ORIGINAL ARTICLE

Toll-like receptor-associated sequence variants and prostate cancer risk among men of African descent

EN Rogers1, DZ Jones1, NC Kidd1, S Yeyeodu2, G Brock3, C Ragin4, M Jackson5, N McFarlane-Anderson5, M Tulloch-Reid5, K Sean Kimbro2 and LR Kidd1

Recent advances demonstrate a relationship between chronic/recurrent inflammation and prostate cancer (PCA). Among inflammatory regulators, toll-like receptors (TLRs) have a critical role in innate immune responses. However, it remains unclear whether variant TLR genes influence PCA risk among men of African descent. Therefore, we evaluated the impact of 32 TLR-associated single-nucleotide polymorphisms (SNPs) on PCA risk among African Americans and Jamaicans. SNP profiles of 814 subjects were evaluated using Illumina’s Veracode genotyping platform. Single and combined effects of SNPs in relation to PCA risk were assessed using age-adjusted logistic regression and entropy-based multifactor dimensionality reduction (MDR) models. Seven sequence variants detected in TLR6, TOLLIP (Toll-interacting protein), IRAK4 (interleukin-1 receptor-associated kinase 4) and IRF3 (interferon regulatory factor 3) were marginally related to PCA. However, none of these effects remained significant after adjusting for multiple hypothesis testing. Nevertheless, MDR modeling revealed a complex interaction between IRAK4 rs4251545 and TLR2 rs1898830 as a significant predictor of PCA risk among US men (permutation testing P-value = 0.001). However, these findings require further assessment and validation.

Genes and Immunity (2013) 14, 347–355; doi:10.1038/gene.2013.22; published online 9 May 2013

Keywords: prostate cancer; toll-like receptor; single-nucleotide polymorphisms; gene–gene interactions; multifactor dimensionality reduction

INTRODUCTION

Prostate cancer (PCA) is one of the most frequently diagnosed cancers among Western men of African origin and is the second leading cause of their cancer-related deaths.1,2 In the United States, African-American men are more likely to receive a PCA diagnosis and die from the disease than any other racial or ethnic group. Between 2003 and 2007, the average annual PCA incidence and mortality rates among African-American men were 1.6 and 2.4 higher than Caucasian-American men, respectively. Moreover, several studies indicate similar PCA disparities between American and Caribbean men.1,3,4 On the basis of the 2008 worldwide statistics, age-standardized death rates among Caribbean men were 2.65-fold higher than American men.4 Ethnicity, age and family history are a few risk factors that have been implicated in determining PCA risk. More recently, chronic inflammation has also been considered as an important contributor of PCA, yet the precise etiology of this disease still remains unknown.5–7

Prostate inflammation may occur as the result of direct infection by microbial pathogens, chemical irritation caused by urine reflux, hormone imbalances and/or autoimmunity. Thus, any or all of the aforementioned factors may also have a role in inflammation-induced PCA. Evolutionarily conserved toll-like receptors (TLRs) have an essential role in regulating innate immune responses to harmful pathogens, as depicted in Figure 1.8–10 In humans, some TLRs (for example, TLR1, TLR2, TLR4, TLR5 and TLR6) are located on the cell surface, whereas others remain within the intracellular compartments (for example, TLR3, TLR7 and TLR9).11 Cell surface TLRs recognize pathogens either directly or with the aid of extracellular accessory proteins (for example, CD-14 and MD-2). Upon pathogen recognition, the cytoplasmic domain of TLRs is activated, which enables their interaction with adapter molecules (for example, MyD88, TRIM and TRIF). TLR-adaptor molecule complexes then recruit downstream targets [for example, IRAK4 (interleukin-1 receptor-associated kinase 4) and TRAF6 (TNF receptor-associated factor 6)], resulting in the activation of transcription factors, such as nuclear factor-kB and interferon regulatory factors (IRFs) via mitogen-activated protein kinase signaling integrators.8,12–22 Overall, TLR signaling cascades induce the expression of inflammatory chemokines, cytokines (tumor necrosis factor-α and interleukins) and interferons, which prompt local inflammation.

The TLR signaling pathway also impacts apoptosis, a form of ‘programmed cell death’ that influences cell differentiation, proliferation and tumorigenesis. Specifically, TLR2, TLR4, IRAK1, IRAK2, IRAK4 and MyD88 regulate both cell survival and cell death signaling pathways.23–27 Genetic alterations in these TLR genes may dysregulate apoptosis, resulting in tumor escape from cell death, uncontrolled cell proliferation, an increase in cellular damage, accumulation of genetic alterations and ultimately increased susceptibility to PCA.28–30

TLR-related sequence variants have been evaluated in relation to a variety of human inflammatory and immune response-related
RESULTS

Population description

The demographic and other pertinent characteristics of cases and controls for the entire study population and each study center are summarized in Table 1 and Supplementary Tables A and B. Overall, men diagnosed with PCA were 14 years older and had higher PSA levels than controls (*P* < 0.0001). Among controls, Jamaican men were about 9 years older and had higher PSA levels (*P* < 0.0001) and as well as higher median Gleason scores (*P* = 0.018) than US men. There were no significant differences in family history of PCA with respect to the following: (1) cases to controls from the total population (*P* = 0.316) (Table 1), US alone (*P* = 0.592) (Supplementary Table A) or Jamaica alone (*P* = 0.272) (Supplementary Table B), and (2) controls (*P* = 0.757) or cases (*P* = 0.830) comparing the two study centers (data not shown).

Figure 1. The TLR signaling pathway is initiated via activation of TLRs, followed by adaptor complex formation, IRAK and/or TRAF6 activation to induce subsequent mitogen-activated protein kinase, nuclear factor-κB (NF-κB) and IRF activation, nuclear translocation and regulation of pro- or anti-inflammatory gene expression. IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; LTA, lipoteichoic acid; LPS, lipopolysaccharides; MMP, matrix metalloproteinase; PARP, poly (ADP-ribose) polymerase; PGN, proteoglycans; TNF, tumor necrosis factor; TOLLIP, Toll-interacting protein; TRAF6, TNF receptor-associated factor 6.
was linked to a modestly significant 68% reduction in PCA risk associated with a 1.14-fold increase in the risk of developing PCA (ORage-adjusted = 1.14; 95% CI = 1.12, 1.18). Among Jamaican men, IRF3 rs2304206, TLR6 rs2381289 and TLR6 rs5743818 were marginally associated with PCA risk. For instance, inheritance of the IRF3 rs2304206 GG genotype (ORage-adjusted = 0.32; 95% CI = 0.10, 0.98) was linked to a modestly significant 68% reduction in PCA susceptibility. On the other hand, there was a 1.10- to 2.0-fold increase in PCA risk associated with inheriting the TLR6 rs2381289 AA vs (GG + AG) (ORage-adjusted = 0.76; 95% CI = 0.51, 1.11) or TLR6 rs5743818 AC + CC GA (ORage-adjusted = 1.10; 95% CI = 1.06, 1.14) genotypes. Notably, additive genetic models for IRF3 rs2304206 and TLR6 rs2381289 were significantly related to PCA risk, which was modestly suggestive of a significant dose–response effect in relation to the number of inherited minor alleles (P-trend 0.037 and 0.019, respectively). These modest associations, however, did not persist after adjustments for multiple hypothesis testing.

### Table 1. Study population characteristics among men of African descent from the United States and Jamaica

| Characteristics                                      | Cases                              | Controls                         | P-value |
|------------------------------------------------------|------------------------------------|----------------------------------|---------|
| Number of participants, n                            | 279                                | 535                              | >0.0001 |
| Age at enrollment (years), median (range)            | 67 (45–91)                         | 53 (27–89)                       |         |
| Family history of prostate cancer, n (%)             |                                    |                                  |         |
| Yes                                                  | 35 (16.1)                          | 21 (12.5)                        | 0.316b  |
| No                                                   | 182 (83.9)                         | 147 (87.5)                       |         |
| Missing                                              | 62 (22.2)                          | 367 (68.6)                       |         |
| PSA (ng ml⁻¹), median (range)                        | 11.7 (0.01–10000)                  | 0.9 (0.0–4.0)                    | <0.0001 |
| PSA (ng ml⁻¹) +, n (%)                               |                                    |                                  |         |
| < 4                                                  | 37 (13.8)                          | 517 (99.8)                       |         |
| ≥4                                                   | 231 (86.2)                         | 1 (0.2)                          |         |
| Missing                                              | 11 (0.04)                          | 17 (0.03)                        |         |
| Gleason score, n (%)                                 |                                    |                                  |         |
| 4                                                    | 12 (5.6)                           |                                  |         |
| 5                                                    | 14 (6.5)                           |                                  |         |
| 6                                                    | 74 (34.2)                          |                                  |         |
| 7                                                    | 70 (32.4)                          |                                  |         |
| 8                                                    | 18 (8.3)                           |                                  |         |
| 9                                                    | 22 (10.2)                          |                                  |         |
| 10                                                   | 6 (2.8)                            |                                  |         |
| Missing                                              | 63 (22.6)                          |                                  |         |
| Global WAA, median (range)                           | 0.79 (0.25–0.94)                   | 0.77 (0.25–0.95)                 | 0.107a  |

Abbreviations: PSA, prostate-specific antigen; WAA, West African ancestry. *Wilcoxon’s rank-sum test was used to examine whether differences exist within median age (years). bχ² Test of heterogeneity was used to determine whether the prevalence of family history or high PSA levels (PSA ≥ 4 ng ml⁻¹) vary between cases and controls, PSA (ng ml⁻¹) and global WAA between cases and controls.

### Table 2. Relationship between TLR- associated SNPs and prostate cancer risk among men of African descent (total population)

| Gene (alleles and position) | Genotype | Cases, N (%) | Controls, N (%) | Unadj. OR (95% CI) | Adj. OR (95% CI) | χ² P-value | Age adj. χ² P-value | P-trend P-value | FDR P-value |
|-----------------------------|----------|--------------|-----------------|--------------------|-----------------|------------|--------------------|-----------------|-------------|
| TLR6 rs2381289 3'-UTR       | GG       | 140 (50.4)   | 302 (56.7)      | 1.00 (referent)    | 1.00 (referent) | 0.164      | 0.198              | 1.00            |             |
|                             | GA       | 124 (44.6)   | 200 (37.7)      | 1.33 (0.98, 1.80)  | 1.46 (1.02, 2.09)| 0.062      | 0.041              |                 |             |
|                             | AA       | 14 (5.04)    | 30 (5.63)       | 1.00 (0.52, 1.96)  | 1.20 (0.52, 2.76)| 0.984      | 0.655              |                 |             |
|                             | GA + AA  | 138 (49.64)  | 230 (43.33)     | 1.28 (0.96, 1.72)  | 1.43 (1.00, 2.00)| 0.088      | 0.046              | 1.00            |             |
|                             | AA vs (GG + AG) | 88 (64.6, 1.70) | 102 (46, 2.29) | 0.724              | 0.956            | 0.853     |                     |                 |             |
| TOLLIP rs3168046 3'-UTR miRNA | GG       | 104 (37.3)   | 189 (35.4)      | 1.00 (referent)    | 1.00 (referent) | 0.363      | 0.261              | 0.798           |             |
|                             | GA       | 131 (46.9)   | 239 (44.8)      | 1.00 (0.72, 1.40)  | 0.85 (0.58, 1.25)| 0.981      | 0.415              |                 |             |
|                             | AA       | 44 (15.8)    | 106 (19.9)      | 0.75 (0.49, 1.15)  | 0.58 (0.35, 0.98)| 0.194      | 0.042              |                 |             |
|                             | GA + AA  | 175 (62.7)   | 345 (64.7)      | 0.92 (0.68, 1.24)  | 0.76 (0.54, 1.10)| 0.596      | 0.155              | 1.00            |             |
|                             | AA vs (GG + AG) | 76 (51.6, 1.11) | 64 (40, 1.02) | 0.155              | 0.628            | 1.000     |                     |                 |             |
| TOLLIP rs5743899 Intron 1    | AA       | 85 (30.8)    | 187 (35.2)      | 1.00 (referent)    | 1.00 (referent) | 0.208      | 0.686              | 1.00            |             |
|                             | AG       | 140 (50.7)   | 253 (44.2)      | 1.31 (0.94, 1.82)  | 1.59 (1.06, 2.38)| 0.109      | 0.023              |                 |             |
|                             | GG       | 51 (18.5)    | 110 (20.7)      | 1.02 (0.67, 1.55)  | 1.29 (0.78, 2.14)| 0.926      | 0.031              |                 |             |
|                             | GA + GG  | 191 (69.2)   | 395 (72.2)      | 1.22 (0.89, 1.66)  | 1.49 (1.02, 2.18)| 0.215      | 0.035              | 0.884           |             |
|                             | GG vs (AA + AG) | 87 (60.6, 1.26) | 98 (64, 1.53) | 0.458              | 0.949            | 0.756     |                     |                 |             |

Abbreviations: CI, confidence interval; FDR, false discovery rate; miRNA, microRNA binding site; OR, odds ratio; SNP, single-nucleotide polymorphism; TLR, Toll-like receptor; TOLLIP, Toll-interacting protein UTR, untranslated region. *Fisher’s P-value was calculated when expected genotype counts were < 5 for both cases and controls. Significant associations are indicated in boldface.
## Table 3. Relationship between TLR-associated SNPs and prostate cancer risk stratified by location

| Gene (allele and position) | Genotype | Unadjusted OR (95% CI) | Unadjusted OR (95% CI) | Age adjusted OR (95% CI) | Age adjusted OR (95% CI) | P-value | P-trend | P-value | P-trend |
|---------------------------|----------|------------------------|------------------------|--------------------------|--------------------------|---------|---------|---------|---------|
|                           | US men   | Jam. men               | US men                 | Jam. men                 | US men                  | Jam. men | Jam. men | US men  | Jam. men |
| IRF3 rs2304206 TFBS        |          |                        |                        |                          |                          |         |         |         |         |
| IRAK4 rs4251473 Intron 5   |          |                        |                        |                          |                          |         |         |         |         |
| IRAK4 rs4251545 Exon 8 or exon 1 splicing |          |                        |                        |                          |                          |         |         |         |         |
| TLR6 rs281289 3'-UTR        |          |                        |                        |                          |                          |         |         |         |         |
| TOLLIP rs1568046 3' UTR mRNA |          |                        |                        |                          |                          |         |         |         |         |
| TOLLIP rs7543899 Intron 1   |          |                        |                        |                          |                          |         |         |         |         |

Abbreviations: CI, confidence interval; ESE, exonic splicing enhancer; ESS, exonic splicing silencers; IRAK4, interleukin-1 receptor-associated kinase 4; Jam., Jamaican; OR, odds ratio; SNP, single-nucleotide polymorphism; TFBS, transcription factor binding site; TLR, Toll-like receptor; TOLLIP, Toll-interacting protein UTR, untranslated region. *Fisher’s P-value was calculated when expected genotype counts were <5 for either cases or controls. Significant associations are indicated in boldface.
Analysis of gene–gene interactions using MDR

MDR modeling was used to efficiently assess and validate age-adjusted gene–gene interactions for the total population, US men alone and Jamaican men alone in relation to PCA risk. The top one-, two- and three-way interaction models for the total population, involving US and Jamaican men combined, displayed 100% cross-validation consistency (CVC) values, 57–65% average testing accuracy (ATA) scores and permutation P-values = 0.001, as shown in Table 4A. However, the three-way interaction among TLR6 rs2381289, TLR10 rs11096957 and IRF3 rs2304206 was selected as the best PCA predictor for men of African descent, as this model had the highest average testing accuracy (ATA = 0.6505). This three-way interaction was primarily driven by a synergistic relationship between TLR6 rs2381289 and IRF3 rs2304206 (data not shown).

CVC and ATA scores for all one-, two- and three-factor models among US men were significant and characterized as 90–100% and 57–62%, respectively, as described in Table 4B. However, interaction between TLR2 rs1898830 and IRAK4 rs4251545 was chosen as the best PCA predictor, based on a higher ATA (61.94%) and lower permutation testing value (P = 0.001) relative to the best three-factor model (P = 0.015). The two-way interaction, as shown in Figure 2, was highly synergistic since the joint information gain score (2.33%) exceeded the mutual information gain scores for TLR2 rs1898830 alone (0.04%) and IRAK4 rs4251545 alone (1.30%).

For the Jamaican population, the one-way model containing the TLR6 rs2381289 locus was the best PCA-related MDR model based on a 62.7% prediction accuracy score and significant permutation P-value (P = 0.0018). Although the two- and three-way models both had high CVC scores (≥80%), these models failed to reach statistical significance.

**DISCUSSION**

Dysregulation and genetic alterations in immune system function are linked to many cancers. In particular, it is estimated that approximately 20% of all human cancers, including PCA, are associated with chronic inflammation. TLR activation, a key initiator of inflammation and dysregulation of TLR-responsive pathways, has been associated with cancer susceptibility. This study evaluated 32 TLR-associated sequence variants to determine their individual and joint modifying effects on PCA risk among 279 cases and 535 disease-free men of African descent. Out of the 32 minor variant alleles, 7 were modestly associated with a 1.14- to 2.05-fold increase (TLR6 rs2381289 and TOLIP rs5743899) or a 39–68% decrease (TOLLIP rs3168046, IRAK4 rs4251473, IRF4 rs4251545, IRF3 rs2304206 and TLR6 rs5743818) in the risk of developing PCA either in the total population and/or the stratified analysis, after adjusting for age. Among US men, there was a nominal 40–66% reduction in PCA susceptibility among those who possessed one or more IRAK4 (rs4251545 and rs4251473) or TLR6 rs5743818 minor alleles. We also found that the TOLLIP rs5743899 SNP under the recessive genetic model (GG vs AG + AA) was modestly associated with a 1.14-fold increase in the risk of developing PCA (ORage-adjusted = 1.14; 95% CI = 1.12, 1.18) among men of African descent from the United States. The TOLLIP rs3168046 SNP was unique to the total population, whereas the three markers (IRAK4 rs4251473, IRF4 rs4251545 and TLR6 rs2381289) were unique to the US men. Jamaican and US subgroups each had one SNP in common with the total population, namely TLR6 rs2381289 and TOLLIP rs5743899, respectively. Among Jamaicans, both the IRF3 rs2304206 and TLR6 rs2381289 loci were significant under the additive genetic model. However, only the TLR6 rs2381289 SNP for the Jamaican population remained statistically significant after adjusting for age and multiple hypothesis testing (permutation P-value = 0.018).

Finally, we examined main effects and interactions of TLR-associated SNPs as predictors of PCA using age-adjusted MDR and found that the best model varied depending on the composition of the study population. Among US men of African descent, the best predictor of PCA risk was the two-factor interaction between IRAK4 rs4251545 and TLR2 rs1898830. Several investigators have evaluated the link between PCA outcomes and TLR-associated (TLR1, TLR4, TLR6, TLR10 and IRAK4) sequence variants. Collectively, nine studies evaluated 14 out of the 32 SNPs considered in this study with mixed findings. In the Cancer of the Prostate Study (CAPS), inheritance of one or more TLR1 rs4833095 and TLR10 rs11096955 and rs11096957 minor alleles were associated with an increased risk in PCA risk among Caucasians. However, the American Cancer Society Cancer Prevention II Nutrition Cohort (CPS-II) study reported protective effects and null findings were observed for Caucasians of the Health Professionals Follow-up Study, PLCO Study as well as a meta-analysis of 3142 cases and 2567 controls. Consistent with this study, five independent studies reported null findings in relation to PCA risk and possession of TLR1 rs46224663 and TLR6 (rs5743814, rs5743810, rs1039559 and rs3281985) variant alleles. Inheritance of one or more minor alleles were associated with either an increase (TLR1 rs5743604 and TLR4 rs4274855) or decrease (TLR1 rs5743595, TLR1 rs2149356 and TLR4 rs1927911) in the risk of developing PCA among Caucasians in four separate observational studies. However, these same markers were not significantly related to PCA among men of African and European descent in the current study and other independent studies.

The TLR4 rs1927911 CC genotype was linked to an increase in PCA.

---

**Table 4. Interactions and main effects of TLR-associated SNPs as predictors of prostate cancer using age-adjusted MDR: (A) MDR models for US and Jamaican men of African descent combined and (B) MDR models for US men**

| (A) Best total population model (dSNPID no.) | CVC | ATA | Permutation testing P-value |
|---------------------------------------------|-----|-----|----------------------------|
| One factor TLR6 rs2381289                    | 10/10 | 0.5722 | 0.001                      |
| Two factor TLR10 rs11096957 TLR6 rs2381289  | 10/10 | 0.6113 | 0.001                      |
| Three factor TLR10 rs11096957 TLR6 rs2381289 IRF3 rs2304206 | 10/10 | 0.6505 | 0.001                      |

| (B) Best US men model (dSNPID no.) | CVC | ATA | Permutation testing P-value |
|-----------------------------------|-----|-----|----------------------------|
| One-factor IRAK4 rs4251473        | 10/10 | 0.5744 | 0.179                      |
| Two-factor TLR2 rs1898830 IRAK4 rs4251545 | 10/10 | 0.6194 | 0.001                      |
| Three-factor TLR6 rs3821985 TLR4 rs1927906 TLR2 rs3804099 | 9/10 | 0.6184 | 0.015                      |

Abbreviations: ATA, average testing accuracy; CVC, cross-validation consistency; IRAK4, interleukin-1 receptor-associated kinase 4; MDR, multifactor dimensionality reduction; SNP, single-nucleotide polymorphism; TLR, Toll-like receptor; UTR, untranslated region.

© 2013 Macmillan Publishers Limited
risk in one small Korean case–control study, whereas another Korean study did not reveal a statistically significant relationship. Discrent findings for these two Korean studies may be attributed to differences in methods used for allelic discrimination. Utilization of restriction fragment length polymorphism-polymerase chain reaction method used in the former study may not have the same capacity to discern between homozgyous and heterozygous genotypes, relative to a more advance method used in the study performed by Kim and co-workers. Other explanations for differences in the directionality of PCA risk estimates in the aforementioned TLR-related SNPs may include: inadequate statistical power to detect significant differences between PCA cases and controls among Koreans; failure to adjust for multiple hypothesis testing bias by adjusting our risk models for West African ancestry ultimately may modify the relationship between TLR SNPs and PCA risk. For this study, adjustments were restricted to men with ≥25% West African ancestry. Adjustment of our risk models for West African ancestry ultimately had no significant bearing on calculated risk estimates among African-American men. Because of chance alone, it is estimated that 5% of the 744 SNP interactions among 32 TLR SNPs on prostate tumor biology. We are aware of both strengths and limitations of our approach. Although we observed nominal relationships between selected TLR-associated SNPs and PCA risk, we cannot rule out the possibility that anomalies within the innate immune pathway will influence disease prognosis. Consequently, future studies will enable us to evaluate the relationship between TLR SNPs and Gleason score, tumor stage, biochemical/disease recurrence and overall/disease-specific mortality. Future multicenter pooled genetic studies with thousands of cases and controls may enable us to confirm and refine our small effect sizes. It is plausible that other targets immediately downstream of the TLRs in innate immune signaling pathways may have a role in PCA risk among men of African descent, as shown in Figure 1. Moreover, PCA susceptibility may also be influenced by polymorphisms of some genes even further down the TLR signaling pathway, including caspases (CASP 3, 7, 8 and 10), mitogen-activated protein kinases, interferon-regulatory factors, interferons, inflammatory cytokines and chemokines. This represents an area of active research within future collaborative studies in our lab.

Analysis of mRNA and protein levels of TLR-related SNPs and investigation of the relative expression and activity of downstream targets are needed to define the biological mechanisms that give rise to PCA disparities among men of African and European descent. The genetic admixture among African-American men, as documented by other published reports, may modify the relationship between TLR SNPs and PCA risk. For this study, analyses were restricted to men with ≥25% West African ancestry. Adjustment of our risk models for West African ancestry ultimately had no significant bearing on calculated risk estimates among African-American men. Because of chance alone, it is estimated that 5% of the 744 SNP interactions among 32 TLR sequence variants will result in 37 significant relationships. However, we controlled for multiple hypothesis testing bias by adjusting our
MDR findings with permutation testing. Given the low prediction accuracy (that is, 61.9%) between TLR2 and IRAK4, our study findings require replication within independent study sets. However, recent simulation studies demonstrate that even modest disparities in genotype frequencies among study participants of independent study sets may interfere with the capacity to replicate complex interactions. Consequently, to replicate our findings, it is critical that future replicate studies should have the same genetic architecture (that is, ancestry identification markers and TLR SNPs) as the African Americans in this study. Caution is recommended in the interpretation of our study findings because of a modest marginal effect between TLR signaling sequence variants and PCA risk. However, enthusiasm for the relationship between PCA and the innate immune signaling pathway was slightly elevated in our exhaustive two- and three-way interactions. In particular, our exploratory analysis revealed a synergistic relationship between IRAK4 and TLR2 as significant PCA markers among men of African descent in the United States. We speculate that genetic variations in TLR-related genes may influence the PCA risk by modulating cell survival, proliferation and/or inflammation. Mechanistic studies are needed to corroborate these findings and explore the functional consequences of TLR-related SNPs on PCA development. By combining our genetic variation analysis of TLR-related polymorphisms with biological studies, we hope to develop a level of understanding that will allow us to accurately predict and eventually offset the increased genetic risk factors for PCA that threaten men of African descent.

**MATERIALS AND METHODS**

**Study population**

Two independent case–control study sets with participants from the PCA Clinical Outcome Study and the PCA Case–Control Study were used in this study (Table 1 and Supplementary Tables A and B). Among all 814 men of African descent, germ-line DNA samples were collected for 279 PCA cases and 535 disease-free men, as shown in Table 1. In the PCA Clinical Outcome Study, 603 unrelated men of African descent were recruited between 2001 and 2005 from Columbia, South Carolina and Howard University Hospital (HUH) Division of Urology in Washington, DC. Self-identified African-American, East African-American, West African-American or Afro-Caribbean American men from the United States were participants of the PCA Clinical Outcome Study, consisting of 170 incident PCA cases and 433 controls, as shown in Table 1. In the PCA Case–Control Study, 211 unrelated Jamaican men (109 incident PCA cases and 102 controls) were consecutively enrolled between 2005 and 2007 during a first time urological clinic visit, as depicted in Supplementary Table B. The examination and inclusion criteria of all subjects have been described in detail previously. Genetic analysis of variant TLR-associated SNPs

De-identified germ-line DNA was obtained from incident PCA male cases (n = 279) and controls (n = 535). SNPs detected in TLRs (1, 2, 4, 6 and 10), IRAK4, TOLLIP and IRF3 were genotyped using Illumina’s GoldenGate genotyping assay system combined with Veracode Technology (Illumina Inc., San Diego, CA, USA). Allelic discrimination was performed using a BeadXpress Reader (Illumina) according to the manufacturer’s instructions. Quality control analyses and data management were performed using SNP Variation Software (SVS) 7.0 (Golden Helix Inc., Bozeman, MT, USA). To ensure high-quality data, SNPs were excluded if: genotype call rates were <95% (n = 1); genotypic distribution between controls deviated substantially from the HWE with a significant cutoff value of X2 test of homogeneity. OR and corresponding 95% CIs for PCA risk in association with TLR SNPs were estimated using unconditional multivariate LR models after adjusting for age, race and disease status. Genotypic analysis was performed using the SNPs validated in the major or common genotype as the referent category. X2 Test and LR analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, NC, USA) and SVS software (Golden Helix Inc.). Adjustments for multiple hypothesis testing was achieved using false discovery rate (FDR). Statistically significant data was based on a P-value cutoff of 0.05.

Statistical power for single gene effects

We calculated the odds of developing PCA among carriers of at least one or more minor allele based on the average minor allele frequency 21.9–23.3% for the three study sets (that is, US men, Jamaican men and US and Jamaican men combined), a PCA disease prevalence of 0.740%, a significance level (α) of 5% and 100% linkage disequilibrium between the casual and the predisposing variant. According to our sample size for the combined population (279 cases and 535 controls), US (170 cases and 433 controls) and Jamaican men (109 cases and 102 controls), we had >80% power to detect ORs of >1.4, >1.6 and >1.9 for PCA risk, respectively. Statistical power calculations were performed using Power for Genetic Association Version 2 Software (National Institute of Health, Rockville, MD, USA), as described previously.

Analysis of gene interactions using MDR

To evaluate the single- and joint-modifying effects of 32 candidate TLR-associated SNPs within a large data set is computationally challenging. To overcome this problem, open source and freely available MDR 2.0 was used to detect and characterize all possible one-, two- and three-way interacting models in relation to PCA (http://www.sphinxbase.org). To reduce computation time needed to process thousands of SNP combinations in relation to PCA risk, we distributed MDR on a workstation with 12 hyper-threaded cores across two central processing units (total of 24 simultaneous threads of execution) and 24 GB of RAM. Although MDR has been described elsewhere, for convenience we provide a brief summary. With MDR, reduced genetic information is reduced to a one-dimensional multilocus genotype variable. Information from various disease loci were grouped and labeled as ‘high risk’ or ‘low risk’ based on whether or not the control ratio met or exceeded a particular threshold. Subsequently, the resulting one-dimensional multilocus genotype variable was examined for its capability to categorize and predict disease outcome through cross-validation and permutation testing procedures. A 10-fold cross-validation was achieved by dividing the entire data set into a training set and an independent testing set. The training set involved 9/10th of the data; the remaining 1/10th, known as the independent testing set, was evaluated against the training set. Evaluation of each independent testing set predicted average testing accuracy values for each MDR model. The greatest cross-validation consistency (that is, CVC = 8/10) and highest prediction accuracy (that is, ATA) were used to identify the best predictors of disease outcome. Sensitivity and specificity were determined as functions of true negatives (TN), false positives (FP) and false negatives (FN). Sensitivity, specificity and balanced accuracy values were calculated as follows: sensitivity = (TP)/(TP + FN); specificity = (TN)/(FP + TN); and balanced accuracy = ([sensitivity + specificity]/2. ATAs were previously. Individual genetic ancestry was determined for each person using 100 ancestry informative markers for West African and European genetic ancestry. Individual genetic ancestry was estimated from the genotype data using the Bayesian Markov Chain-Monte Carlo method implemented in the program STRUCTURE 2.1, as detailed elsewhere. Study participants were grouped from lowest to highest genetic West African ancestry, with scores ranging from 0 to 100%. These 100 markers were evaluated using DNA from self-identified African-Americans (Correll Institute for Medical Research, n = 96), Yoruban West Africans (HapMap, n = 60), West Africans (Bantu and Nilo Saharan speakers, n = 72), Europeans (New York City, n = 24) and CEPH Europeans (HapMap Panel, n = 60), as reported previously. Previous studies with a West African ancestry score >25% and available TLR genotype data were included in the final analysis.
averaged across all 10 pieces of the data, to provide an estimate of the predictive ability of the loci in relation to the outcome of interest. We used CVC to determine the degree to which the same best MDR model was selected across the 10 divisions of the data. Models with a CVC of \( \geq 8/10 \) using a 10-fold cross-validation were considered more carefully. CVCs and ATAs were calculated across 1000 random seeds to ensure reproducibility in model selection. If the MDR model met the CVC criteria, we selected models that had the highest ATAs. Multiple hypothesis testing was controlled by CVC in combination with permutation testing. Permutation testing results \( \leq 0.05 \), generated using random seed 500, were considered statistically significant. Age-group covariate effects were removed by integrating over- and undersampling methods.

Visualization of interaction models using hierarchical interaction entropy graphs

Hierarchical interaction entropy graphs, based on information theory, were used to visualize and interpret complex interactions among selected TLR SNPs and PCA risk.59–62 With this approach, individual and all possible pairwise loci were assigned a joint or mutual information percentage score based on disease risk, respectively. Joint mutual information and mutual information gain scores were based on a number system, ranging from 0 to 100%. However, these scores rarely exceed 5–6%. When the pairwise or joint mutual information exceeded the mutual information gain scores, then the pairwise interaction was considered more informative in relation to PCA risk when compared with each locus considered separately. Potential interactions were assessed using interaction entropy graphs, which uses a color-coding system to depict redundant or synergistic interactions. The distinction between the synergistic or redundant epistasis models was based on a color coding system. Within the entropy graph, lines depicted between SNP pairs that were color-coded red, orange, green, blue and gold represented highly synergistic, moderately synergistic, and undersampling methods.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Tiva T VanCleave and Dr Nicole A Lavender for preparing DNA samples used in this study. We also thank Dr Rick A Kittles for providing US African-American DNA samples. We appreciate the contract services of Expression Analysis Inc. (http://expressionanalysis.com/) for the generation of genotype data. This work was supported by the following grants: Clinical Translational Science Pilot Grant (to LRK); the JGBCC Bucks for Brains ‘Our Highest Potential’ in Cancer Research Endowment (to KSK).

REFERENCES

1 American Cancer Society. Cancer Facts and Figures 2012. American Cancer Society: Atlanta, GA, USA, 2012.
2 Hsing AW, Tsao L, Devesa SS. International trends and patterns of prostate cancer incidence and mortality. Int J Cancer 2000; 85: 60–67.
3 American Cancer Society. Cancer Facts and Figures for African Americans 2011–2012. American Cancer Society: Atlanta, GA, USA, 2011.
4 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127: 2893–2917.
5 Nickel CJ. Prostatitis syndromes: an update for urologic practice. Can J Urol 2000; 7: 1091–1098.
6 Palapattu GS, Sutcliffe SJ, Bastian PJ, Plazet EA, De Marzo AM, Isaacs WB et al. Prostate carcinogenesis and inflammation: emerging insights. Carcinogenesis 2005; 26: 1170–1181.
7 Sfanos KS, De Marzo AM. Prostate cancer and inflammation: the evidence. Histochemistry and Embryology 2012; 60: 199–215.
8 Latz E, Viscidi A, Lien E, Fitzgerald KA, Monks BG, Kurt-Jones EA et al. Lipopolysaccharide rapidly traffic to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CDC14 complex in a process that is distinct from the initiation of signal transduction. J Biol Chem 2002; 277: 47834–47843.
9 Frantz S, Ertl G, Bauersachs J. Mechanisms of disease: Toll-like receptors in cardiovascular disease. Nat Clin Pract Cardiovasc Med 2007; 4: 444–454.
10 Tsujimoto H, Ono S, Efron PA, Scumpia PO, Moldawer LL, Mochizuki H. Role of Toll-like receptors in the development of sepsis. Shock 2008; 29: 315–321.
11 Blasius AL, Beutler B. Intracellular toll-like receptors. Immunity 2010; 32: 305–315.
12 Akiro S, Yamamoto M, Takeda K. Role of adapters in Toll-like receptor signaling. Biochem Soc Trans 2003; 31(3): 637–642.
13 Beutler B. The Toll-like receptors: analysis by forward genetic methods. Immunogenetics 2005; 57: 385–392.
14 Beutler B. Innate immune responses to microbial poisons: discovery and function of the Toll-like receptors. Annu Rev Pharmacol Toxicol 2003; 43: 609–628.
15 Gay NJ, Keith FJ. Drosophila Toll and Il-1 receptor. Nature 1991; 351: 355–356.
16 Gay NJ, Packman LC, Weldon MA, Bara JC. A leucine-rich repeat peptide derived from the Drosophila Toll receptor forms extended filaments with a beta-sheet structure. FEBS Lett 1991; 291: 87–91.
17 Nakata T, Yasuda M, Fujita M, Katoaka H, Kiura K, Sano H et al. CD14 directly binds to triacylated lipopolysaccharides and facilitates recognition of the lipopolysaccharide by the receptor complex of Toll-like receptors 2 and 1 without binding to the complex. Cell Microbiol 2006; 8: 1899–1909.
18 Muzio M, Polentarutti N, Bossio D, Prabhладан MK, Mantovani A. Toll-like receptors: a growing family of immune receptors that are differentially expressed and regulated by different leukocytes. J Leukoc Biol 2000; 67: 450–456.
19 Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG et al. Crystal structure of the TLR1–TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell 2007; 130: 1071–1082.
20 Farhat K, Kienberg S, Heine H, Debarby J, Lang R, Mages J et al. Hetero-dimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. J Leukoc Biol 2008; 83: 692–701.
21 Beutler B, Hobe K, Georl F, Tabeta K, Du X. Genetic analysis of innate immunity: identification and function of the TLR adiogen proteins. Adv Exp Med Biol 2005; 560: 25–39.
22 Salaun B, Romero P, Lebecque S. Toll-like receptors’ two-edged sword: when immunity meets apoptosis. Eur J Immunol 2007; 37: 3311–3318.
23 Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. Nat Rev Cancer 2007; 9: 57–63.
24 Creighton CJ, Benham AL, Zhu H, Khan MF, Reid NG, Nagaraja AK et al. Discovery of novel microRNAs in female reproductive tract using next generation sequencing, PLoS One 2010; 5: e9637.
25 Hinzx LX, Rebsamen M, Rossi DC, Staehli F, Schroder K, Quadroni M et al. The death domain-containing protein Unc5C1 is a novel MyD88-independent activator of the pro-inflammatory IRAK signaling cascade. Cell Death Differ 2012; 19: 722–731.
26 Romoser AA, Chen PL, Berg JM, Seabury C, Ivanov I, Criscitiello MF et al. Quantum dots trigger immunomodulation of the NFkappaB pathway in human cancer cells. Mol Immunol 2011; 48: 1349–1359.
27 De Marzo AM, Plazet EA, Sutcliffe SJ, Xu J, Gronion H, Drake CG et al. Inflammation in prostate carcinogenesis. Nat Rev Cancer 2007; 7: 256–269.
28 Huang B, Zhao J, Unkeless JC, Feng ZH, Xiong H. Toll signaling by tumor and immune cells: a double-edged sword. Oncogene 2008; 27: 218–224.
29 Uno K, Kato K, Atsumi T, Suzuki T, Yohitake J, Morita H et al. Toll-like receptor (TLR) 2 induced through TLR4 signaling initiated by Helicobacter pylori cooperatively amplifies iNOS induction in gastric epithelial cells. Am J Physiol Gastrointest Liver Physiol 2007; 293: G1006–G1012.
30 Song Z, Yin J, Yao C, Sun Z, Shao M, Zhang Y et al. Variants in the Toll-interacting protein gene are associated with susceptibility to sepsis in the Chinese Han population. Crit Care 2011; 15: R12.
31 Qian FH, Zhang Q, Zhou LF, Jin GF, Bai JL, Yin KS. Polymorphisms in the toll-like receptor 2 subfamily and risk of asthma: a case-control analysis in a Chinese population. J Invest Allergol Clin Immunol 2010; 20: 340–346.
32 Slattery ML, Herrick JS, Bondurant KL, Wolff FK. Toll-like receptor genes and their association with colon and rectal cancer development and prognosis. Int J Cancer 2011; 129: 2974–2980.
33 Pandey S, Mittal RD, Srivastava M, Srivastava K, Singh S, Srivastava SK et al. Impact of Toll-like receptors (TLR) 2 (–196 to –174 del) and TLR 4 (Asp299Gly, Thr399Ile) in cervical cancer susceptibility in North Indian women. Gynecol Oncol 2009; 114: 501–505.
34 Hold GL, Rakbin CS, Chow WH, Smith MG, Gammon MD, Risch HA et al. A functional polymorphism of toll-like receptor 4 gene increases risk of gastric carcinoma and its precursors. Gastroenterology 2007; 132: 905–912.
35 Rajaraman P, Brenner AV, Neta G, Pfeiffer R, Wang SS, Yeager M et al. Risk of meningioma and common variation in genes related to innate immunity. Cancer Epidemiol Biomarkers Prev 2010; 19: 1356–1361.
37 Srivastava K, Srivastava A, Kumar A, Mittal B. Gallbladder cancer predisposition: a multigenic approach to DNA-repair, apoptotic and inflammatory pathway genes. *PLoS One* 2011; 6: e16449.

38 Song J, Kim DY, Kim CS, Kim HJ, Lee DH, Lee HM et al. The association between Toll-like receptor 4 (TLR4) polymorphisms and the risk of prostate cancer in Korean men. *Cancer Genet Cytogenet* 2009; **190**: 88–92.

39 Sun J, Wiklund F, Hsu FC, Balter K, Zheng SL, Johansson JE et al. Interactions of sequence variants in interleukin-1 receptor-associated kinase4 and the toll-like receptor 6-1-10 gene cluster increase prostate cancer risk2. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 480–485.

40 Cheng I, Plummer SJ, Casey G, Witte JS. Toll-like receptor 4 genetic variation and advanced prostate cancer risk1. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 352–355.

41 Kim HJ, Bae JS, Chang IH, Kim KD, Lee J, Shin HD et al. Sequence variants of toll-like receptor 4 (TLR4) and the risk of prostate cancer in Korean men. *World J Urol* 2011; **30**: 225–232.

42 Zheng SL, Augustsson-Balter K, Chang B, Hedelin M, Li L, Adami HO et al. Sequence variants of toll-like receptor 4 are associated with prostate cancer risk: results from the Cancer Prostate in Sweden study3. *Cancer Res* 2004; **64**: 2918–2922.

43 Chen YC, Giovannucci E, Lazarus R, Kraft P, Ketkar S, Hunter DJ. Sequence variants of Toll-like receptor 4 and susceptibility to prostate cancer. *Cancer Res* 2005; **65**: 11771–11778.

44 Stevens VL, Hsing AW, Talbot JT, Zheng SL, Sun J, Chen J et al. Genetic variation in the toll-like receptor gene cluster (TLR10–TLR1–TLR6) and prostate cancer risk. *Int J Cancer* 2008; **123**: 2644–2650.

45 Lindstrom S, Hunter DJ, Gronberg H, Stattin P, Wiklund F, Xu J et al. Sequence variants in the TLR4 and TLR6-1-10 genes and prostate cancer risk. Results based on pooled analysis from three independent studies. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 873–886.

46 Chen YC, Giovannucci E, Kraft P, Lazarus R, Hunter DJ. Association between Toll-like receptor gene cluster (TLR6, TLR1, and TLR10) and prostate cancer. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 1982–1989.

47 Gir VN, Egleston B, Ruth K, Uzzo RG, Chen DY, Buyyounouski M et al. Race, genetic West African ancestry, and prostate cancer prediction by prostate-specific antigen in prospectively screened high-risk men. *Cancer Prev Rev (Phila, PA)* 2009; **2**: 244–250.

48 Tian C, Hinds DA, Shigeta R, Kittles R, Ballinger DG, Sel din MF. A genomewide single-nucleotide-polymorphism panel with high ancestry information for African American admixture mapping. *Am J Hum Genet* 2006; **79**: 640–649.

49 Falush D, Stephens M, Pritchard JK. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* 2003; **164**: 1567–1587.

50 Huang QX, Cheng XY, Mao ZC, Wang YS, Zhao L, Yan X et al. MicroRNA discovery and analysis of pinewood nematode *Bursaphelenchus xylophilus* by deep sequencing. *PLoS One* 2010; **5**: e13271.

51 Sun J, Wiklund F, Zheng SL, Chang B, Balter K, Li L et al. Sequence variants in Toll-like receptor gene cluster (TLR6–TLR1–TLR10) and prostate cancer risk4. *J Natl Cancer Inst* 2005; **97**: 525–532.

52 Greene CS, Penrod NM, Williams SM, Moore JH. Failure to replicate a genetic association may provide important clues about genetic architecture. *PLoS One* 2009; **4**: e6539.

53 Kidd LC, Vancleave TT, Doll MA, Srivastava DS, Thacker B, Komolafe O et al. No association between variant N-acetyltransferase genes, cigarette smoking and prostate cancer susceptibility among men of African descent. *Biomark Cancer* 2011; **3**: 1–13.

54 Jackson MD, Walker SP, Simpson-Smith CM, Lindsay CM, Smith G, McFarlane-Anderson N et al. Associations of whole-blood fatty acids and dietary intakes with prostate cancer in Jamaica. *Cancer Causes Control* 2012; **23**: 23–33.

55 Menashe I, Rosenberg PS, Chen BE. PGA: power calculator for case-control genetic association analyses. *BMJ Genet* 2008; **9**: 36.

56 Gui J, Andrew AS, Andrews P, Nelson HM, Kelsey KT, Karagas MR et al. A simple and computationally efficient sampling approach to covariate adjustment for multifactor dimensionality reduction analysis of epistasis. *Hum Hered* 2010; **70**: 219–225.

57 Greene CS, Penrod NM, Ki ralis J, Moore JH. Spatially uniform relief (SURF) for computationally-efficient filtering of gene-gene interactions. *BioData Min* 2009; **2**: 5.

58 Moore JH, Asselbergs FW, Williams SM. Bioinformatics challenges for genome-wide association studies. *Bioinformatics* 2010; **26**: 445–455.

59 Moore JH, Gilbert JC, Tsai CT, Chiang FT, Holden T, Barney N et al. A flexible computational framework for detecting, characterizing, and interpreting statistical patterns of epistasis in genetic studies of human disease susceptibility. *J Theor Biol* 2006; **241**: 252–261.

60 Jakulin A, Bratko I, Smri te D, Demsar J, Zupan B. Attribute Interactions in Medical Data Analysis. *Protaurus*, Cyprus, pp 229–238, 2003.

61 Jakulin A, Bratko I. Analyzing attribute interactions. *Lect Notes Artif Intell* 2003; **2838**: 229.

62 McGill WL. Multivariate information transmission. *Psychometrika* 1954; **19**: 97–116.

63 Mramor M, Leban G, Demsar J, Zupan B. Visualization-based cancer microarray data classification analysis. *Bioinformatics* 2007; **23**: 2147–2154.

Supplementary Information accompanies this paper on Genes and Immunity website (http://www.nature.com/gene)