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ORIGINAL PAPER

Prospective study of effect modification by *Toll-like receptor 4* variation on the association between *Trichomonas vaginalis* serostatus and prostate cancer

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Abstract
Purpose In previous studies, we observed a positive association between *Trichomonas vaginalis* serostatus and risk of prostate cancer, particularly aggressive cancer, which we hypothesized might be due to *T. vaginalis*-mediated intraprostatic inflammation and cell damage. To explore this hypothesis further, we investigated effect modification by *Toll-like receptor 4* (*TLR4*) variation on this association. We hypothesized that *TLR4* variation might serve a marker of the anti-trichomonad immune response because *T. vaginalis* has been shown to elicit inflammation through this receptor.
Methods We previously genotyped the non-synonymous *TLR4* single nucleotide polymorphism (SNP), rs4986790, and determined *T. vaginalis* serostatus for 690 incident prostate cancer cases and 692 controls in a nested case–control study within the Health Professionals Follow-up Study.
Results A non-significant suggestion of effect modification was observed by rs4986790 carrier status on the association between *T. vaginalis* serostatus and prostate cancer risk (p interaction = 0.07). While no association was observed among men homozygous wildtype for this SNP (odds ratio (OR) = 1.23, 95 % confidence interval (CI): 0.86–1.77), a positive association was observed among variant carriers (OR = 4.16, 95 % CI: 1.32–13.1).
Conclusions Although not statistically significant, *TLR4* variation appeared to influence the association between *T. vaginalis* serostatus and prostate cancer risk consistent with the hypothesis that inflammation plays a role in this

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association. Larger studies will be necessary to explore this possible effect modification further.

Keywords *Toll-like receptor 4* · *T. vaginalis* · Prostate cancer · SNP · Aspirin

Introduction

Trichomonas vaginalis is a common, sexually transmitted protozoan known to cause non-gonococcal urethritis, and occasionally clinical prostatitis, in a small proportion of infected men [1]. This sexually transmitted agent has been proposed as a possible cause of prostate cancer, primarily because of its ability to infect and elicit inflammation within the prostate, and because of its common occurrence [2–5]. We previously investigated possible associations between a history of *T. vaginalis* infection and prostate cancer by performing three epidemiologic studies, two of which observed a positive association between *T. vaginalis* serostatus and risk of prostate cancer, particularly high-grade [5] and advanced-stage/fatal disease [6], while the other observed no association between *T. vaginalis* serostatus and early-stage disease [7]. Collectively, these findings suggest that *T. vaginalis* may be involved in the development of aggressive prostate cancer.

Although we have not yet investigated underlying biologic mechanisms for our observed associations, some insight into these mechanisms may come from examination of variation in potentially relevant immune response genes. Recent evidence suggests that *T. vaginalis* may elicit inflammation, at least in part, through activation of the innate immune response receptor, Toll-like receptor 4 (TLR4). Cervico-lavage specimens from women infected with *T. vaginalis* were found to stimulate cytokine production by TLR4-responsive mouse splenic cells, but not TLR4-unresponsive cells [8]. Upregulated expression of *TLR4*, as well as *TLR2* and *TLR9*, was also observed in HeLa cells treated with *T. vaginalis* [9]. Therefore, we hypothesize that variation in human *TLR4* may alter signaling of the inflammatory immune response toward *T. vaginalis*, which may then, in turn, influence the risk of prostate cancer among infected men.

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Some evidence exists to suggest that variant carriers of the *TLR4* SNP, rs4986790 (also known as Asp299Gly or 896A/G), which results in an amino acid change from aspartic acid to glycine, have lower levels of circulating inflammatory markers, a lesser inflammatory response to infectious stimuli, and a greater likelihood of severe infections [10]. These findings suggest that variant carriers may have a lesser ability to fight infections. In other studies, rs4986790 variant carriers have also been found to have a lower risk of cardiovascular disease [11] and allograft rejection [10], possibly further suggesting that variant carriers have a lesser degree of inflammatory immune damage or sequelae. Considering these findings in the context of *T. vaginalis* infection and prostate cancer, they might imply either a greater risk of prostate cancer for rs4986790 variant carriers because of a possibly lesser ability to clear their infection or confine it to the urethra, or they might imply a lower risk of prostate cancer because of a possibly lesser degree of inflammatory immune damage if *T. vaginalis* ascends to the prostate. To explore these hypotheses further, we investigated effect modification by *TLR4* SNP, rs4986790, on the association between *T. vaginalis* serostatus and prostate cancer risk. To our knowledge, our study is the first such investigation.

Methods and materials

Study population

In 1986, 51,529 US men ages 40–75 years were recruited into the Health Professionals Follow-up Study (HPFS). At baseline, participants completed a mailed questionnaire on demographics, lifestyle, and medical history, as well as a semiquantitative food frequency questionnaire. Exposure and disease information was updated every other year, and diet information was updated every 4 years. Blood samples were obtained from 18,018 participants between 1993 and 1995. Information on participant deaths was obtained from family members in response to follow-up questionnaires, or from the National Death Index.

All men who provided a blood sample between 1993 and 1995, and who had no report of cancer, except non-melanoma skin cancer, as of their date of blood draw were eligible for inclusion in the nested case–control study. Prostate cancer cases were defined as men with a diagnosis of prostate cancer between their date of blood draw and 31 January 2000 ($n = 700$). For each reported case, investigators reviewed medical and pathology records or death certificates to confirm their diagnosis of adenocarcinoma of the prostate, and to obtain information on the clinical presentation, stage, and Gleason sum of their tumor. Men with incidental microscopic focal tumors (stage T1a) were not included in

the analyses because these tumors are generally indolent and may be susceptible to detection bias caused by differential rates of surgery for benign prostatic hyperplasia. Cases were categorized as organ-confined/minimal extraprostatic extension (T1b-T3a and N0M0) or regionally invasive/metastatic (stage ≥T3b, N1, or M1), and as low (Gleason sum <7) or high (Gleason sum ≥7) grade.

Controls ($n = 700$) were selected from among men who were alive and free of reported prostate and other cancers, except non-melanoma skin cancer, as of the date of the case diagnosis. To allow for detection of occult prostate cancer, controls were also required to have had a prostate-specific antigen (PSA) test after their date of blood draw, typically within 2.5 years of the date of diagnosis of their matched case. Cases and controls were matched on year of birth, PSA testing before blood draw (yes/no), and time, season, and exact year of blood draw.

Laboratory assays

Additional details of laboratory methods used for *TLR4* genotyping [12] and assessment of *T. vaginalis* serostatus [5] are described in prior publications. Briefly, we selected *TLR4* SNP, rs4986790, as a candidate SNP for genotyping because it is non-synonymous. This relatively uncommon SNP (~4 % in Caucasian populations) results in an amino acid change from aspartic acid to glycine. We also selected 20 common *TLR4* SNPs with minor allele frequencies >5 % in Caucasians listed in the innate immunity in heart, lung, and blood disease programs for genomic application database (http://www.pharmgat.org/IIPGA2/index_html). The methods and results for these SNPs, which are now considered to be tag SNPs, are described in the supplemental materials. We analyzed matched case–control pairs together using the Sequenom system and genotyped SNPs in three plexes at the Harvard Partners Center for Genetics and Genomics (Boston, MA). Genotyping data were missing in <5 % of study participants.

Trichomonas vaginalis serostatus was assessed by an enzyme-linked immunosorbent assay in the laboratory of Dr. Alderete. This assay detects IgG antibodies against purified, recombinant *T. vaginalis* α-actinin protein. Seropositivity was defined as an absorbance value ≥0.4 [5].

Statistical analysis

We investigated effect modification by *TLR4* variation on the association between *T. vaginalis* serostatus and prostate cancer risk by stratifying the analyses by rs4986790 carrier status (homozygous wildtype vs. variant carrier). Stratum-specific odds ratios (ORs) were calculated by logistic regression adjusting for the matching factor, age. We determined the statistical significance of differences in the stratum-specific ORs by comparing a model with main effect terms and an interaction term to a model including only main effect terms using the likelihood ratio test.

In our previous study of *T. vaginalis* serostatus and prostate cancer risk in this cohort [5], we observed a slightly stronger positive association for high- than low-grade prostate cancer. Therefore, in the present analysis, we performed additional separate analyses by Gleason sum (<7, ≥7). Very few men were diagnosed with advanced-stage disease to perform separate analyses by stage. Also, in our previous study in this cohort [5], we observed stronger, positive associations between *T. vaginalis* serostatus and prostate cancer risk among infrequent and moderate lifetime aspirin users than among regular users, who we hypothesized might have had an attenuated intraprostatic inflammatory response to infection. Therefore, we also evaluated the possible interaction between *TLR4* genotype and *T. vaginalis* serostatus on prostate cancer risk among infrequent/moderate lifetime aspirin users. We defined lifetime reported aspirin use as the percentage of time that aspirin was used with any frequency from age 20 to blood draw, and we defined infrequent users as those who had used aspirin 0–19 % of their lifetime and moderate users as those who had used aspirin 20–79 % of their lifetime.

Analyses were conducted using SAS release 9.1 (SAS Institute, Cary, NC). Statistical tests were two sided.

Results

Of the 700 prostate cancer cases and 700 matched controls included in our prior study of *TLR4* variation [12], 690 cases and 692 controls also had information on *T. vaginalis* serostatus (Table 1). In general, cases were similar to controls by most factors examined except for a family history of prostate cancer; cases were more likely to have a family history than controls. Among cases, the mean PSA concentration at diagnosis was 11.6 ng/mL, 72 % had stage T1b to T3a disease, and 34 % had a Gleason sum ≥7.

Considering effect modification by *TLR4* variation, a non-significant suggestion of effect modification was observed by rs4986790 carrier status on the association between *T. vaginalis* serostatus and prostate cancer risk ($p = 0.07$). While no association was observed among men homozygous wildtype for this SNP (OR = 1.23, 95 % CI: 0.86–1.77), a positive association was observed among variant carriers (OR = 4.16, 95 % CI: 1.32–13.1). Similar patterns of association were observed for high- and low-grade prostate cancer. When the analyses were restricted to infrequent/moderate aspirin users, a slightly stronger, albeit less stable, association was observed among variant carriers and a null association was observed among wildtype homozygotes (Table 2).

Table 1 Baseline characteristics of prostate cancer cases and controls, Health Professionals Follow-Up Study, 1993

	Cases ($n = 690$)	Controls ($n = 692$)
Age at blood draw (years, mean ± SD)[a]	65.7 ± 7.4	65.7 ± 7.4
Family history of prostate cancer (%)	20	14
Body mass index (kg/m^2, %)		
<25	62	60
25–29	33	33
≥30	5	7
Cigarette smoking status (%)		
Never smoker	44	44
Former smoker	46	48
Current smoker	6	5
Missing	4	3
Alcohol intake (g/day, mean ± SD)	11.4 ± 14.9	10.3 ± 14.7
Prostate-specific antigen concentration (ng/mL, mean ± SD)[b]	11.6 ± 21	Not applicable
Stage (%)[b]		
T1b–T3a	72	Not applicable
T3b, T4, N1, M1, or prostate cancer death	14	
Missing	14	
Gleason sum (%)[b]		
<7	58	Not applicable
≥7	34	
Missing	8	

SD standard deviation

[a] Matching factor

[b] $n = 501$

Table 2 Stratified analyses of the association between *Trichomonas vaginalis* serostatus and prostate cancer risk by *Toll-like receptor 4* (*TLR4*) SNP rs4986790 carrier status, Health Professionals Follow-up Study, 1993–2000

	T. vaginalis serostatus				$p_{interaction}$
	Seronegative		Seropositive		
	Case/control	OR (ref)	Case/control	OR (95 % CI)	
Total prostate cancer					
Homozygous wildtype (A)	507/539	1.0	71/61	1.23 (0.86–1.77)	0.07
Variant carrier (G)	96/88	1.0	16/4	4.16 (1.32–13.1)	
Low-grade prostate cancer (*Gleason sum* <7)					
Homozygous wildtype (A)	272/531	1.0	38/59	1.26 (0.82–1.94)	0.13
Variant carrier (G)	60/86	1.0	9/4	3.23 (0.95–10.96)	
High-grade prostate cancer (*Gleason sum* ≥7)					
Homozygous wildtype (A)	184/531	1.00	28/59	1.37 (0.85–2.21)	0.15
Variant carrier (G)	27/86	1.00	5/4	3.98 (1.00–15.9)	
Only among infrequent/moderate lifetime aspirin users					
Homozygous wildtype (A)	311/328	1.00	48/37	1.36 (0.86–2.15)	0.05
Variant carrier (G)	58/52	1.00	13/2	6.63 (1.39–31.7)	

CI confidence interval; *OR* odds ratio; *SNP* single nucleotide polymorphism

Discussion

In this large nested case–control study of US male health professionals, we observed a non-statistically significant suggestion of effect modification by *TLR4* variation on the association between *T. vaginalis* serostatus and prostate cancer risk. Specifically, we found that *T. vaginalis* serostatus was positively associated with prostate cancer risk

among variant carriers of the non-synonymous *TLR4* SNP, rs4986790, but not among wildtype homozygotes.

As some evidence exists to suggest that rs4986790 variant carriers mount a weaker immune response to infectious stimuli and have more severe infections than wildtype homozygotes [10], one possible interpretation of our findings is that variant carriers may mount a less effective innate immune response against *T. vaginalis* than wildtype homozygotes and thus may be more likely to develop persistent *T. vaginalis* infections that ultimately ascend to the prostate. Low-grade anti-*T. vaginalis* intraprostatic inflammation or prostatic *T. vaginalis* infections themselves may then raise carriers' risk of prostate cancer, resulting in a positive association between *T. vaginalis* serostatus and prostate cancer risk. In contrast, men homozygous wildtype for SNP rs4986790 may mount a more effective immune response against *T. vaginalis* than variant carriers, and thus may be more likely to clear their infection and prevent it from ascending to the prostate. This hypothesized lesser likelihood of prostate infection may then reduce their risk of *T. vaginalis*-mediated prostate cancer, leading to a null association between *T. vaginalis* serostatus and risk among homozygotes. This interpretation is, however, speculative because studies have not consistently observed a weaker immune response in rs4986790 variant carriers [10].

As an additional consideration for interpreting study findings, our analysis relied on two surrogate markers (i.e., *T. vaginalis* serostatus and *TLR4* variation) to capture a number of different factors, including the likelihood of *T. vaginalis* prostatic involvement, *T. vaginalis*-mediated cell damage or other mechanisms related to carcinogenesis, and anti-trichomonad inflammation-mediated cell damage. In the future studies, it would also be useful to know additional characteristics about both participants' *T. vaginalis* infection history and their anti-*T. vaginalis* immune response. For instance, with respect to *T. vaginalis* infection history, it might also be important to know whether or not the infecting isolate was itself infected with double-stranded RNA (dsRNA) viruses and/or small-sized satellite dsRNAs [13, 14]; the iron status of trichomonads at the site of infection [15–18]; the availability of host spermine for trichomonad polyamine metabolism [19]; trichomonad production of immunoglobulin-degrading cysteine proteinases [18, 20]; and trichomonad ability to colonize the basement membrane [21], all of which have been hypothesized to contribute to *T. vaginalis* persistence and thus to possible prostate involvement, or to *T. vaginalis* virulence. However, no methods currently exist, to our knowledge, to investigate these aspects of *T. vaginalis* infection history in large population-based studies.

With respect to *TLR4* genotype as a surrogate of the anti-trichomonad immune response, although *T. vaginalis* has been shown to stimulate inflammatory cytokine production through TLR4 [8], other immune receptors are also undoubtedly involved, as well as other possible mechanisms of *T. vaginalis*-mediated inflammation, such as tissue irritation by cysteine proteinases [22]. The specific immunogens or byproducts of trichomonad infection that interact with TLR4 or increase its expression are also largely unknown. For instance, although the prominent trichomonad adhesin, AP65, an anchorless surface-associated protein, is known to signal upregulated expression of numerous inflammation-associated host epithelial cell proteins, such as interleukin 8 and cyclooxygenase-2 [23], whether this interaction involves TLR4 has not been investigated. Surface expression or secretion of TLR4-binding trichomonad immunogens or byproducts may also possibly vary according to parasite and host factors (e.g., iron status), as well as in response to host cell contact [24]. Nevertheless, despite the complexity of the host-parasite interrelation and the use of surrogate markers to capture this complexity, interesting, suggestive effect modification was observed in the present analysis supportive of a role for inflammation in the association between *T. vaginalis* infection and prostate carcinogenesis.

As a final consideration, in our previous analysis of *TLR4* variation and prostate cancer risk in the HPFS, we observed no association between rs4986790 carrier status and prostate cancer risk, consistent with other studies of *TLR4* variation and PCa [25–30]. However, this lack of association does not preclude effect modification by rs4986790 carrier status because *T. vaginalis* seroprevalence is relatively low; therefore, it is unlikely that interactions between *TLR4* genotype and *T. vaginalis* serostatus would influence marginal genetic associations to a notable degree. Moreover, it is also possible that other infections or factors might interact with *TLR4* genotype to influence prostate cancer risk; therefore, marginal genetic associations might reflect interactions with several possible factors, including *T. vaginalis* infection.

In summary, while many studies will be necessary to unravel the possible biologic mechanisms underlying observed positive associations between *T. vaginalis* serostatus and prostate cancer risk [5, 6], our novel study of the influence of *TLR4* genotype on the association between *T. vaginalis* serostatus and prostate cancer risk suggests that inflammation may play a role.

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