Short Communication

Toll-Like Receptor 4 Genetic Variation and Advanced Prostate Cancer Risk

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Abstract

Toll-like receptor 4 (TLR4) is a key innate immunity receptor that initiates an inflammatory response primarily against Gram-negative bacteria. Two recent publications reported that variants in *TLR4* were associated with risk of prostate cancer. To further investigate the role of *TLR4* in prostate cancer susceptibility, we identified six tagging single-nucleotide polymorphisms that comprehensively captured the common genetic variation of the locus and tested these polymorphisms in our case-control study of 1,012 men. Two single-nucleotide polymorphisms showed nominally statistically significant associations with prostate cancer risk, with the strongest (rs10759932) associated with a 4-fold increased risk of disease (P = 0.006). We estimated through permutation analysis that a similarly strong result would occur by chance 2.5% of the time. Our findings support previous studies and suggest that inherited differences in *TLR4* influence prostate cancer risk. (Cancer Epidemiol Biomarkers Prev 2007;16(2):352–5)

Introduction

Toll-like receptors (TLR) comprise a family of cell surface proteins that recognize a variety of pathogens, including bacteria, fungi, and viruses (1). TLRs are the primary molecular mechanism by which the host responds to invading microorganisms through recognition of conserved motifs termed pathogen-associated molecular patterns. The interaction of these motifs with the TLRs triggers a cascade of signaling events that stimulate the production of proinflammatory cytokines and chemokines (2, 3). *TLR4* encodes a major endotoxin signaling receptor that plays a fundamental role in pathogen recognition and activation of innate immunity. *TLR4* recognizes a wide array of ligands, including lipopolysaccharide, a cell wall component of Gram-negative bacteria, which in turn activates transcription factors, such as nuclear factor-κB, resulting in the induction of inflammatory genes, such as *tumor necrosis factor-α, interleukin-1, interleukin-6*, and *interleukin-8* (3).

Emerging evidence from association studies has shown that certain polymorphisms in TLRs are associated with increased susceptibility to infections and common diseases, such as atherosclerosis and cancer (4-9). Recently, two studies showed that variants in *TLR4* were associated with the risk of prostate cancer (7, 8). In one study, eight *TLR4* variants were evaluated in a Swedish population and the 11381G/C variant was positively associated with prostate cancer [odds ratio (OR), 1.26; P = 0.02; ref. 7]. In a second report, 16 *TLR4* variants were examined in the Health Professionals Follow-up Study (HPFS) and 8 variants showed an inverse association with prostate cancer (ORs, 0.38-0.73; P = 0.01-0.06), although the 11381G/C variant was not associated with disease (8).

In light of the strong biological support for a role for *TLR4* in carcinogenesis, and intriguing initial findings of others, we further evaluated the role of genetic variation in *TLR4* in prostate cancer susceptibility in a case-control study of advanced prostate cancers. In particular, we comprehensively examined the genetic diversity of *TLR4* and tested whether inherited differences at this locus influence the risk of prostate cancer.

Materials and Methods

Study Subjects. We recruited 506 advanced incident prostate cancer cases and 506 controls from the major medical institutions in Cleveland, Ohio (The Cleveland Clinic, University Hospitals of Cleveland, and their affiliates). Advanced prostate cancer cases were confirmed histologically and defined as having either a Gleason score of ≥7 or tumor-node-metastasis stage of $\geq T_{2c}$ or prostate-specific antigen at diagnosis of >10 ng/mL. Cases were contacted shortly following diagnosis (median time between diagnosis and recruitment, 4.7 months). Restricting the cases to men diagnosed with advanced disease allows us to focus on the most clinically relevant prostate cancers. To help ensure that the controls were representative of the source population of the cases, controls were men who underwent standard annual medical exams at the collaborating medical institutions. Controls had no diagnosis of prostate cancer or any other nonskin cancers. All controls received a prostate-specific antigen test to detect occult prostate cancer. Controls were frequency matched to cases by age (within 5 years), ethnicity, and medical institution. Detailed information on this case-control study has been reported previously (10).

Institutional Review Board approval was obtained from the participating medical institutions, and informed consent was obtained from all study participants. Note that these institutions serve the large majority of men diagnosed with prostate cancer in the Greater Cleveland area. Hence, although not formally population based, the cases are fairly representative of men diagnosed with prostate cancer in the Cleveland region.

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Haplotype Structure and Tag Single-Nucleotide Polymorphism Selection. We determined the haplotype structure of *TLR4* by using publicly available genotype data from the International HapMap project (11).[3] We downloaded the data for 51 single-nucleotide polymorphisms (SNP) genotyped in 120 chromosomes from a multigenerational panel of Centre d'Etude du Polymorphisme Humain Caucasian pedigrees that spanned ~2 kb upstream of the *TLR4* transcription start site and ~1 kb downstream of the 3′-untranslated region (UTR). Thirty-nine SNPs that either displayed poor genotyping results [genotyped <75% or failed to meet Hardy-Weinberg equilibrium ($P < 0.01$)] or were of minor allele frequencies (MAF) <5% (with the exception of the $Asp^{299}Gly$ missense SNP, rs4986790, MAF, 3%) were eliminated from subsequent analysis. The remaining 12 SNPs were used for haplotype characterization, having an average density of 1 SNP every 1.2 kb. We did not attempt to capture the haplotype variation of African populations because our sample size did not have sufficient power to conduct African-American–specific analyses.

We identified tag SNPs using the Tagger software[4] developed by de Bakker et al. (12). To capture all SNPs with MAF ≥5% among the Centre d'Etude du Polymorphisme Humain panel, tag SNPs were selected to construct single-marker and multimarker (haplotype) tests that had a minimum r^2 of >0.8 with unmeasured SNPs. We constrained tag SNPs for multimarker tests to be in strong linkage disequilibrium with each other (logarithm of odds score, >2.0). We ''forced in'' the $Asp^{299}Gly$ missense SNP (rs4986790) to be selected as a tag SNP to ensure that this potentially functional SNP was examined. The $Asp^{299}Gly$ missense SNP (rs4986790) is in perfect linkage disequilibrium ($r^2 = 1$) with the $Thr^{399}Ile$ missense SNP (rs4986791).[5]

Genotyping. Genotyping was done by the 5′ nuclease Taqman allelic discrimination assay using the manufacturer's predesigned primer/probe sets, and assays were read on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). All assays were undertaken by individuals blinded to case-control status. For quality control, 9% replicate samples were included. The concordance rate for replicate samples was 100%. The average genotyping success rate was 99.9%. Further details of genotyping methods are described elsewhere (10).

Statistical Analysis. We tested for Hardy-Weinberg equilibrium for each SNP among cases and controls of each racial/ethnic group. OR and 95% confidence intervals (95% CI) were estimated by unconditional logistic regression to examine the association between *TLR4* SNPs/multimarker haplotypes and prostate cancer risk. We estimated multimarker haplotype frequencies by the expectation-maximization algorithm using the tagSNP software (13). OR estimates were adjusted for the matching variables: age, racial/ethnic group, and medical institution. In addition, we adjusted for family history of prostate cancer; this did not materially alter our results, so we present those unadjusted for family history. All reported *P* values are two sided.

We conducted permutation testing to guide interpretation of nominally statistically significant associations. Case-control status within strata of age, racial/ethnic group, and medical institution was randomly permuted 10,000 times for the seven tests. Permutation *P* values were determined by examining where the nominal *P* value for an ''associated'' SNP fell in relation to the distribution of minimal *P* values generated from the permuted data. For example, if a nominal *P* value of 0.05 marked the 25th percentile of this distribution, then the permutation *P* value would be 0.25.

Results

Characteristics of the 506 prostate cancer cases and 506 controls are presented in Table 1. The mean age was similar for cases and controls, 65.7 and 65.6 years, respectively. Eighty-two percent of the study population was Caucasian and 18% was African-American. As expected, cases were more likely than controls to report a family history of prostate cancer (two or more first-degree relatives or one first-degree and two or more second-degree relatives; $P < 0.001$). Among cases, 84% had a Gleason score of >7 and 36% had tumor stage of $\geq T_2$.

The *TLR4* locus is characterized by a high degree of linkage disequilibrium and limited haplotype diversity (Supplementary Fig. S1), which is consistent with the prior study of *TLR4* sequence variants among Caucasians (8). The six tag SNPs used in this study captured all 12 common variants (MAF, ≥5%), with an r^2 of >0.8 (mean $r^2 = 0.95$; Table 2). All tag SNPs genotyped in the case-control study were in Hardy-Weinberg equilibrium within ethnic and disease status groups (at $P > 0.01$ level).

Table 3 presents the association between *TLR4* variants and prostate cancer risk. Men carrying the CC genotype for the rs10759932 SNP had a nominally statistically significant increased risk of prostate cancer (OR, 4.62; 95% CI, 1.55-13.78) in comparison with men carrying the TT genotype. Racial/ethnic stratified analysis revealed an overall consistent pattern of the CC genotype among Caucasians and African-Americans (although power was decreased due to smaller sample size; Supplementary Table S1). Carriers of the AA genotype of rs5030728 in comparison with GG carriers had a nominally significant association with decreased prostate cancer risk (OR, 0.60; 95% CI, 0.37-0.97), and a nonsignificant association was observed among AG carriers (OR, 0.91; 95% CI, 0.70-1.19). We observed no association between prostate cancer risk and the remaining four single-marker and one multimarker tests.

Table 1. Study characteristics of prostate cancer cases and controls

	Cases (n = 506)	Controls (n = 506)
Age (mean ± SD)	65.7 ± 8.2	65.6 ± 8.3
Ethnicity, *n* (%)		
Caucasian	417 (82)	417 (82)
African-American	89 (18)	89 (18)
Institution, *n* (%)		
Cleveland Clinic Foundation	407 (80)	407 (80)
University Hospitals, Cleveland	99 (20)	99 (20)
Family history of prostate cancer, *n* (%)		
Yes*	31 (6)	7 (1)
No	475 (94)	499 (99)
Gleason score, *n* (%)		
5	3 (1)	
6	79 (16)	
7	314 (62)	
8	67 (13)	
9	39 (8)	
10	4 (1)	
Tumor stage, *n* (%)		
T_1	305 (64)	
T_2	145 (30)	
T_3	30 (6)	
PSA, ng/mL (mean ± SD)	14.1 ± 27.4	1.7 ± 1.7

Abbreviation: PSA, prostate-specific antigen.
*Two or more first-degree relatives or one first-degree and two or more second-degree relatives.

[3] http://www.hapmap.org
[4] http://www.broad.mit.edu/tagger
[5] http://innateimmunity.net/

Table 2. Common SNPs across *TLR4* and correlation with tagging SNPs

Captured SNPs	Position*	Location	MAF	Tagging SNPs †	r^2
rs1927914	117544279	5′-UTR	0.31	rs2149356	0.89
rs10759932 ‡	117544698	5′-UTR	0.16	—	1
rs1927911	117549608	Intron	0.25	rs2149356	0.84
rs12377632	117552284	Intron	0.37	rs2149356, rs5030728	1
rs1927907	117552318	Intron	0.16	rs10759932	0.85
rs2770146	117552892	Intron	0.34	rs5030728	1
rs5030717	117553388	Intron	0.13	rs10759932	0.87
rs2149356 ‡	117553753	Intron	0.28	—	1
rs5030728 ‡	117553836	Intron	0.34	—	1
rs4986790 ‡	117554856	Exon	0.03	—	1
rs11536889 ‡	117557685	3′-UTR	0.13	—	1
rs7873784 ‡	117558490	3′-UTR	0.14	—	1

*SNP position based on May 2004 (University of California at Santa Cruz version human genome 17).
†Tagging SNP that predicts other captured SNP.
‡Tag SNP.

We conducted permutation testing to determine how often the strongest association would have occurred by chance by randomly permuting case-control status for the seven tests within strata of age, racial/ethnic group, and medical institution to obtain a null distribution of *P* values. The rs10759932 SNP displayed the smallest nominal *P* value (0.006), and similar levels of significance were observed in 2.5% of the simulated null data sets.

Discussion

In this report, we comprehensively examined the genetic diversity in *TLR4* and tested the previous hypothesis that inherited differences in *TLR4* were associated with prostate cancer risk. We confirmed the extensive linkage disequilibrium among common variants in the *TLR4* gene (8) and selected six tag SNPs that reconstructed all common variants at the *TLR4* locus. Our results suggest that inherited variation in *TLR4* influences prostate cancer risk. Specifically, we observed a positive association between a 5′-UTR polymorphism (rs10759932) and prostate cancer risk. Using permutation analyses to evaluate the possibility of a spurious effect, we found that a similarly strong effect would occur by chance only 2.5% of the time.

The initial Swedish study of *TLR4* reported an association between the 11381G/C variant (rs11536889) and prostate cancer, although no association with this polymorphism was observed in the HPFS (7, 8). Our study could not replicate an association with this polymorphism but corroborated the findings of the HPFS (7). The HPFS reported associations between eight *TLR4* variants and prostate cancer. We examined five polymorphisms that were tested in the HPFS (rs10759932, rs2149356, rs4986790, rs11536889, and rs7873784), two of which were associated with prostate cancer risk (rs10759932 and rs2149356) and only one (rs10759932) was associated with risk of prostate cancer in our study (CC genotype versus TT: OR, 4.62; 95% CI, 1.55-13.78). In contrast, the CC genotype of this polymorphism in the HPFS was not associated with prostate cancer (CC versus TT: OR, 0.84; 95% CI, 0.37-1.92), although the CT genotype was inversely associated with disease (CT versus TT: OR, 0.73; 95% CI, 0.57-0.93; ref. 8). The CC frequency of rs10759932 among controls in our study was similar to that of controls in HPFS (~1% versus 2%, respectively). We examined the linkage disequilibrium of three polymorphisms (rs11536889,

Table 3. Associations between *TLR4* variants and prostate cancer risk

SNP	IIPGA name	Genotype	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)*	*P*
rs10759932	TLR4_2856	TT	358 (70.9)	370 (73.1)	1.00	
		CT	143 (28.3)	117 (23.1)	0.80 (0.60-1.06)	0.111
		CC	4 (0.8)	19 (3.8)	4.62 (1.55-13.78)	0.006
rs2149356	TLR4_11912	GG	210 (41.6)	197 (39.0)	1.00	
		GT	213 (42.2)	223 (44.2)	1.12 (0.85-1.48)	0.415
		TT	82 (16.2)	85 (16.8)	1.12 (0.75-1.68)	0.575
rs5030728	TLR4_11995	GG	280 (55.3)	260 (51.5)	1.00	
		AG	194 (38.3)	196 (38.8)	0.91 (0.70-1.19)	0.504
		AA	32 (6.3)	49 (9.7)	0.60 (0.37-0.97)	0.037
rs4986790	TLR4_13015	AA	456 (90.1)	439 (86.8)	1.00	
		AG	48 (9.5)	66 (13.0)	1.43 (0.97-2.13)	0.075
		GG	2 (0.4)	1 (0.2)	0.52 (0.05-5.73)	0.591
rs11536889 †	TLR4_15844	GG	401 (79.2)	385 (76.1)	1.00	
		CG	93 (18.4)	105 (20.8)	1.18 (0.86-1.63)	0.296
		CC	12 (2.4)	16 (3.2)	1.40 (0.65-3.01)	0.389
rs7873784	TLR4_16649	GG	346 (68.4)	362 (71.7)	1.00	
		CG	146 (28.9)	130 (25.7)	0.85 (0.64-1.13)	0.256
		CC	14 (2.8)	13 (2.6)	0.89 (0.41-1.92)	0.759
Multimarker haplotype		Haplotype				
rs2149356, rs5030728: GG	—	2 copies	232 (45.8)	216 (42.7)	1.00	
		1 copy	209 (41.3)	220 (43.5)	1.14 (0.86-1.50)	0.358
		0 copy	65 (12.8)	70 (13.8)	1.18 (0.79-1.75)	0.421

Abbreviation: IIPGA, Innate Immunity Programs for Genomic Applications.
*Adjusted for age, ethnicity, and institution.
†11381G/C polymorphism.

rs10759932, and rs2149356) that were genotyped in our study and were associated with prostate cancer in the Swedish study, in the HPFS, and/or in our study. The variant alleles of these polymorphisms were dispersed across four common haplotypes, ranging in OR from 1.05 to 1.31 and P values of >0.07. The different haplotype backgrounds for these associated variants indicate that further localization of the causal variant is needed.

The discrepancies in findings may reflect heterogeneity in study populations, population stratification, and/or chance findings. With regard to heterogeneity, our study population consisted of only advanced prostate cancer in contrast to the other studies of predominately nonadvanced disease (7, 8). In addition, differences in the use of prostate-specific antigen testing among control subjects may contribute to differences in study populations. Heterogeneity in allele frequency is also possible, although the MAF for rs10759932 was relatively similar both in our study (African-Americans, 20%; Caucasians, 14%) and in the HPFS (Caucasians, 16%). Population stratification is also worth exploring as a potential source of the difference across studies. Given the higher incidence rate of prostate cancer among African-Americans, false-positive associations could occur if there was an overrepresentation of African-American alleles among cases compared with controls. By matching on, and controlling for ethnicity as well as medical institution source, our study should have limited potential for population stratification bias. Furthermore, our consistent findings across racial/ethnic groups argue against large-scale population stratification; of course, one cannot rule out mild stratification due to population substructure (14). Finally, the wide confidence interval in our estimate of the OR for rs10759932 indicates that chance could explain our findings. Our permutation analyses, which empirically assessed the robustness of nominally significant P values and corrected for multiple hypothesis testing (14), suggest that a similarly strong result would have occurred by chance only 2.5% of the time.

Due to the strong regional correlation across *TLR4*, rs10759932 may serve as a proxy for the predisposing variant or the polymorphism itself may have functional consequences on *TLR4* activity. We hypothesize that genetic variation in *TLR4* may affect *TLR4* expression or signaling activity. Alteration in *TLR4* activity influences innate immunity and inflammation, which in turn may affect prostate cancer susceptibility. Chronic intraprostatic inflammation is believed to play a role in prostate cancer susceptibility (15). Sexually transmitted diseases and prostatitis have been associated with prostate cancer risk (16, 17), and long-term use of nonsteroidal anti-inflammatory drugs seems to lower the risk of prostate cancer (18). Recently, a newly identified virus has been reported among prostate tumors of men carrying the variant of the antiviral gene *RNASEL* (19), further supporting the hypothesis that prostate cancer risk may be directly affected by variations in immune response to invading pathogens.

Our study has several limitations. We did not select tag SNPs to capture the genetic variation among Africans because of insufficient study power for African-American–specific analysis. Thus, we have not comprehensively evaluated the genetic variation of this group in relation to prostate cancer risk. Thorough evaluation of this locus among African-Americans is needed and may be useful in identifying the causal variant. In addition, our study cannot exclude the possibility that rare variants in *TLR4* may influence the risk of prostate cancer.

Our study suggests that common genetic variation in *TLR4* contributes to prostate cancer risk. This finding coupled with prior evidence of an association between *TLR4* and prostate cancer provides solid reasoning to further replicate this work in larger studies. Our work builds support for the role of innate immunity and inflammation in prostate cancer susceptibility. Additional research into the mechanisms by which inherited differences in innate immunity and inflammation genes influence the risk of disease will further our understanding of prostate cancer development.

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