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14. ABSTRACT Prostate cancer is the most common noncutaneous cancer in males in the U.S. While the major indolent form may not even require treatment, about 10-15% of PCa cases of aggressive form requiring intensive treatment. However, our inability to reliably distinguish between aggressive and indolent PCa early on in the course of the disease has resulted in the over-treatment of many and under treatment of some. We hypothesize that inherited sequence variants in the genome are associated with PCa aggressiveness. Currently, we conducted single variant analysis and gene-based analysis to identify rare variants that have strong effects on aggressive PCa risk in exome-array data among a total of 1,902 PCa cases of European descent, including 464 aggressive PCa cases and 1,438 indolent PCa cases. We successfully identified 11 novel variants associated with PCa aggressiveness in Caucasians and further confirmation in additional Caucasians and African American men are to be conducted. Our study will provide more insight into the etiology of aggressive PCa and provide potential effective targets for therapy of aggressive PCa.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Project Summary.....	4-18
4. Accomplishments.....	18
5. Conclusion.....	18
6. Reportable Outcomes.....	19
7. References.....	19-22
8. Training Activities.....	22
8. Appendices.....	n/a

INTRODUCTION

While most prostate cancer (PCa) patients have an indolent form of the disease that may not even require treatment, about 10-15% of PCa patients have an aggressive form that may progress to metastases and death, thus requiring intensive treatment. Several clinical variables such as PSA levels, Gleason grade, and TNM stage are good predictors for disease with poor clinical outcomes; however, their predictive performance needs to be improved. Our inability to reliably distinguish between these two forms of PCa, early on in the course of the disease has resulted in the over-treatment of many and under treatment of some. Another dilemma is a large difference in PCa risk, especially aggressive PCa, between races. African Americans (AAs) have the world's highest incidence of PCa and are twice as likely, as compared with Caucasians to die of the disease. Inherited markers of aggressive PCa could be used for screening and diagnosis of aggressive PCa at an early stage while reducing over-diagnosis and treatment for others. The overall hypothesis is that inherited sequence variants in the genome are associated with a lethal (aggressive) form of PCa but not indolent PCa, and the difference in these variants between races may contribute to higher incidence of and mortality from aggressive PCa in AA.

In this DOD proposal, we proposed: **1)** To identify novel inherited rare variants (MAF<5%) in the exome that are associated with aggressive but not indolent PCa in a case-control population of 1,000 aggressive and 1,000 indolent PCa patients of European descent from John Hopkins Hospital (JHH); **2)** To replicate the rare variants identified in Aim1 in additional 1,000 aggressive and 1,000 indolent PCa patients of European descent from JHH; and **3)** To evaluate the effect of rare mutations confirmed in Aim2 in an African American (AA) population with 500 aggressive and 500 indolent PCa patients from JHH.

KEYWORDS

prostate cancer, aggressiveness, association, rare variants, INPP5D, HINFP, exome

OVERALL PROJECT SUMMARY

Approved Statement of Work:

Aim 1. To identify novel inherited rare variants (MAF<5%) in the exome that are associated with aggressive but not indolent PCa in a case-control population of 1,000 aggressive and 1,000 indolent PCa patients of European descent from John Hopkins Hospital (JHH).

Tasks

Month 1-4: Preparation of the study, including regulatory review, IRB approval and other logistical issues.

Month 4-8: 1) Genotype 1,000 aggressive and 1,000 indolent PCa patients of European descent from JHH using Illumina Human Exome BeadChip platform. 2) Perform bioinformatics analysis for all nonsynonymous variants on Illumina Human Exome BeadChip by PolyPhen2.

Month 8-12: Perform single variant analysis using logistic regression and gene-based analysis by SKAT. Select rare variants with a p-value of $1E-5$ in single variant analyses and variants in genes with reach a p-value of $1E-4$ in gene-based analyses by SKAT.

Outcome and deliverables

We expect to identify 2-10 rare variants with a P-value of $1E-5$ based on single variant analysis, and also 2-10 genes with a P-value of $1E-4$ based on gene-based analysis by SKAT.

Detailed report

Study design modification. In our initial proposal, we proposed to genotype 1,000 aggressive and 1,000 indolent PCa patients of European descent from JHH using Illumina Human Exome BeadChip platform. During month 4-8, we genotyped 791 subjects from JHH study, including 142 subjects with aggressive PCa and 635 subjects with non-aggressive PCa using Illumina Human Exome BeadChip platform. In addition, we were able to obtain access to the Exome BeadChip array data for two additional Caucasian populations (Michigan and CAPS) with a total of 328 aggressive PCa cases and 814 indolent PCa cases. Therefore, we also conducted a genome-wide association analysis for rare variants with PCa aggressiveness in those two populations. Compared with our original study design, the new design greatly improved our statistical power since we were able to evaluate the replication results for all the rare variants. We were also able to decrease the number of false positive results by including two more populations to compare the association results.

Study Subjects. Subjects included in the John Hopkins (JHH) study were recruited during Jan. 1999 to Dec. 2008. All of them underwent radical prostatectomy for treatment of prostate cancer. Details of this study have been described in previous publications. In this study, aggressive prostate cancer was defined as: 1) Gleason Score ≥ 8 ; or 2) Gleason Score = 7, with the most prevalent pattern being 4; or 3) stage T3b or higher; or 4) involvement of regional lymph nodes; or 5) presence of distant metastasis. Otherwise, the cancers were classified as non-aggressive prostate cancer. In this study, we genotyped 791 subjects from the JHH study, including 142 subjects with aggressive PCa and 635 subjects with non-aggressive PCa using Illumina Human Exome BeadChip platform.

The second population included subjects recruited in Sweden from the CAPS study, which were diagnosed from Jul. 2001 and Oct. 2003. Details of this study have been described in previous publications. In the CAPS study, aggressive prostate cancers were defined as: 1) Gleason Score

≥ 8 ; or 2) stage T3 or higher; or 3) involvement of regional lymph nodes; or 4) presence of distant metastasis; or 5) serum PSA >50 ng/ml. Otherwise, the cancers were classified as non-aggressive prostate cancer. In this study, 446 subjects from the CAPS study were genotyped by ExomeArray. Among them, 149 subjects were aggressive prostate cancer patients while 297 patients had a non-aggressive form of the disease.

The third study population included subjects recruited by the University of Michigan. The definition of prostate cancer aggressiveness in the Michigan population was exactly the same as in the JHH study. In this study, 696 subjects from the Michigan study were genotyped using the Human Exome BeadChip platform. Among them, 179 subjects were aggressive prostate cancer patients while 517 subjects had a non-aggressive form of the disease.

Genotyping and Quality Control. Genotyping of samples in the first stage was conducted using the Illumina Human Exome BeadChip at the Center for Cancer Genomics, Wake Forest University School of Medicine. A total of 247,870 genetic variants were included in the ExomeArray. Those polymorphic SNPs were used for sex and an IBS check was performed for all subjects using PLINK software (Purcell 2007). In addition, polymorphic SNPs were also used to estimate the missing rate per individual. In each stage, subjects with a genotyping missing rate $>5\%$ were removed from further analysis. For subjects in stage 1 with exome data available, an IBS check and sex check were also performed. SNPs with a missing rate $>2\%$ in subjects passed quality control (QC) were removed from further analysis.

Bioinformatics analysis (Variant effect prediction). All coding nonsynonymous variants were assessed for potential effect by Polymorphism Phenotyping version 2 (PolyPhen2), which is a tool for predicting the possible impact of an amino acid substitution on the structure and function of a human protein. For a given variant, PolyPhen2 calculates a Naïve Bayes posterior probability that the mutation is damaging and then appraised qualitatively as benign, possibly damaging, or probably damaging (Adzhubei 2010).

Statistical analysis for single SNP effect. Principal components analysis was conducted to detect potential population stratification by EIGENSTRAT software (Price 2006). The top 5 eigenvectors which indicates ancestral heterogeneity within a group of individuals were adjusted as covariates in multivariate logistic regression analysis.

All polymorphic genetic variants that passed QC were evaluated for associations with prostate cancer aggressiveness. For genetic variants with any of the genotype counts ≤ 5 , Fisher's exact test was applied to investigate potential association. For genetic variants with genotype counts >5 , multivariate logistic regression analysis was conducted assuming an additive genetic model, adjusting for age-at-diagnosis and the top 5 eigenvectors. All analyses were performed using the PLINK software package (Purcell 2007).

Gene-based analysis. We used a novel statistical approach called Sequence Kernel Association Test (SKAT), to conduct gene-based analysis of rare variants for aggressive PCa. SKAT is a supervised and flexible regression method to test for association between rare variants in a gene or genetic region and a continuous or dichotomous trait. Compared to other methods of estimating the joint effect of a subset of SNPs, SKAT is able to deal with variants that have different direction and magnitude of effects, and allows for covariate adjustment (Wu 2011). In addition, SKAT can also avoid arbitrary selection of threshold in burden test. Moreover, SKAT is computationally efficient, compared to a permutation test, making it feasible to analyze the large dataset in our study.

Results

Detailed clinical and demographic characteristics for the study populations were presented in Table 1.

Table 1. Clinical and Demographic Characteristics of Subjects in Stage 1.

Characteristics	JHH # (%)		MI # (%)		CAPS # (%)	
	Agg (N=142)	Non-Agg (N=635)	Agg (N=179)	Non-Agg (N=517)	Agg (N=149)	Non-Agg (N=297)
<i>Age at enrollment (Year)</i>						
Mean (sd)	51.5 (3.9)	49.29 (4.44)	NA	NA	NA	NA
<i>Age at diagnosis</i>						
≤ 55	NA	NA	178 (99.4)	517 (100)	48 (32.2)	93 (31.3)
> 55	NA	NA	1 (0.6)	0	101 (67.8)	204 (68.7)
Missing	NA	NA	0	0	0	0
<i>Family History (first-degree relatives)</i>						
No	125 (88.0)	551 (86.8)	NA	NA	105 (70.5)	184 (62.0)
Yes	15 (10.6)	66 (10.4)	NA	NA	41 (27.5)	109 (36.7)
Missing	2 (1.4)	18 (2.8)	NA	NA	3 (2.0)	4 (1.3)
<i>PSA levels at diagnosis for cases or at enrollment for controls (ng/ml)</i>						
≤ 4	21 (14.8)	224 (35.3)	12 (6.7)	164 (31.7)	7 (4.7)	60 (20.2)
4.01-9.99	78 (54.9)	357 (56.2)	89 (49.7)	281 (54.4)	25 (16.8)	157 (52.9)
10-19.99	23 (16.2)	45 (7.1)	30 (16.8)	39 (7.5)	22 (14.8)	55 (18.5)
20-49.99	18 (12.7)	4 (0.6)	20 (11.2)	4 (0.8)	25 (16.8)	23 (7.7)
50-99.99	0	0	19 (10.6)	1 (0.2)	25 (16.8)	0
≥100	0	0	0	0	43 (28.9)	0
Missing	2 (1.4)	5 (0.8)	9 (5.0)	28 (5.4)	2 (1.3)	2 (0.7)
<i>T-stage</i>						
T1	0	0	0	1 (0.2)	20 (13.4)	173 (58.2)
T2	47 (33.1)	512 (80.6)	71 (39.7)	467 (90.3)	26 (17.4)	122 (41.1)
T3a	53 (37.3)	123 (19.4)	33 (18.4)	49 (9.5)	0	0
T3b	41 (28.9)	0	33 (18.4)	0	0	0
T3c	0	0	0	0	0	0

T3x	1 (0.7)	0	0	0	83 (55.7)	0
T4	0	0	3	0	18 (12.1)	0
TX	0	0	0	0	0	0
Missing	0	0	39 (21.8)	0	2 (1.3)	2 (0.7)
<i>N-stage</i>						
N0	119 (83.8)	627 (98.7)	119 (66.5)	410 (79.3)	36 (24.2)	60 (20.2)
N1	16 (11.3)	0	26 (14.5)	0	22 (14.8)	0
NX	1 (0.7)	8 (1.3)	20 (11.2)	107 (20.7)	91 (61.1)	237 (79.8)
Missing	0	0	14 (7.8)	0	0	0
<i>M-stage</i>						
M0	0	0	81 (45.3)	257 (49.7)	76 (51.0)	110 (37.0)
M1	0	0	15 (8.4)	0	45 (30.2)	0
MX	142 (100)	635 (100)	72 (40.2)	260 (50.3)	28 (18.8)	187 (63.0)
Missing	0	0	11 (6.1)	0	0	0
<i>Gleason (biopsy)</i>						
≤ 4	0	0	0	6 (1.2)	0	21 (6.7)
5	0	8 (1.3)	0	21 (4.1)	9 (6.0)	49 (16.5)
6	1 (0.7)	420 (66.1)	6 (3.4)	272 (52.6)	25 (16.8)	163 (54.9)
7 (3+4)	16 (11.3)	207 (32.6)	16 (8.9)	218 (42.2)	0	60 (20.2)
7 (4+3)	75 (52.8)	0	84 (46.9)	0	48 (32.2)	0
7 (total)	91 (64.1)	207 (32.9)	100 (55.9)	218 (42.2)	48 (32.2)	60 (20.2)
8	31 (21.8)	0	31 (17.3)	0	22 (14.8)	0
9	19 (13.4)	0	35 (19.6)	0	31 (20.8)	0
10	0	0	3 (1.7)	0	3 (2.0)	0
Missing	0	0	0	0	11 (7.4)	4 (1.3)

A total of 247,870 genetic variants were included in this ExomeArray. Among them, 92,173, 88,087 and 71,435 genetic variants were polymorphic in JHH, Michigan and CAPS population, respectively. For polymorphic genetic variants, only those with a missing rate >0.98 in subjects passed QC were kept for further statistical analyses, including 91,998 variants in JHH, 87,879 variants in MI and 71,220 variants in CAPS. 79,729, 60,243, 57,126 genetic variants had an MAF < 0.1 in the JHH, Michigan and CAPS population, respectively.

Association Analysis for single variant

We did not observe any association between genetic variants and PCa aggressiveness achieved genome-wide significance ($p\text{-value} < 5E-3$) in JHH, Michigan or CAPS populations. In the JHH population, 47 variants were significantly associated with PCa aggressiveness with a $p\text{-value} < 1E-3$, including 13 rare variants with minor allele frequency (MAF) < 0.05, and 34 common ones (MAF ≥ 0.05) (Table 2). In the Michigan population, we found 27 variants significantly associated with PCa aggressiveness ($p\text{-value} < 1E-3$), including 11 rare ones and 16 common ones (Table 3). In the CAPS population, we identified 18 variants significantly associated with

PCa aggressiveness (p-value < 1E-3), including 7 rare ones and 11 common ones (Table 4). No variants were significantly associated with PCa aggressiveness with p-value < 1E-3 in all three populations.

Table 2. Associations between genetic variants and PCa aggressiveness in the JHH population with p-value < 1E-3.

SNP	CHR	BP	Gene	A1/A2	MAF Agg	MAF Non-Agg	Genotype Agg	Genotype Non-Agg	OR	P-value
rs16830693	1	43,805,240	MPL	G/A	0.060	0.021	1/15/120	0/26/599	3.14	7.62E-04
rs61818256	1	201,294,910	PKP1	A/G	0.059	0.018	0/16/120	0/22/602	3.48	3.67E-04
rs17851681	1	227,954,677	SNAP47	A/G	0.048	0.113	0/13/123	6/129/490	0.39	7.97E-04
rs17228441	2	186,627,943	FSIP2	A/G	0.603	0.447	48/68/20	119/319/185	1.88	1.10E-05
rs992822	2	186,654,592	FSIP2	A/G	0.603	0.448	48/68/20	119/322/184	1.88	1.15E-05
rs17229201	2	186,656,956	FSIP2	A/G	0.603	0.448	48/68/20	119/322/184	1.88	1.15E-05
rs60029104	2	186,658,056	FSIP2	G/A	0.613	0.455	50/66/20	126/317/182	1.85	1.53E-05
rs10490391	2	186,658,438	FSIP2	G/A	0.603	0.448	48/68/20	119/322/184	1.88	1.15E-05
rs10490392	2	186,658,565	FSIP2	C/A	0.603	0.448	48/68/20	119/322/184	1.88	1.15E-05
rs2161036	2	186,659,359	FSIP2	C/A	0.603	0.448	48/68/20	119/322/184	1.88	1.15E-05
rs10931200	2	186,664,963	FSIP2	C/A	0.603	0.448	48/68/20	119/322/184	1.88	1.15E-05
rs36004074	2	186,665,432	FSIP2	G/A	0.603	0.448	48/68/20	119/322/184	1.88	1.15E-05
rs11695215	2	186,665,824	FSIP2	A/G	0.603	0.448	48/68/20	119/322/184	1.88	1.15E-05
rs7605884	2	186,667,121	FSIP2	A/G	0.603	0.448	48/68/20	119/322/184	1.88	1.15E-05
rs16827154	2	186,670,780	FSIP2	T/A	0.603	0.453	48/68/20	120/325/179	1.87	1.66E-05
rs17826534	2	186,671,357	FSIP2	G/A	0.603	0.448	48/68/20	119/322/184	1.88	1.15E-05
rs1862066	2	186,671,912	FSIP2	G/A	0.599	0.447	47/69/20	118/323/184	1.87	1.57E-05
rs10804178	2	210,849,283	UNC80	G/A	0.401	0.512	23/63/50	153/334/138	0.59	2.52E-04
rs61729839	2	238,277,379	COL6A3	A/G	0.044	0.011	0/12/124	0/14/611	4.08	7.50E-04
bs2_242593011	2	242,593,011	ATG4B	A/G	0.033	0.006	0/9/127	0/7/618	6.08	5.82E-04
rs877859	3	107,714,075	CD47	G/A	0.412	0.297	23/66/47	52/267/306	1.66	4.55E-04
rs11921691	3	113,673,125	ZDHHC23	A/G	0.375	0.506	22/58/56	168/297/160	0.62	6.26E-04
rs6883840	5	40,286,410	PTGER4	A/G	0.129	0.240	2/31/103	31/238/356	0.47	3.48E-05
rs10057851	5	64,565,261	ADAMTS6	G/A	0.375	0.502	13/76/47	153/321/151	0.60	5.24E-04
rs78649652	5	96,124,373	ERAP1	A/G	0.040	0.006	0/11/125	0/8/617	6.54	9.59E-05
rs2122554	5	165,957,086	ODZ2	A/C	0.081	0.031	2/18/116	1/37/587	2.73	4.93E-04
rs11955074	5	178,294,060	ZNF354B	A/G	0.184	0.108	4/42/90	9/117/499	1.86	9.75E-04
rs4712653	6	22,125,964	LINC00340	G/A	0.559	0.452	40/72/24	122/321/182	1.64	5.73E-04
rs6939340	6	22,140,004	LINC00340	G/A	0.581	0.464	45/68/23	134/312/179	1.67	2.99E-04
rs3095250	6	31,208,340	HLA-C	G/A	0.353	0.437	21/54/61	114/318/193	0.53	6.99E-04
rs3130688	6	31,210,216	HLA-C	G/A	0.353	0.435	21/54/61	113/318/194	0.54	9.98E-04
rs10274334	7	47,925,331	PKD1L1	G/C	0.500	0.382	31/72/31	95/287/243	1.62	5.25E-04
rs2247572	8	73,633,028	KCNB2	A/G	0.206	0.12	5/46/85	6/138/481	1.90	3.89E-04
rs3133745	8	96,534,806	LOC100616530	A/G	0.206	0.125	5/46/85	11/134/480	1.82	9.45E-04
rs34075341	9	91,616,843	S1PR3	A/G	0.004	0.045	0/1/135	0/56/569	0.08	2.82E-04
rs2418135	9	113,901,309	OR2K2	A/G	0.382	0.520	19/66/51	172/306/147	0.55	2.20E-05
rs56224008	9	131,107,634	SLC27A4	A/G	0.066	0.023	0/18/118	1/27/597	2.98	7.09E-04
rs61734605	11	34,916,657	APIP	A/G	0.422	0.307	25/65/46	60/264/301	1.75	1.10E-04
rs938886	14	20,837,701	TEP1	G/C	0.140	0.234	2/34/100	33/226/366	0.53	4.71E-04
rs1713449	14	20,841,707	TEP1	A/G	0.136	0.228	2/33/101	31/223/371	0.53	5.65E-04
rs2069541	14	23,901,012	MYH7	G/A	0.033	0.006	0/9/127	0/7/618	6.08	5.82E-04
rs17101661	14	64,564,680	SYNE2	A/G	0.048	0.011	0/13/123	0/14/611	4.43	2.66E-04

rs12918952	16	78,420,775	WVOX	G/A	0.294	0.423	15/50/71	110/309/206	0.58	2.99E-04
rs79954845	17	36,483,889	GPR179	C/G	0.030	0.003	2/4/130	0/4/621	9.44	2.42E-04
rs16950981	18	6,992,683	LAMA1	A/T	0.037	0.007	0/10/126	0/9/616	5.26	5.76E-04
rs12961939	18	6,997,818	LAMA1	C/A	0.191	0.290	4/44/88	59/245/321	0.58	7.12E-04
bs19_9068458	19	9,068,458	MUC16	A/T	0.026	0.002	0/7/129	0/3/622	10.98	3.99E-04

Table 3. Associations between genetic variants and PCa aggressiveness in the MI population with p-value < 1E-3.

SNP	CHR	BP	Gene	A1/A2	MAF Agg	MAF Non-Agg	Genotype Agg	Genotype Non-Agg	OR	P-value
rs9701796	1	19,186,129	TAS1R2	C/G	0.279	0.190	16/68/95	16/164/337	1.67	4.31E-04
rs2272994	1	40,923,019	ZNF643	A/G	0.288	0.193	10/83/86	20/159/338	1.72	2.06E-04
rs28568406	1	158,687,163	OR6K3	A/G	0.464	0.365	38/90/51	62/253/202	1.53	9.20E-04
rs7530895	1	203,260,756	LOC730227	G/A	0.008	0.044	0/3/176	1/43/473	0.19	6.72E-04
rs669408	1	232,519,150	SIPA1L2	C/A	0.489	0.378	44/85/48	79/233/205	1.54	4.46E-04
rs2924461	5	8,012,069	MTRR	G/A	0.528	0.416	48/93/38	94/242/181	1.57	2.98E-04
rs10499052	6	109,885,475	AKD1	A/G	0.355	0.252	29/69/81	33/194/290	1.61	2.60E-04
rs41289902	6	112,460,365	LAMA4	A/G	0.028	0.004	0/10/169	0/4/513	7.40	4.09E-04
rs117497357	7	20,768,077	ABCB5	A/T	0.073	0.030	1/24/154	0/31/486	2.53	9.60E-04
rs741301	7	36,917,995	ELMO1	G/A	0.413	0.321	25/98/56	53/226/238	1.56	7.59E-04
rs28382644	7	44,118,394	POLM	G/C	0.034	0.004	0/12/167	0/4/513	8.93	4.64E-05
rs7787531	7	129,023,597	AHCYL2	G/A	0.123	0.065	3/38/138	3/61/453	2.02	9.25E-04
rs118014343	8	623,906	ERICH1	A/G	0.056	0.019	0/20/159	0/20/497	3.00	7.95E-04
rs7008113	8	111,438,655	KCNV1	A/G	0.271	0.185	10/77/92	17/157/343	1.68	4.96E-04
rs10090835	8	130,789,767	GSDMC	A/G	0.011	0.056	0/4/175	3/52/462	0.19	1.44E-04
rs4919060	10	98,699,136	LCOR	A/C	0.221	0.148	8/63/108	10/133/374	1.74	6.61E-04
rs61898615	11	103,019,260	DYN2H1	A/G	0.031	0.005	0/11/168	0/5/512	6.52	3.44E-04
rs10841496	12	20,521,654	PDE3A	A/C	0.578	0.462	62/83/34	113/252/152	1.56	3.42E-04
rs8176345	12	58,158,558	CYP27B1	A/G	0.061	0.023	1/20/158	0/24/493	2.76	9.73E-04
rs11116595	12	85,165,879	SLC6A15	A/G	0.377	0.485	31/73/75	119/263/135	0.64	4.55E-04
rs117476305	12	101,761,753	UTP20	G/A	0.031	0.006	0/11/168	0/6/511	5.43	7.52E-04
rs912969	13	103,867,104	SLC10A2	A/G	0.028	0.076	0/10/169	3/73/441	0.35	6.72E-04
rs4790404	17	2,886,642	RAP1GAP2	G/A	0.391	0.502	27/86/66	135/248/133	0.63	2.23E-04
rs1901187	17	38,646,147	TNS4	G/A	0.346	0.458	19/86/74	99/276/142	0.61	1.85E-04
rs7236632	18	55,434,202	ATP8B1	G/A	0.224	0.139	9/62/108	7/130/380	1.83	1.87E-04
rs34070230	19	4,844,790	PLIN3	C/G	0.061	0.023	1/20/158	1/22/494	2.76	9.73E-04
rs11881700	19	52,538,428	ZNF432	G/A	0.187	0.115	5/57/117	7/105/405	1.77	8.20E-04

Table 4. Associations between genetic variants and PCa aggressiveness in the CAPS population with p-value < 1E-3.

SNP	CHR	BP	Gene	A1/A2	MAF Agg	MAF Non-Agg	Genotype Agg	Genotype Non-Agg	OR	P-value
rs10797449	1	233,490,138	KIAA1804	A/G	0.513	0.365	44/65/40	30/156/110	1.88	3.78E-05
rs6757496	2	8,978,676	KIDINS220	A/G	0.299	0.412	10/69/70	41/162/93	0.56	4.45E-04
rs34276015	3	16,419,309	RFTN1	A/G	0.007	0.049	0/2/147	0/29/267	0.13	6.82E-04
rs13141997	4	99,478,756	TSPAN5	A/G	0.440	0.319	26/79/44	27/135/134	1.73	4.37E-04
rs1650697	5	79,950,781	MSH3	A/G	0.188	0.291	2/52/95	23/126/147	0.57	8.49E-04
rs17601580	6	132,061,420	ENPP3	A/G	0.121	0.215	2/32/115	13/101/182	0.50	6.45E-04
rs723874	8	20,107,601	LZTS1	C/G	0.010	0.059	0/3/146	1/33/261	0.16	3.11E-04
rs6475797	9	24,545,513	ELAVL2	G/A	0.383	0.530	22/70/57	80/154/62	0.49	5.53E-06
rs2071348	11	5,264,146	HBBP1	C/A	0.242	0.372	8/56/85	36/148/112	0.50	5.53E-05
rs10895391	11	103,158,278	DYNC2H1	A/G	0.453	0.334	25/85/39	31/135/129	1.71	5.32E-04
rs111874833	12	70,953,189	PTPRB	A/G	0.034	0.003	0/10/139	0/2/294	10.24	5.22E-04
rs117710037	13	115,091,073	CHAMP1	A/C	0.007	0.051	0/2/147	1/28/267	0.13	4.08E-04
rs35572669	14	77,141,290	VASH1	C/A	0.493	0.367	40/67/42	36/145/115	1.73	2.34E-04
rs117555414	16	57,113,168	NLRC5	A/G	0.030	0.002	1/7/141	0/1/295	18.40	3.44E-04
bs16_75269325	16	75,269,325	BCAR1	A/C	0.050	0.012	1/13/135	0/7/289	4.43	9.15E-04
bs16_75301838	16	75,301,838	BCAR1	C/G	0.050	0.012	1/13/135	0/7/289	4.43	9.15E-04
rs1559806	18	72,108,787	FAM69C	A/G	0.500	0.370	35/79/35	33/152/110	1.76	3.52E-04
rs266849	19	51,349,090	KLK3	G/A	0.185	0.100	4/47/98	2/55/239	2.05	6.14E-04

We therefore conducted association analysis in a pooled sample of all three populations. A total of 39 genetic variants achieved a P-value < 1E-3 in the pooled analysis. Among them, 31 variants had effects in the same direction in all three populations. We further examined the 31 variants and found that 11 variants showed significant association ($P < 0.05$) in at least two populations. The association results of the 11 variants in each population and the pooled analysis were presented in Table 2.

Among those 11 variants, a rare but recurrent missense genetic variant bs2_233990575 in INPP5D region had consistent effect on PCa aggressiveness among all three populations at a liberal P-value of 0.05 (Table 4). It is located at 233,990,575 bp of chromosome 2, the bs2_233990575 rare allele 'A' appeared only in aggressive PCa subjects, with a minor allele frequency of 0.008, 0.008 and 0.010 in JHH, MI and CAPS population, respectively. On contrast, this rare allele was not observed among the 625, 517 and 296 non-aggressive PCa subjects in JHH, MI and CAPS population respectively (Table 4).

We therefore examined the other genetic variants located in the INPP5D gene region. We found that another rare missense variant rs115393439, which was 17 bp upstream of bs2_233990575, was significantly associated with PCa aggressiveness in the JHH and CAPS populations, with P of 0.012 and 0.050, respectively (Table 4). In addition, rs115393439 was associated with PCa aggressiveness with a marginal $P = 0.055$ in the MI population (Table 4). Similar with bs2_233990575, the minor allele frequency of rs115393439 was higher in aggressive PCa

subjects than in non-aggressive PCa cases, resulting in an odds ratio (OR) of 5.83, 8.73 and 3.54 in JHH, MI and CAPS, respectively (Table 4).

Table 5. Associations between 11 selected variants and prostate cancer aggressiveness in stage 1.

Population	SNP	CHR	BP	Gene	A1/A2	MAF Agg	MAF Non- Agg	Genotype Agg	Genotype Non-Agg	OR	P-value
JHH	bs1_156907115	1	156,907,115	ARHGEF11	G/A	0.408	0.346	17/77/42	80/272/273	1.38	2.30E-02
	bs2_233990575	2	233,990,575	INPP5D	A/G	0.007	0.000	0/2/134	0/0/625		3.18E-02
	rs115393439	2	233,990,592	INPP5D	C/A	0.018	0.003	0/5/131	0/4/621	5.83	1.19E-02
	rs464494	5	76,003,258	IQGAP2	A/G	0.375	0.457	22/58/56	120/331/174	0.70	1.42E-02
	rs61740965	5	81,608,563	ATP6AP1L	G/A	0.022	0.004	0/6/130	0/5/620	5.62	6.40E-03
	rs10274334	7	47,925,331	PKD1L1	G/C	0.500	0.382	31/72/31	95/287/243	1.62	5.25E-04
	rs7385804	7	100,235,970	TFR2	C/A	0.456	0.378	27/70/39	91/290/244	1.42	1.35E-02
	rs2418135	9	113,901,309	OR2K2	G/A	0.618	0.480	19/66/51	172/306/147	1.82	2.20E-05
	rs61753080	11	119,005,003	HINFP	A/G	0.007	0.004	0/2/133	0/5/616	1.85	3.64E-01
	rs11116595	12	85,165,879	SLC6A15	A/G	0.382	0.436	22/60/54	114/317/194	0.79	9.77E-02
	rs17474506	17	38,990,780	TMEM99	G/C	0.085	0.050	3/17/116	3/57/565	1.74	4.10E-02
MI	bs1_156907115	1	156,907,115	ARHGEF11	G/A	0.422	0.336	29/93/57	56/235/225	1.46	3.35E-03
	bs2_233990575	2	233,990,575	INPP5D	A/G	0.008	0.000	0/3/176	0/0/517		1.69E-02
	rs115393439	2	233,990,592	INPP5D	C/A	0.008	0.001	0/3/176	0/1/516	8.73	5.47E-02
	rs464494	5	76,003,258	IQGAP2	A/G	0.358	0.460	20/88/71	113/250/154	0.66	1.16E-03
	rs61740965	5	81,608,563	ATP6AP1L	G/A	0.017	0.004	0/6/173	0/4/513	4.39	2.23E-02
	rs10274334	7	47,925,331	PKD1L1	G/C	0.441	0.431	38/82/59	89/268/160	1.02	8.65E-01
	rs7385804	7	100,235,970	TFR2	C/A	0.455	0.374	42/79/58	85/217/215	1.34	1.40E-02
	rs2418135	9	113,901,309	OR2K2	G/A	0.500	0.480	45/89/45	116/263/137	1.09	4.71E-01
	rs61753080	11	119,005,003	HINFP	A/G	0.020	0.005	0/7/170	0/5/510	4.14	1.58E-02
	rs11116595	12	85,165,879	SLC6A15	A/G	0.377	0.485	31/73/75	119/263/135	0.64	4.55E-04
	rs17474506	17	38,990,780	TMEM99	G/C	0.089	0.052	1/30/148	1/52/463	1.78	1.53E-02
CAPS	bs1_156907115	1	156,907,115	ARHGEF11	G/A	0.403	0.368	23/74/52	39/140/117	1.17	2.96E-01
	bs2_233990575	2	233,990,575	INPP5D	A/G	0.010	0.000	0/3/146	0/0/296		3.73E-02
	rs115393439	2	233,990,592	INPP5D	C/A	0.023	0.007	0/7/142	0/4/292	3.54	4.96E-02
	rs464494	5	76,003,258	IQGAP2	A/G	0.366	0.411	19/71/59	47/149/100	0.84	2.50E-01
	rs61740965	5	81,608,563	ATP6AP1L	G/A	0.003	0.002	0/1/148	0/1/295	1.99	1
	rs10274334	7	47,925,331	PKD1L1	G/C	0.534	0.444	45/69/35	60/143/93	1.46	8.54E-03
	rs7385804	7	100,235,970	TFR2	C/A	0.453	0.439	26/83/40	50/160/86	1.13	4.32E-01
	rs2418135	9	113,901,309	OR2K2	G/A	0.540	0.453	43/75/31	65/138/93	1.39	2.05E-02
	rs61753080	11	119,005,003	HINFP	A/G	0.023	0.007	1/5/143	0/4/291	3.52	4.99E-02
	rs11116595	12	85,165,879	SLC6A15	A/G	0.389	0.476	25/66/58	63/155/77	0.72	2.81E-02
	rs17474506	17	38,990,780	TMEM99	G/C	0.074	0.052	1/20/128	1/29/266	1.44	2.30E-01
Pooled	bs1_156907115	1	156,907,115	ARHGEF11	G/A	0.412	0.347	69/244/151	175/646/615	1.33	3.68E-04
	bs2_233990575	2	233,990,575	INPP5D	A/G	0.009	0.000	0/8/456	0/0/1437		1.23E-05
	rs115393439	2	233,990,592	INPP5D	C/A	0.016	0.003	0/15/449	0/9/1428	5.23	7.86E-05
	rs464494	5	76,003,258	IQGAP2	A/G	0.365	0.449	61/217/186	280/730/427	0.72	5.01E-05
	rs61740965	5	81,608,563	ATP6AP1L	G/A	0.014	0.003	0/13/451	0/10/1427	4.07	9.41E-04

rs10274334	7	47,925,331	PKD1L1	G/C	0.488	0.412	114/223/125	244/697/496	1.32	2.83E-04
rs7385804	7	100,235,970	TFR2	C/A	0.455	0.389	95/232/137	226/666/545	1.29	9.36E-04
rs2418135	9	113,901,309	OR2K2	G/A	0.547	0.474	139/230/95	328/706/402	1.37	4.81E-05
rs61753080	11	119,005,003	HINFP	A/G	0.017	0.005	1/14/446	0/14/1416	3.59	7.98E-04
rs11116595	12	85,165,879	SLC6A15	A/G	0.383	0.462	78/199/187	296/735/406	0.72	2.92E-05
rs17474506	17	38,990,780	TMEM99	G/C	0.083	0.052	5/67/392	5/138/1293	1.67	7.34E-04

Gene-based association Analysis.

In addition to single variant analysis, we performed the gene-based association analysis in each population using SKAT. All polymorphic variants that passed quality control were included in the analysis. We found there were four genes, three genes and one gene significantly associated with PCa aggressiveness (p-value < 1E-4) in the JHH, MI and CAPS populations, respectively (Table 6). In the JHH population, the genes CREB3L1 (cAMP Responsive Element Binding Protein 3-like 1), KLF13 (Kruppel-like Factor 13), ROBO4 (Roundabout, Axon Guidance Receptor, Homolog 4), and ZCCHC6 (Zinc Finger, CCHC Domain Containing 6) presented significant association; in the Michigan population, the significant genes were TEK (Tyrosine Kinase, Endothelial), CDH2 (Cadherin 2), and BEST2 (Bestrophin 2); while in the CAPS population, the gene that showed significant association with PCa aggressiveness was actually a pseudogene LOC100128542. We then explored if there were genes contributing to PCa aggressiveness in at least two populations, setting a p-value threshold of 1E-3. We found 30 genes significantly associated with PCa aggressiveness in the JHH population (p-value < 1E-3), 22 genes in the MI population, and 70 genes in the CAPS population (Table 6). However, none of these genes that were significant at a P-value of 1E-03 were shared in more than 1 population.

Table 6. Gene-based analysis in JHH, MI and CAPS using SKAT.

Population	SetID	P.value	N.Marker.All	N.Marker.Test
JHH				
	CREB3L1	2.55E-06	9	9
	KLF13	1.43E-05	1	1
	ROBO4	2.63E-05	9	9
	ZCCHC6	4.17E-05	7	7
	RNF208	1.01E-04	2	2
	LOC152742	1.24E-04	2	2
	TRIM17	1.72E-04	3	3
	L3MBTL2	1.78E-04	4	4

SNX10	2.01E-04	2	2
CXorf68	2.01E-04	1	1
ZSCAN23	2.01E-04	1	1
F8	2.01E-04	2	2
FAM45A	2.28E-04	1	1
KRTAP22-1	2.63E-04	2	2
RSG1	2.88E-04	3	3
TMEM177	3.11E-04	7	7
CDH6	3.32E-04	4	4
SPAG7	3.40E-04	2	2
RAB26	3.48E-04	3	3
IL16	3.70E-04	12	12
ZNF829	4.24E-04	3	3
EXOC3L2	4.32E-04	2	2
RIMS3	5.03E-04	3	3
MIR4697	5.60E-04	1	1
ARHGEF10	6.36E-04	12	12
C9orf135	6.41E-04	8	8
MLXIPL	6.65E-04	6	6
PNMA2	6.73E-04	3	3
CCL16	9.79E-04	1	1
AHNAK	9.98E-04	32	32

MI

TEK	1.47E-05	9	9
CDH2	5.24E-05	14	14
BEST2	7.97E-05	4	4
LOC100130581	1.45E-04	1	1

OR11L1	2.38E-04	8	8
S100PBP	2.59E-04	4	4
LOC643339	4.25E-04	2	2
INMT	5.07E-04	11	11
LOC148145	5.51E-04	1	1
NRIP3	5.80E-04	3	3
PPARGC1B	6.33E-04	13	13
TIMM44	7.05E-04	8	8
LOC401164	7.18E-04	3	3
RFC1	7.47E-04	2	2
SLC16A5	7.47E-04	2	2
SRSF1	7.71E-04	1	1
MORN3	7.79E-04	4	4
CDH3	7.79E-04	10	10
DDHD1	8.71E-04	2	2
TMEM106C	9.02E-04	5	5
KLK15	9.52E-04	4	4
LINC00284	9.80E-04	1	1
CAPS			
LOC100128542	6.03E-05	2	2
HS3ST2	2.04E-04	2	2
PCDH8	2.47E-04	4	4
MRPL9	2.55E-04	7	7
PIGA	2.85E-04	1	1
CCNI2	3.29E-04	1	1
MGC45800	3.40E-04	3	3
ZNF624	3.79E-04	2	2

MAD2L1BP	5.16E-04	1	1
KIAA1462	5.41E-04	9	9
BTN2A1	5.95E-04	4	4
LOC645206	9.17E-04	2	2
WDR72	9.19E-04	13	13

In addition to single variant and gene based association analysis, we also assessed the potential effects for all coding nonsynonymous variants by PolyPhen2. The possible impact of an amino acid substitution on the structure and function of a human protein was appraised quantitatively as benign, possibly damaging, or probably damaging. In table 7, we listed the prediction for the 7 missense variants of the 11 significant variants associated with PCa aggressiveness. According to PolyPhen2, 3 variants were predicted with benign effect, 2 with possibly damaging and 2 with probably damaging.

Table 7. Effect prediction for 11 significant variants associated with PCa aggressiveness by PolyPhen2.

SNP	CHR	BP	Gene	A1/A2	Annotation	Amino Acid Change	PolyPhen2 Prediction
bs1_156907115	1	156,907,115	ARHGEF11	G/A	missense	S(AGC) to G(GGC)	benign
bs2_233990575	2	233,990,575	INPP5D	A/G	missense	R(CGC) to H (CAC)	possibly damaging
rs115393439	2	233,990,592	INPP5D	C/A	missense	T(ACA) to P(CCA)	probably damaging
rs464494	5	76,003,258	IQGAP2	A/G	utr3		
rs61740965	5	81,608,563	ATP6AP1L	G/A	missense	Y(TAC) to H(CAC)	probably damaging
rs10274334	7	47,925,331	PKD1L1	G/C	missense	R(CGC) to P(CCC)	benign
rs7385804	7	100,235,970	TFR2	C/A	Intron		
rs2418135	9	113,901,309	OR2K2	G/A	Intergenic		
rs61753080	11	119,005,003	HINFP	A/G	missense	G(GGG) to E(GAG)	benign
rs11116595	12	85,165,879	SLC6A15	A/G	Intergenic		
rs17474506	17	38,990,780	TMEM99	G/C	missense	I(ATC) to M(ATG)	possibly damaging

Discussion

To our knowledge, our study represents one of the first comprehensive studies to identify rare variants that are associated with aggressive PCa. Our data generated from the first 12-month funding period identified novel rare variants that are associated with aggressive PCa in Caucasians. We plan to conduct confirmation studies for the 11 significant variants in additional Caucasians and African American men.

We selected the Illumina Human Exome BeadChip (ExomeArray) as our genotyping platform to study rare variants. The ExomeArray chip represents the newest gene chip that delivers unparalleled coverage of putative functional exonic variants. The relatively cheaper cost makes it possible to study larger sample sizes. The Exome Beadchip is comprised of >240,000 markers,

including >200,000 nonsynonymous SNPs, nonsense mutations, SNPs in splice sites and promoter regions, as well as thousands of GWAS tag markers. Nearly 90% of the SNPs on the exome arrays are rare, with a MAF<5%. In addition, the markers on the Illumina Human Exome BeadChips are selected from over 12,000 individual exome and whole-genome sequences, representing diverse populations, including those of European and African descent. Therefore, it is more efficient and economical to use exome arrays to identify rare variants associated with aggressive PCa, compared with whole genome sequencing.

As presented above, we found that two rare but recurrent variants that were 17 bp apart, in the INPP5D gene, were significantly associated with PCa aggressiveness. The INPP5D gene (Inositol Polyphosphate-5-Phosphatase 1) is a member of the INPP5 family. It encodes a Phosphatidylinositol (PtdIns) phosphatase that specifically hydrolyzes the 5-phosphate of phosphatidylinositol (3,4,5)-triphosphate (PtdIns (3,4,5) P3) to produce PtdIns(3,4)P2, and therefore negatively regulating the PI3K (phosphoinositide 3-kinase) pathways (Dunant et al. 2000). Acting as an inhibitor of the PI3K pathway, INPP5D is considered as a tumor suppressor in acute myeloid leukemia, Hodgkin's lymphoma, and acute lymphoblastic leukemia (Luo et al. 2004; Metzner et al. 2009; Tiacci et al. 2012). Besides, INPP5D has been found as the target of the cellular tumor antigen p53 in human breast cancer adenocarcinoma MCF7 cells and testicular germ cell tumor-derived human embryonal carcinoma cells (Kerley-Hamilton et al. 2005; Lion et al. 2013). Although the role INPP5D plays in prostate tumor cells has not been established, it is possible that INPP5D contributes to prostate cancer progression through the PI3K or p53 pathway.

Besides the single variant analysis, we also performed gene-based approach to identify genes that were associated with PCa aggressiveness. The gene-based approach (SKAT) we adopted is a novel statistical approach. SKAT is a supervised and flexible regression method to test for association between rare variants in a gene or genetic region and a continuous or dichotomous trait. Compared to other methods of estimating the joint effect of a subset of SNPs, SKAT is able to deal with variants that have different direction and magnitude of effects, and allows for covariate adjustment (Wu 2011). In addition, SKAT can also avoid arbitrary selection of threshold in burden test. Moreover, SKAT is computationally efficient, compared to a permutation test, making it feasible to analyze the large dataset in our study. Interestingly, several of the top targets identified by SKAT analysis (CREB3L1 and KLF13) encode transcription factors.

Besides all the above findings, we have also carefully calculated the study power based on our modified study design. We have >80% power to detect an OR of 1.7 (2.8) for variants with a MAF of 0.05 (0.01), at an alpha level of 1E-05 (2-sided). Therefore, we have sufficient power to identify novel rare mutations with relatively large effect based on our proposed sample size. We also considered several procedures to control for multiple test correction and SNP selection to be confirmed in additional independent samples. The Bonferroni corrected P-values are 2E-7 (0.05/200,000 variants) and 2E-6 (0.05/20,000 genes), for single variant analysis and gene-based

analysis, respectively. However, not all the tests for single variants are independent due to linkage disequilibrium (LD) structure among variants. In addition, previous studies also showed that the true associations do not necessarily reach the stringent Bonferroni corrected P-value cutoffs. Therefore, to balance study power and false positives, rare variants in Aim 1 that meet either of the following criteria with less stringent P-value cutoffs will be selected for replication: 1) variants reach a pooled analysis p-value of 1E-3 in single variant analysis; 2) variants with same effect direction in the JHH, Michigan and CAPS population; and 3) variants reached a p-value of 0.05 in at least two of the three populations. The adoption of the two-stage study design will further help to remove false positives.

In conclusion, we have identified several novel rare variants and genes that are associated with aggressive PCa in Caucasians. We expect that some of these significant variants could be confirmed in Caucasians and African American men in the next 12-month funding period. In that condition, the newly identified variants can provide more insight into the etiology of aggressive PCa and provide potential effective targets for therapy of aggressive PCa.

KEY RESEARCH ACCOMPLISHMENTS

- 1) Completed IRB and other logistical issues
- 2) Performed single rare variant analysis, bioinformatics analysis, and gene-based analysis (SKAT) to identify rare variants that have strong effects on aggressive PCa risk in exome-array data among a total of 1,902 PCa cases, including 464 aggressive PCa cases and 1,438 indolent PCa cases.
- 3) Successfully identified 11 novel variants associated with PCa aggressiveness in Caucasians and further confirmation in additional Caucasians and African American men are to be conducted.

CONCLUSION

- 1) We have made great progress in achieving the goals described in the approved Statement of Work.
- 2) We have identified 11 variants that are associated with aggressive PCa in Caucasians.
- 3) We plan to replicate the rare variants identified in Aim1 in additional 1,000 aggressive and 1,000 indolent PCa patients of European descent from JHH using Sequenom iPLEX MassARRAY platform.
- 4) We plan to evaluate the effect of rare mutations confirmed in Aim2 in an African American (AA) population with 500 aggressive and 500 indolent PCa patients from JHH.

- 5) We expect some of the variants identified in the first stage could be further confirmed, and therefore provide more insight into the etiology of aggressive PCa and provide potential effective targets for therapy of aggressive PCa.

REPORTABLE OUTCOMES

- 1) Top variants in the genome that are significantly associated with aggressive PCa in EAs (Table 2 - Table 5)
- 2) Top genes in the genome that are significantly associated with aggressive PCa in EAs (Table 6)

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TRAINING ACTIVITIES

My training was carried out following the training plan in the statement of work. The completed training activities include the several aspects: 1) reviewed literature on PCa genetics and genetic epidemiology: including but not limited to: clinical pathological characteristics of PCa, etiology of PCa, study designs in genetic epidemiology, on rare mutations and complex diseases; 2) received online education on ethics issues by Institutional Review Board (IRB) at WFUHS; 3) took two courses at WFUHS, including Introduction to Biostatistics (course code: CPTS730) and Epidemiology (CPTS720); 4) learned a set of software tools that are commonly used to manage and/or analyze genetic data, including PLINK, SKAT, EIGENSTRAT, STRUTURE, and SAS; 5) learned to use key bioinformatics tools, such as Polymorphism, and bioinformatics programming language, such as Perl and Python; 6) attended the 2013 American Society of Human Genetics meeting and earning continuing education credits; 7) attended a weekly journal club organized at the Center for Cancer Genomics, WFUSH.