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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 410 prostate cancer patients to date identified during follow-up of the Hawaii/Los Angeles Multiethnic Cohort study. One-hundred eight-five tissue samples have been received and processed histopathologically by Project C, which has begun immunohistochemical staining for p27, p21 and p16 markers with additional markers to follow. Project B, studying the androgen receptor (AR) gene in detail, has identified 21 sequence variants in 58 samples analyzed to date; one mutation was shown in culture to be twice as active as wild-type AR. In Project A, studying the SRD5A2 gene in detail, 24 sequence variants were found in 30 tumors with three mutations (A495, G692A and V63M occurring on a recurring basis.

14. SUBJECT TERMS
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Overall Introduction

We report below our first 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; in fact these were just recently resolved in Hawaii. Nonetheless, we have signed releases from 410 subjects and have secured tissue from 185. 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 begun and are now going full volume in conducting the immunohistochemical studies of molecular markers of progression as proposed in this Project (see below).

Table of Contents – Project A

Cover 1

SF 298 2

Table of Contents 4

Introduction 5

Body 5

Key Research Accomplishments 5

Reportable Outcomes 6

Conclusions 6

References 6

Appendices 6

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 in three areas:

i) We sequenced the putative promoter region, 5' and 3' UTR (untranslated regions) and the protein coding region of the human SRD5A2 gene in 30 tumors to identify somatic mutations on an automated DNA sequencer. At least 24 nucleotide changes were identified in these samples. We also sequenced the same regions in constitutional DNA to determine which of these were in fact *de novo* somatic mutations (and which were pre-existing constitutional DNA variants). These data then narrowed the number to 19 *de novo* somatic mutations. Finally, we determined which mutations occurred more than once in our sample since these recurrent substitutions are the most sensible to screen for in specific aim 2.

ii) A single recurrent somatic mutations was identified in the 5' UTR:

- G692A (guanine at nucleotide 692 replaced by adenine) was found in 2/30 (~7 %) of the tumors sequenced.

iii) We identified two recurrent mutations in the protein-coding region:

- A49T (alanine at codon 49 substituted with threonine) was found in 4/30 (~13 %) tumors sequenced;
- V63M (valine-63 to methionine) was identified in 3/30 samples (~10 %).

REPORTABLE OUTCOMES

None thus far.

CONCLUSIONS

This laboratory has completed the investigation of specific aim 1. We are, therefore, poised to begin with the investigation of specific aim 2. We -in fact- have already begun screening for the A49T recurrent protein-coding region in tumor samples obtained through this grant. We will add into our screens other substitutions, such as V63M and others such as G692A, as quickly as possible and as warranted by other data.

REFERENCES

None

APPENDICES

None

Table of Contents – Project B

Cover 1

SF 298 2

Table of Contents 7

Introduction 8

Body 8

Key Research Accomplishments 12

Reportable Outcomes 12

Conclusions 13

References 13

Appendices 13

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

INTRODUCTION

The androgen receptor (AR) enhances prostate epithelial cell proliferation and differentiation and may influence prostate cancer progression including establishment of hormone independence. Previously we have shown that two microsatellite repeats (CAG and GGC) in exon 1 of the AR gene are associated with prostate cancer risk. The two microsatellites encode poly-glutamine (poly-Q) and poly-glycine (poly-G) stretches in the N-terminal transactivation domain (TAD) of the AR. The AR-TAD domain contains, in addition, two non-overlapping discrete transactivation subdomains situated between the poly amino acid stretches. We have discovered a third functional subdomain in the AR-TAD, further downstream from the previously identified transactivation subdomains and overlapping with the poly-G stretch. The novel subdomain interacts with transcription coactivators and is a hotspot for somatic mutations in prostate tumors. **Our hypothesis is that progression to advanced prostate cancer is partly due to gain-of-function somatic mutations in the AR gene and that many of these mutations occur in the novel AR/co-activator subdomain we have discovered**. Although AR somatic mutations were once considered to occur rarely in prostate tumors, recent data indicate that they occur much more frequently in advanced disease than previously thought. The mutations so far analyzed are often of the gain-of-function kind, and may contribute to the progression to hormone resistant disease. In order to explore our working hypothesis, we propose here the following two specific aims. (i) We will measure the frequency of somatic AR mutations in prostate tumors across four racial-ethnic groups and as a function of disease stage. This will allow for an assessment of mutated AR in disease progression and a comparison of mutation frequencies across racial-ethnic groups. (ii) Next, we will determine the functional significance of the identified somatic AR mutations. We will reconstruct each and model their effects in transfection studies using prostate epithelial cells. We intend to investigate ligand specificities and coactivation-mediated transactivation of the reconstructed AR somatic mutants. Overall results from this study will define the role of the AR in prostate cancer progression and might provide insights into mechanisms that govern evolution to hormone independence, as well as to which prostate cancers should be treated most aggressively.

BODY

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

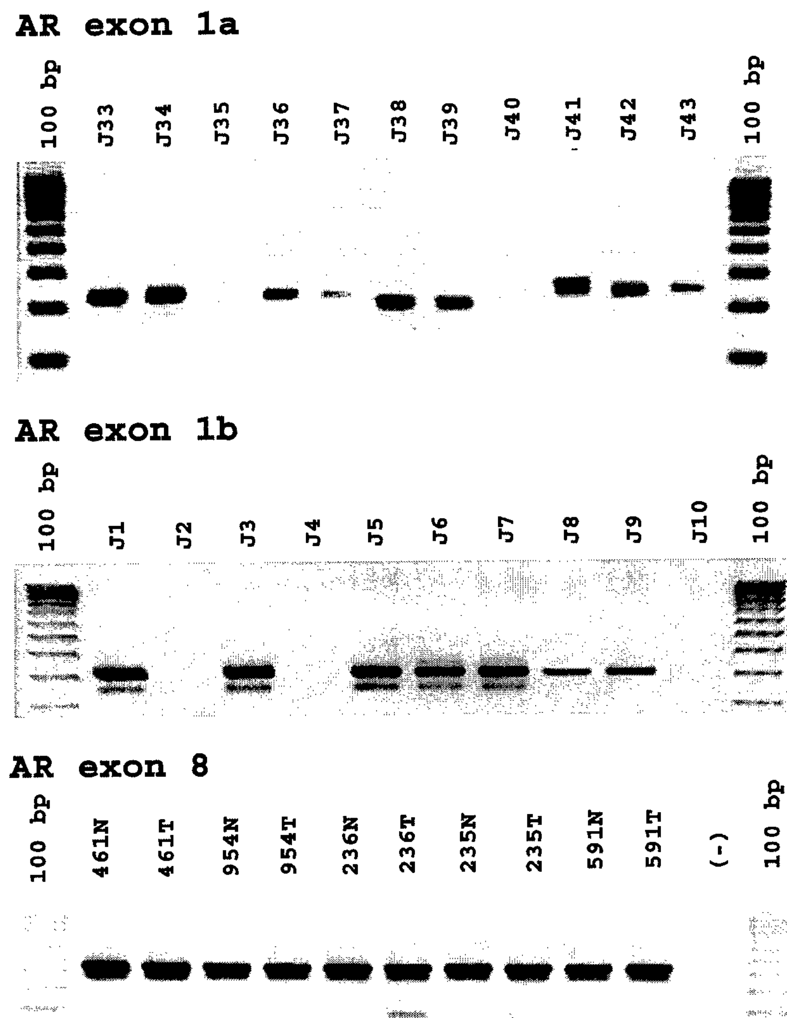
Task 1. To measure the frequency of somatic AR mutations in tumors from prostate cancer patients with 'localized' and 'advanced' disease, and to determine if the frequency differs between the two groups among different racial-ethnic groups (weeks 1-24). Microdissect samples (week 1). Extract DNA (week 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).

Due to an initial lag in obtaining tissue blocks and due to difficulties related to efficient PCR amplification of AR fragments (see below), we have to date extracted DNA from 58 samples (1 tumor and 1 surrounding normal tissue for each sample) and have begun to PCR amplify AR mutational hotspots, exon 1a, exon 1b and exon 8 (Fig 1). We have detected 21 mutations. However, we were able to amplify only 64%, 55% and 98% of the samples for hotspot exons 1a, 1b, and 8 respectively. Due to this variability, we are in the process of recalibrating PCR conditions for all hotspots (initially we used published information). To this end we have purchased a specialized PCR instrument (Hybaid PCR Express equipped with a gradient block 0.2) to allow for more efficient determination of optimal annealing temperatures. We are confident that this will allow more efficient amplification of all AR fragments. For example we know there are enough DNA present in our extracts since we are able to amplify exon 8 in nearly all samples. The 21 novel mutations we have detected are currently being evaluated for sequencing/PCR artifacts and we should be able to assess them soon for functional effects as well.

Since our overall aim in the present study is to gain insights into the mechanism by which genetic variation/changes at the AR locus affect the function of AR signaling, we have proceeded to assess the poly-Q (CAG repeat) variation in exon 1a using two related but separate approaches. In the first approach we assessed, in collaboration with the Ingles lab (USC/Norris Cancer Center), how two polymorphisms, the AR-CAG (poly-Q variation), and another in an androgen response element of the prostate specific antigen (PSA ARE1, G/A) gene, influence serum PSA levels in healthy men. Serum PSA and the two genotypes were assayed for 420 healthy men from a multi-ethnic cohort, and regression models were fit to estimate the effects of AR-CAG genotype and PSA ARE1 genotype on serum PSA levels. Predicted serum PSA decreased 3.5% with each additional AR-CAG repeat decile ($p=0.01$). Serum PSA was also associated with PSA ARE1 genotype, with PSA levels being higher among men with the PSA AA genotype compared to men with the AG or GG genotypes ($p=0.02$). The relationship between serum PSA level and AR-CAG length differed according to PSA genotype ($p=0.049$): for genotype GG, the slope was not significantly different from zero ($p=0.74$); for genotype AG, serum PSA increased 4.5% with each decrease of one CAG repeat decile ($p=0.03$); for genotype AA serum PSA increased 7% with each decrease of one CAG repeat decile ($p=0.02$). These results indicate that in healthy men, genetic variants in the PSA and AR genes contribute to variation in serum PSA levels. Men with the PSA AA genotype and short AR CAG alleles have, on average, higher serum PSA levels. (Manuscript accepted for publication in CEBP, a copy in Appendix A).



Success rate of PCR amplification from slide DNA:

AR exon 1a:	**34/53 = 64%**
AR exon 1b:	**29/53 = 55%**
AR exon 8:	**71/72 = 98%**

Figure 1

The second approach was to model a previously detected somatic mutation (from an advanced prostate tumor), in collaboration with the lab of Dr. Wayne Tilley (Adelaide, Australia) using transfected PC3 cells as outlined in the methods section of our original application. The mutation and results are depicted in Figure 2.

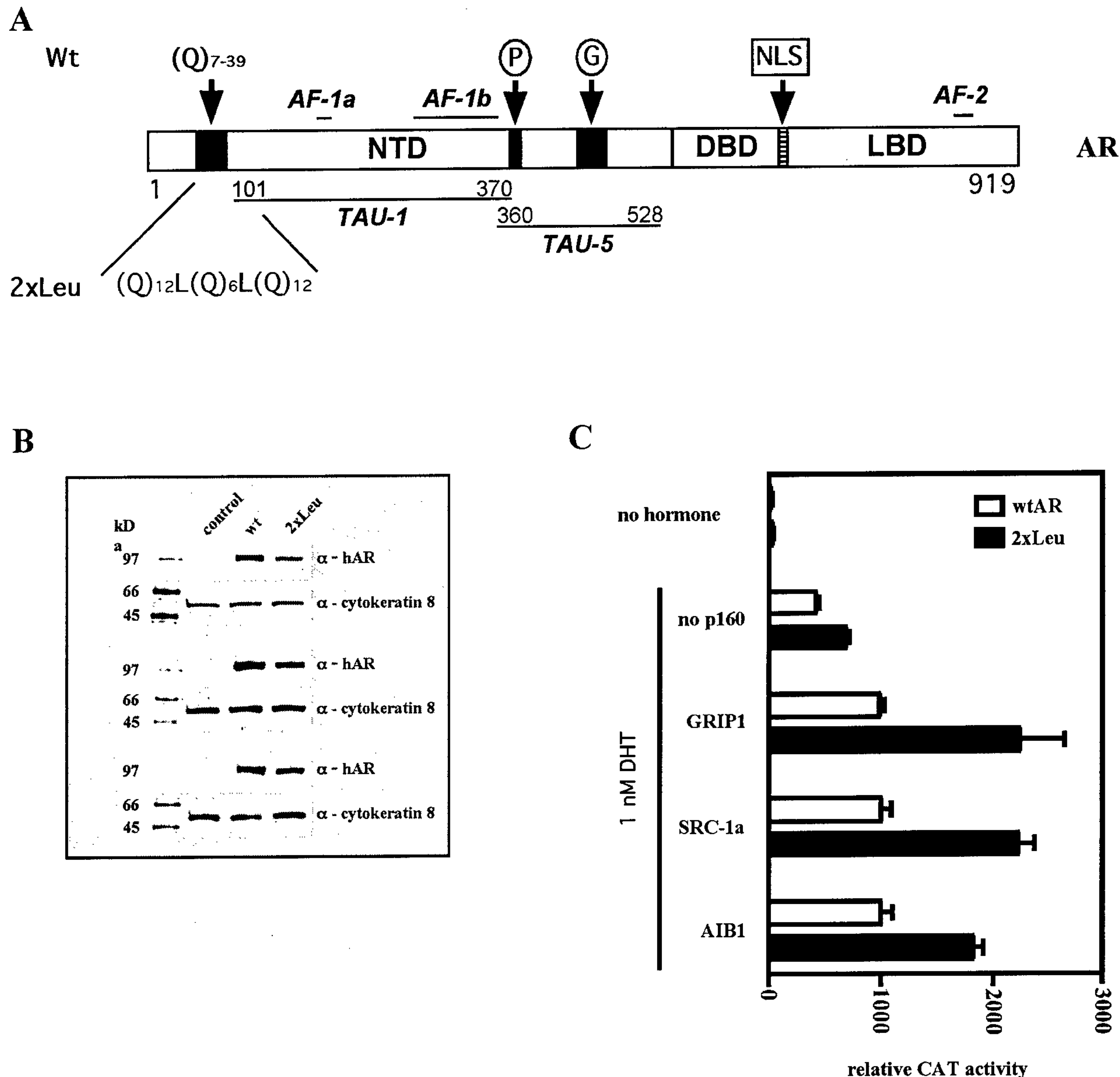


Figure 2: Relative expression and transactivation activity of the AR (2xLeu) mutant. (A) Schematic representation of the AR protein depicting important domains and subdomains. (B) PC-3 cells were transiently cotransfected with 3.0 ug pCMV-hAR or pCMV-hAR(2xLeu) and 2.0 ug pcDNA3.1(+). 24h after transfection, cells received fresh medium that contained 10% charcoal/dextran-stripped FBS and 10 nM DHT. 24h later, whole cell extracts were prepared and normalized for total cell protein. Equivalent amounts of each extract were probed with anti-AR or anti-CK8 antibody after western transfer of SDS-PAGE gels. Autoradiograms from three independent experiments were analyzed by scanning densitometry. (C) PC-3 cells were transiently transfected with 0.1 ug pCMV-hAR, 2.0 ug of the indicated coactivator expression vector, and 2.0 ug of the ARR3TK-CAT reporter plasmid. 24h after transfection, cells received fresh medium that contained 10% charcoal/dextran-stripped FBS and 1 nM DHT or ethanolic vehicle. 24h later, whole-cell extracts were prepared and assayed for CAT activity and corrected for protein expression. Data presented are the mean +/-SE of three independent dishes.

The mutant is the so-called AR 2XLeu cloned by Dr. Tilley and colleagues (Flinders Cancer Centre, Australia) from an adenocarcinoma of the prostate (Tilley *et al.*, unpublished results). It has two CTG codons inserted into the CAG microsatellite [i.e., 5'-CTG$(CAG)_{12}$**CTG**-$(CAG)_6$**CTG**$(CAG)_2$CAA-3' instead of 5'-CTG$(CAG)_{20}$CAA-3'] and, therefore, encodes a poly-Q disrupted by two interspersed leucine residues. To assess the effect of this 2XLeu insertion mutation on AR function, the relative expression level and transactivation activity of the AR (2XLeu) mutant were investigated. Expression levels of AR (2XLeu) and AR (wt) were determined by anti-AR Western blots carried out on whole-cell extracts from transfected PC-3 cells, and the resultant autoradiograms were quantified by densitometric scanning (Fig. 2). In subsequent transactivation experiments, the corrected, DHT-dependent activity of AR (2XLeu) was nearly 2-fold higher than that of AR (wt) on the ARR_3TK-CAT reporter. Likewise, potentiation of AR (2XLeu) activity by p160-coactivators was markedly enhanced relative to AR (wt).

KEY RESEARCH ACCOMPLISHMENTS

- AR variants (poly-Q variation, encoded for by a CAG repeat microsatellite in exon 1) were associated with PSA levels in men with a particular polymorphism in their PSA promoter androgen response element.

- A somatic mutation in the CAG repeat of the AR (Q→L), found in an advanced prostate tumor resulted in an AR protein that was twice as active as wild type AR.

REPORTABLE OUTCOMES

Manuscripts:

Wen-Mei Xue, Gerhard A Coetzee, Ronald K Ross, Ryan Irvine, Laurence Kolonel, Brian E Henderson, Sue Ann Ingles: ***Genetic Determinants of Serum PSA levels in Healthy Men from a Multiethnic Cohort***., CEBP in press (2001). Appendix A

Presentations:

Gerhard A Coetzee: ***Molecular Biology of Predisposition to, and Hormone Therapy of Prostate Cancer,*** Current Issues in Hormone Therapy for Prostate Cancer, First Annual Charles Huggins Symposium Friday, June 1, 2001 Dinner Program. Appendix B

Funding applied for based on work supported by this award:

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.

CONCLUSIONS

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

REFERENCES

None

APPENDICES

Appendix A: Xue et al, Manuscript CEBP in press (2001).

Appendix B: Coetzee, Lecture summary First Annual Charles Huggins Symposium.

Table of Contents – Project C

Cover 1

SF 298 2

Table of Contents 14

Introduction 15

Body 16

Key Research Accomplishments 16

Reportable Outcomes 17

Conclusions 17

References 17

Appendices 17

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

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer in the United States, having surpassed female breast cancer in 1994. 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.

Prostate cancer is a highly heterogeneous disease with an unpredictable course. 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.

We proposed 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). Our longer-term goal is to be in a position to eventually relate these changes to clinical outcome (survival and mortality across racial-ethnic groups).

BODY

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 using the SEER registry and highly multiethnic population of Los Angeles, California in planning and executing this project. 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. To date we have received formalin-fixed, paraffin-embedded tissue from 148 cases of prostate cancer from our Epidemiology Core 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 103 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 addition, we have examined the tissue and assessed its suitability for DNA extraction. We have identified 58 of the 103 specimens that most likely contain sufficient tumor for successful extraction. Tissue from these cases has been supplied to Dr. Coetzee (Project 2) for analysis. To date we have stained 92 of the 148 cases received with antibody against p27. These slides are currently under review by our laboratory. We are in the process of immunostaining these for the other factors hypothesized to be involved in tumor progression. These include cox-2, bcl-2, E-cadherin p53, Rb, CD34, p21, p16, Ki67, PCNA, Topoisomerase-II and thrombospondin-1.

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.

KEY RESEARCH ACCOMPLISHMENTS

- Pathologic assessment and distribution of 58 tumors for AR and SRD5A2 sequencing.
- Immunostaining for p27 on 92 specimens.

REPORTABLE OUTCOMES

None to date.

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 will investigate understudied populations of contrasting risk (African-American, Asian, Latino and white men). It will directly expand on ongoing epidemiologic studies (LA County SEER registry will research in a complementary epidemiologic model of prostate cancer etiology).

REFERENCES

None

APPENDICES

None

Table of Contents – Epidemiology Core

Cover 1

SF 298 2

Table of Contents 18

Introduction 19

Body 19

Key Research Accomplishments 20

Reportable Outcomes 20

Conclusions 20

References 20

Appendices 20

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. 1205 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. 560 men (300 African-American and 260 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 subjects have died and we are trying to secure tissue release forms signed by next-of-kin. Sixty subjects have refused participation.

We have given 410 (200 African-American and 210 Latinos) tissue release request forms to date to the Tissue Procurement Core Resource at USC/Norris Comprehensive Cancer Center. We have received tissue on 100 African-American and 85 Latinos. One hundred and eighty five (100 African-American and 85 Latinos) samples have been forwarded to Dr. Richard Cote's lab. Sixty tissue samples have been forwarded to Dr. Gerhard Coetzee's lab and 60 tissue samples have been forwarded to Dr. Juergen Reichardt's lab.

We have not received any tissue from the University of Hawaii. They have had delays in gaining local IRB approval but have begun contacting pathologists at local hospitals for support regarding this tissue retrieval, and we expect the first batch of tissues to arrive soon. As with those from Los Angeles, they will be processed, sent to Dr. Cote's lab for processing and then 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

None

APPENDICES

None

CEBP in press 01

Genetic Determinants of Serum PSA levels in Healthy Men from a Multiethnic Cohort

Wen-Mei Xue[1], Gerhard A Coetzee[2], Ronald K Ross[1], Ryan Irvine[2], Laurence Kolonel[3], Brian E Henderson[1], Sue Ann Ingles[1]

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Running title: Genetic determinants of serum PSA levels

Keywords: serum PSA, androgen receptor, androgen response element

We recently reported an association between prostate cancer risk and polymorphisms in the prostate-specific antigen (PSA) and androgen receptor (AR) genes. The purpose of this study is to test whether these two polymorphisms, AR CAG, and PSA ARE1, influence serum PSA levels in healthy men. Serum PSA and the two genotypes were assayed for 420 healthy men from a multi-ethnic cohort, and regression models were fit to estimate the effects of AR CAG genotype and PSA ARE1 genotype on serum PSA levels. Predicted serum PSA decreased 3.5% with each additional AR CAG repeat decile (p=0.01). Serum PSA was also associated with PSA ARE1 genotype, with PSA levels being higher among men with the PSA AA genotype compared to men with the AG or GG genotypes (p=0.02). The relationship between serum PSA level and AR CAG length differed according to PSA genotype (p=0.049): for genotype GG, the slope was not significantly different from zero (p=0.74); for genotype AG, serum PSA increased 4.5% with each decrease of one CAG repeat decile (p=0.03); for genotype AA serum PSA increased 7% with each decrease of one CAG repeat decile (p=0.02). These results indicate that in healthy men, genetic variants in the PSA and AR genes contribute to variation in serum PSA levels. Men with the PSA AA genotype and short AR CAG alleles have, on average, higher serum PSA levels.

Introduction

Serum prostate specific antigen (PSA) is widely used as a tumor marker for early detection of prostate cancer. PSA, however, is not cancer specific. Benign prostatic epithelial cells also produce PSA. Any condition that increases prostate size, such as benign prostatic hyperplasia, or that disrupts the prostatic architecture, such as prostatitis, prostatic ischemia or infarction, can elevate serum PSA. Serum PSA gradually increases with age, due to a progressive increase in prostate size (1). Racial differences in serum PSA levels have been noted, with black men having markedly higher serum PSA levels than their white counterparts (2-3), perhaps due in part to larger average prostate volume. Other factors that might influence PSA levels include those factors that directly or indirectly regulate PSA gene expression.

The major regulator of PSA gene expression is androgen. The androgen receptor (AR), after binding to ligand (androgen), recognizes and binds to specific nucleotide sequences, called androgen response elements (AREs), in the promoter regions of androgen-regulated genes. At least three AREs have been identified in the PSA gene promoter (4). The one nearest the transcription start site is referred to as ARE1. We recently reported that a single-nucleotide polymorphism in the ARE1 sequence was associated with prostate cancer risk, and furthermore, that this association may be modified by allelic variation in the androgen receptor gene (5). In this study, we set out to test whether polymorphisms in these two genes, PSA and AR, influence serum PSA levels in healthy men.

Materials and Methods

Subjects. Subjects were 456 men participating in the Hawaii-Los Angeles Multiethnic Cohort Study of diet and cancer. The male cohort consists of approximately 13,000 African-Americans, 23,000 Latinos, 27,000 Japanese-Americans, and 23,000 non-Hispanic whites who were between the ages of 45 and 75 at entry into the cohort. All

cohort members have completed a detailed health and dietary questionnaire, and are periodically traced, primarily through population-based cancer registries, for occurrence of all incident cancers. Blood and urine specimens are collected from all incident cancer cases and from a 3% random sample of the cohort. AR CAG genotypes had been performed on approximately the first 1000 samples (cases and controls) collected. Men eligible for the current study were those who have not been diagnosed with prostate cancer and for whom AR CAG genotypes were already available. Because our aim was to study men having normal prostate function, we excluded 36 men who had serum PSA levels above 4 ng/ml, leaving 420 men (100 African-Americans, 113 non-Hipanic whites, 108 Hispanics, and 99 Japanese-Americans) in the study. Forty men (9.5%) reported a history of prostate enlargement. Exluding these men did not alter our results. Written informed consent was obtained from each subject. The study was approved by the University of Southern California School of Medicine Institutional Review Board.

Genotyping. Two genes, AR and PSA, were examined in this study. In the AR gene, two microsatellite polymorphisms (CAG and GGC) in exon 1 were genotyped using methods described in our previous report (6). These microsatellites are length polymorphisms, with individual alleles defined by the number of repeated units (CAG or GGC repeats) that they contain. Genotypes were assayed by separating radioactively-labeled PCR products on polyacrylamide gels. GGC genotype was missing for 10 subjects due to PCR failure.

In the PSA gene promoter, a G/A substitution polymorphism in the ARE1 sequence was genotyped using methods described in our previous report (5). PCR products were digested with the NheI enzyme (New England Biolabs, Beverly MA) and genotypes were distinguished by running digested products on agarose gels: AA (300bp), AG (150, 300), and GG (150bp). Additionally, a 560 bp region surrounding this polymorphism was sequenced for all subjects using primers GTTGGGAGTGCAAGGAAAAG (forward) and GGACAGGGTGAGGAAGACAA (reverse). For eighteen subjects, the complete sequence was not readable due to poor template quality.

Serum PSA levels. Serum PSA levels were performed by the University of Southern California Norris Cancer Hospital Clinical Laboratory using a two-site immunoenzymometric assay with a Hybritech anti-PSA mouse monoclonal antibody (TOSOH Medics, Inc., Foster City, CA). The minimal detectable PSA concentration was 0.05 ng/ml, with intra-assay and inter-assay coefficient of variations of 2.9% and 2.1%, respectively.

Statistical Methods. Serum PSA levels were log transformed, and linear regression models were fit to estimate the effects of AR and/or PSA genotypes on serum PSA levels, adjusting for age and ethnicity. Because a few observations with extremely long or extremely short AR CAG length were highly influential in determining regression coefficients, CAG length was grouped into approximate deciles to improve robustness of the models. Decile 1 corresponds to 7-16 CAG repeats, decile 2 to 17-18 CAG repeats, deciles 3 through 8 each correspond to a single CAG repeat category (19 to 24 repeats, respectively), decile 9 corresponds to 25-26 CAG repeats, and decile 10 to 27-37 CAG repeats. The medians of the decile groups: 15, 17, 19, 20, 21, 22, 23, 24, 25, and 28 CAG repeats, were used as scores for coding CAG length in the regression equations. The resulting regression coefficient can be interpreted as representing the additive increase in ln(PSA), and the exponentiated coefficient as the multiplicative increase in PSA for each decrease of one CAG unit. Heterogeneity tests were performed by calculating the likelihood ratio statistic, comparing the model with a single regression line to a model with separate regression lines for each genotypic group. All p-values were two-sided.

Because all AR GGC genotypes other than genotype 16 were relatively uncommon, GGC alleles were categorized as <16, 16, and >16, roughly corresponding to the bottom quartile, middle 50%, and upper quartile, respectively. GGC genotype group was modeled by including two indicator variables in the regression model.

Results

The age of the subjects at the time of blood collection ranged from 47 to 80 years, with a mean of 62.8 years. Mean and median ages were slightly higher for Hispanics than for other ethnic groups, but these differences were not statistically significant (Table 1). There were no significant ethnic differences in serum PSA (Table 1), either before or after adjusting for age. Age was weakly correlated with serum PSA ($R = 0.16, p<0.01$).

The distributions of AR and PSA allele frequencies are shown in Table 2. Ethnic-specific allele

frequencies were similar to those previously reported (*5-6*). In the AR gene, short CAG length (<19 CAG repeats) and short GGC length (<16 GGC repeats) were more common, and GGC length of 16 was less common among African-Americans than among men of other ethnic groups. At the PSA ARE1 locus, the G allele was most frequent among Japanese-Americans, intermediate among Hispanics, and least frequent among blacks and non-Hispanic whites. Genotype frequencies were in agreement with Hardy-Weinberg equilibrium in all ethnic groups (data not shown).

In the PSA gene promoter, two new polymorphisms were identified. An A/G polymorphism at -252, 79 bp upstream from ARE1, and a single-base deletion polymorphism (A/)A) at -232. The two polymorphisms were in perfect linkage disequilibrium, with the -252A allele always corresponding to the -232 A allele and the -252G to the -232)A allele (see Figure 1). The -252A/-232A allele was much less common among African-Americans than among men of other ethnic groups; in fact no -252A/-232A homozygotes were observed among African-Americans (Table 2). The two polymorphisms were also in linkage disequilibrium with the ARE1 polymorphism, with the -252A/-232A allele occuring only in combination with the ARE1 G allele (Figure 1). Thus there exist three PSA promoter haplotypes, -252G/-232)A/ARE1A, -252G/-232)A/ARE1G, and -252A/-232A/ARE1G, which we have designated as PSA*1, PSA*2, and PSA*3, respectively (Figure 1), according to a recently recommended nomenclature system (*7*).

Of the two AR polymorphisms, only CAG length was associated with serum PSA levels. Serum PSA is shown as a function of AR CAG length in Figure 2. There was a subtle but significant (p=0.01) decrease in serum PSA with increasing CAG length. Serum PSA was predicted to decrease by 3.5% for each additional CAG. In the lowest decile (CAG<17), geometric mean PSA was 83% higher (1.19 ng/ml) than in the highest decile (CAG>26; geometric mean PSA=0.65 ng/ml). There was no evidence of heterogeneity in slope by ethnicity (p=0.33). There was no association between serum PSA level and GGC length, either before (p=0.25) or after (p=0.29) adjusting for CAG length.

Figure 3 shows the distribution of serum PSA by ethnicity and by PSA ARE1 genotype. Combining all ethnic groups, geometric mean PSA levels were higher among men with the AA genotype compared to men with the AG or GG genotypes (p=0.02). PSA levels did not differ between genotypes AG and GG (p=0.80). This same pattern was seen among African-Americans (p=0.06), non-Hispanic whites (p=0.28), and Hispanics (p=0.11) but was not statistically significant within group, due to smaller sample. The association of the AA genotype with higher PSA levels could not be evaluated among Japanese-Americans (p=0.85), since only five Japanese-Americans had the AA genotype. Serum PSA levels were not associated with the newly identified -252A/G and -232A/)A polymorphisms either alone (p=0.62) or after adjusting for ARE1 genotype (p=0.33). Haplotypes (-252/-232/ARE1) did not predict serum PSA better than ARE1 genotype alone (p=0.67).

Because the AR regulates PSA transcription by binding the ARE1 sequence, we further examined the data for evidence of interaction between AR and PSA genotypes. Stratification on PSA ARE1 genotype (Figure 4) provided a significantly better fit to the data than did the unstratified model (Figure 2) (p=0.049). For genotype GG, the slope was not significantly different from zero (p=0.74). For genotype AG, serum PSA increased 4.5% with each decrease of one CAG repeat decile (p=0.03), while for genotype AA serum PSA increased 7% with each decrease of one CAG repeat decile (p=0.02).

Discussion

This is the first report to identify genetic determinants of serum PSA levels. We found that variation in serum PSA levels among healthy men can be influenced by polymorphic variation in both the AR and the PSA genes. Log-transformed PSA levels were linearly and inversely associated with AR CAG length. Moreover, AR CAG length influenced serum PSA most strongly among men having PSA genotype AA, only modestly among men having genotype AG, and not at all among men with genotype GG. In other words, PSA and AR genotypes interact to influence serum PSA levels.

Physical interaction of the AR transciption complex with AREs in the PSA gene promoter activates PSA gene transcription. *In vitro* studies have established that ARs encoded by short CAG alleles are more efficient

transactivators than those encoded by long CAG alleles (8-9). The reduction in AR transactivation activity observed *in vitro* with increasing CAG length is modest (10), and is consistent with the subtle decrease in serum PSA levels observed in the present study.

The PSA ARE1 sequence lies 170 bp upstream of the transcription start site and has two allelic variants: AGAACAnnnAGT*A*CT and AGAACAnnnAGT*G*CT. Experimental studies addressing the functional differences between these two alleles have not been reported. The allelic differences observed in this study were subtle and may be difficult to detect in an *in vitro* system. Nevertheless, our data suggest that the A and G alleles interact differently with the AR, leading to quantitative differences in PSA expression. Alternatively, the ARE1 polymorphism may be in linkage disquilibrium with undefined coding polymorphisms that influence PSA activity or with upstream or downstream regulatory elements that affect transcription efficiency. To address the possibility that the ARE1 polymorphism may simply mark a nearby functional promoter polymorphism, we sequenced a 560 base-pair region surrounding the ARE1. Although we found two additional polymorphic sites, these sites do not appear to influence serum PSA levels.

Even though PSA has been used as a tumor marker for many years, the role of PSA in prostate physiology is still unclear. Both protective and pathogenic functions have been attributed to PSA. PSA cleaves the major IGF-binding protein, IGFBP-3, and increases bioavailable IGF-I and IGF-II, potentially having a stimulatory effect on prostatic epithelial cell proliferation (11). On the other hand, PSA has been reported to be antiangiogenic (12). This function could help prevent progression of localized prostate cancers to a more advanced stage. In our previous study (5), we found that the PSA GG genotype, which is associated with lower serum PSA levels in the current study, was associated with increased risk of advanced prostate cancer. This result supports a protective role for PSA against prostate cancer progression.

The role of the AR CAG repeat polymorphism is less clear. The genotype associated with higher serum PSA levels, namely CAG short, was associated with increased risk of prostate cancer in our previous study (5) and in several other studies (13-15). This apparent inconsistency might be explained by multiple downsteam effects of androgen signalling. The AR, by transactivating other genes in addition to PSA, might influence prostate cancer risk through several pathways, some which confer risk and others, such as PSA, which are protective. Both the AR CAG and the PSA ARE1 polymorphisms need to be examined in large numbers of advanced and localized prostate cancer cases and controls to shed light on this situation.

One strength of this study is that subjects were chosen from a well-characterized cohort of healthy men. Men with elevated PSA levels (>4 ng/ml) were excluded. The remaining men are unlikely to have significant disruption of prostatic barriers, thus differences in serum PSA levels are likely to be due to PSA production. Higher PSA production among certain genotypic groups might be due to either increased production by individual cells or to prostatic hyperplasia. We cannot rule out the possibility that the higher PSA levels among men with short CAG alleles might be due to an increase in BPH among this group. However, our results were not changed by eliminating forty men who reported a history of prostate enlargement. Further studies will be necessary to determine whether intra-prostatic PSA expression is associated with genotype.

In summary, we have shown that in healthy men, genetic variants in the PSA and AR genes contribute to variation in serum PSA levels. Men with the PSA AA genotype and short AR alleles have, on average, higher serum PSA levels. Together with our previous finding that the AA genotype protects against advanced prostate cancer, these results suggest that higher PSA levels associated with the AA genotype may actually be beneficial.

Acknowledgements

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Table 1: Age and serum PSA levels in study population by ethnicity

	Blacks	Whites	Hispanics	Asians
N	100	113	108	99
Age				
mean (SD)	62.2 (8.2)	62.2 (8.7)	64.1 (8.0)	62.5 (9.7)
median (25th, 75th percentile)	62 (57, 68)	62 (55, 69)	65 (58, 72)	62 (54, 71)
PSA				
mean (SD)	1.3 (0.9)	1.2 (0.9)	1.3 (1.0)	1.2 (0.8)
median (25th, 75th percentile)	1.0 (0.6, 1.7)	1.0 (0.5, 1.6)	1.0 (0.6, 1.7)	1.0 (0.6, 1.7)

Table 2: Distribution of AR and PSA genotypes by ethnicity

Genotype	Blacks (N=100)	Whites (N=113)	Hispanics (N=108)	Asians (N=99)	All groups (N=420)
AR CAG					
7-16	23 (23%)	4 (4%)	5 (5%)	2 (2%)	34 (8%)
17-18	23 (23%)	10 (9%)	9 (8%)	5 (5%)	47 (11%)
19	8 (8%)	18 (16%)	6 (6%)	6 (6%)	38 (9%)
20	13 (13%)	17 (15%)	19 (18%)	9 (9%)	58 (14%)
21	13 (13%)	12 (11%)	15 (14%)	19 (19%)	59 (14%)
22	5 (5%)	15 (13%)	14 (13%)	26 (26%)	60 (14%)
23	1 (1%)	14 (12%)	13 (12%)	15 (15%)	43 (10%)
24	5 (5%)	13 (12%)	12 (11%)	8 (8%)	38 (9%)
25-26	5 (5%)	8 (7%)	7 (6%)	5 (5%)	25 (6%)
27-37	4 (4%)	2 (2%)	8 (7%)	4 (4%)	18 (4%)
AR GGC[1]					
3-15	42 (45%)	14 (12%)	12 (11%)	19 (19%)	87 (21%)
16	21 (23%)	66 (59%)	65 (61%)	56 (57%)	208 (51%)
17-20	30 (32%)	32 (29%)	30 (28%)	23 (24%)	115 (28%)
PSA ARE1					
AA	28 (28%)	28 (25%)	12 (11%)	5 (5%)	73 (17%)
AG	48 (48%)	52 (46%)	56 (52%)	31 (31%)	187 (45%)
GG	24 (24%)	33 (29%)	40 (37%)	63 (64%)	160 (38%)
PSA -252/-203[2]					
GG/ΔAΔA	84 (87%)	52 (49%)	49 (47%)	38 (40%)	223 (56%)
GA/AΔA	12 (13%)	46 (43%)	46 (44%)	50 (52%)	154 (38%)
AA/AA	0 (0%)	8 (8%)	9 (9%)	8 (8%)	25 (6%)

[1]AR GGC genotype missing for 10 samples
[2]PSA -252/-203 genotypes missing for 18 samples

Table 3: PSA promoter haplotype frequencies by ethnicity

	Black (N=96)	**White** (N=106)	**Hispanic** (N=104)	**Asian** (N=96)
PSA*1	53%	46%	37%	20%
PSA*2	41%	25%	32%	45%
PSA*3	6%	29%	31%	35%

FIGURE LEGENDS

Figure 1: Correspondence between PSA genotypes and haplotypes.
(A) Classification of 402 subjects according to PSA genotypes at positions -252, -232, and -158; (B) Classification of 804 chromosomes according to PSA haplotypes (PSA*1, PSA*2, and PSA*3)

Figure 2: Serum PSA as a function of AR CAG length.
Each circle represents a single subject. The line represents regression of ln(PSA) on AR CAG length, adjusted for age and ethnicity.

Figure 3: Distribution of serum PSA levels by PSA ARE1 genotype and ethnicity.
The width of each box is proportional to the square root of the number of observations in the group.

Figure 4: Serum PSA as a function of AR CAG length, stratified by PSA genotype.
Each circle represents a single subject. The line represents regression of ln(PSA) on AR CAG length, adjusted for age and ethnicity. (A) PSA genotype AA; (B) PSA genotype AG; (C) PSA genotype GG

Fig 1

A. Genotypes

-252	-232	-158 (ARE1) AA	AG	GG
GG	ΔA/ΔA	69 (17%) *PSA*1/*1*	104 (26%) *PSA*1/*2*	50 (12%) *PSA*2/*2*
GA	ΔA/A	0 (0%)	74 (18%) *PSA*1/*3*	80 (20%) *PSA*2/*3*
AA	A/A	0 (0%)	0 (0%)	25 (6%) *PSA*3/*3*

B. Haplotypes

		-252 A/G	-232 [A]	-158 A/G	+1
PSA*1	316 (39%)	G	ΔA	A	
PSA*2	284 (35%)	G	ΔA	G	
PSA*3	204 (26%)	A	A	G	

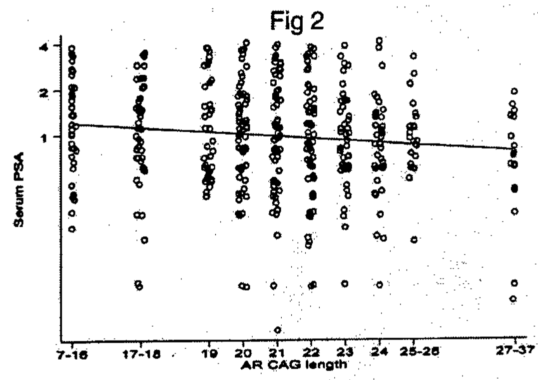

Fig 2
Serum PSA
4
2
1
7-16
17-18
19
20
21
22
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24
25-26
27-37
AR CAG length

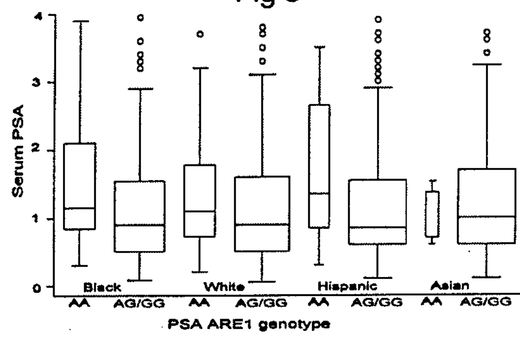
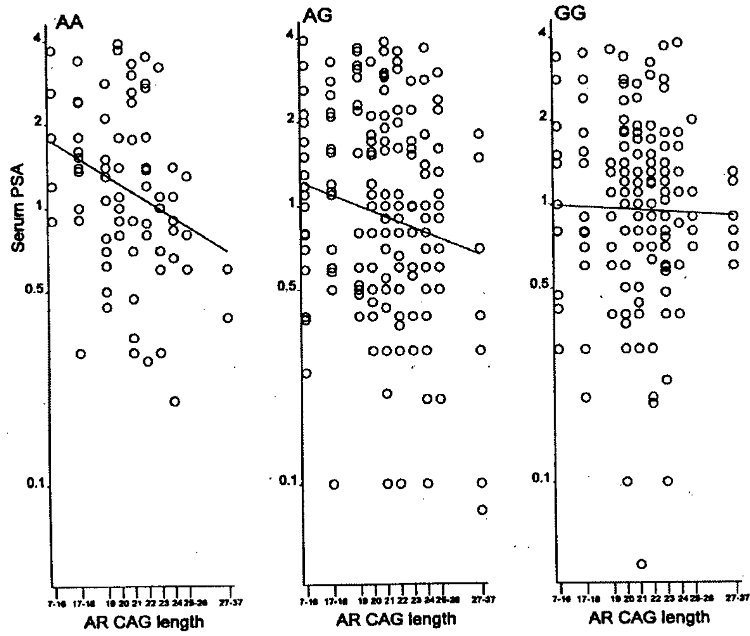


Fig 3

Fig 4



Current Issues in Hormone Therapy for Prostate Cancer
First Annual Charles Huggins Symposium
Friday, June 1, 2001
Dinner Program

Molecular Biology of Predisposition to, and Hormone Therapy of Prostate Cancer

GA Coetzee, Ph.D.

Hormone action in prostate cells primarily involves the binding of the male hormone dihydrotestosterone to cytoplasmic androgen receptor (AR), followed by the translocation of the hormone-receptor complex to the nucleus where it activates the transcription of a wide range of target genes in both the normal and malignant prostate. This activity culminates in epithelial cell proliferation and/or differentiation. It is postulated that the AR influences both prostate cancer predisposition and progression to hormone independence by playing a role in normal prostate development as well as in androgen-dependent and–independent tumor growth. Coupled with the fact that substantial genetic variability exists at the AR locus and that it is located on the X chromosome (one copy per male cell), the AR is considered an important candidate gene involved in all phases of prostate cancer development.

Predisposition: The AR locus displays substantial germline genetic variation. Two polymorphic microsatellites lie within the coding sequence of exon 1 of the gene. As a consequence size variation in either microsatellite results in corresponding changes in AR amino acid composition. For one of these, the AR CAG repeat, the resultant changes in the AR protein (poly-glutamine variation) have been shown to influence its function, indicating that it is perhaps directly involved in modifying prostate cancer risk at the AR locus. Thus, the possible association of AR CAG microsatellite repeat length variation with prostate cancer risk has been studied extensively in recent years (for review see Ross *et al.*, 1998). In a 1995 study (Irvine *et al.*, 1995), we showed that racial-ethnic variations in CAG repeat length correlated with prostate cancer risk among men in Los Angeles such that an excess of AR alleles with short CAGs was found in high-risk African-Americans relative to intermediate-risk Whites and low-risk Asian-Americans. Consistent with this finding, we subsequently demonstrated a 2-fold increased risk of prostate cancer among White men with short CAGs (i.e., <20 repeats); this genotype–prostate cancer-relationship was especially pronounced for advanced disease (Ingles *et al.*, 1997). These novel observations were later confirmed by two large-scale studies on prostate cancer risk in US Whites (Giovannucci *et al.*, 1997; Stanford *et al.*, 1997). In particular, Giovannucci *et al.* (1997) detailed a highly significant inverse linear relationship between CAG size and risk for prostate cancer. Thus, based on epidemiological studies, a general paradigm has emerged to explain AR function in prostate cancer risk: longer CAG alleles encode less active receptors that protect against tumorogenesis by causing decreased epithelial cell proliferation. However, the molecular mechanisms that underlie changes in AR transactivation activity due to CAG repeat size variation remain largely unknown although clues related to coactivator action have been forthcoming (Irvine *et al.*, 2000).

One of the classic target genes of the AR is the PSA gene and expression of this important prostate cancer marker is under the control of androgen and the AR. The AR, after binding to ligand (androgen), recognizes and binds to specific nucleotide sequences [androgen response elements (ARE)] in promoter regions of androgen-regulated genes. At least three AREs have been identified in the PSA gene promoter; the one nearest to the transcription start site (ARE1) contains a single nucleotide polymorphism (-158A/G). We have recently found that in healthy men this genetic variation in the PSA gene and the CAG repeat variation in the AR gene both contribute in an interactive fashion to variation in serum PSA levels (Xue *et al.* 2001). How these results might influence prostate cancer risk or the use of PSA levels as a marker of tumor growth are still unclear.

Progression: To date, many laboratories have collectively identified at least 77 individual AR gene mutations in clinically relevant prostate tumors; 56 of them (73%) are confined to 6 discrete regions ('hotspots') comprising only 14% of the receptor codons (Tilley, personal communication). The 6 regions comprise small non-contiguous areas in the N-terminal domain (NTD) and hormone-binding domain (HBD) of the receptor (3 regions in each). When the phenotypic effects of some of these somatic mutations were studied, it was found that they impose a gain of function to the receptor to allow AR signaling to occur under altered or decreased androgen levels. Thus 'androgen independence' of tumor growth in most cases is not necessarily androgen receptor independent. The realization of this fact will lead to better design strategies for the therapy of hormone independent prostate cancer.

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