

## Admixture Mapping Links RACGAP1 Regulation to Prostate Cancer in African Americans

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**Abstract.** *Background/Aim: Prostate cancer is the most common malignancy in US males. African American men have higher incidence and mortality rates than European Americans. Five single nucleotide polymorphisms are associated with PCa. We hypothesized haplotypes inferred from these SNPs are also associated with PCa. Patients and Methods: We genotyped SNPs in a case-control admixture mapping study. SNP haplotypes inferred for 157 PCa cases and 150 controls were used in the regression analysis. Results: We found an association between “GTCCC”, “ATTCT”, and “ACCCC” haplotypes and PCa after ancestry adjustment (OR=3.62, 95%CI=1.42-9.21, p=0.0070; OR=7.89, 95%CI=2.36-26.31, p=0.0008; OR=4.34, 95%CI=1.75-10.78, p=0.0016). The rs615382 variant disrupts the recombination signal binding protein with immunoglobulin kappa J binding site in Rac GTPase activating protein 1 (RACGAP1). Conclusion: Disruption of notch 1 mediated-repression of RACGAP1 may contribute to PCa in African Americans.*

Prostate cancer (PCa) is the third most prevalent cancer in the world after breast and colorectal cancers, and the most common cancer affecting men in the US (1-9). PCa incidence and mortality rates are disproportionately higher among African American (AA) men compared to their European American counterparts (2-9). This disparity is greatest in the nation’s capital, Washington D.C., which has

the highest incidence and mortality rates in the country (10). It is unclear what factors account for the observed disparities. Risk factors for PCa include age, race/ethnicity, family history and environmental factors; however, a strong genetic association has yet to be identified (11-13). The observed disparities warrant the use of an approach that leverages allele frequency differences to identify loci contributing to disease pathology.

The approaches and methods used to identify genes that are causal or predispose an individual to PCa have evolved over time. Early efforts studied the disease in familial clusters and twin cohorts (13-24). Study designs then shifted to case-control and candidate gene studies. This shift was facilitated by the completion of the working draft of the genome map, a deliverable of the Human Genome Project. Advances in genotyping technology and the availability of the genome map facilitated the use of single nucleotide polymorphisms (SNPs) in association studies. The International HapMap project leveraged the information provided in the patterns of linkage disequilibrium (LD) to enhance SNP association studies. The differences observed in SNP allele frequencies between populations were later used as a tool in admixture mapping (24-29). Genome-wide association studies (GWAS) have become the standard for SNP association (30-34). Additionally, PCa GWAS results have been pooled in meta-analyses, reanalyzed using new statistical tools and re-sequenced in attempts to replicate findings (35-39).

To date over 70 variant loci have been implicated in PCa etiology *via* admixture mapping and GWAS (24-39). However, the identified loci do not appear to account for a majority of genetic susceptibility to PCa. In addition, these findings have not been reported with a high degree of consistency. A screening of 21 AIMs (Ancestry Informative Markers) on chromosome 12 identified a cancer susceptibility locus for PCa in African American men (29). A larger follow-

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up study was conducted to explore the findings of the screening and the results were published by Ricks-Santi *et al.* (40) The data from the follow-up study were re-examined to investigate the biological nature of the statistical association. Local and regional patterns of LD, evidence of selective pressure, and disruption of regulatory sequence motifs were among the local sequence features interrogated in this study.

## Patients and Methods

**Study population.** We selected 157 AA men who were older than 40 years of age from the Washington, DC area with histologically diagnosed adenocarcinoma of the prostate, prostate specific antigen (PSA) of >4.0 ng/ml, and a positive digital rectal examination (DRE). We then identified 150 ethnicities matched (AA) controls who were regularly screened with PSA levels <4.0 ng/ml, normal DRE and with no history of PCa among first-degree relatives. PSA values and blood samples were obtained at the time of diagnosis for case subjects and at the time of study enrollment for control subjects. Participants were recruited for a Howard University Institutional Review Board (IRB) approved study through the Division of Urology at the Howard University Hospital (HUH) and from the free prostate cancer screening program at the Howard University Cancer Center (HUCC) from 2000 to 2004.

**AIMs selection and genotyping.** We selected a total of 21 AIMs on 12p12–q14 to cover an average of 25 Mb upstream and/or downstream of VDR (vitamin D receptor). Allele frequency differences between putative parental populations for African Americans (West African and European) parental populations for all AIMs was >0.30 (based on HapMap data). Genomic DNA extraction was performed on whole blood using the Genra Puregene Blood Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. DNA from 157 PCa and 150 control subjects was genotyped to study the association of 21 AIMs with PCa risk. Polymerase chain reaction (PCR) and pyrosequencing primers were designed using PyroMark Assay Design software ver. 20.0 (Qiagen, Hilden, Germany). DNA samples were polymerase chain reaction (PCR)-amplified using whole-genome amplified DNA, forward and reverse primers, MgCl<sub>2</sub>, deoxy-nucleotide triphosphates and platinum Taq DNA polymerase (Invitrogen, Grand Island, NY, USA). Results were analyzed with the PyroMark Q24 software (Qiagen, Hilden, Germany). Duplicate test samples and negative controls were included in each 96-well plate.

**Association analysis.** The association of disease status with AIM genotype and haplotype was analyzed by binary logistic regression using the R statistical software (version 2.9.0) (Vienna, Austria) and SAS/STAT version 9.1 (SAS Institute, Cary, NC, USA) software packages. Fisher’s exact test and  $\chi^2$  analysis were used to test the alleles for Hardy-Weinberg equilibrium in controls when appropriate. For this study, the major allele found in people of African descent was considered the reference allele. For each SNP genotype the dominant, co-dominant, recessive and log-additive genetic models were conducted. Confidence intervals (CIs) of 95% were set on the odds ratios (ORs) calculated after adjusting for age and ancestry. Two sided *p*-values of  $\leq 0.05$  were considered to be statistically significant. The adjustment for multiple comparisons was made using the Bonferroni test.

Table I. Study participant descriptive statistics.

Characteristics	Cases	Controls	<i>p</i> -Value
Number of participants	157	150	-
Mean Age ( $\pm$ SD)	65.6 ( $\pm$ 9.1)	56.0 ( $\pm$ 14.6)	<0.001
Mean PSA level ( $\pm$ SD)	51.8 ( $\pm$ 180.9)	3.5 ( $\pm$ 13.8)	<0.001
Mean % West African ancestry	73.0	67.0	0.047

**Genetic ancestry estimation.** West African ancestry was estimated in cases and controls using AIMs to control for population stratification. Individual ancestry was determined for each participant using the genotype data from the original 21 AIMs listed in Table I. Global individual ancestry (% West African and % European) was calculated from the genotype data using the Bayesian Markov Chain Monte Carlo method implemented in the program STRUCTURE 2.1.24 These ancestry estimates were used as covariates in the regression models.

**Additional analyses.** Haplotypes that were inferred using *fastPHASE* version 2.1 from the AIMs which showed an association with PCa after application of dominant, co-dominant, recessive, and log-additive genetic models (41). For AIMs located in putative promoter or other non-coding regions, Alibaba 2.1 (BIOBASE, Beverly, MA, USA) and HaploReg (HaploReg v3, Broad Institute of MIT, Boston MA) programs were used to identify whether variants disrupted or created regulatory factor binding sites. SNP@Evolution and dbCLINE were used to test for the evidence of selection and association with environmental factors respectively (42-44).

## Results

The demographic, clinical, and ancestry data of the human subjects are shown in Table I. The student’s *t*-test reveals significant differences in age between the PCa and control subjects (*p*<0.001). The mean age of patients with PCa (65.6 $\pm$ 9.1) was significantly older than the controls (56.0 $\pm$ 14.6). The mean PSA levels were significantly higher in PCa patients (51.8 $\pm$ 180.9) than the controls (3.5 $\pm$ 13.8) (*p*<0.001). The percentage of West African Ancestry was significantly different between PCa (73.0) cases and controls (67.0) (*p*<0.047).

**Association between AIMs and PCa risk.** The studied AIMs were in Hardy-Weinberg equilibrium (*p*<0.05). The allele frequencies in PCa patients were similar to the controls (Table II). Five (rs1993973, rs1561131, rs1843321, rs1963562, and rs615382) of the twenty-one AIMs studied were found to be associated with PCa using the four genetic models (*p*<0.05) (Table III). AIM rs1993973 was found to be associated with PCa risk under the five genetic models; AIM rs1561131 under the co-dominant and recessive models; AIM rs1843321 only under the log-additive model; AIM rs1963562 under the dominant model only; and AIM

Table II. Description of the studied 21 AIMs and their allele frequency in the PCa cases and controls.

AIMs	Chromosomal position (bp)	Gene	Location	Major/Minor alleles	Delta	Cases	Controls
rs989278	23773750	<i>SOX5</i>	intron 7	C/G	0.08	0.34	0.33
*rs1993973	26201374	-	-	G/A	0.63	0.45	0.38
rs1027089	26362337	-	-	C/G	0.33	0.16	0.14
rs895964	26749333	<i>ITPR2</i>	intron 10	G/A	0.36	0.21	0.26
rs1010096	27735497	<i>PPFIBP1</i>	intron 28	C/T	0.32	0.44	0.43
*rs1561131	29199198	-	-	C/T	0.39	0.52	0.48
*rs1843321	30245161	-	-	C/T	0.57	0.37	0.41
*rs1963562	36949163	-	-	C/T	0.39	0.11	0.10
rs870431	37239340	-	-	A/G	0.55	0.15	0.14
rs278906	39704904	<i>CNTN1</i>	intron 21	C/T	0.24	0.17	0.23
rs180438	45473527	<i>SLC38A4</i>	intron 2	G/A	0.67	0.23	0.24
rs739857	46254553	-	-	A/C	0.40	0.14	0.11
rs1235153	46396592	<i>P11</i>	intron 5	C/T	0.05	0.01	0.00
*rs615382	48699178	<i>RACGAP1</i>	intron 2	C/A	0.73	0.32	0.28
rs303810	50449515	<i>SCN8A</i>	intron 18	T/C	0.49	0.48	0.47
rs585224	51209139	-	-	A/G	0.51	0.28	0.23
rs1683151	52232486	<i>ATF7</i>	intron 5	C/G	0.51	0.11	0.09
rs1867299	52616242	-	-	T/C	0.50	0.30	0.33
rs1078109	56272633	<i>PIP4K2C</i>	intron 2	G/A	0.07	0.01	0.01
rs1157239	63814994	-	-	G/A	0.52	0.32	0.27
rs343087	64547191	<i>HMGA2</i>	intron 4	A/G	0.78	0.19	0.16

\* AIMs associated with PCa risk.

Table III. AIM association with PCa risk.

SNP ID	Alleles	Gene	Wild-type allele	Risk allele	Co-Dominant	Dominant	Recessive	Log-additive
rs989278	C:G	<i>SOX5</i>	C	G	0.66	0.36	0.63	0.4
*rs1993973	G:A	<i>IVS</i>	G	A	0.0018	0.0076	0.0022	0.0004
rs1027089	C:G	<i>IVS</i>	C	G	0.68	0.88	0.38	0.69
rs895964	G:A	<i>ITPR2</i>	A	G	0.32	0.92	0.15	0.65
rs1010096	C:T	<i>PPFIBP1</i>	C	T	0.36	0.21	0.26	0.15
*rs1561131	C:T	<i>IVS</i>	C	T	0.0052	0.14	0.022	0.62
*rs1843321	C:T	<i>IVS</i>	C	T	0.074	0.078	0.059	0.025
*rs1963562	C:T	<i>IVS</i>	C	T	0.052	0.031	0.53	0.07
rs870431	A:G	<i>IVS</i>	A	G	0.74	0.88	0.44	0.77
†rs278906	C:T	<i>CNTN1</i>	C	T	-	-	-	-
rs180438	G:A	<i>SLC38A4</i>	G	A	0.77	0.47	0.85	0.54
rs739857	A:C	<i>IVS</i>	A	C	0.54	0.35	0.75	0.48
†rs1235153	C:T	<i>ENDOU</i>	C	T	-	-	-	-
*rs615382	C:A	<i>RACGAP1</i>	C	A	0.04	0.23	0.012	0.04
rs303810	T:C	<i>SCN8A</i>	T	C	0.42	0.44	0.44	0.95
rs585224	A:G	<i>IVS</i>	A	G	0.052	0.016	0.6	0.033
rs1683151	G:C	<i>ATF7</i>	G	C	0.92	0.99	0.69	0.92
rs1867299	T:C	<i>IVS</i>	T	C	0.7	0.89	0.46	0.82
rs1078109	G:A	<i>PIP4K2C</i>	G	A	0.32	0.25	0.18	0.18
rs1157239	G:A	<i>IVS</i>	G	A	0.43	0.53	0.21	0.31
rs343087	A:G	<i>HMGA2</i>	A	G	0.92	0.69	0.98	0.72

\*AIMs associated with PCa; all *p*-Values shown corrected for multiple comparisons. †AIMs with no associations with PCa under any genetic model

rs615382 under the co-dominant, recessive and log-additive models. Only AIM rs615382 is located within a genic region and lies within intron two of the *RACGAP1* gene.

*Haplotype analyses.* The haplotypes were constructed using *fastPHASE* v2.1 and are shown in Table IV. Three haplotypes 5 “GTCCC”, 9 “ATTCT”, and 11 “ACCCC” showed an

Table IV. *fastPHASE* haplotype unadjusted association with PCa risk.

	Haplotype	Controls	%	Cases	%	OR (95% CI)	p-Value
1	GCCCT	57	19.0%	42	13.4%	-	-
2	GCCCC	44	14.7%	46	14.6%	1.4 (0.8-2.5)	0.25
3	ACCCT	29	9.7%	13	4.1%	0.6 (0.3-1.3)	0.26
4	GTCCT	25	8.3%	23	7.3%	1.2 (0.6-2.5)	0.60
5	GTCCC	12	4.0%	27	8.6%	3.1 (1.4-6.7)	0.01
6	ATTTC	14	4.7%	9	2.9%	0.9 (0.3-2.2)	0.82
7	ATTCC	24	8.0%	27	8.6%	1.5 (0.8-3.0)	0.23
8	ATCCT	17	5.7%	14	4.5%	1.1 (0.5-2.5)	0.84
9	ATTCT	6	2.0%	26	8.3%	5.9 (2.2-15.6)	0.0002
10	GTTCT	9	3.0%	9	2.9%	1.4 (0.5-3.4)	0.61
11	ACCCC	14	4.7%	25	8.0%	2.4 (1.1-5.2)	0.03
	Other*	49	16.3%	53	16.9%	1.5 (0.8-2.6)	0.20

\*Other refers to haplotypes inferred with a frequency of less than 1%.

Table V. *fastPHASE* haplotype association with PCa adjusted for age and ancestry.

Haplotype	Age adjusted		Age and Ancestry adjusted		
	OR (95% CI)	p-Value	OR(95% CI)	p-Value	
1	GCCCT	-	-	-	
2	GCCCC	1.98 (0.96-4.07)	0.0636	1.75 (0.84-3.64)	0.1361
3	ACCCT	0.81 (0.33-1.98)	0.634	0.8 (0.32-1.98)	0.6298
4	GTCCT	1.74 (0.74-4.07)	0.2016	1.75 (0.74-4.15)	0.2012
5	GTCCC	3.97 (1.57-10.05)	0.0037	3.62 (1.42-9.21)	0.0070*
6	ATTTC	0.69 (0.17-2.82)	0.6071	0.61 (0.15-2.50)	0.4907
7	ATTCC	2.23 (0.96-5.16)	0.0620	2.36 (1.0-5.54)	0.05
8	ATCCT	1.69 (0.61-4.67)	0.31	1.68 (0.61-4.66)	0.3176
9	ATTCT	8.30 (2.51-27.45)	0.0005	7.89 (2.37-26.31)	0.0008*
10	GTTCT	1.08 (0.24-4.81)	0.9230	0.92 (0.21-4.14)	0.9143
11	ACCCC	4.62 (1.03-4.33)	0.0009	4.34 (1.75-10.78)	0.0016*
	*Other	2.11 (1.05-0.09)	0.0408	1.9 (0.92-3.95)	0.0845

\*Other refers to haplotypes inferred with a frequency of less than 1%.

association of haplotypes with risk for PCa (OR=3.1; 95%CI=1.4-6.7;  $p=0.01$ , OR=5.9; 95%CI=2.2-15.6;  $p=0.0002$  and OR=2.4; 95%CI=1.1-5.2;  $p=0.03$ ; respectively). After adjusting for age and ancestry, these three haplotypes remained significantly associated with PCa risk (Table V).

The search for alteration of regulatory mechanisms using Alibaba and HaploReg yielded the modification of a number of regulatory binding sites. The most notable being the disruption of a binding site for the notch signaling pathway regulated transcriptional modulator RBP-Jkappa in intron 2 of the *RACGAP1* gene (Figure 1).

## Discussion

Five AIMs were associated with PCa after the application of different genetic models as well as haplotypes constructed

using them. The “ACCCC” haplotype showed a statistically significant association with PCa after adjusting for age and ancestry. Only one AIM in the haplotype is located within a genic region. SNP rs615382 is located in an intron of the proto-oncogene *RACGAP1*. *RACGAP1*, Rac GTPase activating protein (GAP) 1, is under regulation of the Notch signaling pathway. SNP rs615382 disrupts the binding site for recombination signal binding protein for immunoglobulin kappa J (RBP-Jkappa) (44). RBP-Jkappa acts as a transcriptional repressor or activator when bound or unbound to Notch proteins respectively. Loss of the RBP-Jkappa binding site eliminates the ability of RBP-Jkappa to repress *RACGAP1* transcription. GAPs bind activated forms of Rho GTPases and stimulate GTP hydrolysis. GAPs negatively regulate Rho-mediated signals and are involved in downstream signaling of Ras-like GTPases.

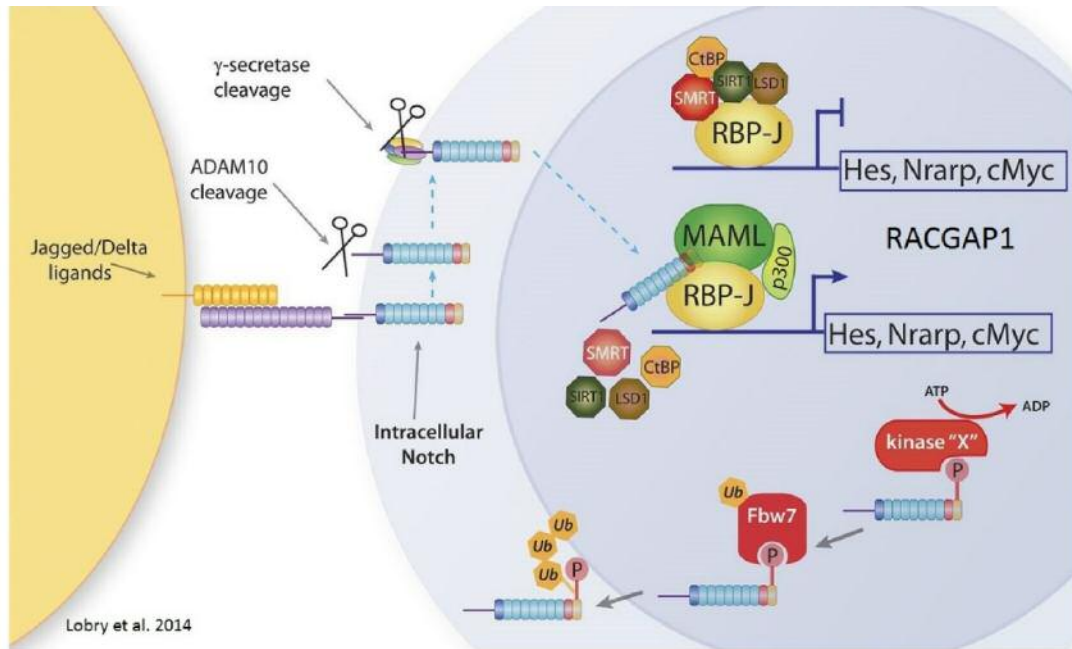


Figure 1. Adopted from Lobry *et al.* 2014. Mammalian Notch canonical activation pathway. Cognate interaction between Notch receptors and Notch ligands (Jagged/Delta) triggers 2 consecutive proteolytic cleavages by the ADAM10 metalloprotease and the  $\gamma$ -secretase complex. This generates ICN, the signaling portion of the receptor, which enters the nucleus and displaces corepressors (SMRT and CtBP1) and recruits the coactivator MAML1 and the acetyltransferase p300. ICN activating complex is short-lived, and ICN gets phosphorylated on its PEST domain and subsequently ubiquitinated by Fbw7 and targeted for degradation by the proteasome. ADP: Adenosine diphosphate; ATP: adenosine triphosphate.

RACGAP1 has been implicated in multiple malignancies including breast, gastric, hepatocellular, and non small-cell lung carcinoma (45-47). Up-regulation of RACGAP1 has been associated with early recurrence of hepatocellular carcinoma and is overexpressed in non small-cell lung and luminal breast cancer. RACGAP1 expression was also correlated with tumor progression and poor prognosis in gastric cancer. To date, there have been no reports of RACGAP association with PCa.

We understand that cancer results from an accumulation of genetic mutations. We also understand that although many cancers share common mutations, they are discrete entities that are capable of evolving over time. Loss of RACGAP1 regulation may be one of the mutations acquired in the development of PCa. A larger study of African American tumor samples would reveal whether RACGAP1 overexpression is a common feature of PCa.

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