in low-risk Asians (i.e., 70%). This is suggestive of a protective role for this allele in prostate cancer risk. It is possible that the 16-repeat GGC allele encodes an AR containing a poly-G tract of 'optimal' length for normal receptor function in prostatic epithelial cells. While this is speculative, as it is not known whether variation in poly-G length modulates AR activity, a weak though non-significant paucity of the 16-repeat GGC allele was observed among white Caucasian prostate cancer patients compared to control subjects, suggesting that there is enrichment of putative risk alleles (i.e., non-16-repeat GGC alleles) among cases [42].

Because the AR gene is X-linked, with each male inheriting a single maternal copy, it is possible to define a putative AR prostate cancer risk allelotype of short CAG (i.e., <22 repeats) and non-16-repeat GGC. As expected, we observed that the distribution of this allelotype was significantly different among control subjects, with African-Americans and Asians having the highest and lowest prevalence, respectively. Among white Caucasian prostate cancer patients, the <22 CAG/non-16-repeat GGC haplotype conferred a 2-fold increase in risk of prostate cancer, although statistical significance was not reached [42]. Among prostate cancer patients, a nonrandom distribution of CAG and GGC alleles was observed; 66% of patients

with a short CAG allele also had a non-16-repeat GGC allele, while only 25% of patients with long CAG alleles had a non-16-repeat GGC allele. As the CAG and GGC microsatellites are in close proximity at the AR locus, it was not surprising to find evidence of linkage disequilibrium between the intragenic markers in patient samples. In contrast, there was no evidence of linkage disequilibrium between control samples when assessed either together or by ethnicity. This indicates that in normal men, either one or both of the microsatellites are hypermutable, resulting in a random distribution of CAG and GGC alleles at the AR locus. Indeed, when the rate of mutation at the CAG microsatellite was measured using single-cell assays of sperm, an exceptionally high rate of 1–4% was observed [62]. Collectively, this data suggests that a non-random subset of CAG and GGC AR alleles occur in men with prostate cancer.

In three matched case-control studies (Table 1), a positive association between AR-GGC repeat length variation and prostate cancer risk was found [47,63,64]. The failure to consistently demonstrate this association in other studies (Table 1), might be due to the lack of statistical power and/or failure to appropriately match cases with controls. A more detailed assessment of the effects of the AR-GGC repeat on prostate cancer risk awaits elucidation of the effects of alterations in poly-G tract length on AR function.

AR and prostate cancer progression: Localized disease

Although the maintenance of AR immunoreactivity has been demonstrated in the majority of prostate tumors in both localized and metastatic disease [65–73], only recently has the role of AR in progression of clinically localized prostate cancer been addressed [71,73]. Henshall et al. [73] reported that AR was expressed in more than 70% of the tumor cells in localized prostate cancer, but that there was a loss of AR immunoreactivity in the adjacent peritumoral stroma which was associated with earlier relapse after radical prostatectomy. Another study by Sweat et al. [71] found no association between AR expression and disease progression in a highly selected cohort of tumors with a Gleason score of 6–9. In a recent study, we found that the level of AR protein in tumor foci determined by video image analysis is a strong predictor of the risk of relapse following radical prostatectomy (unpublished data). ~~However, the percentage AR-positive cells determined by visual assessment was not associated with an increased risk of relapse following radical prostatectomy, suggesting that video image analysis of nuclear AR immunoreactivity provides additional information regarding critical features of nuclear AR immunoreactivity associated with disease progression.~~ While further studies are necessary to determine how AR influences disease progression in clinically localized prostate cancer, a number of mechanisms have been identified in prostatic tumors that potentially explain the increase in levels of AR immunostaining observed in tumor cells in our study. These mechanisms include amplification of the AR gene [74], changes in the methylation status of the AR promoter and hence transcription of the AR gene [75,76], altered stability of AR mRNA [77] and LIA [11,78]. Irrespective of the mechanism, increased AR levels likely result in altered expression profiles of androgen-regulated proteins, including angiogenic factors, cell adhesion molecules and cell cycle regulators (e.g. vascular endothelial growth factor, integrins and cyclin-dependent kinases and their inhibitors [79–81], which collectively contribute to disease progression.

AR related mechanisms contributing to the failure of androgen-ablation therapy in advanced prostate cancer

Recent studies in clinical prostate cancer have identified several mechanisms that potentially explain how prostate tumors progress following initiation of androgen ablation, including amplification or mutation of the AR gene, and LIA of the AR ([9,82,83]; reviewed in [12,84–86]). These studies suggest that resistance to conventional hormonal therapy is not due to a loss of androgen sensitivity but rather may be a consequence of a deregulated androgen-signaling axis ([87]; reviewed in [84,85]. Although initial studies using the Dunning animal model suggested that loss of AR gene expression could be a mechanism for failure of androgen ablation [88,89], subsequent immunohistochemical studies of clinical prostate cancer have demonstrated that the AR is expressed in essentially all metastatic tumors, including those that continue to grow following androgen ablation [90]. Moreover, amplification of the AR gene has been reported in 22% of prostate cancer metastases [82], and in 23–28% of primary tumors following androgen deprivation [74,91]. An average 2-fold increased level of both AR and PSA proteins has been reported

in prostate tumor samples with AR gene amplification compared to samples where no AR amplification was found [9,83]. Increased AR levels may augment the sensitivity of the androgen-signaling axis, and has the potential to contribute to disease progression during the course of androgen ablation.

The first indication that AR gene mutations might contribute to the failure of androgen-ablation therapies came from studies of the androgen-responsive human prostate cancer cell line, LNCaP. The AR in LNCaP cells contains a single amino acid substitution (Thr–Ala877) that facilitates inappropriate activation by glucocorticoids, progestins, adrenal androgens, estradiol and the anti-androgen hydroxyflutamide [92,93]. Subsequently, somatic missense mutations have been detected throughout the AR coding sequence at frequencies of up to 50% in advanced primary tumors and metastatic deposits (reviewed in [12,94]). These mutations consistently result in receptors that exhibit decreased specificity of ligand-binding and enhanced receptor activation by androgens and non-classical ligands compared to wild-type AR (wtAR; reviewed in [85,95]). More recently in collaboration with Dr Norman Greenberg at Baylor College of Medicine, Houston, TX, we reported the identification of AR gene mutations in the autochthonous transgenic adenocarcinoma of mouse prostate (TRAMP) model [96]. Analogous to the findings in clinical prostate cancer, AR gene mutations detected in TRAMP tumors also result in receptors that contribute to altered androgen signaling [96].

Structural and functional collocation of AR variants

We recently reported that nearly 80% of missense AR gene mutations identified in clinical prostate cancer cluster to discrete regions of the receptor that collectively span less than 15% of the coding sequence ([85]; Figure 2; Table 2).

Ligand-binding domain variants. In the LBD, mutations collocate to (i) the 'signature sequence', a conserved 20-amino-acid region of nuclear receptors involved in ligand recognition and specificity [97], (ii) AF-2, a binding site for the p160 cofactors, and (iii) a region at the boundary of the hinge and LBD containing a 4-amino-acid tetrapeptide (^{668}QPIF^{671}) that may define a protein–protein interaction surface. Many of the AR gene mutations identified in the LBD of the AR in the TRAMP model occur in the same three regions as mutations in clinical prostate cancer. For example, a Phe–Ile671 mutation identified in an intact TRAMP mouse collocated to the ^{668}QPIF^{671} tetrapeptide with mutations identified human prostate cancer [10]. AR gene mutations identified in both clinical prostate cancer and TRAMP tumors in this region exhibit a 2–4-fold greater transactivation activity in response to DHT, non-classical ligands and hydroxyflutamide compared to wtAR (Figure 3A; [10]), without altering ligand-binding kinetics, receptor levels or DNA-binding capacity. Homology modeling revealed that the ^{668}QPIF^{671} tetrapeptide residues form a potential protein–protein interaction surface that is markedly disrupted by the naturally occurring mutations, providing a mechanism that could explain the observed gain in transcriptional activity [10].

Another AR missense mutation identified in the TRAMP model, Phe–Ser697, is located adjacent to the signature sequence. The Ser697 AR variant exhibits markedly reduced transactivation responses to progesterone and 17β-estradiol, but enhanced response to R1881 compared to wtAR (Figure 3B; [96]). These results are consistent with the role of the signature sequence in ligand recognition and specificity, and with previous reports of mutations in this region in clinical disease (reviewed in [12,85]). Analysis of the Thr–Ala877 AR variant, identified in a significant proportion of clinical prostate tumors and in the human prostate cancer cell line, LNCaP, has confirmed that this mutation exhibits increased transactivation activity in response to progesterone, 17β-estradiol, adrenal androgens and hydroxyflutamide compared to wtAR (Figure 3C; [98]). The recently determined AR-LBD crystal structure [99,100] allowed us to use homology modeling to demonstrate that this mutation results in changes to the shape and volume of the ligand-binding pocket such that bulkier ligands like progesterone can be accommodated [96].

DNA-binding domain variants. Five somatic missense mutations have been identified in clinical prostate tumors that collocate to a 14-amino-acid region at the carboxyl-terminal end of the first zinc finger motif in the DBD of the AR [101,102]. The effect of each of these mutations is unknown, but none of the codons in which they occur have been reported to contain mutations that cause receptor inactivation in the clinical syndrome of androgen insensitivity. Mutations in the AR-DBD have been shown to selectively

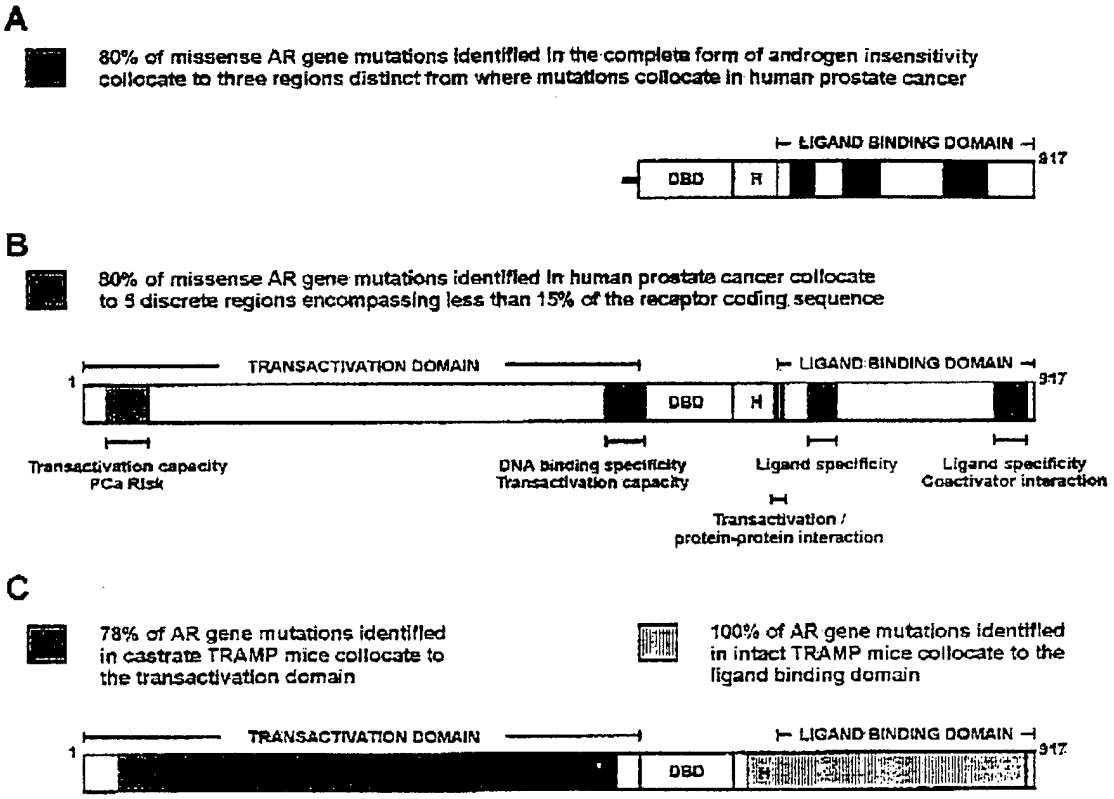

Figure 2. Collocation of AR variants. (A) Collocation of 80% of inactivating AR gene mutations detected in the clinical syndrome of complete androgen insensitivity. (B) Collocation of 80% of AR gene mutations detected in clinical prostate cancer to five discrete regions of the receptor (shaded), which account for less than 15% of the coding sequence. (C) AR gene mutations detected in prostate tumors derived from the TRAMP model segregate to the amino-terminal transactivation domain or to the LBD in castrated and intact mice respectively.

affect transactivation and transrepression functions of the AR on different promoters despite a reduced DNA-binding ability [103,104], and may represent a predisposing factor for male breast cancer [105]. Due to the high homology of the DBD across members of the nuclear receptor superfamily, the cell and promoter specificity of different receptors is, in part, mediated by only a few changes in DBD sequence [106]. It has been speculated that mutations in the DBD could result in AR variants that bind to response elements normally specific for other nuclear receptors [105], leading to inappropriate activation or repression of growth regulatory pathways. In an analogous manner, mutations in androgen receptor response elements have been shown to increase the sensitivity of the enhancer for the glucocorticoid receptor [107]. Modeling experiments suggest that residues in the AR-DBD could form a protein interaction surface [105], and several AR coactivators that interact with the DBD in a ligand-dependent manner [108–110] are predicted to alter receptor activity via local chromatin remodeling [108], interaction with components of the transcriptional machinery [109], or inhibiting nuclear export [111]. It is also possible that mutations in the DBD of the AR gene identified in prostate cancer could alter the affinity of receptor binding to response elements, resulting in altered expression of a range of target genes regulated by the AR.

Amino-terminal transactivation domain variants. Nearly half of the AR gene mutations identified in clinical prostate cancer are located in the NTD of the

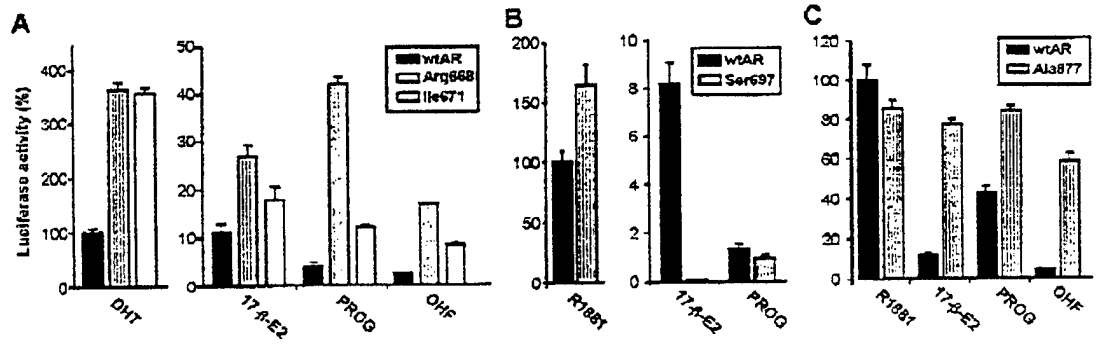

Figure 3. Transactivation capacity of AR variants identified in tumors derived from clinical prostate cancer (Arg668, Ala877) and TRAMP mice (Ile671, Ser697). Transactivation assays were performed in the human prostate cancer cell line, PC-3 with the minimal androgen-responsive probasin promoter, tk81-PB3 as previously described (10). Data is expressed as a percentage of the luciferase activity induced by wtAR in the presence of 1 nM DHT, and represents the mean (±sem) of 3–7 independent experiments. (A) Mutations occurring in ^{668}QPIF^{671} residues. Treatment was with 1 nM of either DHT, 17β-estradiol (17β-E2), or progesterone (PROG), or with 1 μM hydroxyflutamide (OHF). (B) Mutation occurring adjacent to the signature sequence. Treatment was with 1 nM R1881 or 10 nM of either 17β-E2 or PROG. (C) LNCaP AR variant in the region of AF-2. Treatment was with 1 nM of R1881 or 10 nM of either 17β-E2 or PROG, or with 1000 nM OHF.

receptor. AR gene mutations in this domain have also been identified in the TRAMP model and, analogous to the observations for the LBD, these mutations cluster with those identified in clinical prostate cancer to discrete regions within the NTD that are implicated in receptor function. The main regions of collocation in the NTD are (i) within and adjacent to the poly-Q tract (codons 54–78), which as discussed above has been implicated in modulating receptor activity and prostate cancer risk, and (ii) a region amino-terminal to the DBD (codons 502–535) known to modulate the transactivation capacity of the receptor in both ligand-dependent and ligand-independent manner (i.e., a LIA function or LIAF) [112,113] (Figure 2).

Somatic contractions in the CAG repeat of the AR gene, which potentially increase AR activity in a subpopulation of cells and thereby contribute to disease progression, have been identified in three independent studies of clinical prostate tumors [114–116]. In addition, we have recently identified a somatic mutation within the CAG repeat of the AR gene in a primary prostate tumor that results in interruption of the polyglutamine repeat by two leucine residues. This AR variant has a 2–4-fold greater ability to transactivate target genes compared to wtAR in the presence of physiological concentrations of DHT [117]. Four additional somatic mutations have been identified in or adjacent to the CAG repeat region of the AR gene in human prostate cancer [101], but have not yet been characterized. Further analysis of inherited and somatic alterations in this region of the AR gene in prostate cancer is warranted to determine the contribution of this motif to AR activity, and its potential to influence the development and/or progression of the disease.

A second region of the NTD where amino acid substitutions have been identified in hormone-refractory prostate tumors is the LIAF. In a recent study, we examined the complete coding sequence of the AR gene for mutations in metastatic tissue biopsies from 12 patients who exhibited the clinical syndrome of steroid-hormone and anti-antiandrogen withdrawal response to hydroxyflutamide. Four of seven mutations identified in the tumor samples collocated to a small carboxyl-terminal portion (codons 502–535) of the NTD of the AR. An additional mutation (Asp–Gly526) previously identified in our studies of primary prostate tumors [101], and another identified in the TRAMP model [96], collocate to this region of the AR. This carboxyl-terminal region of the amino-terminal domain of the AR is known to modulate the transactivation capacity of the receptor in both a ligand-dependent and ligand-independent manner [112,113], and has recently been shown to be involved in direct interactions with the p160 coactivators and the transcription regulator p300/CBP [29,118,119]. This region of the NTD also contains the binding site for the receptor accessory factor (RAF), which enhances the specific DNA binding of rat AR [120]. Further evidence in support of

the LIAF region being important in AR transactivation has been provided by recent studies demonstrating that mutation of Ser513, which is located in a consensus MAP kinase phosphorylation site, partially inhibits LIA of the receptor by HER-2/*neu*. Collectively, these observations suggest that the LIAF region may contain an interaction surface for accessory proteins that promote ligand-independent transactivation, and that mutations in this region may alter the ability of the receptor to respond to these and other cofactors, thereby altering the transactivation capacity of the AR in a manner that could provide a growth advantage to prostate cancer cells in an appropriate hormonal environment.

Contribution of cofactors to AR signaling

Two additional AR NTD missense mutations (Met–Thr265; Pro–Ser268; [101]) identified in clinical prostate cancer are located in close proximity to two mutations (Ala–Thr234; Glu–Gly236; [96]) identified in TRAMP tumors. Characterization of the Glu–Gly236 substitution revealed that the variant receptor had increased transactivation function compared to wtAR in response to R1881 and 17β-estradiol only in the presence of the coactivator, ARA70, and increased response to R1881 but not 17β-estradiol in the presence of the coactivator, ARA160 [96]. Similarly, Gregory et al. [121] have shown that overexpression of the p160 coactivators, GRIP1 and SRC-1, observed in recurrent tumors from CWR22 human prostate xenografts and clinical prostate cancer, increases AR transactivation capacity at physiological concentrations of non-classical ligands (adrenal androgens, estradiol and progesterone). Collectively, these findings suggest that the phenotype of some AR gene mutations may only be apparent in the presence of the appropriate milieu of coregulators, and that altered expression and/or structure of AR accessory proteins in prostate cancer cells could provide another mechanism contributing to the recurrent growth of prostate tumors in an androgen-depleted environment.

Ligand-independent activation of the AR

Another potentially important mechanism contributing to the failure of androgen ablation is LIA of the AR. The AR can be activated in the absence of ligand by growth factors (keratinocyte growth factor, insulin-like growth factor-1 and epidermal growth factor), cytokines (Interleukin-6), protein kinase-A, components of the MAP kinase pathway (MEKK1), differentiation agents such as butyrate and other factors that directly or indirectly increase intracellular kinase activity or decrease phosphatase activity (reviewed in [84]).

Aberrant expression of growth factor receptors also contributes to development and progression of prostate cancers [12,85]. HER2 (neu/c-erbB-2), a transmembrane glycoprotein member of the epidermal growth factor receptor family, is overexpressed in carcinomas of the breast, ovary, and stomach [17,42,122]. Unlike other epidermal growth factor receptor members, HER2 has intrinsic tyrosine kinase activity and mediates signal transduction in the absence of ligand [123]. Recent studies suggest that HER2 expression is increased in hormone-refractory prostate tumors compared to earlier stages of disease [124,125]. Over-expression of HER2 in androgen-responsive prostate cancer cell lines enhances AR transactivation of androgen-regulated genes such as PSA, in a ligand-independent manner, and increases cell survival during androgen deprivation [11,126]. Although the mechanism involved in HER2 modulation of AR transactivation has not been fully characterized, HER2 expression is associated with activation of MAP kinase and Akt (protein kinase B) pathways which have been implicated in LIA of the AR [11,78]. It was recently shown that the tyrosine kinase receptor, HER-2/*neu*, can promote LIA of the AR via both the PI3K/Akt and MAP kinase pathways [78,127]. In those studies, LIA by HER-2/*neu* could be partially blocked by an ~~Akt inhibitor~~ [78], or mutation of AR-Ser513, which is located in a consensus MAP kinase phosphorylation site. HER-2/*neu* activation of Akt results in binding of Akt to the AR and phosphorylation of the receptor at two residues (Ser212, Ser791). Another study demonstrated that a specific inhibitor of protein kinase A could block LIA of the AR induced by butyrate [127]. These data suggest that both ligand-dependent and ligand-independent signals converge upon the AR, with at least three signal transduction pathways having the potential to activate the AR.

Tumor cells with increased HER2 expression and high AR may have a selective growth advantage. For example HER2 activation of an Akt–AR pathway [11,78,126] may confer a clonal advantage by promoting cancer cell survival via the androgen-signaling axis [78] or by the induction of Akt-dependent pathways [123]. In a recent study using prostate cancer xenograft models, Herceptin (a monoclonal antibody directed

TIF2/

inhibitor the PI3 Akt path

Similarly, Ye et al. [143] have shown that coactivators (eg ARA70, ARA160) can enhance the androgenic activity of 17β-estradiol and hydroxyflutamide, suggesting that the effect of agonists and antiandrogens can be modulated by accessory proteins

against activated HER2) monotherapy resulted in antiproliferative activity in androgen-dependent LNCaP and CWR22 tumors, but no significant growth inhibition was observed in androgen-independent CWR22 tumors [128]. The lack of response of the androgen-independent tumors to Herceptin in the presence of androgen indicates that signaling through the AR is a requisite for Herceptin response in these prostate tumors [128]. Thus, patients with elevated levels of both HER2 and AR immunostaining may benefit from early treatment targeting both the AR and HER2 signaling cascades.

Therapy selects for AR gene mutations with a phenotype permissive for growth

In the late 1980s, Labrie et al. [129] reported that administration of LHRH agonists in combination with hydroxyflutamide (~~maximal~~ androgen blockade; MAB) could prolong survival of patients with metastatic prostate cancer by about 17 months. However, subsequent reports were conflicting, and currently most patients initially are treated with monotherapy, usually LHRH agonists. Indeed, a recent systematic review of MAB, encompassing 20 individual trials and more than 6000 patients, concluded that MAB results in only a modest increase in survival compared to monotherapy alone, but is more likely to be associated with adverse events and reduced quality of life [130]. Recent evidence regarding the mechanisms contributing to therapy failure (see above) suggests that combinational approaches with LHRH agonists and receptor antagonists cannot completely abrogate androgen action, and may select for cells with a growth state permissive for a particular hormonal environment. The potential clinical importance of a therapy-mediated selective pressure is illustrated by the syndrome of steroid-hormone and anti-antiandrogen withdrawal, which is characterized by tumor regression and decreasing serum levels of PSA when treatment with an anti-androgen, progestational agent or estrogen is selectively discontinued at a time of clinical progression [131]. A withdrawal response has been observed in up to 30% of patients with hormone-refractory prostate cancer when treatment with the antiandrogen, hydroxyflutamide is terminated [131–133], and has also been documented following withdrawal of the AR antagonists, nilutamide and bicalutamide, the estrogens, diethylstilbestrol and megestrol acetate, and the progestational agent, chlormadinone acetate [134–139]. Withdrawal responses have been reported at a higher incidence following combined therapy, consisting of castration or LHRH agonists in combination with an AR antagonist, compared to antagonist alone, leading the authors to conclude that prolonged exposure to antiandrogens was the predominant factor in the withdrawal response rather than a low level of androgens [135,140]. In one study, inhibition of adrenal steroid production with ketoconazole following discontinuation of antiandrogen therapy resulted in a higher proportion of patients (55%) exhibiting a withdrawal response and an increased duration of response [141] than reported for withdrawal of the antiandrogen alone [140].

Selection for AR gene mutations with a phenotype permissive for growth is also evident from a number of other studies. AR gene mutations detected in patients who were treated with hydroxyflutamide in conjunction with androgen-ablation therapy result in receptors exhibiting a marked increase in activity in response to hydroxyflutamide, but not to DHT or other androgenic ligands [142]. In the TRAMP model, we recently reported that different hormonal environments result in the selection of AR variants with mutations in distinctly different regions of the receptor [96]. In tumors derived from TRAMP mice at 24–28 weeks of age, 7/7 of the missense AR gene mutations identified in the amino-terminal transactivation domain were derived from mice castrated at 12 weeks of age, whereas 6/8 of the mutations identified in the LBD were from intact animals ([96]; Figure 2C). Moreover, 4/9 of the mutations identified in castrated TRAMP mice resulted in receptors with increased transactivation function in the absence of ligand [96]. Therefore, AR gene mutations identified in prostate cancer could provide a selective growth advantage given the appropriate hormonal environment, resulting in the re-emergence of tumor growth during the course of hormone-ablation therapies. In addition, AR gene amplification and overexpression of the AR has been reported in hormone-refractory prostate cancer following monotherapy but not in primary prostate tumors [9], suggesting an alternative mechanism by which hormonal therapies could select for cells with an ability to maintain growth during treatment.

Conclusions

Collectively, the above evidence suggests that AR signaling plays a key role in many phases of prostate

cancer biology. Carefully matched case-control and other studies suggest that length variation of the AR-CAG and AR-GGC polymorphic microsatellite repeats contributes to prostate cancer risk, and may also influence age of onset and tumor pathology, by altering AR transcriptional activity and/or interaction with AR coregulators. AR gene mutations have frequently been reported in clinical prostate cancer and consistently exhibit a gain-of-function phenotype that, along with AR gene amplification and/or activation of the AR by growth factors and cytokines, could facilitate continued AR signaling in an androgen-depleted environment. Thus, resistance to androgen ablation and survival of prostate cancer cells is not necessarily due to the evolution of a growth state that circumvents the androgen-signaling axis, but could be explained in part by increased activity of the AR in the presence of native ligands, inappropriate activation of the AR by non-classical ligands due to mutations in the AR gene or inappropriate expression of AR coregulators, or ligand-independent mechanisms. This represents a paradigm shift in our understanding of hormone-refractory prostate cancer. Further analysis of AR target genes and how their transcription is influenced by non-classical ligands, AR coregulators or variation of the polymorphic AR-CAG and AR-GGC repeats is necessary in order to develop new treatment strategies that target androgen signaling, irrespective of the structure and level of expression of the receptor. This potentially could result in a more complete blockade of androgen signaling, which would represent a significant advance in the treatment of metastatic prostate cancer by preventing or delaying the onset of resistance to androgen-ablation therapies.

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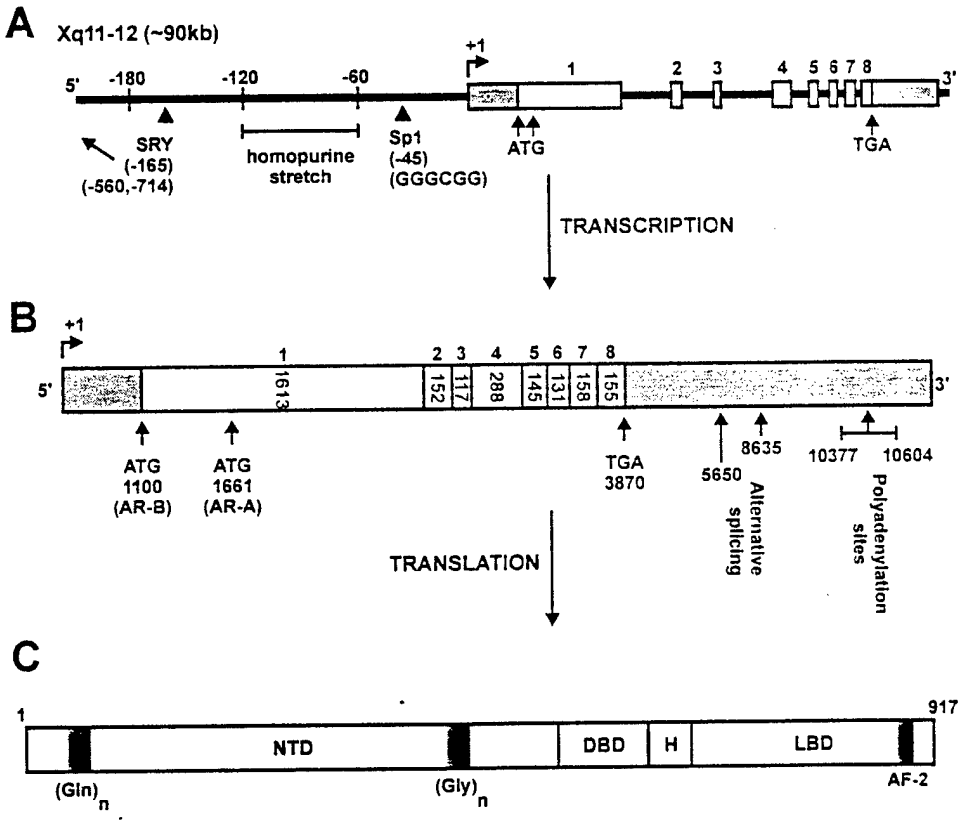
Fig 1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