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DHPLC Elution Patterns of *VDR* PCR Products Can Predict Prostate Cancer Susceptibility in African American Men

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Abstract. *Background/Aim: Denaturing high-performance liquid chromatography (DHPLC) is a technique that is used to detect mutations. The aim of the present study was to determine whether DHPLC elution patterns of vitamin D receptor (VDR) gene PCR products can serve as indicators of susceptibility to prostate cancer (PCa) risk. Materials and Methods: DNA samples of PCa cases and controls were screened for mutations and/or polymorphisms in coding exons of VDR gene using DHPLC analysis. Logistic regression, phi-coefficient (ϕ), and Backward Wald models were used to analyze the data. Results: Similar elution patterns of exons 1, 6, 7 and 9 along with higher prevalence of heteroduplex DNA were observed in PCa samples than in controls. Exons 4 and 8 had highly significant protective effects ($p<0.05$). Whereas, exons 5, 7, and 9 were perfectly positively correlated with PCa risk ($\phi=1$), thus presenting candidate exons significantly associated with susceptibility to PCa. Conclusion: DHPLC elution patterns of the selected exons could be useful to predict susceptibility to develop PCa.*

Prostate cancer (PCa) is one of the most commonly diagnosed forms of cancer among men in the developed world (1, 2). In the United States, it is the second leading cause of death in males. In 2014, it was estimated that 161,360 men were diagnosed and 26,730 men died of PCa in the United States (3). There are well-established risk-factors such as genetic pre-disposition, age, ethnicity, family history, diet and environmental that contribute to the etiology of PCa (4, 5).

Epidemiological studies suggest that PCa risk is determined by interactions between environment and genetic predisposition (6-8). There is accumulating evidence that vitamin D may play an important role in the occurrence and progression of prostate cancer. Vitamin D deficiency is widespread throughout the United States and the world. The suggestion has been forwarded that high incidence of prostate cancer in African American men might be related to the deficiency of vitamin D. Therefore, vitamin D deficiency has become a public health concern especially in the African American population in the United States. Sunlight exposure may increase vitamin D synthesis in the skin and is thought to provide protection against prostate cancer (8-11).

Since genetic predisposition is considered a major risk factor, there are many studies suggesting an association between vitamin D receptor (*VDR*) gene polymorphisms and the development of PCa (12-20). Due to the abundance of *VDR* polymorphisms in the human genome as well as their high frequency in the human population, they have often been studied with the aim of explaining variations in the risk for PCa (21, 22). Most of the published studies utilized different single nucleotides polymorphisms (SNPs) detection methods, such as TaqMan® SNP Genotyping Assays, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), Illumina Human arrays and denaturing high-performance liquid chromatography (DHPLC) techniques.

DHPLC has been described as a method for screening DNA samples by comparing two or more chromosomes as a mixture of denatured and reannealed PCR amplicons. When the PCR product is heated and then slowly cooled, the DNA strands separate and randomly reanneal to form a mixture of three

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Table I. *List of forward and reverse primers used for PCR amplification of the exons 1-9 of vitamin D receptor gene.*

Exon	Region length (bp) (chromosomal location)*	Fragment length (bp)	Primer sequence
Exon 1	244 (47882608-47882851)	230	5' CGTGCCCACTTCCTTAGAGACTG 3' 5' CCACCACCTTCTTATGCCCCT 3'
Exon 2	338 (47878917-47879254)	396	5' GATGCCCACCCTTGCTGAG 3' 5' TGCTTCTTCTCCCTCCCTTTC 3'
Exon 3	307 (47864938-47865244)	395	5' TCCGTGATGACAGGGTGAGG 3' 5' TACAGAGGAAGGGCAGGCAGA 3'
Exon 4	494 (47857387-47857880)	348	5' CTTCCTTTTCACCATAGCAAACCC 3' 5' CGTCCCTACCCCAGTTCTGTTC 3'
Exon 5	314 (47857040-47857353)	349	5' GCCTTCCTGTAGACCTTCCTCAA 3' 5' ACCTCCTTCCATCCAGCAGC 3'
Exon 6	444 (47855490-47855933)	333	5' ACCTGTGGAGTCACTGTGGGATTC3' 5' AGCCTGCGTGACAGAGCAAGA 3'
Exon 7	434 (47846537-47846970)	403	5' GAACACTCTTGTCCCTTCCAGCC 3' 5' TCTCTCCCTGTTGGTGCCTAACTC 3'
Exon 8	348 (47846266-47846613)	353	5' AGATTCTGGCTCCACCCGTC 3' 5' CAGCAGGTCTTTGTCCTTCATACTC3'
Exon 9	525 (47844654-47845178)	402	5' AGTCACTGGAGGGCTTTGGG 3' 5' TGAGGAGGGCTGCTGAGTAGC 3'

*Chromosomal locations and exon sequences were obtained from UCSC database. (https://www.ncbi.nlm.nih.gov/genome/gdv/browser/?context=gene&acc=7421). For accuracy, the primer sequences were checked using USSC In-Silico PCR tool (https://genome.ucsc.edu/cgi-bin/hgPcr). bp, Base pair.

species: a mutant homoduplex, a heteroduplex, and a wild-type homoduplex. This reveals the presence of a mutation by the differential retention of homo- and heteroduplex DNA on reversed-phase chromatography supports under partial denaturation (http://www.transgenomic.com/). Individuals who are heterozygous in a single-nucleotide mutation or polymorphism have a 1:1 ratio of wild type and mutant DNA. The heteroduplex DNA fragments form as a result of base pairing of the single-stranded mutated DNA with single-stranded wild type DNA. Heteroduplex profiles are easily distinguished from homoduplex peaks and thereby provide a reliable means for mutation scanning and discovery (23).

This is the first study to investigate the association of the DHPLC elution patterns (homoduplex, heteroduplex) of PCR products corresponding to *VDR* coding exons with PCa risk in high-risk population of African American men. Therefore, we have screened a total of 183 DNA samples of PCa cases and controls for the detection of mutations and /or polymorphisms in all coding exons of *VDR* using the DHPLC technique. Furthermore, we investigated whether a specific elution pattern, age, prostate-specific antigen (PSA) level and family history might exhibit a greater or less association with PCa risk.

Materials and Methods

Study population. The study cohort consisted of 91 African-American men aged more than 40 years old from the Washington DC area with histologically diagnosed adenocarcinoma of the prostate, PSA of more than 3.5 ng/ml, and a positive digital rectal examination (DRE). From our previous studies (8, 24), we have also identified 92 age- and ethnicity- matched (African-American) healthy individuals (control group) who were regularly screened, with PSA levels of less than 3.5 ng/ml, normal DRE and with no history of prostate cancer among first-degree relatives. PSA values were obtained at the time of diagnosis for cases and at the time of study enrollment for the control group. The study was approved by the Howard University Institutional Review Board (IRB-02-MED-42) and signed informed consent forms have been obtained from each participant. Detailed information about demographics and medical history has previously been described (8, 24).

DNA extraction and PCR. Genomic DNA was extracted using the QIAmp DNA Blood Maxi Kit (Qiagen, Inc., Valencia, CA) according to manufacturer's instructions. Different primer sets for exon (E1-E9) fragments (Table I) were used for PCR amplification. The VDR chromosome location is chr12:47,831,537-47,953,048. (http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr12%3A47831537-47953048&hgsid=602911891_He50lFkDr5dXtxf5kcZnwFb5BeDu).

PCR was performed in a total volume of 25 μl, containing 30 ng of genomic DNA as template, 1X PCR buffer II, 20 pmole each of exon specific forward and reverse primers (Table I), 2 mM MgCl2, 0.2 mM dNTP mix (Applied Biosystems, Foster City, CA) and 2 μl AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA). PCR was performed in an AmpGene 9700 thermal cycler (Perkin-Elmer 600, Foster City, CA), under the conditions described in Table II. Crude PCR products were checked by 2% (wt/vol) agarose gel electrophoresis before DHPLC analysis, to ensure the absence of non-specific bands, which could lead to artificial heteroduplex conformation. Crude PCR products were subjected to

Table II. *Alternative PCR conditions that were used for the improvement of the PCR protocol in DHPLC analysis.*

94˚C for 20 s; exon specific temperature ˚C for 40 s and 72˚C for 20 s for 5 cycles at each increment. 94˚C for 20 s; optimum temperature ˚C for 40 s and 72˚C for 20 s for 35 cycles	
VDR exons	Increment annealing temperature (˚C)
Exon 1	53.6, 55.6, 57.6, 59.6
Exon 2	54.9, 56.9, 58.9, 60.9
Exon 3	54.7, 56.7, 58.7, 60.7
Exon 4	55. 57, 59, 61
Exon 5	53.6, 55.6, 57.6, 59.6
Exon 6	52.5, 54.5, 56.5, 58.5
Exon 7	53.8, 55.8, 57.8, 59.8
Exon 8	54.6, 56.6, 58.6, 60.6
Exon 9	56.1, 58.1, 60.1, 62.1

an additional 10 min at 95˚C denaturing step, followed by 10 min at room temperature for reannealing prior to analysis.

DHPLC. DHPLC was used in order to detect mutations and /or polymorphisms in *VDR* gene. DHPLC was carried out on an automated DHPLC instrument (WAVE® DNA Fragment Analysis System, Transgenomic, Omaha, NE) equipped with a DNASep column (Transgenomic Inc., San Jose, CA). The cartridge is packed with C18 alkalylated, polystyrene-divinylbenzene polymeric beads that allow analysis under a wide range of pH and temperature conditions. DHPLC was performed according to the pre-set conditions including: an initial gradient of 45% buffer A [0.1 M tri-ethyl-ammonium acetate (TEAA) solution, pH 7.0] and 55% buffer B (0.1 M TEAA containing 25% acetonitrile, pH 7.0), followed by a final gradient of 36% buffer A and 64% buffer B, using an acquisition time of 8.7 min. PCR products (25 μl) were eluted at a 0.9 ml/min flow rate. The start- and end-points of the gradient were adjusted according to the size of the PCR products using an algorithm provided by the WAVEMakerTM system control software version 1.5.4 (Transgenomic Inc., San Jose, CA). The temperature required for successful resolution of heteroduplex molecules was determined by use of the DHPLC melting algorithm and pretesting of several temperatures.

Statistical analyses. Phi-coefficient (ϕ) analysis was used to study the correlation among the *VDR* exons in relation to PCa. Logistic regression was used to examine the association between each *VDR* exon, as an independent variable, and PCa as the dependent variable. The results were further investigated using logistic regression model with Backward elimination to examine whether the effect of any of the exons (E) is modulated by another factor (*e.g.* Age, PSA level, family history). In particular, we assessed selected "E4 x E7"; "E4 x E7 x age"; "E4 x E7 x age x PSA"; and "E4 x E7 x age x PSA x family history" interactions in 4 models (Model, 1, Model 2, Model 3, and Model 4; respectively) that may include PCa as a factor. A p-value>0.05 was considered as statistically significant (SPSS IBM ver 23).

Table III. *Elution profiles associated with the DHPLC analysis of PCR amplicons corresponding to VDR exons, for control and PCa DNA samples.*

Exons	Homoduplex sample number	Heteroduplex sample number	Total number
Exon 1			
Control	60	33	93
Cases	54	37	91
Exon 2			
Control	76	17	93
Cases	86	5	91
Exon 3			
Control	72	21	93
Cases	90	1	91
Exon 4			
Control	27	66	93
Cases	63	28	91
Exon 5			
Control	64	29	93
Cases	70	21	91
Exon 6			
Control	91	2	93
Cases	86	5	91
Exon 7			
Control	57	36	93
Cases	51	40	91
Exon 8			
Control	27	66	93
Cases	63	28	91
Exon 9			
Control	51	42	93
Cases	46	45	91

Homoduplex, Single peak; Heteroduplex, double or more peaks.

Results

The elution patterns of VDR PCR products and their association with PCa risk. We have screened a total of 183 DNA samples of PCa cases and controls for mutations and SNPs detection in all coding exons of *VDR* using DHPLC analysis. The elution patterns associated with the DHPLC analysis of PCR amplicons containing *VDR* exons were further investigated to examine whether a specific elution pattern, age, PSA level and family history may exhibit a greater or less PCa risk association.

The differential retention of homoduplex (single peak) and heteroduplex (double peaks) DNA species under conditions of partial thermal denaturation within each of the *VDR* exons are listed in Table III. The elution patterns for *VDR* PCR products corresponding to exons 1, 6, 7 and 9 were highly similar with higher prevalence of double peaks of heteroduplex DNA among the PCa cases than controls. While, exons 2, 4, 5, 8 showed a similar elution patterns with

Table IV. *The correlation between the VDR exons and susceptibility to prostate cancer using phi-coefficient (ϕ).*

	Exon 1	Exon 2	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8	Exon 9
Exon 1	1							
Exon 2	0.056	1						
Exon 4	0.095	0.227	1					
Exon 5	0.075	0.038	0.329	1				
Exon 6	0.137	–0.073	–0.090	0.134	1			
Exon 7	0.070	–0.003	0.335	**0.579**	–0.167	1		
Exon 8	0.095	0.227	**1.000**	0.329	–0.090	0.335	1	
Exon 9	0.177	–0.047	0.295	**0.645**	0.210	**0.886**	0.295	1.

Based on Table III data, exon 3 was excluded from analysis since there was only one sample with heteroduplex profile. Bold text indicates statistically significant a correlation (p-value<0.05).

higher prevalence of double peaks among the controls than the cases. Exon 3 showed a distinct pattern of heteroduplex DNA in only one PCa case, therefore, it was excluded from any further analysis.

We further investigated the correlation among the *VDR* exons and susceptibility to PCa using phi-coefficient (ϕ) and logistic regression analysis. Phi-coefficient (ϕ) analysis showed that exons 5, 7, and 9 were moderately correlated (ϕ=0.579, 0.645, 0.886; respectively). Exons 4 and 8 were perfectly correlated (ϕ=1) and they might be candidate exons associated with PCa risk (Table IV). The association of each *VDR* exon, as an independent variable without adjusting for any of the studied factors, with PCa risk was further investigated using logistic regression model. The significant results indicate that exon 2 (β=–1.347, p<0.05), exon 4 (β=–1.705, p<0.05) and exon 8 (β=–1.705, p<0.05) had highly significant protective effects (Table V). On the other hand, exons 1, 6, 7, and 9 showed no statistically significant association with increased PCa risk (p>0.05).

When all of the predictors were included in the regression equation based on the Backward Wald elimination method, only Exons 4 and 7 were the dominant factors; thus exons 2 and 8 were dropped out. The results were further investigated using logistic regression model to examine whether the effect of any of the exons (E) is modulated by another factor (*e.g.* Age, PSA level, family history). In particular, we assessed selected "E4 x E7"; "E4 x E7 x age"; "E4 x E7 x age x PSA"; and "E4 x E7 x age x PSA x family history" interactions in 4 models (Model, 1, Model 2, Model 3, and Model 4; respectively) that may include PCa as a factor. When a significant interaction was observed, we examined the effect of one factor at each level of the other factor (Table VI). Model 1 showed that exon 4 was a disease protective (β=–2.206, OR=0.110, p<0.05). Whereas, exon 7 was significantly associated with PCa risk (β=1.150, OR=3.159, p<0.05) and the heteroduplex profile of exon 7 was highly correlated with PCa risk. The outcome of models 2, 3 and 4 was inconsistent with model 1 data, exons 4 and 7 maintained the protective and risk associations with PCa; respectively. Moreover, the heteroduplex profile of exon 7 was highly correlated with PCa risk, and as expected age and PSA level and family history were significantly associated with increasing prostate cancer risk (p<0.05).

Discussion

Studies analyzing association of *VDR* gene polymorphisms with cancer or other diseases, such as immune system related disorders, have been reported in the literature (13, 25). To date, more than 60 *VDR* polymorphisms have been discovered that are located in the promoter region, within and around exons 2-9, and in the 3' untranslated region (UTR) region (26, 27). Analysis of the importance of these *VDR* polymorphisms for various diseases has proven difficult. As a result, only few polymorphisms of this large gene have been studied. Most of them are RFLP with an unknown functional effect (26). In some cases, it has been indicated that they may be linked to truly functional polymorphisms elsewhere in the *VDR* gene (or in a nearby gene), which explains some of the associations observed (27). However, there is still controversy about the importance of *VDR* polymorphisms for individual malignancies. There are many genetic studies that have examined the *VDR* gene and PCa risk in which many reported statistically significant associations (5, 7), weaker associations (4, 28), or no associations (29-31) between common VDR variants and PCa risk. The *VDR* Bsm I polymorphism (rs5444410) does not affect the amino acid sequence of VDR, but many studies have suggested that it is closely related to PCa risk (32-35). Also, Taq I (rs731236) polymorphism has been associated with PCa risk (28-30, 32, 36-38). In the present study, we investigated the DHPLC elution patterns of PCR amplicons corresponding to VDR exons and their association with PCa risk. The elution patterns of *VDR* exons 1, 6, 7 and 9 were all highly similar

Table V. *The association of each VDR exon, as an independent variable without adjusting to any of the studied factors, with prostate cancer risk using standard logistic regression model.*

Outcome Cases/control	β-value	SD	*p*-Value	OR
Constant	–0.105	0.188	0.574	0.900
Exon 1	0.220	0.304	0.470	1.246
Constant	0.124	0.157	0.432	1.132
Exon 2	**–1.347**	0.533	**0.011**	0.260
Constant	0.847	0.230	0.000	2.333
Exon 4	**–1.705**	0.322	**0.000**	0.182
Constant	0.090	0.173	0.604	1.094
Exon 5	0.412	0.335	0.218	0.662
Constant	–0.057	0.150	0.707	0.945
Exon 6	0.973	0.850	0.252	2.645
Constant	–0.111	0.193	0.564	0.895
Exon 7	0.217	0.300	0.470	1.242
Constant	0.847	0.230	0.000	2.333
Exon 8	**–1.705**	0.322	**0.000**	0.182
Constant	–0.103	0.203	0.612	0.902
Exon 9	0.172	0.296	0.560	1.188

SD, Standard deviation; OR, odd ratio. Bold text indicates a *p*-value less than 0.05.

Table VI. *Logistic regression model to determine association of the exons and risk factors (age, PSA and family history) with prostate cancer risk (outcome).*

	Model 1	Model 2	Model 3	Model 4
Exon 4				
β-Value	–2.206	–2.491	–2.814	–2.760
p-Value	0.000	0.000	0.000	0.000
Odds ratio	0.110	0.083	0.060	0.063
Exon 7				
β-Value	1.150	1.510	1.804	1.811
p-Value	0.000	0.000	0.000	0.000
Odds ratio	3.159	4.529	6.074	6.117
Age				
β-Value		0.123	0.116	0.117
p-Value		0.000	0.000	0.000
Odds ratio		1.131	1.122	1.124
PSA				
β-Value			0.235	0.229
p-Value			0.000	0.000
Odds ratio			1.265	1.257
Fam History				
β-Value				0.413
p-Value				0.000
Odds ratio				1.511
Constant				
β-Value	0.619	–7.252	–7.308	–7.505
p-Value	0.010	0.000	0.000	0.000

Model 1: "prostate cancer x E4 x E7"; Model 2: "prostate cancer x E4 x E7 x age"; Model 3: "prostate cancer x E4 x E7 x age x PSA"; Model 4: "prostate cancer x E4 x E7 x age x PSA x family history. Exon, E; PSA, prostate-specific antigen.

with higher prevalence of double peaks of heteroduplex DNA among the PCa cases than controls. While, exons 2, 4, 5 and 8 showed similar patterns with higher prevalence of double peaks among the controls than the cases. Our further analysis suggested that exon 7 is associated with PCa risk; whereas, exons 4, and 8 are protective. These positive and negative association results are based on the DHPLC patterns and other PCa risk factors such as age, PSA level, and family history of PCa. Previously, we have reported an association of rs731236 (exon 9) and rs7975232 with PCa risk (15). In the analysis of clinical phenotypes, rs731236, rs1544410, and rs3782905 are strongly associated with high PSA level, whereas rs1544410 and rs2239185 show a statistically significant association with high Gleason score (15). A previous publication has reported that the Taq I (rs731236) polymorphism affects VDR transcriptional activity and mRNA stability, thus altering the abundance of VDR protein, and in turn affecting vitamin D levels (39).

Liu S. and colleagues (40) have conducted a meta-analysis to retrieve genetic association analyses of rs731236 and rs1544410 polymorphisms with PCa from studies published between 2006 and 2016. Their findings suggest a significant association between rs731236 and PCa risk in Asians and African Americans, but rs1544410 was not associated with PCa under three genetic models.

In our previous study, we have found an association between Taq I, Apa I and rs2239185 with PCa risk (15), while Oakley-Girvan *et al*. (41) did not find an association among Bsm I, Apa I, or Taq I and poly-A microsatellite with PCa risk in the family or case-control data.

Our previous haplotype analysis for SNPs in the VDR gene revealed that rs2239185 is in linkage disequilibrium with rs2289179 and Apa I, while Taq I and Apa I are in linkage disequilibrium and are located 80 bps apart. Furthermore, rs52853563 is in linkage disequilibrium with Bsm I and with rs4516035, rs10783218, and rs3782905. It has been reported that the Apa I, Taq I, Bsm I SNPs are all located in one block, which is thought to contain other variants at 3'-untranslated region of the VDR gene that may affect VDR expression by altering the mRNA stability and thus have an effect on vitamin D metabolism and activity (42, 43). However, the Taq I polymorphism is not functional but is in linkage disequilibrium with a poly-A microsatellite repeat in the 3' UTR that is thought to be important for the post-transcriptional regulation of gene expression. The SNP rs3782905 is in the DNA binding domain responsible for interaction with vitamin D response elements (VDREs) in target genes (44).

In general, SNPs tend to be inherited in groups and these groups of SNPs and their various possible combinations might have a significant association with the disease phenotype. Haplotype analysis is likely to continue to play a key role in genetic epidemiology studies because it effectively captures both the joint marker correlations and the evolutionary history (45). Therefore, the DHPLC elution patterns of each exon could predict the risk of PCa.

Conclusion

To our knowledge, this is the first study to evaluate the relation between the DHPLC elution patterns of *VDR* PCR amplicons and the risk of PCa in African American men. Further investigation of this association, in larger studies could reveal the usefulness of DHPLC analysis in fast screening and treatment, aiming to reduce the disease impact on health disparity, mainly in African American men.

Disclosure

The Authors have no personal or financial conflicts of interest and have not entered into any agreement that could interfere with our access to the data on the research, or upon our ability to analyze the data independently, to prepare manuscripts, and to publish them.

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