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14. ABSTRACT: We utilized 97 healthy, 77 benign prostatic hyperplasia (BPH) and 156 prostate cancer (PC) samples that are pre-existing from African-American and Caucasian patients. Due to human subjects issues, the project has been on hold from Dec 2005 till Jan 2008 and thus, results reported are based on a period of approximately two years. In the first hypothesis, we have determined CYP1B1 protein to be localized to the cytoplasm of PC cells, with levels being much higher in PC than BPH. In the second hypothesis, single nucleotide polymorphisms (SNPs) of CYP1B1 have been evaluated to determine if they are risk factors for race-related PC. Racial differences are observed in allele frequencies as the variant at codons 119 and 432 are greater among Blacks (P<0.001) whereas the 453 variant is predominant in Whites (P<0.001). Within race, a case-control study shows the variant at codon 453 plays a protective role for PC among Blacks (P<0.05). Interestingly, SNPs at codons 432 and 449 are determined to be linked and the 432G-449C haplotype was observed to be a risk for PC (P<0.05). In a sampling of cases, no differences were observed between stages (<T2c vs >T2c) and grades (<7 vs >7) of PC in either race. A no-cost extension has been submitted for the 3rd year for which continuation of SNP studies with additional samples to be collected; as well as further experimentation with aim #1 to be performed.					
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INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of death among men with cancer in the USA. When comparing races, the incidence and mortality rates of prostate cancer in African-Americans is higher than in Caucasians and Asians. Cytochrome P450 (CYP) 1B1 converts estrogens to the 4-hydroxy-catechol-estrogens. Studies show this catechol-estrogen to be mutagenic and may lead to prostate cancer. Polymorphisms of CYP1B1 have been associated with various types of cancers and recently, we have shown that CYP1B1 polymorphisms have higher risks for prostate cancer (Abstract; J. Urol. 171(Suppl. 4):111, 2004). However, such studies are lacking in race-related prostate cancer. There are at least 4 polymorphisms that have been identified on the CYP1B1 gene that results in a structural change in the enzyme and are at the following locations: codons 48 (C to G), 119 (G to T), 432 (C to G), and 453 (A to G). The main goal of this project is to investigate whether polymorphisms of the CYP1B1 gene can be a risk factor for race-related prostate cancer. To determine this, two specific aims are tested. In specific aim #1, the hypothesis that CYP1B1 gene is hyper-activated during malignant transformation of race-related prostate cells is tested. In the 2nd aim, the hypothesis that single nucleotide polymorphisms (SNPs) of the CYP1B1 gene have higher risk for race-related prostate cancer and correlate with hyper-activated CYP1B1 gene is tested. Data generated from these experiments will determine whether CYP1B1 gene expression differs between Caucasian and African-American prostate cancer samples. Also, these experiments will determine whether CYP1B1 SNPs are involved in race-related prostate cancer. This knowledge will help to understand the genetic basis for racial differences as well as identify the subjects who are at higher risk for prostate cancer.

NOTE: Due to human subjects protocol issues, the current project has been placed on hold from Dec 2005 till Jan 2008. Thus, this report is based on experiments performed during a span of approximately two years, from Jun 2004 to Nov 2005, and from Jan to May 2008. Earlier, we have submitted a request for a 1-year no-cost extension so that we could continue work on this project.

BODY

Samples: From our previously funded projects, we have obtained a total of 97 normal healthy, 77 benign prostatic hyperplasia (BPH) and 156 prostate cancer specimens that were pre-existing archival specimens from African-Americans and Caucasians. Experimental results are based on these samples.

Task #1. To determine if the CYP1B1 gene is differentially expressed between races (African-Americans and Whites) and in different stages and grades of prostate cancer.

The procedure for immunostaining of CYP1B1 protein has been developed and evaluated in formalin-fixed, paraffin-embedded prostate specimens that have been sliced into 5 µm sections. The protocol utilizes a rabbit polyclonal antibody (1:300 dilution) against human CYP1B1 (BD Gentest Corporation, Woburn, MA). The staining procedure is based on a commercial kit from Santa Cruz Biotechnology (Santa Cruz, CA) and immunoperoxidase activity was developed with 3,3'-diaminobenzidine. The sections were then counterstained with hematoxylin. Based on a few BPH (n=5) and cancerous (n=5) tissue sections evaluated, the CYP1B1 protein is determined to be localized to the cytoplasm of cancer cells (Figure 1). Protein expression was also observed in the smooth muscle cells of both BPH and prostate cancer specimens. Using Image J software to evaluate intensity of staining, the cancerous tissues stain much higher compared to BPH.

Task #2. To determine if single nucleotide polymorphisms (SNPs) of the CYP1B1 gene are risk factors for the etiology of race-related prostate cancer and correlate with hyper-activity of its gene.

From the pre-existing BPH and prostate cancer obtained from African-American and Caucasian patients, and normal healthy from African-American, DNA was collected by using a DNA extraction kit (Qiagen, Valencia, CA). Quantity and quality of DNA was measured at 260 nm and 280 nm by the

use of a spectrophotometer. A two-step polymerase chain reaction (PCR) procedure was designed for the analysis of CYP1B1 polymorphisms. The primers of four of the polymorphic sites studied so far (codons 119, 432, 449, and 453) and PCR conditions are summarized in Table 1. In the first PCR, DNA (10 ng) was amplified in a 20 μ l reaction containing 1.5 mM MgCl₂, 0.8 mM dNTP mix, PCR buffer, and 0.5 units of Red-Taq polymerase (Sigma-Aldrich, St. Louis, MO), along with primer sets designed to contain the polymorphic sites (Table 1). In the sequence-specific PCR (SSP), each polymorphic fragment was further amplified under similar conditions as the first-step PCR except for the use of SSP primer sets (Table 1). Each of the SSP products were electrophoretically separated on 3% agarose gels using 180 volts at ambient temperature. The products were then visualized by ethidium bromide staining under UV light. To confirm genotyping, products of the first PCR were subjected to direct DNA sequencing. In the case of the codon 449 polymorphism, direct sequencing was performed on all samples. DNA was purified from gels using a QIAquick PCR purification kit (Qiagen; Valencia, CA). Sequence analysis of purified products was then determined by using the first PCR primers and ABI 377 Sequencer and Dye Terminator Cycle sequencing kit (Applied Biosystems Inc.; Foster City, CA). Confirmation of DNA sequence was done on at least 3 representative samples for each of the polymorphic types. Frequencies of the various genotypes and allele types of CYP1B1 polymorphisms in the different categories of samples were determined and tabulated. Chi-square analysis was used to test each of the polymorphisms for differences in genotypic and allelic frequencies between Whites and Blacks as well as between BPH and prostate cancer. Relative risk associated with a particular genotype or allele was estimated by calculating odds ratios (OR) along with 95% confidence intervals (CI). In African-American samples, further analyses included case-control, linkage disequilibrium, and haplotype evaluation using the SNPalyze software (Dynacom, Japan).

Results of the genotypic and allelic frequencies of three SNP sites of the CYP1B1 gene between African-Americans and Caucasians for BPH and prostate cancer, and total patients (including African-American controls) are shown in Tables 2 and 3, respectively. Interestingly in total patients, the variant genotype at all 3 codons differ significantly between African-Americans and Caucasians. The T/T genotype at codon 119 are highly predominant in African-Americans as compared to Caucasians (Chi-square, $P < 0.001$). OR (95% CI) were 2.71 (1.61-4.55) and 3.55 (1.96-6.43) for the G/T and T/T genotypes, respectively, in Blacks compared to Whites. At codon 432, the C/G and G/G genotypes were much greater in African-Americans with OR (95% CI) values of 3.90 (2.03-7.52) and 12.09 (5.95-24.58) as compared to Caucasians (Chi-square, $P < 0.001$). The polymorphism as codon 453 also proved to be different between Blacks and Whites although the variant A/G and G/G genotypes were much greater in Caucasians (Chi-square, $P < 0.001$). Thus the OR (95% CI) values were lower for Blacks compared to Whites with values of 0.17 (0.09-0.34) for A/G and 0.10 (0.01-1.02) for the G/G genotypes as compared to Caucasians. Significant differences in genotype frequencies between Blacks and Whites also occurred when data were evaluated just among the prostate cancer patients at all 3 codons (Table 2). When classified among the BPH patients, significant differences were observed at the codon 432 and 449 sites but not the 119 site (Table 2). This may be due to the small N size of BPH patients.

Likewise when evaluating allele frequencies between African-Americans and Caucasians, significant differences were observed at all 3 SNP sites in total patients ($P < 0.001$; Table 3). This was also true in prostate cancer patients at all 3 sites ($P < 0.001$; Table 3). In BPH patients, significance was only found at the codon 432 site and may be due to the small BPH patient N size.

A case-control study was performed on African-Americans as 97 healthy controls and 97 age-matched prostate cancer specimens were obtained. In addition to the 3 polymorphic sites, the codon 449 SNP site was also evaluated. Table 4 shows the results of genotype and allele frequencies in prostate cancer and controls at these 4 polymorphic sites. The polymorphism at codon 453 was relatively low in frequency as the homozygous variant (G/G) was not observed in any patient. Frequencies in healthy controls were in Hardy-Weinberg equilibrium at these sites. When compared to healthy volunteers, no differences in polymorphic frequencies were observed in prostate cancer cases

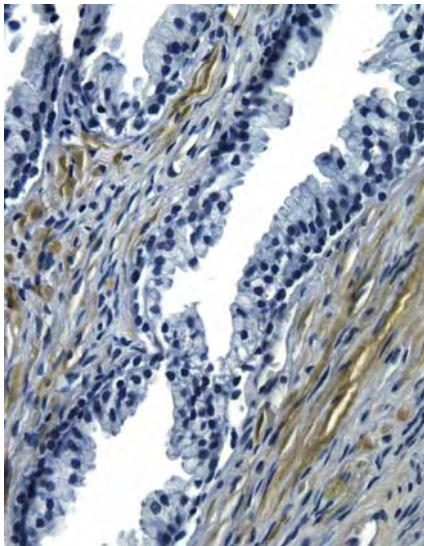
at codons 119, 432, and 449. However, the SNP at codon 453 proved to play a protective role as cancer cases had a significantly lower frequency of the variant A/G genotype when compared to healthy controls ($P=0.035$). The OR was 0.16 with a 95% CI of 0.05 to 0.51 for the A/G genotype as compared to A/A. In concordance, the allele frequency also differed with the variant G being much lower in prostate cancer cases ($P=0.024$).

Linkage disequilibrium analysis was performed to determine any linkage between the 4 polymorphic sites. Healthy control samples were analyzed and interestingly, a strong linkage was observed between the codon 432 and codon 449 sites (Table 5). The D-value between these two sites was 0.1905. No linkages were observed between other polymorphic sites. Since these two sites were linked, haplotype analysis was performed between prostate cancer cases and controls. Interestingly as shown in Table 6, the 432G – 449C haplotype proved to be significantly associated for cancer as 6.3% of this combination was found in cases compared to just 1.0% in controls ($P=0.016$).

Clinical and pathological information were obtained for a portion of the prostate cancer patients. Cancer samples were classified in terms of stage and due to the small N size when divided between races, stage classifications were made as $< T2c$ or $\geq T2c$. Results for each race is shown in Table 7. No differences were observed between stages for either race although sample size is small. Likewise, cancer samples were classified in terms of pathological grade and were based as < 7 or ≥ 7 . Table 8 shows the results of samples based on grade for both Blacks and Whites and no differences were observed.

Figure 1. Immunohistochemistry staining of CYP1B1 protein in BPH and prostate cancer tissue.

BPH



Prostate Cancer

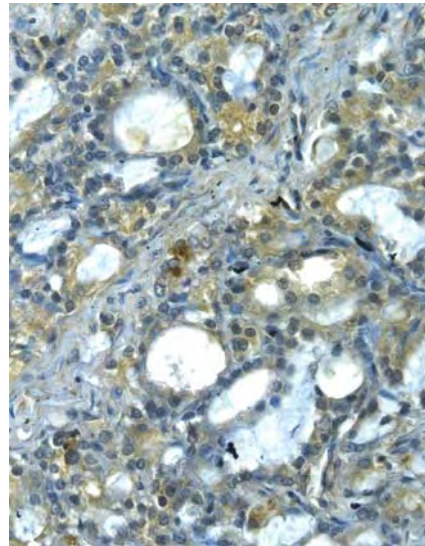


Table 1. First step PCR and SSP primers utilized to determine SNP in BPH and prostate cancer samples

CODON 119

1st PCR

<u>Primer</u>	<u>Sequence</u>	<u>Anneal Temp</u>
C119-Rev	ccttcagtgctccgagtag	47 C
C119-For	cccatagtggtgctgaatg	

SSP

<u>Primer</u>	<u>Sequence</u>	<u>Anneal Temp</u>
C119 G-Rev with C119-For	acggaaggaggcgaaggc	65 C
C119 T-Rev with C119-For	acggaaggaggcgaagga	65 C

CODON 432

1ST PCR

<u>Primer</u>	<u>Sequence</u>	<u>Anneal Temp</u>
C432-Rev	tcatcactctgctggtcagg	47 C
C432-For	gtcttgggtaccacattcc	

SSP

<u>Primer</u>	<u>Sequence</u>	<u>Anneal Temp</u>
C432 C-Rev with C432-For	tccgggttaggccacttcag	65 C
C432 G-Rev with C432-For	tccgggttaggccacttcac	65 C

CODON 449

Determined by direct sequencing using 1ST PCR primers of codon 432 (C432-Rev and C432-For)

CODON 453

1ST PCR

<u>Primer</u>	<u>Sequence</u>	<u>Anneal Temp</u>
C453-Rev	agaaagttcttcgcaatgc	47 C
C453-For	gaccactgaagtggcctaa	

SSP

<u>Primer</u>	<u>Sequence</u>	<u>Anneal Temp</u>
C453 A-Rev with C453-For	tctgctggtcaggtccttgt	64 C
C453 G-Rev with C453-For	tctgctggtcaggtccttgc	64 C

Table 2. Genotypic frequencies of CYP1B1 SNPs between races for BPH and Prostate cancer and Total Patients. P-value reflect chi-square test.

Type	Codon	Gene	White	Black	OR (95% CI)
BPH	119	G/G	21	16	Ref
		G/T	12	17	1.86 (0.71-4.88)
		T/T	6	5	1.09 (0.29-4.19)
	432	C/C	11	2	Ref
		C/G	20	14	3.85 (1.01-14.62)
		G/G	8	22	15.13 (4.14-55.23)
	453	A/A	28	33	Ref
		A/G	11	4	0.31 (0.10-0.95)
		G/G	0	1	-
PC	119	G/G	38	33	Ref
		G/T	14	34	2.80 (1.34-5.83)
		T/T	7	30	4.94 (2.21-11.00)
	432	C/C	23	7	Ref
		C/G	22	30	4.48 (1.83-10.99)
		G/G	14	60	14.08 (5.83-34.01)
	453	A/A	37	95	Ref
		A/G	19	2	0.04 (0.02-0.11)
		G/G	3	0	-
Total Patients (Includes Healthy Blacks)	119	G/G	59	78	Ref
		G/T	26	93	2.71 (1.61-4.55)
		T/T	13	61	3.55 (1.96-6.43)
	432	C/C	34	17	Ref
		C/G	42	82	3.90 (2.03-7.52)
		G/G	22	133	12.09 (5.95-24.58)
	453	A/A	65	214	Ref
		A/G	30	17	0.17 (0.09-0.34)
		G/G	3	1	0.10 (0.01-1.02)

Table 3. Allele frequencies of CYP1B1 SNPs between races for BPH and Prostate cancer. P-value reflect chi-square test.

Type	Codon	Allele	White	Black
BPH	119	G	54	49
		T	24	27
	432	C	42	18
		G	36	58
	453	A	67	70
		G	11	6
PC	119	G	90	100
		T	28	94
	432	C	68	44
		G	50	150
	453	A	93	192
		G	25	2
Total Patients (Includes Healthy Blacks)	119	G	144	249
		T	52	215
	432	C	110	116
		G	86	348
	453	A	160	445
		G	36	19

Table 4. Genotypic and allelic frequencies of CYP1B1 SNPs between normal healthy and prostate cancer patients among Blacks. P-value reflect chi-square test.

Type	Codon	Gene	Healthy	Cancer	OR (95% CI)
Genotype	119	G/G	29	33	
		G/T	42	34	0.71 (0.36-1.39)
		T/T	26	30	1.01 (0.49-2.09)
	432	C/C	8	7	
		C/G	38	30	0.90 (0.29-2.76)
		G/G	51	60	1.34 (0.46-3.94)
	449	C/C	9	10	
		C/T	36	33	0.83 (0.30-2.27)
		T/T	52	54	0.93 (0.35-2.47)
	453	A/A	86	95	
	P=0.035	A/G	11	2	0.16 (0.05-0.51)
		G/G	0	0	-
Allele	119	G	100	100	
		T	94	94	
	432	C	54	44	
		G	140	150	
	449	C	54	53	
		T	140	141	
	453	A	183	192	
	P=0.024	G	11	2	

Table 5. Linkage disequilibrium among 4 polymorphic sites of CYP1B1 in healthy controls of Black volunteers. D-values are shown.

<u>Codon</u>	<u>119</u>	<u>432</u>	<u>449</u>	<u>453</u>
119	-	0.0195	0.0305	-0.0181
432	0.0195	-	0.1905	0.0409
449	0.0305	0.1905	-	0.0409
453	-0.0181	0.0409	0.0409	-

Table 6. Haplotype frequencies of codons 432 and 449 of CYP1B1 among Black healthy controls and prostate cancer patients.

<u>Haplotype</u>	<u>Control</u>	<u>Cancer</u>	<u>P-value</u>
432G-449T	71.1%	71.0%	
432C-449C	26.8%	21.0%	
432G-449C	1.0%	6.3%	0.016
432G-449T	1.0%	1.6%	

Table 7. Genotypic frequencies of CYP1B1 SNPs between stages of cancer (< T2c vs $\geq$ T2c) for Blacks and Whites. Note: partial # of Black samples. P-value reflect chi-square test.

Race	Codon	Gene	< T2c	$\geq$ T2c	P-value
Blacks	119	G/G	2	10	0.17
		G/T	3	6	
		T/T	5	4	
	432	C/C	2	1	0.43
		C/G	2	5	
		G/G	6	14	
	453	A/A	10	18	0.59
		A/G	0	2	
		G/G	0	0	
Whites	119	G/G	15	19	0.96
		G/T	6	7	
		T/T	3	3	
	432	C/C	12	9	0.37
		C/G	8	13	
		G/G	4	7	
	453	A/A	15	17	0.64
		A/G	7	11	
		G/G	2	1	

Table 8. Genotypic frequencies of CYP1B1 SNPs between grades of cancer (< 7 vs ≥ 7) for Blacks and Whites. Note: partial # of Black samples. P-value reflect chi-square test.

Race	Codon	Gene	< 7	≥ 7	P-value
Blacks	119	G/G	7	10	0.86
		G/T	5	5	
		T/T	5	5	
	432	C/C	1	2	0.76
		C/G	5	4	
		G/G	11	14	
	453	A/A	16	19	0.99
		A/G	1	1	
		G/G	0	0	
Whites	119	G/G	20	17	0.27
		G/T	11	3	
		T/T	4	3	
	432	C/C	14	9	0.44
		C/G	15	7	
		G/G	6	7	
	453	A/A	22	14	0.95
		A/G	11	8	
		G/G	2	1	

KEY RESEARCH ACCOPLISHMENTS:

- Established protocol to measure CYP1B1 in prostate tissue by immunohistochemistry.
- Evaluated CYP1B1 protein in BPH and prostate cancer samples.
- Established primers and protocol to measure four CYP1B1 SNPs.
- Evaluated CYP1B1 SNPs in healthy controls, BPH and prostate cancer samples.
- Evaluated linkages between SNP sites.
- Evaluated SNP haplotypes between cases and controls.
- Evaluated CYP1B1 SNPs in stages and grade of prostate cancer samples.

REPORTABLE OUTCOMES:

Abstract presented at the American Association for Cancer Research (April, 2006).

The significances of the research performed to date are the following:

- 1) CYP1B1 protein is localized in the cytoplasm of prostate cancer cells with some expression in smooth muscle cells.
- 2) CYP1B1 protein has a much higher level in prostate cancer compared to BPH.
- 3) CYP1B1 codon 119 T variant genotype and allele are significantly higher in African-Americans with prostate cancer compared to Caucasians.
- 4) CYP1B1 codon 432 G variant genotype and allele frequency are significantly higher in African-Americans compared to Caucasians with BPH or prostate cancer.
- 5) CYP1B1 codon 453 G variant genotype and allele frequency are significantly lower in African-Americans compared to Caucasians with prostate cancer.
- 6) In African-Americans, no associations found for prostate cancer at codons 119, 432, and 449 SNP sites. However, the G variant at codon 453 played a protective role against cancer.
- 7) In African-Americans, polymorphisms at codons 432 and 449 were in linkage disequilibrium.
- 8) In African-Americans, the 432G-449C haplotype is observed to be associated with prostate cancer.
- 9) CYP1B1 polymorphisms did not correlate with stage or grade of prostate cancer in African-Americans and Caucasians.

CONCLUSIONS:

Racial differences in polymorphisms of the CYP1B1 gene exist and therefore, can identify the population with higher risk for prostate cancer.

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- Tanaka Y.,** M. Sasaki, H. Shiina, M. Igawa, M. Kaneuchi, C.J. Kane, P.R. Carroll, and R. Dahiya. 2004. Polymorphisms of Cytochrome P450 1B1 are risk factors for prostate cancer. J. Urol. 171(Suppl. 4):111, Abstract.

PERSONNEL:

Hideki Enokida

APPENDICES:

Abstract presented at the American Association for Cancer Research in April, 2006.

#3687 Cytochrome P450 1B1 polymorphisms in African-Americans and Caucasians with prostate cancer. Yuichiro Tanaka, Hiroshi Hirata, Toshifumi Kawakami, Deepa Pookot, Zhong Chen, Shinji Urakami, Ken Kawamoto, Hiroyuki Enokida, Rajvir Dahiya. *VA Medical Center, San Francisco, CA.*

Differential rates in incidence and mortality due to prostate cancer occur between races. A factor that may play a role in the carcinogenesis process is catechol-estrogen and 4-hydroxy-estrogen has been shown to be tumorigenic and mutagenic. The enzyme capable of producing 4-hydroxy-estrogen is cytochrome P450 (CYP) 1B1 and polymorphisms of this enzyme have been demonstrated to be a risk factor for prostate cancer. We thus hypothesize that CYP1B1 polymorphisms could contribute to racial differences in prostate cancer risk. To test this hypothesis, the gene distribution of three different CYP1B1 polymorphisms at codons 119(G→T), 432(C→G), and 453(A→G), and their association with prostate cancer in African-American and Caucasian populations were investigated by using a sequence-specific PCR technique. For each race, prostate cancer and benign prostatic hyperplasia (BPH) control specimens were obtained. Results of preliminary experiments demonstrate the codon 119T, 432G and 453A alleles to be significantly higher in African-Americans compared to Caucasians among prostate cancer cases ($P < 0.01$). When assessing the risk for prostate cancer as compared to BPH controls within races, no differences were observed for any of the polymorphic sites. Also, differences were not detected between stages and grades of cancer. Thus, the experimental results demonstrate differences in CYP1B1 polymorphisms between races and may be important in understanding race-related prostate cancer.