PLOS ONE

# Polymorphisms of an Innate Immune Gene, Toll-Like Receptor 4, and Aggressive Prostate Cancer Risk: A Systematic Review and Meta-analysis

--Manuscript Draft--

| | |
|---|---|
| **Manuscript Number:** | PONE-D-14-21052R1 |
| **Article Type:** | Research Article |
| **Full Title:** | Polymorphisms of an Innate Immune Gene, Toll-Like Receptor 4, and Aggressive Prostate Cancer Risk: A Systematic Review and Meta-analysis |
| **Short Title:** | TLR4 polymorphisms and aggressive prostate cancer |
| **Corresponding Author:** | Yen-Ching Chen, ScD<br>Institute of Epidemiology and Preventive Medicine<br>Taipei, NA TAIWAN |
| **Keywords:** | TLR4 polymorphisms; aggressive prostate cancer; meta-analysis |
| **Abstract:** | Background: Toll-like receptor 4 (TLR4) is one of the best known TLR members expressed on the surface of several leukocytes and tissue cells and has a key function in detecting pathogen and danger-associated molecular patterns. The role of TLR4 in the pathophysiology of several age-related diseases is also well recognized, such as prostate cancer (PCa). TLR4 polymorphisms have been related to PCa risk, but the relationship between TLR4 genotypes and aggressive PCa risk has not been evaluated by any systematic reviews.<br>Methods: We performed a systematic review and meta-analysis of candidate-gene and genome-wide association studies analyzing this relationship and included only white population. Considering appropriate criteria, only nine studies were analyzed in the meta-analysis, including 3,937 aggressive PCa and 7,382 controls.<br>Results: Using random effects model, no significant association was found in the ten TLR4 SNPs reported by at least four included studies under any inheritance model (rs2737191, rs1927914, rs10759932, rs1927911, rs11536879, rs2149356, rs4986790, rs11536889, rs7873784, and rs1554973). Pooled estimates from another ten TLR4 SNPs reported by three studies also showed no significant association (rs10759930, rs10116253, rs11536869, rs5030717, rs4986791, rs11536897, rs1927906, rs913930, rs1927905, and rs7045953). Meta-regression revealed that study type was not a significant source of between-study heterogeneity.<br>Conclusions: TLR4 polymorphisms were not significantly associated with the risk of aggressive PCa. |
| **Order of Authors:** | Pei-Hsuan Weng |
| | Yi-Ling Huang |
| | John H. Page |
| | Jen-Hau Chen |
| | Jianfeng Xu |
| | Stella Koutros |
| | Sonja Berndt |
| | Stephen Chanock |
| | Meredith Yeager |
| | John S. Witte |
| | Rosalind A. Eeles |
| | Douglas F. Easton |
| | David E. Neal |

| | |
|---|---|
| | Jenny Donovan |
| | Freddie C. Hamdy |
| | Kenneth R. Muir |
| | Graham Giles |
| | Gianluca Severi |
| | Jeffrey R. Smith |
| | Carmela R. Balistreri |
| | Irene M. Shui |
| | Yen-Ching Chen, ScD |
| **Suggested Reviewers:** | Shuangyue Zhang<br>Central Lab, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, China<br>mahongxia927@gmail.com<br>author of a similar meta-analysis |
| | Jing-Jing Jing<br>Tumor Etiology and Screening Department of Cancer Institute and General Surgery, The First Affiliated Hospital of China Medical University<br>yyuan@mail.cmu.edu.cn<br>author of a similar meta-analysis |
| **Opposed Reviewers:** | none none<br>none<br><br>none |
| **Response to Reviewers:** | PONE-D-14-21052<br>Reviewer #1<br>1.Is the manuscript technically sound, and do the data support the conclusions?<br>Reviewer #1: Yes<br><br>2.Has the statistical analysis been performed appropriately and rigorously?<br>Reviewer #1: Yes<br><br>3.Does the manuscript adhere to the PLOS Data Policy?<br>Reviewer #1: Yes<br><br>4.Is the manuscript presented in an intelligible fashion and written in standard English?<br>Reviewer #1: Yes<br><br>5.Review Comments to the Author<br>Reviewer #1: The authors have adequately addressed the concerns raised in the initial review. But I would like to suggest the authors to label and mark the revisions in the revised version of the paper next time to make it more readable. In general, the manuscript was well written, and there are only a few technical points that need to be addressed.<br>1.Figures 1 and 3 are illegible, and Figure3 is too busy and irregular, it should be more standardized when combining multiple forest plots for a better presentation.<br>Response: Thank you for reviewer's suggestion. We have provided a manuscript with track changes this time. Figure 1 and Figure 3 were revised. The figure resolution was increased and the page layout was improved.<br><br>2.In Figure 4, TLR4 should be marked.<br>Response: TLR4 was added in the revised Figure 4.<br><br>3.Supplemental figures and tables are not available.<br>Response: We have uploaded the supplementary figures and tables during submission. The files are available online.<br><br>4.A full description of the study characteristics has been showed in the table, such as |

the population, exposure, genotyping methods, comparison group, and outcome, etc. If you do not conduct stratification by such factors, I think it's important to explain the reasons in detail in Discussion section.
Response: Thank you for reviewer’s suggestion. Type of study (candidate gene studies vs. GWASs) was the pre-specified covariate for meta-regression in this analysis. Most of the other study characteristics were very similar across the nine studies (e.g. publication year, ancestry, definition of aggressive prostate cancer). The major between-study differences were the methods of control selection and case selection. For example, using prevalent PCa cases or hospital-based design might introduce selection bias. However, stratification analysis was not performed because the influences of such biases are complex and usually not unidirectional. Another between-study difference is the different genotyping platforms. We did not perform stratification analysis according to this covariate because previous studies revealed high concordance rate across different genotyping platforms [1]. The method part was revised to address this issue.

Reviewers' comments:
Reviewer #2
1.Is the manuscript technically sound, and do the data support the conclusions?
Reviewer #2: Partly

2.Has the statistical analysis been performed appropriately and rigorously?
Reviewer #2: N/A

3.Does the manuscript adhere to the PLOS Data Policy?
Reviewer #2: Yes

4.Is the manuscript presented in an intelligible fashion and written in standard English?
Reviewer #2: Yes

5.Review Comments to the Author
Reviewer #2: This article aims to quantify the relationship between TLR4 and the risk of aggressive PCa by meta-analysis of all genetic epidemiologic studies in the literature. The results indicate that none of the examined TLR4 SNPs was associated with risk of aggressive PCa under any inheritance model.
This article is well organized and the methods they employed are appropriate. Most of the concerns from reviewers have been addressed.

The authors attempted to state that there is no significant association via nine studies, my concern is that whether these nine studies are sufficient to draw this conclusion or not. The authors pick out 31 studies from 40 in the literature, and only kept 9 studies. Although some external condition exists, it seems that some information has been lost. Do the authors consider to search over the unpublished results and put them in?

Response: Thank you for reviewer’s advice. To retrieve unpublished studies, we additionally searched the Scopus database. We also searched the abstract books or websites of three major conference proceedings in this field: American Urological Association (AUA), European Association of Urology (EAU), and the Societe Internationale D'Urologie (SIU). However, no new studies met the inclusion criteria. We did not include the process of searching unpublished studies in this meta-analysis because of the questionable validity of grey literature.
In this meta-analysis, publication bias was assessed by Funnel plots and the Egger linear regression test. We found no obvious publication bias among the included studies except a borderline significant Egger test P value of 0.06 in rs1554973. Therefore, there is little evidence that the results are biased by excluding unpublished studies.

Reviewer #2: More, it seems there still exist some typos in the paper. I would suggest the authors to carefully read the paper again before next submission.
Response: Thank you for reviewer’s suggestion. We have proof read the manuscript and corrected these mistakes carefully.

References
1.Hong H, Xu L, Liu J, Jones WD, Su Z, et al. Technical reproducibility of genotyping

| | |
|---|---|
| | SNP arrays used in genome-wide association studies. PLoS One 7: e44483. |
| **Additional Information:** | |
| **Question** | **Response** |
| **Financial Disclosure**<br>Please describe all sources of funding that have supported your work. A complete funding statement should do the following:<br>Include **grant numbers and the URLs** of any funder's website. Use the full name, not acronyms, of funding institutions, and use initials to identify authors who received the funding.<br>**Describe the role** of any sponsors or funders in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. If they had no role in any of the above, include this sentence at the end of your statement: "*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*"<br>If the study was **unfunded**, provide a statement that clearly indicates this, for example: "*The author(s) received no specific funding for this work.*"<br>* typeset | The authors received no specific funding for this work |
| **Competing Interests**<br>You are responsible for recognizing and disclosing on behalf of all authors any competing interest that could be perceived to bias their work, acknowledging all financial support and any other relevant financial or non-financial competing interests.<br>Do any authors of this manuscript have competing interests (as described in the PLOS Policy on Declaration and Evaluation of Competing Interests)?<br>**If yes**, please provide details about any and all competing interests in the box below. Your response should begin with this statement: *I have read the journal's* | The authors have declared that no competing interests exist |

| | |
|---|---|
| *policy and the authors of this manuscript have the following competing interests:*<br>**If no** authors have any competing interests to declare, please enter this statement in the box: "*The authors have declared that no competing interests exist.*"<br>* typeset | |
| **Ethics Statement**<br>You must provide an ethics statement if your study involved human participants, specimens or tissue samples, or vertebrate animals, embryos or tissues. All information entered here should **also be included in the Methods section** of your manuscript. Please write "N/A" if your study does not require an ethics statement.<br>**Human Subject Research (involved human participants and/or tissue)**<br>All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or an equivalent committee, and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. Informed consent, written or oral, should also have been obtained from the participants. If no consent was given, the reason must be explained (e.g. the data were analyzed anonymously) and reported. The form of consent (written/oral), or reason for lack of consent, should be indicated in the Methods section of your manuscript.<br>Please enter the name of the IRB or Ethics Committee that approved this study in the space below. Include the approval number and/or a statement indicating approval of this research.<br>**Animal Research (involved vertebrate animals, embryos or tissues)**<br>All animal work must have been conducted according to relevant national and international guidelines. If your study | The execution of each individual study was previously approved by the respective institution. This systematic review was performed at the study level without access to individual-level data, and therefore, institutional review board approval was not necessary. Informed consent was obtained from each participant before the start of each individual study. |

| | |
|---|---|
| involved non-human primates, you must provide details regarding animal welfare and steps taken to ameliorate suffering; this is in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research." The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement.<br>If anesthesia, euthanasia or any kind of animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied.<br>Please enter the name of your Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained.<br>**Field Permit**<br>Please indicate the name of the institution or the relevant body that granted permission. | |
| **Data Availability**<br>PLOS journals require authors to make all data underlying the findings described in their manuscript fully available, without restriction and from the time of publication, with only rare exceptions to address legal and ethical concerns (see the PLOS Data Policy and FAQ for further details). When submitting a manuscript, authors must provide a Data Availability Statement that describes where the data underlying their manuscript can be found.<br>Your answers to the following constitute your statement about data availability and will be included with the article in the event of publication. **Please note that simply stating 'data available on request from the author' is not acceptable.** ***If, however, your data are only available upon request from the author(s), you must answer "No" to the first question below, and explain your exceptional situation in the text box provided.***<br>Do the authors confirm that all data underlying the findings described in their | No - some restrictions will apply |

| | |
|---|---|
| manuscript are fully available without restriction? | |
| Please describe where your data may be found, writing in full sentences. **Your answers should be entered into the box below and will be published in the form you provide them, if your manuscript is accepted.** If you are copying our sample text below, please ensure you replace any instances of XXX with the appropriate details.<br>If your data are all contained within the paper and/or Supporting Information files, please state this in your answer below. For example, “All relevant data are within the paper and its Supporting Information files.”<br>If your data are held or will be held in a public repository, include URLs, accession numbers or DOIs. For example, “All XXX files are available from the XXX database (accession number(s) XXX, XXX)." If this information will only be available after acceptance, please indicate this by ticking the box below.<br>If neither of these applies but you are able to provide details of access elsewhere, with or without limitations, please do so in the box below. For example:<br>“Data are available from the XXX Institutional Data Access / Ethics Committee for researchers who meet the criteria for access to confidential data.”<br>“Data are from the XXX study whose authors may be contacted at XXX.”<br>* typeset | Some of the details (i.e.: frequencies of variant carriers in cases and controls) used for conducting this meta-analysis was obtained by E-mail request to the original authors of each individual studies. We cannot distribute these original data without their agreement.Readers may contact the corresponding authors of each original studies to request the original data. |
| Additional data availability information: | |

August 29, 2014

Damian Pattinson, PhD
Editorial director
PLoS ONE
1160 Battery Street,
Koshland Building East, Suite 100
San Francisco, CA 94111
United States

**Re:** Response to reviewers' comments
PONE-D-14-21052: Polymorphisms of an Innate Immune Gene, Toll-Like Receptor 4, and Aggressive Prostate Cancer Risk: A Systematic Review and Meta-analysis

Dear Dr. Pattinson:

Attached please find our response to reviewers' comments on the manuscript PONE-D-14-21052 'Polymorphisms of an Innate Immune Gene, Toll-Like Receptor 4, and Aggressive Prostate Cancer Risk: A Systematic Review and Meta-analysis', which was submitted to PLoS ONE.

In the previous decision letter, the editor asked for amended statements of Competing Interests and Financial disclosure because one co-author was employed by a company "SAIC-Frederick, Inc.". We have confirmed the statement of competing interests with the co-author. SAIC-Frederick is not a commercial company. It is a sole-customer government contract for the US National Cancer Institute. They are instructed to fill out all forms as NIH researchers and are in turn restricted by the same rules as US government employees. Therefore, all the authors declared that no competing interests exist.

The editor also asked for explanations about the statement "data are available upon request". Because some of the details (i.e.: frequencies of variant carriers in cases and controls) used for conducting this meta-analysis was obtained by E-mail request to the original authors of each individual studies. We cannot distribute these original data without their agreement. Readers may contact the corresponding authors of each original studies to request the original data.

We have responded to the reviewers’ comments in detail. We look forward to your decision. Please let us know if we can provide any further information or details of the study.

Sincerely,

Yen-Ching (Karen) Chen, ScD
Associate Director
Program of Master of Public Health
Associate Professor
Institute of Epidemiology and Preventive Medicine
Department of Public Health
Research Center for Genes, Environment, and Human Health
National Taiwan University
No.17, Xu-Zhou Road, Taipei 10055, Taiwan
E-mail: karenchen@ntu.edu.tw
chenkaren@post.harvard.edu

# Polymorphisms of an Innate Immune Gene, Toll-Like Receptor 4, and Aggressive Prostate Cancer Risk: A Systematic Review and Meta-analysis

**Pei-Hsuan Weng[1,2]*, Yi-Ling Huang[3]*, John H. Page[4], Jen-Hau Chen[2,5], Jianfeng Xu[6], Stella Koutros[7], Sonja Berndt[7], Stephen Chanock[7], Meredith Yeager[8,9], John S. Witte[10], Rosalind A. Eeles[11], Douglas F. Easton[12], David E. Neal[13], Jenny Donovan[14], Freddie C. Hamdy[15], Kenneth R. Muir[16], Graham Giles[17], Gianluca Severi[17], Jeffrey R. Smith[18], Carmela R. Balistreri[19] , Irene M. Shui[20], Yen-Ching Chen[2,21,22]**

**1** Department of Family Medicine, Taiwan Adventist Hospital, Taipei, Taiwan;
**2** Institute of Epidemiology and Preventive Medicine, College of Public Health, National Taiwan University, Taipei, Taiwan;
**3** Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging, Tufts University, Boston MA, USA;
**4** Channing Laboratory, Department of Epidemiology, Brigham and Women's Hospital, Boston MA, USA;
5 Department of Geriatrics and Gerontology, National Taiwan University Hospital, Taipei, Taiwan;
**6** Center for Human Genomics, Wake Forest University School of Medicine, Winston-Salem, NC, USA;
**7** Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Bethesda, Maryland, USA;
**8** Core Genotyping Facility, SAIC-Frederick, Inc., NCI-Frederick, Frederick, Maryland, USA;
**9** Division of Cancer Epidemiology and Genetics, NCI, NIH, DHHS, Bethesda, Maryland, USA;
**10** Department of Epidemiology and Biostatistics and Center of Human Genetics, University of California, San Francisco, California, USA;
**11** The Institute of Cancer Research, Sutton, UK;
**12** Centre for Cancer Epidemiology, Departments of Public Health and Primary Care and Oncology, University of Cambridge, Strangeways Laboratory, Worts Causeway, Cambridge, CB1 8RN, UK;
**13** Surgical Oncology (Uro-Oncology: S4), Departments of Oncology and Surgery, University of Cambridge, Box 279, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2QQ, UK;
**14** Department of Social Medicine, University of Bristol, Canynge Hall, Whiteladies Road, Bristol, UK;
**15** Academic Urology Unit, University of Sheffield, Sheffield, S10 2JF, UK;
**16** University of Nottingham Medical School, Queens Medical Centre, Nottingham, NG7 2UH, UK;
**17** Cancer Epidemiology Centre, The Cancer Council Victoria, 1 Rathdowne Street, Carlton VIC 3053, Australia;
**18** Department of Medicine, Vanderbil University School of Medicine, Nashville, Tennessee, USA;
**19** Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Palermo, Italy;
**20** Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts, USA;
**21** Research Center for Genes, Environment and Human Health, College of Public Health, National Taiwan University, Taipei, Taiwan;
**22** Department of Public Health, College of Public Health, National Taiwan University, Taipei, Taiwan.

*First two authors contributed equally to this work.

**Correspondence:**

Prof. Yen-Ching Chen

Institute of Epidemiology and Preventive Medicine
Department of Public Health
National Taiwan University
No.17, Xu-Zhou Road,
Taipei 10055,
Taiwan
Telephone: 886-2-3366-8019
Fax: 886-2-2351-1955
E-mail: karenchen@ntu.edu.tw

## Abstract

***Background:*** Toll-like receptor 4 (TLR4) is one of the best known TLR members expressed on the surface of several leukocytes and tissue cells and has a key function in detecting pathogen and danger-associated molecular patterns. The role of TLR4 in the pathophysiology of several age-related diseases is also well recognized, such as prostate cancer (PCa). *TLR4* polymorphisms have been related to PCa risk, but the relationship between *TLR4* genotypes and aggressive PCa risk has not been evaluated by any systematic reviews.

***Methods:*** We performed a systematic review and meta-analysis of candidate-gene and genome-wide association studies analyzing this relationship and included only white population. Considering appropriate criteria, only nine studies were analyzed in the meta-analysis, including 3,937 aggressive PCa and 7,382 controls.

***Results:*** Using random effects model, no significant association was found in the ten *TLR4* SNPs reported by at least four included studies under any inheritance model (rs2737191, rs1927914, rs10759932, rs1927911, rs11536879, rs2149356, rs4986790, rs11536889, rs7873784, and rs1554973). Pooled estimates from another ten *TLR4* SNPs reported by three studies also showed no significant association (rs10759930, rs10116253, rs11536869, rs5030717, rs4986791, rs11536897, rs1927906, rs913930, rs1927905, and rs7045953). Meta-regression revealed that study type was not a significant source of between-study heterogeneity.

***Conclusions***: *TLR4* polymorphisms were not significantly associated with the risk of aggressive PCa.

## Introduction

Prostate cancer (PCa) is the most common malignancy since 1984, the most frequently diagnosed cancer, and the second leading cause of cancer-related deaths in 2013 among men in the USA [1]. The risk of PCa is related to family history, race, and genetic factors. Several other causes have been associated with PCa pathogenesis, including infectious agents, chronic non-infectious inflammatory diseases, diet, environmental carcinogens, imbalance of sex hormone, obesity, and urine reflux [2-4]. Chronic inflammation has been linked to the pathogenesis of PCa in both epidemiologic studies and molecular pathology investigations [5,6]. In particular, several studies have suggested that sexually transmitted infections may be a risk factor for PCa through causing inflammation, even though not all the studies are consistent [7,8]. Chronic inflammation seems to induce prostate carcinogenesis and also promote neoplastic progression [9]. Furthermore, several pathways linking inflammation and PCa have been identified: an intrinsic one driven by genetic events that cause neoplasia, and an extrinsic one driven by inflammatory conditions that predispose to cancer [9]. Among these, the eicosanoid pathway activated by cyclooxygenase 2 (COX-2) has been suggested to be involved in the pathogenesis of aggressive PCa by a recent study [10]. COX-2 was over-expressed in PCa tumors and the intensity of immunostaining was correlated with prostate tumor grade [11]. Despite the available evidence on the role of the inflammatory response in PCa onset and progression, the association between genetic variants of innate immune genes and the risk of aggressive PCa remains unclear.

Toll-like receptor 4 (TLR4) is an important pathogen recognition receptor involved in detection of lipopolysaccharide (LPS) of Gram-negative bacteria and other exogenous or endogenous ligands [12]. The TLR4 encoding gene is located on chromosome 9q32-q33. Through nuclear factor kappa B (NF-κB), TLR4 initiates the production of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNF-α) [13]. TLR4 also mediates signaling related to tumor cell invasion, survival, and metastasis in various cancers [14,15]. Its activity and function seems to be modulated by genetic variations, principally single nucleotide polymorphisms (SNPs). Mice with deficiency or mutation of TLR4 had a weaker inflammatory immune response to viral, bacterial [16,17], and protozoal [18] infections than that of wild-type mice. Therefore, variations in *TLR4* gene may modify the signaling of the immune response, which in turn may have effects on the pathogenesis of PCa.

Three recent meta-analyses have explored the association between *TLR4* SNPs and PCa [19-21]. They all reported non-significant findings after stratification by ethnicity. However, these studies focused their attention on overall PCa and did not contain genome-wide association studies (GWASs). In addition, they did not analyze the association between *TLR4* SNPs and the aggressive type of PCa. Thus, we conducted a systematic review and meta-analysis of all genetic epidemiologic association studies that have evaluated the relationship between *TLR4* polymorphisms and risk of aggressive PCa. Both candidate-gene studies and GWASs were included. The primary research questions are: (1) is there an association between *TLR4* SNPs and risk of aggressive PCa and if so, what is the size of the relationship? (2) what is the validity of the evidence of association between *TLR4* polymorphisms and risk of aggressive PCa?

## Materials and Methods

### *Ethics Statement*

The execution of each individual study was previously approved by the respective institution. This systematic review was performed at the study level without access to individual-level data, and therefore, institutional review board approval was not necessary. Informed consent was obtained from each participant before the start of each individual study.

### *Study Selection*

The study was performed using pre-specified research objectives, search strategy, study eligibility criteria, methods of data extraction, and statistical analyses. Relevant studies were identified by searching the MEDLINE (http://gateway.ovid.com/), EMBASE (http://www.embase.com), Science Citation Index (http://science.thomsonreuters.com/cgi-bin/jrnlst/jlsearch.cgi?PC=K), and Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/omim) databases for all genetic association studies published before February 2013, using combinations of the search terms "toll-like receptor 4," OR "toll-like receptor 4 gene," OR "*TLR,"* OR *"TLR* gene," OR "*TLR4,"* OR *"TLR4* gene," AND "prostate cancer," OR "prostatic neoplasms." GWASs were searched using combinations of the search terms "genome-wide association study," OR "GWAS," AND "prostate cancer," OR "prostatic neoplasms." In addition, we manually searched the reference lists from reviews and original articles to retrieve other papers relevant to the topic. Where there was overlap in the study populations of published papers, only the largest study was included. No language restriction was placed on the literature search strategies. Unpublished findings were not identified.

### *Exposure Measures*

The main exposure variables were *TLR4* genotypes as measured in blood DNA samples from men in the respective studies. This meta-analysis summarized *TLR4* SNPs which were reported by at least three included studies. Because many *TLR4* SNPs were explored by two studies only, and the respective sample sizes were small, these SNPs were not analyzed in this meta-analysis.

### ***Outcome Measures***

The outcome measure was aggressive PCa as defined by Gleason score greater than or equal to seven, or TNM stage greater than or equal to T3b or any nodal involvement or any distant metastases. However, some included studies extended this definition. Controls for aggressive PCa are ideally men without aggressive PCa chosen from the population at risk, although some studies selected controls from men without screening for occult PCa (Table 1).

### ***Data Extraction***

Three of us (PH Weng, YL Huang, and YC Chen) independently reviewed each published paper and extracted relevant information examining the associations between *TLR4* polymorphisms and risk of aggressive PCa. Inter-observer differences, if any existed, were reconciled through group discussion. In order to pool data from different studies, we requested data from each study based on the definition for aggressive PCa in this meta-analysis, which may be slightly different from their original design. For GWASs that did not report detailed information of *TLR4*, we contacted the investigators to obtain data on advanced PCa counts and the corresponding *TLR4* genotyping frequencies. To avoid population stratification, this meta-analysis was restricted to samples taken from European ancestry. We evaluated selection bias based on the extent to which controls are representative of the “person-time population” from which the cases were sampled, and the extent to which cases are a random sample of that latter population.

### ***Statistical Analyses***

Meta-analyses were performed for SNPs that were reported by at least three included studies. The pooled odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between *TLR4* genotypes and risk of aggressive PCa were calculated using random effects models. Random effects models are preferred to fixed effect models because of the differences in study designs and study populations [22]. To incorporate both within-study and between-study variability, we used DerSimonian and Laird’s [23] random effects models to pool the estimates of log OR from each individual study (unadjusted for covariates). Between-study heterogeneity was quantified by using the $I^2$ statistic [24,25], which indicates the proportion of variability across studies attributable to heterogeneity. Tests of heterogeneity were assessed by a $\chi^2$ statistic. To explore the inheritance mode for the effect of *TLR4* polymorphisms, we evaluated the following genotype contrasts (where a and A denote minor and major alleles, respectively): (1) a/a and A/a combined versus A/A (dominant model); (2) a/a versus A/a and A/A combined (recessive model); (3) a/a versus A/A and A/a versus A/A (co-dominant model); (4) the increment of one minor allele (additive model). The Hardy-Weinberg equilibrium (HWE) was assessed via $\chi^2$ test. We did not perform haplotype analysis because none of the previous studies performed haplotype analysis specific for these SNPs. Because most GWASs did not adjust for covariates, this meta-analysis reported unadjusted pooled results.

To evaluate the presence of publication bias, we examined the funnel plot, by plotting the reciprocal of the standard error of log OR versus the log OR, for symmetry. The Egger linear regression test was also performed to assess funnel plot’s asymmetry [26]. Random effects meta-regression was performed under dominant model to explore possible sources of between-study heterogeneity. Study type (candidate-gene studies vs. GWASs) was the pre-specified covariate. We did not perform stratification analysis according to differences in control and case selection, because such influences are complex and are usually not unidirectional. Because previous studies revealed high concordance rate across genotyping platforms [27], stratification analysis was not carried out according to this covariate. Analyses were performed with Stata version 11.0 software (Stata Corporation, College Station, TX, USA). All *P* values were two-sided. QUANTO program (http://hydra.usc.edu/gxe/) was used to evaluate statistical power of the association between *TLR4* polymorphisms and aggressive PCa.

## Results

### Characteristics of Association Studies

Using the pre-specified search methodology we retrieved forty relevant publications (Figure 1). After excluding duplicates (n=10), seventeen studies were further excluded due to the following reasons: (1) not European ancestry (n=5), (2) partially overlapped populations (n=9), (3) lack of controls (n=1), and (4) GWAS which did not include *TLR4* gene (n=2).

We contacted the authors of the remaining 13 relevant studies for necessary details, and authors of three of the GWASs [28-30] didn't respond and were thus excluded. One GWAS was excluded because it didn't contain the information of PCa aggressiveness [31]. For studies composed of multiple cohorts (e.g., Lindstrom et al. [32] ), we tried to obtain data from each cohort and used the original study to represent each cohort (e. g., Chen et al. [33] for HPFS, Dunggan et al. [34] for CAPS, and Yeager et al. [35] for PLCO). For the CAPS study, the GWAS by Dunggan et al. [34] was selected instead of the candidate-gene study done by Zheng et al. [36] because the former was composed of aggressive PCa cases from Zheng's study and evaluated more SNPs. In sum, nine studies were included for the meta-analysis.

A total of 3,937 aggressive PCa cases and 7,382 controls were included in this work. Six studies were candidate-gene studies [33,37-41], and three of them were GWASs [34,35,42]. Six papers studied US populations [33,35,37-39], one studied a Swedish population [34], one studied the combination of UK and Australian population [42], and one studied an Italian population [40]. Details of the studies analyzed in this meta-analysis were summarized in Table 1, including first author, year of publication, type of study, ancestry, sample size, control selection, possible sources of selection bias, definition of PCa aggressiveness, genotyping methods and quality control.

For the association between *TLR4* SNPs and aggressive PCa, seven studies assessed rs4986790 [33-35,37,39,40,42]; five studies investigated rs2149356 [33,34,37,39,41], rs11536889 [33,34,37,39,41], rs7873784 [33,34,37,39,41]; and four studies explored rs2737191[34,35,41,42] , rs1927914 [33,34,38,39], rs10759932 [33,34,37,41], rs1927911 [33,34,38,39], rs11536879 [34,35,38,42], and rs1554973 [34,35,41,42].

### Allele Frequencies of *TLR4* SNPs

Ten *TLR4* SNPs had been evaluated by at least 4 included studies. The minor allele frequencies (MAF) between case and controls were shown in Table 2, along with the test for HWE in controls. Among them, three SNPs are located on 5’ untranslated region (UTR, rs2737191, rs1927914 and rs10759932), three are intronic SNPs (rs1927911, rs11536879, and rs2149356), one is non-synonymous exonic SNP (rs4986790), and three SNPs are located on 3’ UTR (rs11536889, rs7873784, and rs1554973). Another 10 *TLR4* SNPs were reported by 3 studies, including one SNP located on the promoter region (rs10759930), one SNP located on 5’UTR (rs10116253), two intronic SNPs (rs11536869 and rs5030717), one non-synonymous exonic SNP (rs4986791), and five SNPs located on 3’ UTR (rs11536897, rs1927906, rs913930, rs1927905, and rs7045953). The locations of the explored SNPs (10 SNPs with ≧4 studies, 10 SNPs with 3 studies) are shown in Figure 2. rs2149356, rs4986790 and rs7873784 in Chen’s study and rs1927911 in Wang’s study were out of HWE ($P = 0.01$-$0.03$) but were kept in the analysis because the HWE tests were not significant after correction for multiple tests.

### Meta-Analysis

Using random effects meta-analysis, the ten *TLR4* SNPs (rs2737191, rs1927914, rs10759932, rs1927911, rs11536879, rs2149356, rs4986790, rs11536889, rs7873784, and rs1444973) were not associated with the risk of aggressive PCa regardless of the inheritance model used (Table 3, Figure 3). The meta-analysis was also performed for another ten SNPs which were reported by three included studies (rs10759930, rs10116253, rs11536869, rs5030717, rs4986791, rs11536897, rs1927906, rs913930, rs1927905, and rs7045953) (Table S1). None of the SNPs revealed significant association with aggressive PCa. This meta-analysis was reported according to the PRISMA checklist [43] (Table S2).

### Publication Bias

Funnel plots were used to assess the relationship between the ten *TLR4* SNPs and aggressive PCa

(Figure S1). Using the Egger linear regression test, possible publication bias was found among the included studies on rs1554973 (Egger test *P* = 0.06). For the other 9 SNPs, *P* values ranged from 0.2 to 0.77.

### Meta-regression

Random effects meta-regression was performed under dominant model. Different study type (candidate-gene studies vs. GWASs) was not a significant source of between-study heterogeneity (*P* value ranged from 0.15 to 0.79 for the ten *TLR4* SNPs).

### Power Calculation

For people of European ancestry, given a MAF of 0.15 and $\alpha$ of 0.05, this study had over 95% power to detect an OR of 1.20 for 3,937 cases and 7,382 controls.

## Discussion

Recently, some researchers hypothesized that PCa is the result of a chronic inflammatory process [44]. Proliferative inflammatory atrophy (PIA), proposed as a potential precursor to PCa, occurs frequently in the periphery of the prostate gland where PCa occurs [5]. PIA lesions seem to be the result of different conditions, including infections, chronic non-infectious inflammatory diseases, dietary carcinogens, physical trauma, imbalance of sex hormone and urine reflux [9].Chronic infections may contribute to PIA and lead to onset of PCa [45-47]. Several innate inflammatory pathways seem to be involved. Among these, TLR4 pathway plays a crucial role [48].

TLR4 recognizes pathogen-associated molecular patterns, i.e. LPS [46]. Damage-associated molecular pattern molecules may also interact with TLR4, i.e. oxidized low-density lipoprotein (LDL) [49], one of the atherogenic lipoproteins associated with atherosclerosis [50] and insulin resistance [51,52]. Their interaction leads to the initiation of inflammatory response via NF-κB (Figure 4) [53]. TLR4 can also promote PCa development through releasing inflammatory mediators. Associations between *TLR4* SNPs and PCa have been examined in several studies, though discordant data have been reported. However, the relationship between *TLR4* genotypes and aggressive PCa risk has not been evaluated by any systematic reviews. Thus, we performed a systematic review and meta-analysis of candidate-gene studies and GWASs analyzing this relationship and restricted to samples taken from European ancestry.

In the current meta-analysis, none of the examined *TLR4* SNPs was significantly associated with risk of aggressive PCa under any inheritance model. No significant association was found between the *TLR4* SNPs (5’UTR: rs2737191, rs1927914 and rs10759932; intron: rs1927911, rs11536879, and rs2149356; exon: rs4986790; 3’UTR: rs11536889, rs7873784, and rs1554973) and risk of aggressive PCa in the pooled analysis. The non-significant findings may be attributable to (1) failure to adjust for the conventional risk factors of PCa, e.g. family history of PCa, (2) inability to assess the within-population heterogeneity or geographic variation, and (3) the studied *TLR4* SNPs may be more closely related to non-aggressive PCa.

Three recent meta-analyses evaluated the association between *TLR4* SNPs and overall PCa. Jing et al. [19], including four candidate-gene studies [33,37,39,40], examined two *TLR4* SNPs (rs4986790 and rs4986791) and found that rs4986790 showed a protective effect on overall PCa under co-dominant and recessive models. However, the effect was not statistically significant after stratification by ethnicity. Another work by Zhang et al. [20] examined six *TLR4* SNPs (rs1927914, rs4986790, rs4986791, rs11536889, rs1927911, rs2149356) and did not find significant associations with overall PCa. The pooled estimates of Zhang et al. were derived from one Asian study [54] and four other populations of European ancestry [33,36,39,41], which might be confounded by population stratification. Zhu et al. [21] examined rs4986790 and rs4986791 and found no significant association with overall PCa in five populations of European ancestry [33,36,37,39,40]. In summary, our findings on aggressive PCa are consistent with the previous meta-analyses on overall PCa. Our study had several advantages over the previous meta-analyses: (1) this study additionally included GWASs, whereas previous meta-analyses included candidate-gene studies only [19-21] , (2) this study focused on aggressive PCa, which is more clinically relevant, (3) this study was restricted to populations of European ancestry to avoid population stratification, and (4) this study evaluated an additional 14 SNPs, which were not reported in the previous meta-analyses.

Previous candidate-gene studies and GWASs found inconsistent results for the association between *TLR4* polymorphisms and PCa risk. This may be explained by different ethnicity, within-population heterogeneity, case and control selection, gene-gene interactions, and gene-environment interactions. Although most of the relevant medical centers were in the “catchment” area, Cheng and colleagues [37] used controls from medical centers, which differ from the source population in that not all men with potential PCa would go to these centers to be screened and diagnosed.

There were some limitations of this study. One of them is the possibility of publication bias. Though the funnel plots did not reveal obvious publication bias among most of *TLR4* SNPs, the SNPs reported in this study were under the influence of publication bias because only SNPs explored in ≧3 studies were included. We were unable to include three other GWASs because the authors did not respond to our data request [28-30]. After exclusion of men with African and Asian ancestry, there was little evidence that population stratification was a cause of confounding. Though the included studies were conducted separately in the United States, Sweden, Italy, UK and Australia, a prior theoretical calculation on genetic case-control studies showed that ignoring ethnicity among non-Hispanic U.S. Caucasians with ancestries from different European countries resulted in bias of less than 1% [55]. Last, the included studies used different genotyping approaches, which may be associated with different genotyping success rates and data quality. However, genotyping errors are expected to be small, and thus the resulting biases are likely to be small.

This study had some advantages. To the best of our knowledge, this is the first meta-analysis on *TLR4* polymorphisms and aggressive PCa, which shows more clinical relevance. All the included studies were reasonably well-designed epidemiological studies. Genotyping was carried out “blind” to the disease status, and assessment of aggressive PCa was carried out “blind” to the genotypes. This study had sufficient power (> 0.95) to detect a potential OR of aggressive PCa associated with a SNP of 1.20. This study presents the best available evidence on the relationship between *TLR4* polymorphisms and risk of aggressive PCa.

In conclusion, this study found that none of the examined *TLR4* SNPs were significantly associated with risk of aggressive PCa under any mode of inheritance. Control selection, different ancestry, small statistical power in some studies, publication bias, gene-gene and gene-environment interactions, different genotyping approaches, and issues of multiple tests may contribute to the inconsistent findings in previous studies. Meta-regression revealed that different study type (candidate-gene studies vs. GWASs) was not a significant source of between-study heterogeneity. Large-scale and well-designed studies using population-based controls and more studies in each ethnic group are needed to confirm our findings.

## Acknowledgement

Thanks to Dr. Elizabeth Platz, who provided detailed information on aggressive PCa from their study [39].

## Supporting Information

**Table S1 Pooled estimated ORs and 95% CIs for the association of *TLR4* SNPs in aggressive PCa risk**

**Table S2 PRISMA checklist**

**Figure S1 Funnel plot of *TLR4* SNPs**

Funnel plot displays the publication bias for each study (indicated as one dot) exploring the relation between *TLR4* SNPs and aggressive prostate cancer. SNPs reported by at least four studies were shown here

## References

1. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. CA Cancer J Clin 63: 11-30.
2. Bostwick DG, Burke HB, Djakiew D, Euling S, Ho SM, et al. (2004) Human prostate cancer risk factors. Cancer 101: 2371-2490.
3. Haqq C, Li R, Khodabakhsh D, Frolov A, Ginzinger D, et al. (2005) Ethnic and racial differences in prostate stromal estrogen receptor alpha. Prostate 65: 101-109.
4. Platz EA, Giovannucci E (2004) The epidemiology of sex steroid hormones and their signaling and metabolic pathways in the etiology of prostate cancer. J Steroid Biochem Mol Biol 92: 237-253.
5. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, et al. (2007) Inflammation in prostate carcinogenesis. Nat Rev Cancer 7: 256-269.
6. Klein EA, Silverman R (2008) Inflammation, infection, and prostate cancer. Curr Opin Urol 18: 315-319.
7. Sutcliffe S, Platz EA (2007) Inflammation in the etiology of prostate cancer: an epidemiologic perspective. Urol Oncol 25: 242-249.
8. Dennis LK, Dawson DV (2002) Meta-analysis of measures of sexual activity and prostate cancer. Epidemiology 13: 72-79.
9. Balistreri CR, Candore G, Lio D, Carruba G (2014) Prostate cancer: from the pathophysiologic implications of some genetic risk factors to translation in personalized cancer treatments. Cancer Gene Ther 21: 2-11.
10. Cheng I, Liu X, Plummer SJ, Krumroy LM, Casey G, et al. (2007) COX2 genetic variation, NSAIDs, and advanced prostate cancer risk. Br J Cancer 97: 557-561.
11. Lee LM, Pan CC, Cheng CJ, Chi CW, Liu TY (2001) Expression of cyclooxygenase-2 in prostate adenocarcinoma and benign prostatic hyperplasia. Anticancer Research 21: 1291-1294.
12. Balistreri CR, Colonna-Romano G, Lio D, Candore G, Caruso C (2009) TLR4 polymorphisms and ageing: implications for the pathophysiology of age-related diseases. J Clin Immunol 29: 406-415.
13. Ferwerda B, McCall MB, Verheijen K, Kullberg BJ, van der Ven AJ, et al. (2008) Functional consequences of toll-like receptor 4 polymorphisms. Molecular Medicine 14: 346-352.
14. El-Omar EM, Ng MT, Hold GL (2008) Polymorphisms in Toll-like receptor genes and risk of cancer. Oncogene 27: 244-252.
15. Hua D, Liu MY, Cheng ZD, Qin XJ, Zhang HM, et al. (2009) Small interfering RNA-directed targeting of Toll-like receptor 4 inhibits human prostate cancer cell invasion, survival, and tumorigenicity. Mol Immunol 46: 2876-2884.
16. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, et al. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282: 2085-2088.
17. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, et al. (2000) Pattern recognition receptors

TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol 1: 398-401.

18. Kropf P, Freudenberg MA, Modolell M, Price HP, Herath S, et al. (2004) Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite Leishmania major. Infection and Immunity 72: 1920-1928.
19. Jing JJ, Li M, Yuan Y (2012) Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms in cancer: a meta-analysis. Gene 499: 237-242.
20. Zhang K, Zhou B, Wang Y, Rao L, Zhang L (2013) The TLR4 gene polymorphisms and susceptibility to cancer: a systematic review and meta-analysis. Eur J Cancer 49: 946-954.
21. Zhu L, Yuan H, Jiang T, Wang R, Ma H, et al. (2013) Association of TLR2 and TLR4 Polymorphisms with Risk of Cancer: A Meta-Analysis. PLoS One 8: e82858.
22. Egger M, Smith GD, Altman DG (2001) Systematic reviews in health care: meta-analysis in context. London: The BMJ Publishing Group.
23. DerSimonian R, Laird N (1986) Meta-analysis in clinical trials. Controlled Clinical Trials 7: 177-188.
24. Higgins JP, Thompson SG (2004) Controlling the risk of spurious findings from meta-regression. Stat Med 23: 1663-1682.
25. Higgins JP, Thompson SG, Deeks JJ, Altman DG (2003) Measuring inconsistency in meta-analyses. BMJ 327: 557-560.
26. Egger M, Davey Smith G, Schneider M, Minder C (1997) Bias in meta-analysis detected by a simple, graphical test. BMJ 315: 629-634.
27. Hong H, Xu L, Liu J, Jones WD, Su Z, et al. (2012) Technical reproducibility of genotyping SNP arrays used in genome-wide association studies. PLoS One 7: e44483.
28. Schumacher FR, Berndt SI, Siddiq A, Jacobs KB, Wang Z, et al. (2011) Genome-wide association study identifies new prostate cancer susceptibility loci. Hum Mol Genet 20: 3867-3875.
29. FitzGerald LM, Kwon EM, Conomos MP, Kolb S, Holt SK, et al. (2011) Genome-wide association study identifies a genetic variant associated with risk for more aggressive prostate cancer. Cancer Epidemiol Biomarkers Prev 20: 1196-1203.
30. Nam RK, Zhang W, Siminovitch K, Shlien A, Kattan MW, et al. (2011) New variants at 10q26 and 15q21 are associated with aggressive prostate cancer in a genome-wide association study from a prostate biopsy screening cohort. Cancer Biol Ther 12: 997-1004.
31. Murabito JM, Rosenberg CL, Finger D, Kreger BE, Levy D, et al. (2007) A genome-wide association study of breast and prostate cancer in the NHLBI's Framingham Heart Study. BMC Med Genet 8 Suppl 1: S6.
32. Lindstrom S, Hunter DJ, Gronberg H, Stattin P, Wiklund F, et al. (2010) Sequence variants in the TLR4 and TLR6-1-10 genes and prostate cancer risk. Results based on pooled analysis from three independent studies. Cancer Epidemiology, Biomarkers and Prevention 19: 873-876.

33. Chen YC, Giovannucci E, Lazarus R, Kraft P, Ketkar S, et al. (2005) Sequence variants of Toll-like receptor 4 and susceptibility to prostate cancer. Cancer Res 65: 11771-11778.
34. Duggan D, Zheng SL, Knowlton M, Benitez D, Dimitrov L, et al. (2007) Two genome-wide association studies of aggressive prostate cancer implicate putative prostate tumor suppressor gene DAB2IP. J Natl Cancer Inst 99: 1836-1844.
35. Yeager M, Orr N, Hayes RB, Jacobs KB, Kraft P, et al. (2007) Genome-wide association study of prostate cancer identifies a second risk locus at 8q24. Nat Genet 39: 645-649.
36. Zheng SL, Augustsson-Balter K, Chang B, Hedelin M, Li L, et al. (2004) Sequence variants of toll-like receptor 4 are associated with prostate cancer risk: results from the CAncer Prostate in Sweden Study. Cancer Res 64: 2918-2922.
37. Cheng I, Plummer SJ, Casey G, Witte JS (2007) Toll-like receptor 4 genetic variation and advanced prostate cancer risk. Cancer Epidemiol Biomarkers Prev 16: 352-355.
38. Breyer JP, McReynolds KM, Yaspan BL, Bradley KM, Dupont WD, et al. (2009) Genetic variants and prostate cancer risk: candidate replication and exploration of viral restriction genes. Cancer Epidemiol Biomarkers Prev 18: 2137-2144.
39. Wang MH, Helzlsouer KJ, Smith MW, Hoffman-Bolton JA, Clipp SL, et al. (2009) Association of IL10 and other immune response- and obesity-related genes with prostate cancer in CLUE II. Prostate 69: 874-885.
40. Balistreri CR, Caruso C, Carruba G, Miceli V, Campisi I, et al. (2010) A pilot study on prostate cancer risk and pro-inflammatory genotypes: pathophysiology and therapeutic implications. Curr Pharm Des 16: 718-724.
41. Shui IM, Stark JR, Penney KL, Schumacher FR, Epstein MM, et al. (2012) Genetic variation in the toll-like receptor 4 and prostate cancer incidence and mortality. Prostate 72: 209-216.
42. Eeles RA, Kote-Jarai Z, Giles GG, Olama AA, Guy M, et al. (2008) Multiple newly identified loci associated with prostate cancer susceptibility. Nature Genetics 40: 316-321.
43. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gotzsche PC, et al. (2009) The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. PLoS Med 6: e1000100.
44. Sakr WA, Grignon DJ, Crissman JD, Heilbrun LK, Cassin BJ, et al. (1994) High grade prostatic intraepithelial neoplasia (HGPIN) and prostatic adenocarcinoma between the ages of 20-69: an autopsy study of 249 cases. In Vivo 8: 439-443.
45. Das D, Wojno K, Imperiale MJ (2008) BK virus as a cofactor in the etiology of prostate cancer in its early stages. J Virol 82: 2705-2714.
46. Cavallaro S, Meiri N, Yi CL, Musco S, Ma W, et al. (1997) Late memory-related genes in the hippocampus revealed by RNA fingerprinting. Proc Natl Acad Sci U S A 94: 9669-9673.

47. Taylor ML, Mainous AG, 3rd, Wells BJ (2005) Prostate cancer and sexually transmitted diseases: a meta-analysis. Fam Med 37: 506-512.
48. DeFranco AL, Locksley RM, Robertson M (2007) Immunity: the immune response in infectious and inflammatory disease. Corby, Northants, UK: Oxford University Press. 387 p.
49. Miller YI, Viriyakosol S, Binder CJ, Feramisco JR, Kirkland TN, et al. (2003) Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. J Biol Chem 278: 1561-1568.
50. Holvoet P, Mertens A, Verhamme P, Bogaerts K, Beyens G, et al. (2001) Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. Arterioscler Thromb Vasc Biol 21: 844-848.
51. Shashkin PN, Jain N, Miller YI, Rissing BA, Huo Y, et al. (2006) Insulin and glucose play a role in foam cell formation and function. Cardiovasc Diabetol 5: 13.
52. Carantoni M, Abbasi F, Warmerdam F, Klebanov M, Wang PW, et al. (1998) Relationship between insulin resistance and partially oxidized LDL particles in healthy, nondiabetic volunteers. Arterioscler Thromb Vasc Biol 18: 762-767.
53. Madoff LC, Kasper DL (2008) Introduction of Infectious Diseases: Host-Pathogen Interactions. In: Fauci AS, Braunwald E, Kasper DL, Hauser SL, Longo DL et al., editors. Harrison's principles of internal medicine. 17 ed. New York: McGraw-Hill.
54. Song J, Kim DY, Kim CS, Kim HJ, Lee DH, et al. (2009) The association between Toll-like receptor 4 (TLR4) polymorphisms and the risk of prostate cancer in Korean men. Cancer Genetics and Cytogenetics 190: 88-92.
55. Wacholder S, Rothman N, Caporaso N (2000) Population stratification in epidemiologic studies of common genetic variants and cancer: quantification of bias. Journal of the National Cancer Institute 92: 1151-1158.

## Figure legends

### Figure 1. Study selection flowchart

Forty studies were reviewed after literature search. Among them, 31 studies were excluded due to duplication, race other than whites, and insufficient data. A total of 9 studies were included for meta-analysis.

### Figure 2. *TLR4* SNPs evaluated in this meta-analysis

This plot was generated by the Locusview program. The highlighted boxed SNPs were *TLR4* polymorphisms explored by at least four studies. The remaining SNPs were those reported by three studies, discussed in the supplemental data.

### Figure 3. Forest plot examines relationship between *TLR4* SNPs and risk of aggressive prostate cancer

Odds ratios and weights were demonstrated for each individual study and for the pooled analysis, assuming a dominant model. SNPs that were evaluated by at least 4 studies were shown here.

### Figure 4. The role of TLR4 in innate immunity

TLR4 receptors are responsible for the recognition of bacterial lipopolysaccharide (LPS) monomers and partially oxidized LDL (oLDL) on innate immune cells. LPS monomers and oLDL bind to sites on the protein, CD14. CD14 promotes the binding of these ligands to the TLR4-MD-2 complex, which signals the activation of the nuclear factor kappa B (NF-κB) pathway. NF-κB products enter the nucleus and result in transcription followed by the production of cytokines and the activation of multiple inflammatory pathways. This figure was adapted from DeFranco et al. [48].

## Table 1. Characteristics of the study populations that evaluated the relationship between *TLR4* polymorphisms and risk of prostate cancer

| Source, publication year (study year) | Type of study | Country/ ancestry | Aggressive PCa/ control | Control selection | Comments about control selection | Case selection | Definition of aggressive prostate cancer | Outcome assessment "blinded" to genotype | Genotyping procedures | Genotyping quality control |
|---|---|---|---|---|---|---|---|---|---|---|
| Chen et al., 2005<br>(1993-1995) | Candidate gene | U.S. / 97% Caucasians | 260/ 700 | Age- matched controls from prospective cohort | PSA tested in controls | Incident PCa | TNM stage T3b or T4 or N1 or M1 or death due to PCa or Gleason sum ≥ 7 | Yes | MassARRAY system (SEQUENOM)** | 100% concordance, > 95% genotyping success |
| Dunggan et al., 2007<br>(2001-2002) | GWAS | Sweden/ Not mentioned | 505/ 507 | Age-matched population controls from the same geographical region | 74% response rate in cases, 52% in controls. No PSA tested in controls. | PCa from cancer registry | TNM stage T3 or T4 or N+ or M+ or grade III or Gleason sum > 7 or PSA > 100 ng/ml | Yes | MassARRAY system (SEQUENOM) ** | >99 % concordance, >98% genotyping success |
| Yeager et al., 2007<br>(1993-2001) | GWAS | U.S. /White and non-hispanic | 1081/ 1416 | Risk set sampling from a population-based randomized controlled trial | PSA tested in controls | Incident PCa | Gleason sum ≥ 7 or stage ≥ 3 | Yes | Illumina system | >99 % concordance, >99% genotyping success |
| Cheng et al., 2007<br>(2002-2004) | Candidate gene | U.S./ Caucasians | 417/ 417 | From annual medical examinations at the same medical institutions of cases | Hospital-based study. PSA tested in controls | Incident PCa | TNM stage ≥ T2c or Gleason sum ≥ 7 or PSA> 10 ng/ml | Yes | Taqman | 100% concordance, 99.9% genotyping success |
| Eeles et al., 2008<br>(1993-2001) | GWAS | U.K., Australia/ Excluded self-reported "non-white" | 564/ 1894 | Community-based randomized controlled trial/electoral rolls | Controls to be frequency matched to the geographical distribution of the cases. | PCa from cancer registry, urology clinic | Gleason sum ≥ 7 | Yes | Stage 1: Illumina Infinium HumanHap550 array<br>Stage 2: Taqman | >97 % SNPs at a confidence score of ≥ 0.25, 98.8 % concordance |
| Breyer et al.,2009<br>(2002-2008) | Candidate-gene | U.S./ Americans of Northern European decents | 441/ 772 | Age-matched controls from a preventive screening | Hospital-based.<br>PSA tested in controls | Incident PCa | Gleason sum ≥ 7 | Yes | Illumina GoldenGate platform and Taqman | 99.7 % of genotyping success |
| Wang et al., 2009<br>(1992-2002) | Candidate gene | U.S./ White only | 77/ 264 | Age- matched controls from a prospective cohort | No PSA tested in controls | Incident PCa | TNM stage T3 or T4 or N1 or M1 or death due to PCa or Gleason sum ≥ 7 | Not mentioned | Taqman | 93-99 % genotyping success |
| Ballistreri et al., | Candidate | Italy/ | 32/ | Age-matched controls | Hospital-based study. | Prevalent PCa | Gleason sum ≥ 7 | Yes | RFLP-PCR | Not mentioned |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 2010<br>(NA) | gene | European ancestry | 125 | in good health | No clear description on control selection. No PSA tested in controls | | | | | |
| Shui et al., 2012<br>(1982-2004) | Candidate gene | U.S./ White | 560/ 1287 | Risk set sampling from a prospective cohort, matched on age and smoking | No PSA tested in controls | Incident PCa | TNM stage T3 or T4, M1 or N1 or death due to PCa or Gleason sum ≥ 7 | Yes | Sequenom iPLEX matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry technology. | 100% concordance, >95% genotyping success |

Abbreviations: PCa, prostate cancer; TNM, the tumor node metastases classification system; PSA, prostate specific antigen; GWAS, genome-wide association study; RLFP-PCR, restriction fragment length polymorphism-polymerase chain reaction

All studies met the following criteria and they were not listed in the table: (1) clear description of laboratory methods, (2) genotyping identical for cases and controls, (3) genotyping blinded to case control status, and (4) specimen came from peripheral blood sample.

**Table 2. Characteristics of included studies**

| | rs2737191 (A/G) | | rs1927914 (A/G) | | rs10759932 (T/C) | | rs1927911 (G/A) | | rs11536879 (A/G) | | rs2149356 (G/T) | | rs4986790 (A/G) | | rs11536889 (A/G) | | rs7873784 (G/C) | | rs1554973 (T/C) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls |
| Chen et al.,2005 | NA | NA | 0.30/ 0.35 | 0.15 | 0.14/ 0.16 | 0.09 | 0.25/ 0.29 | 0.43 | NA | NA | 0.30/ 0.34 | 0.02 | 0.04/ 0.05 | 0.01 | 0.15/ 0.14 | 0.52 | 0.15/ 0.18 | 0.03 | NA | NA |
| Dunggan et al.,2007 | 0.27/ 0.27 | 0.46 | 0.33/ 0.34 | 0.55 | 0.16/ 0.15 | 0.71 | 0.27/ 0.26 | 0.74 | 0.01/ 0.01 | 0.82 | 0.31/ 0.32 | 0.89 | 0.05/ 0.06 | 0.15 | NA | NA | 0.11/ 0.13 | 0.99 | 0.19/ 0.21 | 0.45 |
| Yeager et al.,2007 | 0.28/ 0.29 | 0.88 | 0.32/ 0.32 | 0.94 | NA | NA | NA | NA | 0.04/ 0.04 | 0.83 | NA | NA | 0.06/ 0.05 | 0.59 | NA | NA | NA | NA | 0.24/ 0.23 | 0.11 |
| Cheng et al.,2007 | NA | NA | NA | NA | 0.13/ 0.14 | 0.04 | NA | NA | NA | NA | 0.32/ 0.30 | 0.68 | 0.06/ 0.05 | 0.98 | 0.15/ 0.14 | 0.09 | 0.15/ 0.16 | 0.82 | NA | NA |
| Eeles et al.,2008 | 0.27 /0.29 | 0.76 | 0.33/ 0.33 | 0.79 | NA | NA | NA | NA | 0.05/ 0.04 | 0.71 | NA | NA | 0.05/ 0.06 | 0.74 | NA | NA | NA | NA | 0.26/ 0.26 | 0.86 |
| Breyer et al, 2009 | NA | NA | NA | NA | NA | NA | 0.27/ 0.26 | 0.34 | 0.04/ 0.03 | 0.92 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| Wang et al.,2009 | NA | NA | 0.32/ 0.32 | 0.24 | NA | NA | 0.27/ 0.24 | 0.02 | NA | NA | 0.35/ 0.32 | 0.18 | 0.06/ 0.07 | 0.24 | 0.16/ 0.16 | 0.76 | 0.11/ 0.12 | 0.91 | NA | NA |
| Ballistreri et al.,2010 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | 0/ 0.06 | 0.38 | NA | NA | NA | NA | NA | NA |
| Shui et al.,2012 | 0.26/ 0.26 | 0.08 | NA | NA | 0.13/ 0.13 | 0.03 | NA | NA | NA | NA | 0.30/ 0.30 | 0.06 | NA | NA | 0.16/ 0.14 | 0.55 | 0.14/ 0.14 | 0.20 | 0.25 /0.25 | 0.18 |

SNPs that were evaluated by at least 4 studies were shown here. Abbreviations: MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; NA, not available.

**Table 3. Pooled estimated ORs and 95% CIs for the association of *TLR4* SNPs in aggressive PCa risk**

| | | Random effects model | | Heterogeneity | | | | Random effects model | | Heterogeneity | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Genetic model | OR (95% CI) | *P* | $I^2$ | *P* | | Genetic model | OR (95% CI) | *P* | $I^2$ | *P* |
| rs2737191 | Dominant | 0.96 (0.84-1.11) | 0.61 | 50.8% | 0.11 | rs2149356 | Dominant | 1.01 (0.89-1.14) | 0.90 | 0% | 0.62 |
| | Recessive | 0.86 (0.72-1.03) | 0.40 | 0% | 0.40 | | Recessive | 0.91 (0.73-1.12) | 0.37 | 6% | 0.37 |
| | AG vs. AA | 1.00 (0.83-1.19) | 0.97 | 65.9% | 0.03 | | GT vs. GG | 1.03 (0.91-1.17) | 0.63 | 0% | 0.86 |
| | GG vs. AA | 0.84 (0.84-1.08) | 0.07 | 35.7% | 0.14 | | TT vs. GG | 0.92 (0.72-1.17) | 0.49 | 16.9% | 0.31 |
| | Additive | 0.95 (0.84-1.07) | 0.41 | 30% | 0.18 | | Additive | 0.99 (0.90-1.08) | 0.83 | 0% | 0.69 |
| rs1927914 | Dominant | 0.95 (0.83-1.08) | 0.43 | 0% | 0.82 | rs4986790 | Dominant | 0.98 (0.83-1.16) | 0.82 | 12.2% | 0.34 |
| | Recessive | 0.88 (0.62-1.24) | 0.46 | 52.9% | 0.10 | | Recessive | 1.29 (0.57-2.95) | 0.55 | 0% | 0.81 |
| | AG vs. AA | 0.96 (0.84-1.10) | 0.53 | 0 | 0.86 | | AG vs. AA | 0.98 (0.83-1.16) | 0.81 | 10% | 0.35 |
| | GG vs. AA | 0.87 (0.63-1.21) | 0.41 | 44.9% | 0.14 | | GG vs. AA | 1.28 (0.56-2.93) | 0.59 | 0% | 0.82 |
| | Additive | 0.96 (0.87-1.06) | 0.44 | 1% | 0.42 | | Additive | 1.02 (0.88-1.17) | 0.83 | 0% | 0.62 |
| rs10759932 | Dominant | 0.97 (0.83-1.14) | 0.70 | 19.8% | 0.29 | rs11536889 | Dominant | 1.05 (0.91-1.21) | 0.48 | 0% | 0.49 |
| | Recessive | 1.33 (0.70-2.54) | 0.38 | 44% | 0.15 | | Recessive | 1.25 (0.84-1.86) | 0.26 | 0% | 0.94 |
| | TC vs. TT | 0.94 (0.79-1.14) | 0.54 | 35.7% | 0.20 | | AG vs. AA | 1.03 (0.89-1.20) | 0.66 | 0% | 0.48 |
| | CC vs. TT | 1.31 (0.70-2.46) | 0.40 | 40.9% | 0.17 | | GG vs. AA | 1.27 (0.85-1.89) | 0.24 | 0% | 0.95 |
| | Additive | 0.96 (0.81-1.13) | 0.60 | 20.5% | 0.27 | | Additive | 1.06 (0.94-1.20) | 0.32 | 0% | 0.87 |
| rs1927911 | Dominant | 0.95 (0.83-1.10) | 0.49 | 0% | 0.50 | rs7873784 | Dominant | 0.91 (0.80-1.05) | 0.19 | 0% | 0.85 |
| | Recessive | 1.06 (0.67-1.67) | 0.80 | 56.2% | 0.08 | | Recessive | 1.03 (0.69-1.52) | 0.90 | 0% | 0.56 |
| | GA vs. GG | 0.93 (0.80-1.08) | 0.35 | 0% | 0.44 | | GC vs. GG | 0.91 (0.79-1.04) | 0.17 | 0% | 0.88 |
| | AA vs. GG | 1.03 (0.67-1.61) | 0.88 | 51.1% | 0.11 | | CC vs. GG | 1.00 (0.67-1.48) | 0.99 | 0% | 0.55 |
| | Additive | 0.99 (0.84-1.17) | 0.92 | 23.9% | 0.24 | | Additive | 0.93 (0.83-1.05) | 0.26 | 0% | 0.84 |
| rs11536879 | Dominant | 1.17 (0.96-1.41) | 0.12 | 0% | 0.93 | rs1554973 | Dominant | 0.98 (0.89-1.08) | 0.71 | 0% | 0.69 |
| | Recessive | 0.82 (0.17-3.86) | 0.80 | 0% | 0.80 | | Recessive | 1.01 (0.83-1.24) | 0.91 | 0% | 0.86 |
| | AG vs. AA | 1.18 (0.97-1.43) | 0.10 | 0% | 0.95 | | TC vs. TT | 0.98 (0.88-1.08) | 0.67 | 0% | 0.75 |
| | GG vs. AA | 0.83 (0.18-3.91) | 0.82 | 0% | 0.45 | | CC vs. TT | 1.01 (0.82-1.23) | 0.96 | 0% | 0.83 |
| | Additive | 1.15 (0.95-1.40) | 0.15 | 0% | 0.95 | | Additive | 0.99 (0.92-1.07) | 0.81 | 0% | 0.95 |

SNPs that were evaluated by at least 4 studies were shown here. Abbreviation: OR, odds ratio; CI, confidence interval; PCa, prostate cancer

Figure1

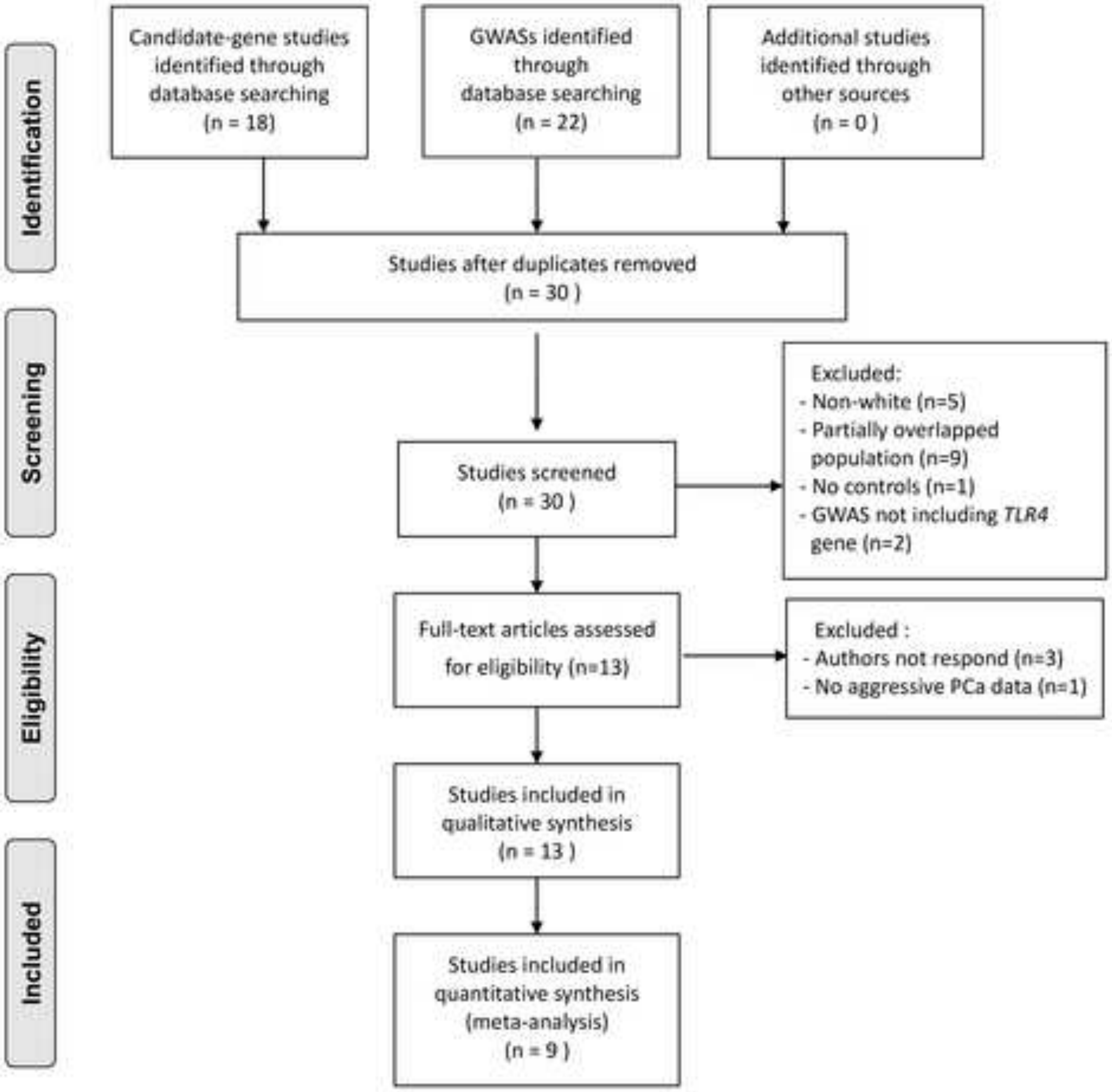

Identification
Candidate-gene studies identified through database searching (n = 18)
GWASs identified through database searching (n = 22)
Additional studies identified through other sources (n = 0 )
Studies after duplicates removed (n = 30 )
Screening
Studies screened (n = 30 )
Excluded:
- Non-white (n=5)
- Partially overlapped population (n=9)
- No controls (n=1)
- GWAS not including *TLR4* gene (n=2)
Eligibility
Full-text articles assessed for eligibility (n=13)
Excluded :
- Authors not respond (n=3)
- No aggressive PCa data (n=1)
Included
Studies included in qualitative synthesis (n = 13 )
Studies included in quantitative synthesis (meta-analysis) (n = 9 )

Figure2

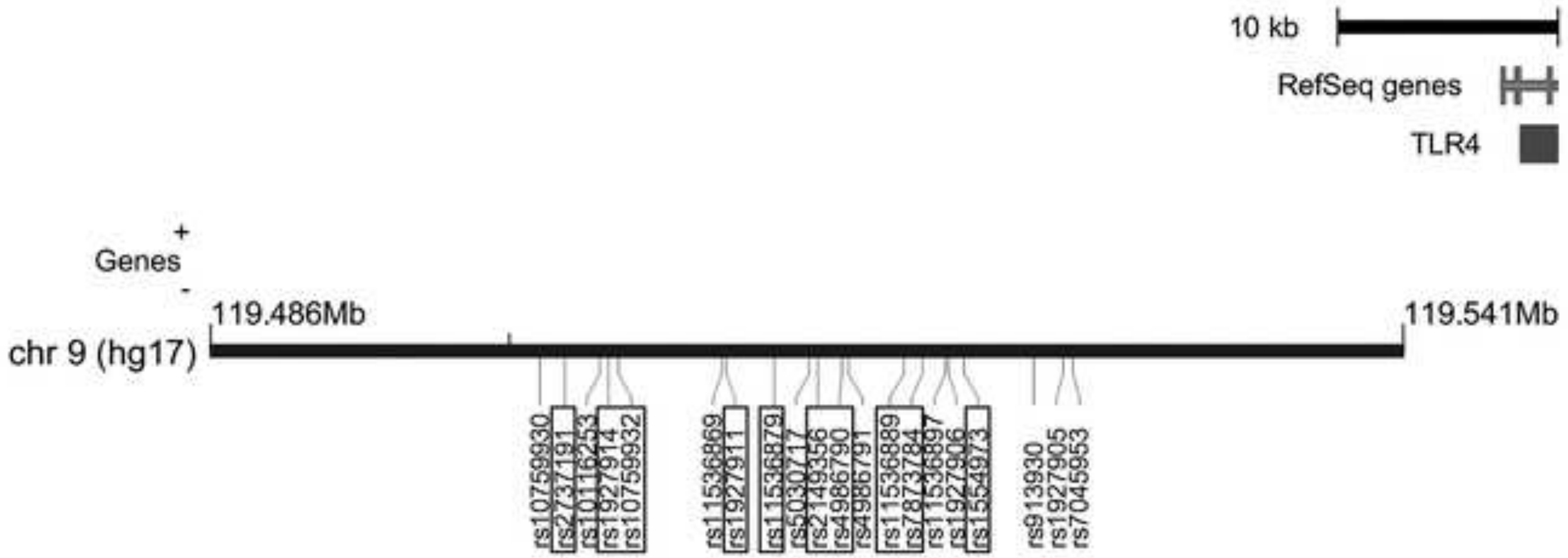
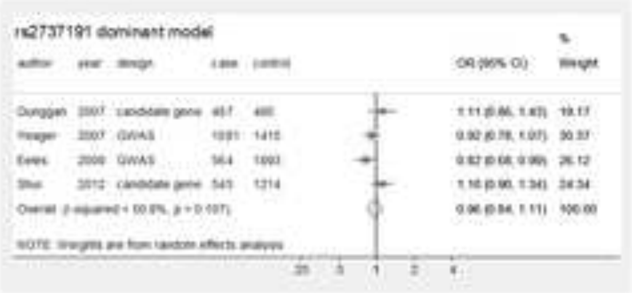
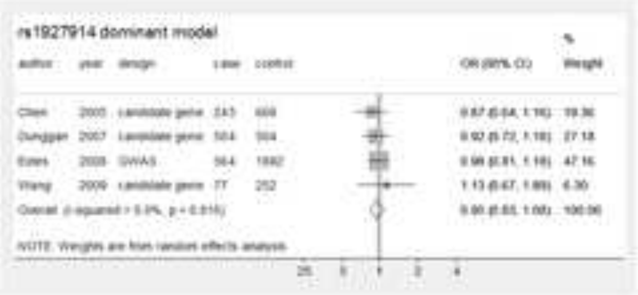

10 kb
RefSeq genes
TLR4
+
Genes
-
119.486Mb
119.541Mb
chr 9 (hg17)
rs10759930
rs2737191
rs10116253
rs1927914
rs10759932
rs11536869
rs1927911
rs11536879
rs5030717
rs2149356
rs4986790
rs4986791
rs11536889
rs7873784
rs11536897
rs1927906
rs1554973
rs913930
rs1927905
rs7045953

Figure3

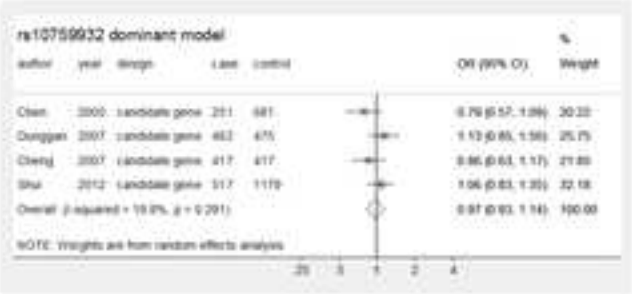
**rs2737191**



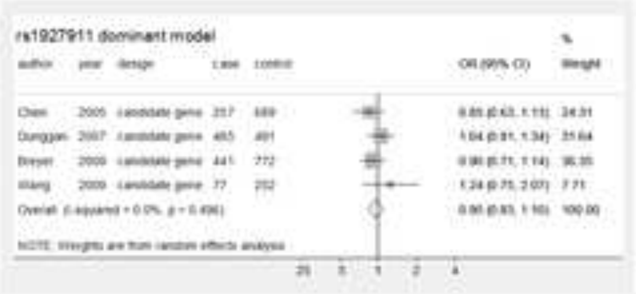
**rs1927914**



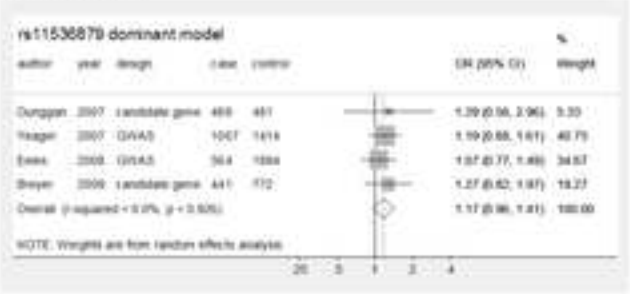
**rs10759932**



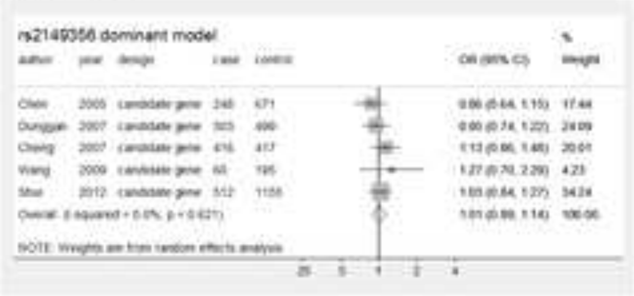
**rs1927911**



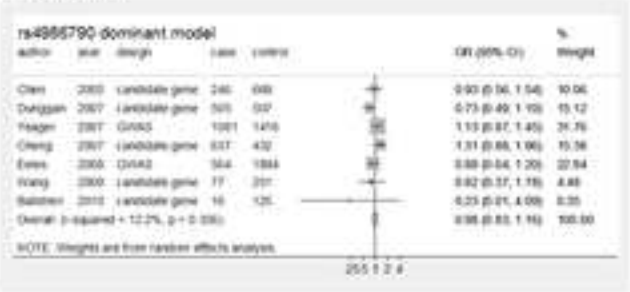
**rs11536889**



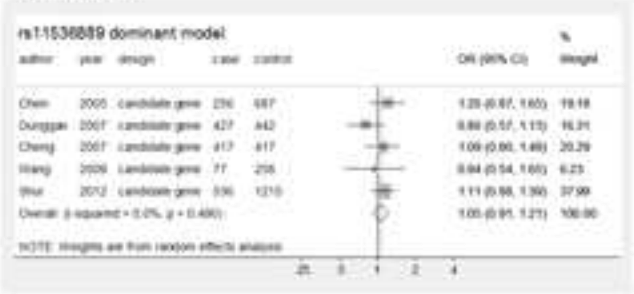
**rs2149356**



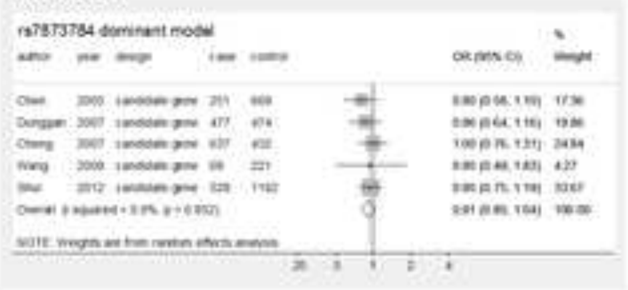
**rs4986790**



**rs11536889**



**rs7873784**



**rs1554973**

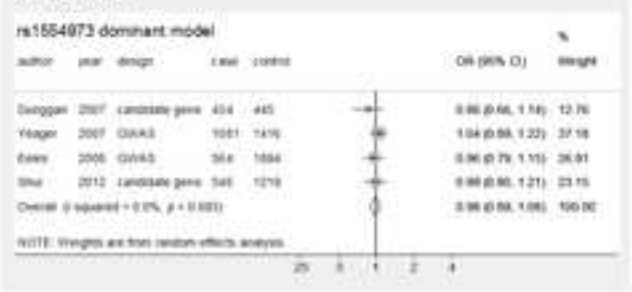
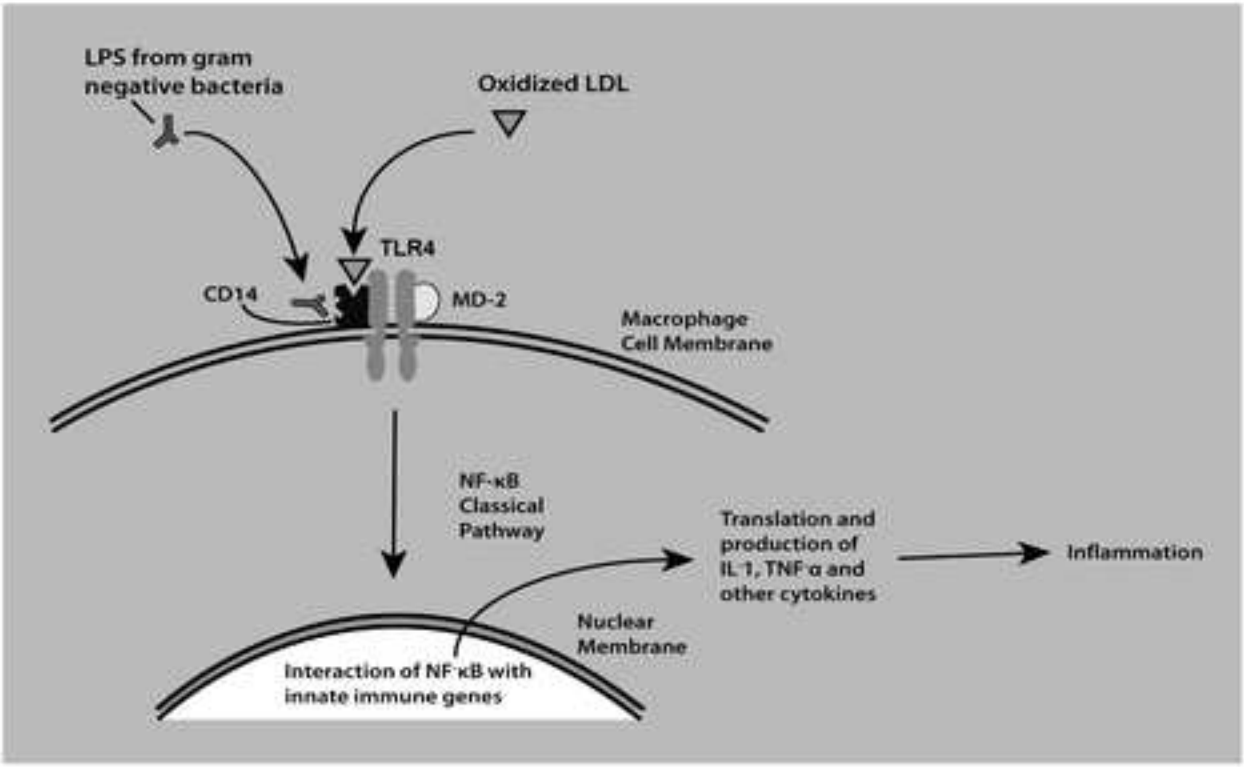


Figure4


LPS from gram negative bacteria
Oxidized LDL
TLR4
CD14
MD-2
Macrophage Cell Membrane
NF-κB Classical Pathway
Translation and production of IL-1, TNF-α and other cytokines
Inflammation
Nuclear Membrane
Interaction of NF-κB with innate immune genes

Supporting Information_Table S1

Supporting Information_Table S2

Supporting Information figure S1

excluded studies and reasons for exclusion meta-analysis on genetic association studies checklist

# Polymorphisms of an Innate Immune Gene, Toll-Like Receptor 4, and Aggressive Prostate Cancer Risk: A Systematic Review and Meta-analysis

**Pei-Hsuan Weng[1,2]*, Yi-Ling Huang[3]*, John H. Page[4], Jen-Hau Chen[2,5], Jianfeng Xu[6], Stella Koutros[7], Sonja Berndt[7], Stephen Chanock[7], Meredith Yeager[8,9], John S. Witte[10], Rosalind A. Eeles[11], Douglas F. Easton[12], David E. Neal[13], Jenny Donovan[14], Freddie C. Hamdy[15], Kenneth R. Muir[16], Graham Giles[17], Gianluca Severi[17], Jeffrey R. Smith[18], Carmela R. Balistreri[19] , Irene M. Shui[20], Yen-Ching Chen[2,21,22]**

**1** Department of Family Medicine, Taiwan Adventist Hospital, Taipei, Taiwan;
**2** Institute of Epidemiology and Preventive Medicine, College of Public Health, National Taiwan University, Taipei, Taiwan;
**3** Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging, Tufts University, Boston MA, USA;
**4** Channing Laboratory, Department of Epidemiology, Brigham and Women's Hospital, Boston MA, USA;
5 Department of Geriatrics and Gerontology, National Taiwan University Hospital, Taipei, Taiwan;
**6** Center for Human Genomics, Wake Forest University School of Medicine, Winston-Salem, NC, USA;
**7** Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Bethesda, Maryland, USA;
**8** Core Genotyping Facility, SAIC-Frederick, Inc., NCI-Frederick, Frederick, Maryland, USA;
**9** Division of Cancer Epidemiology and Genetics, NCI, NIH, DHHS, Bethesda, Maryland, USA;
**10** Department of Epidemiology and Biostatistics and Center of Human Genetics, University of California, San Francisco, California, USA;
**11** The Institute of Cancer Research, Sutton, UK;
**12** Centre for Cancer Epidemiology, Departments of Public Health and Primary Care and Oncology, University of Cambridge, Strangeways Laboratory, Worts Causeway, Cambridge, CB1 8RN, UK;
**13** Surgical Oncology (Uro-Oncology: S4), Departments of Oncology and Surgery, University of Cambridge, Box 279, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2QQ, UK;
**14** Department of Social Medicine, University of Bristol, Canynge Hall, Whiteladies Road, Bristol, UK;
**15** Academic Urology Unit, University of Sheffield, Sheffield, S10 2JF, UK;
**16** University of Nottingham Medical School, Queens Medical Centre, Nottingham, NG7 2UH, UK;
**17** Cancer Epidemiology Centre, The Cancer Council Victoria, 1 Rathdowne Street, Carlton VIC 3053, Australia;
**18** Department of Medicine, Vanderbil University School of Medicine, Nashville, Tennessee, USA;
**19** Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Palermo, Italy;
**20** Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts, USA;
**21** Research Center for Genes, Environment and Human Health, College of Public Health, National Taiwan University, Taipei, Taiwan;
**22** Department of Public Health, College of Public Health, National Taiwan University, Taipei, Taiwan.

*First two authors contributed equally to this work.

**Correspondence:**

Prof. Yen-Ching Chen

Institute of Epidemiology and Preventive Medicine
Department of Public Health
National Taiwan University
No.17, Xu-Zhou Road,
Taipei 10055,
Taiwan
Telephone: 886-2-3366-8019
Fax: 886-2-2351-1955
E-mail: karenchen@ntu.edu.tw

## Abstract

***Background:*** Toll-like receptor 4 (TLR4) is one of the best known TLR members expressed on the surface of several leukocytes and tissue cells and has a ~~having the~~ key function in detecting pathogen and danger-associated molecular patterns. The role of TLR4 in the pathophysiology of several age-related diseases is also well recognized, such as prostate cancer (PCa). *TLR4* polymorphisms have been related to PCa risk, but the relationship between *TLR4* genotypes and aggressive PCa risk has not been evaluated by any systematic reviews.

***Methods:*** We performed a systematic review and meta-analysis of candidate-gene and genome-wide association studies analyzing this relationship and included only white population~~s~~. Considering appropriate criteria, only nine studies were analyzed in the meta-analysis, including 3,937 aggressive PCa and 7,382 controls.

***Results:*** Using random effects model, no significant association was found in the ten *TLR4* SNPs reported by at least four included studies under any inheritance model (rs2737191, rs1927914, rs10759932, rs1927911, rs11536879, rs2149356, rs4986790, rs11536889, rs7873784, and rs1554973). Pooled estimates from another ten *TLR4* SNPs reported by three studies also showed no significant association (rs10759930, rs10116253, rs11536869, rs5030717, rs4986791, rs11536897, rs1927906, rs913930, rs1927905, and rs7045953). Meta-regression revealed that study type was not a significant source of between-study heterogeneity.

***Conclusions*****:** *TLR4* polymorphisms were~~as~~ not significantly associated with the risk of aggressive PCa.

**Formatted:** Font: Italic

## Introduction

Prostate cancer (PCa) is the most common malignancy ~~diagnosed~~ since 1984, the most frequently diagnosed cancer, and the second leading cause of cancer-related deaths in 2013 among men in the USA [1]. The risk of PCa is related to family history, race, and genetic factors. Several other causes have been associated with PCa pathogenesis, including infectious agents, chronic non-infectious inflammatory diseases, diet, environmental carcinogens, imbalance of sex hormone, obesity, and urine reflux [2-4]. Chronic inflammation has been linked to the pathogenesis of PCa in both epidemiologic studies and molecular pathology investigations [5,6]. In particular, several studies have suggested that sexually transmitted infections may be a risk factor for PCa through causing inflammation, even though not all the studies are consistent [7,8]. Chronic inflammation seems to induce prostate carcinogenesis and also promote neoplastic progression [9]. Furthermore, several pathways linking inflammation and PCa have been identified: an intrinsic one driven by genetic events that cause neoplasia, and an extrinsic one driven by inflammatory conditions that predispose to cancer [9]. Among these, the eicosanoid pathway activated by cyclooxygenase 2 (COX-2) has been suggested to be involved in the pathogenesis of aggressive PCa by a recent study [10]. COX-2 was over-expressed in PCa tumors and the intensity of immunostaining was correlated with prostate tumor grade [11]. Despite the available evidence on the role of the inflammatory response in PCa onset and progression, the association between genetic variants of innate immune genes and the risk of aggressive PCa remains unclear.

Toll-like receptor 4 (TLR4) is an important pathogen recognition receptor involved in detection of lipopolysaccharide (LPS) of Gram-negative bacteria~~,~~ and ~~of~~ other exogenous or~~and~~ endogenous ligands – [12]. The TLR4 encoding gene is located on chromosome 9q32-q33. Through nuclear factor kappa B (NF-κB), TLR4 initiates the production of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNF-α) [13]. TLR4 also mediates signaling related to tumor cell invasion, survival, and metastasis in various cancers [14,15]. Its activity and function seems to be modulated by genetic variations, principally single nucleotide polymorphisms (SNPs). Mice with deficiency or mutation

**Formatted:** Font: Not Italic

of TLR4 had a weaker inflammatory immune response to viral, bacterial [16,17], and protozoal [18] infections than that of wild-type mice. Therefore, variations in *TLR4* gene may modify the signaling of the immune response, which in turn may have effects on the pathogenesis of PCa.

**Formatted:** Font: Not Italic

Three recent meta-analyses have explored the association between *TLR4* SNPs and PCa [19-21]. They all reported non-significant findings after stratification by ethnicity. However, these studies focused their attention on overall PCa and did not contain genome-wide association studies (GWASs). In addition, they did not analyze the association between *TLR4* SNPs and the aggressive type of PCa. Thus, we conducted a systematic review and meta-analysis of all genetic epidemiologic association studies that have evaluated the relationship between *TLR4* polymorphisms and risk of aggressive PCa. Both candidate-gene studies and GWASs were included. The primary research questions are: (1) is there an association between *TLR4* SNPs and risk of aggressive PCa and if so, what is the size of the relationship? (2) what is the validity of the evidence of association between *TLR4* polymorphisms and risk of aggressive PCa?

## Materials and Methods

### *Ethics Statement*

The execution of each individual study was previously approved by the respective institution. This systematic review was performed at the study level without access to individual-level data, and therefore, institutional review board approval was not necessary. Informed consent was obtained from each participant before the start of each individual study.

### *Study Selection*

The study was performed using pre-specified research objectives, search strategy, study eligibility criteria, methods of data extraction, and statistical analyses. Relevant studies were identified by searching the MEDLINE (http://gateway.ovid.com/), EMBASE (http://www.embase.com), Science Citation Index (http://science.thomsonreuters.com/cgi-bin/jrnlst/jlsearch.cgi?PC=K), and Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/omim) databases for all genetic association studies published before February 2013, using combinations of the search terms "toll-like receptor 4," OR "toll-like receptor 4 gene," OR "*TLR,"* OR *"TLR* gene," OR "*TLR4,"* OR *"TLR4* gene," AND "prostate cancer," OR "prostatic neoplasms." GWASs were searched using combinations of the search terms "genome-wide association study," OR "GWAS," AND "prostate cancer," OR "prostatic neoplasms." In addition, we manually searched the reference lists from reviews and original articles to retrieve other papers relevant to the topic. Where there was overlap in the study populations of published papers, only the largest study was included. No language restriction was placed on the literature search strategies. Unpublished findings were not identified.

### *Exposure Measures*

The main exposure variables were *TLR4* genotypes as measured in blood DNA samples from men in the respective studies. This meta-analysis summarized *TLR4* SNPs which were reported by at least three included studies. Because many *TLR4* SNPs were explored by two studies only, and the respective sample sizes were small, these SNPs were not analyzed in this meta-analysis.

### *Outcome Measures*

The outcome measure was aggressive PCa as defined by Gleason score greater than or equal to seven, or TNM stage greater than or equal to T3b or any nodal involvement or any distant metastases. However, some included studies extended this definition. Controls for aggressive PCa are ideally men without aggressive PCa chosen from the population at risk, although some studies selected controls from men without screening for occult PCa (Table 1).

### *Data Extraction*

Three of us (PH Weng, YL Huang, and YC Chen) independently reviewed each published paper and extracted relevant information examining the associations between *TLR4* polymorphisms and risk of aggressive PCa. Inter-observer differences, if any existed, were reconciled through group discussion. In order to pool data from different studies, we requested data from each study based on the definition for aggressive PCa in this meta-analysis, which may be slightly different from their original design. For GWASs that did not report detailed information of *TLR4*, we contacted the investigators to obtain data on advanced PCa counts and the corresponding *TLR4* genotyping frequencies. To avoid population stratification, this meta-analysis was restricted to samples taken from European ancestry. We evaluated selection bias based on the extent to which controls are representative of the "person-time population" from which the cases were sampled, and the extent to which cases are a random sample of that latter population.

### *Statistical Analyses*

Meta-analyses were performed for SNPs that were reported by at least three included studies. The pooled odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between *TLR4* genotypes and risk of aggressive PCa were calculated using random effects models. Random effects models are preferred to fixed effect models because of the differences in study designs and study populations [22]. To incorporate both within-study and between-study variability, we used DerSimonian and Laird's [23] random effects models to pool the estimates of log OR from each individual study (unadjusted for covariates). Between-study heterogeneity was quantified by using the $I^2$ statistic [24,25], which indicates the proportion of variability across studies attributable to heterogeneity. Tests of heterogeneity were assessed by a $\chi^2$ statistic. To explore the inheritance mode for the effect of *TLR4* polymorphisms, we evaluated the following genotype contrasts (where a and A denote minor and major alleles, respectively): (1) a/a and A/a combined versus A/A (dominant model); (2) a/a versus A/a and A/A combined (recessive model); (3) a/a versus A/A and A/a versus A/A (co-dominant model); (4) the increment of one minor allele (additive model). The Hardy-Weinberg equilibrium (HWE) was assessed via $\chi^2$ ~~$\chi^2$~~ test. We did not perform haplotype analysis because none of the previous studies performed haplotype analysis specific for these SNPs. Because most GWASs did not adjust for covariates, this meta-analysis reported unadjusted pooled results.

To evaluate the presence of publication bias, we examined the funnel plot, by plotting the reciprocal of the standard error of log OR versus the log OR, for symmetry. The Egger linear regression test was also performed to assess funnel plot's asymmetry [26]. Random effects meta-regression was performed under dominant model to explore possible sources of between-study heterogeneity. Study type (candidate-gene studies vs. GWASs) was the pre-specified covariate. We did not perform stratification analysis according to differences in control and case selection, because such influences are complex and are usually not unidirectional. Because previous studies revealed high concordance rate across genotyping platforms [27], stratification analysis was not carried out according to this covariate. Analyses were performed with Stata version 11.0 software (Stata Corporation, College Station, TX, USA). All *P* values were two-sided. QUANTO program (http://hydra.usc.edu/gxe/) was used to evaluate statistical power of the association between *TLR4* polymorphisms and aggressive PCa.

**Formatted:** Font: Not Bold

**Formatted:** Font: Not Bold

**Field Code Changed**

**Field Code Changed**

**Formatted:** Font: Bold, Underline, Font color: Auto

## Results

### Characteristics of Association Studies

Using the pre-specified search methodology we retrieved forty relevant publications (Figure 1). After excluding duplicates (n=10), seventeen studies were further excluded due to the following reasons: (1) not European ancestry (n=5), (2) partially overlapped populations (n=9), (3) lack of controls (n=1), and (4) GWAS which did not include *TLR4* gene (n=2).

We contacted the authors of the remaining 13 relevant studies for necessary details, and authors ~~for~~ of three of the GWASs [28-30] didn't respond and were thus excluded. One GWAS was excluded because it didn't contain the information of PCa aggressiveness [31]. For studies composed of multiple cohorts (e.g., Lindstrom et al. [32] ), we tried to obtain data from each cohort and used the original study to represent each cohort (e. g., Chen et al. [33] for HPFS, Dunggan et al. [34] for CAPS, and Yeager et al. ~~for PLCO~~ [35] for PLCO). For the CAPS study, the GWAS by Dunggan et al. [34] was selected instead of the candidate-gene study done by Zheng et al. [36] because the former was composed of aggressive PCa cases from Zheng's study and evaluated more SNPs. In sum, nine studies were included for the meta-analysis.

A total of 3,937 aggressive PCa cases and 7,382 controls were included in this work. Six studies were candidate-gene studies [33,37-41], and three of them were GWASs [34,35,42]. Six papers studied US populations [33,35,37-39], one studied a Swedish population [34], one studied the combination of UK and Australian population [42], and one studied an Italian population [40]. Details of the studies analyzed in this meta-analysis were summarized in Table 1, including first author, year of publication, type of study, ancestry, sample size, control selection, possible sources of selection bias, definition of PCa aggressiveness, genotyping methods and quality control.

For the association between *TLR4* SNPs and aggressive PCa, seven studies assessed rs4986790 [33-35,37,39,40,42]; five studies investigated rs2149356 [33,34,37,39,41], rs11536889 [33,34,37,39,41], rs7873784 [33,34,37,39,41]; and four studies explored rs2737191[34,35,41,42] , rs1927914 [33,34,38,39], rs10759932 [33,34,37,41], rs1927911 [33,34,38,39], rs11536879 [34,35,38,42], and rs1554973 [34,35,41,42].

### Allele Frequencies of *TLR4* SNPs

Ten *TLR4* SNPs had been evaluated by at least 4 included studies. The minor allele frequencies (MAF) between case and controls were shown in Table 2, along with the test for HWE in controls. Among them, three SNPs are located on 5’ untranslated region (UTR, rs2737191, rs1927914 and rs10759932), three are intronic SNPs (rs1927911, rs11536879, and rs2149356), one is non-synonymous exonic SNP (rs4986790), and three SNPs are located on 3’ UTR (rs11536889, rs7873784, and rs1554973). Another 10 *TLR4* SNPs were reported by 3 studies, including one SNP located on the promoter region (rs10759930), one SNP located on 5’UTR (rs10116253), two intronic SNPs (rs11536869 and rs5030717), one non-synonymous exonic SNP (rs4986791), and five SNPs located on 3’ UTR (rs11536897, rs1927906, rs913930, rs1927905, and rs7045953). The locations of the explored SNPs (10 SNPs with ≧4 studies, 10 SNPs with 3 studies) are shown in Figure 2. rs2149356, rs4986790 and rs7873784 in Chen’s study and rs1927911 in Wang’s study were out of HWE (*P* = 0.01-0.03) but were kept in the analysis because the HWE tests were not significant after correction for multiple tests.

### Meta-Analysi~~e~~s

Using random effects meta-analysis, the ten *TLR4* SNPs (rs2737191, rs1927914, rs10759932, rs1927911, rs11536879, rs2149356, rs4986790, rs11536889, rs7873784, and rs1444973) were not associated with the risk of aggressive PCa regardless of the inheritance model used (Table 3, Figure 3). The meta-analysis was also performed for another ten SNPs which were reported by three included studies (rs10759930, rs10116253, rs11536869, rs5030717, rs4986791, rs11536897, rs1927906, rs913930, rs1927905, and rs7045953) (Table S1). None of the SNPs revealed significant association with aggressive PCa. This meta-analysis was reported according to the PRISMA checklist [43] (Table S2).

### Publication Bias

Funnel plots were used to assess the relationship between the ten *TLR4* SNPs and aggressive PCa

(Figure S1). Using the Egger linear regression test, possible publication bias was found among the included studies on rs1554973 (Egger test *P* = 0.06). For the other 9 SNPs, *P* values ranged from 0.2 to 0.77.

### Meta-regression

Random effects meta-regression was performed under dominant model. Different study type (candidate-gene studies vs. GWASs) was not a significant source of between-study heterogeneity (*P* value ranged from 0.15 to 0.79 for the ten *TLR4* SNPs).

### Power Calculation

For people of European ancestry, given a MAF of 0.15 and $\alpha$ of 0.05, this study had over 95% power to detect an OR of 1.20 for 3,937 cases and 7,382 controls.

**Formatted:** Indent: First line: 0 ch

## Discussion

Recently, some researchers hypothesized that PCa is the result of a chronic inflammatory process [44]. Proliferative inflammatory atrophy (PIA), proposed as a potential precursor to PCa, occurs frequently in the periphery of the prostate gland where PCa occurs [5]. PIA lesions seem to be the result of different conditions, including infections, chronic non-infectious inflammatory diseases, dietary carcinogens, physical trauma, imbalance of sex hormone and urine reflux [9].Chronic infections may contribute to PIA and lead to onset of PCa [45-47]. Several innate inflammatory pathways seem to be involved. Among these, TLR4 pathway plays a crucial role [48].

TLR4 recognizes pathogen-associated molecular patterns, i.e. LPS [46]. Damage-associated molecular pattern molecules may also interact with TLR4, i.e. oxidized low-density lipoprotein (LDL) [49], one of the atherogenic lipoproteins associated with atherosclerosis [50] and insulin resistance [51,52]. Their interaction leads to the initiation of inflammatory response via NF-κB (Figure 4) [53]. TLR4 can also promote PCa development through releasing inflammatory mediators. Associations between *TLR4* SNPs and PCa have been examined in several studies, though discordant data have been reported. However, the relationship between *TLR4* genotypes and aggressive PCa risk has not been evaluated by any systematic reviews. Thus, we performed a systematic review and meta-analysis of candidate-gene studies and GWASs analyzing this relationship and restricted to samples taken from European ancestry.

In the current meta-analysis, none of the examined *TLR4* SNPs was significantly associated with risk of aggressive PCa under any inheritance model. No significant association was found between the *TLR4* SNPs (5’UTR: rs2737191, rs1927914 and rs10759932; intron: rs1927911, rs11536879, and rs2149356; exon: rs4986790; 3’UTR: rs11536889, rs7873784, and rs1554973) and risk of aggressive PCa in the pooled analysis. The non-significant findings may be attributable to (1) failure to adjust for the conventional risk factors of PCa, e.g. family history of PCa, (2) inability to assess the within-population heterogeneity or geographic variation, and (3) the studied *TLR4* SNPs may be more closely related to non-aggressive PCa.

Three recent meta-analyses evaluated the association between *TLR4* SNPs and overall PCa. Jing et al. [19], including four candidate-gene studies [33,37,39,40], examined two *TLR4* SNPs (rs4986790 and rs4986791) and found that rs4986790 showed a protective effect on overall PCa under co-dominant and recessive models. However, the effect was not statistically significant after stratification by ethnicity. Another work by Zhang et al. [20] examined six *TLR4* SNPs (rs1927914, rs4986790, rs4986791, rs11536889, rs1927911, rs2149356) and did not find significant associations with overall PCa. The pooled estimates of Zhang et al. were derived from one Asian study [54] and four other populations of European ancestry [33,36,39,41], which might be confounded by population stratification. Zhu et al. [21] examined rs4986790 and rs4986791 and found no significant association with overall PCa in five populations of European ancestry [33,36,37,39,40]. In summary, our findings on aggressive PCa are consistent with the previous meta-analyses on overall PCa. Our study had several advantages over the previous meta-analyses: (1) this study additionally included GWASs, whereas previous meta-analyses included candidate-gene studies only [19-21] , (2) this study focused on aggressive PCa, which is more clinically relevant, (3) this study was restricted to populations of European ancestry to avoid population stratification, and (4) this study evaluated an additional 14 SNPs, which were not reported in the previous meta-analyses.

Previous candidate-gene studies and GWASs found inconsistent results for the association between *TLR4* polymorphisms and PCa risk. This may be explained by different ethnicity, within-population heterogeneity, case and control selection, gene-gene interactions, and gene-environment interactions. Although most of the relevant medical centers were in the “catchment” area, Cheng and colleagues [37] used controls from medical centers, which differ from the source population in that not all men with potential PCa would go to these centers to be screened and diagnosed.

There were some limitations of this study. One of them is the possibility of publication bias. Though the funnel plots did not reveal obvious publication bias among most of *TLR4* SNPs, the SNPs reported in this study were under the influence of publication bias because only SNPs explored in ≧3 studies were included. We were unable to include three other GWASs because the authors did not respond to our data request [28-30]. After exclusion of men with African and Asian ancestry, there was little evidence that population stratification was a cause of confounding. Though the included studies were conducted separately in the United States, Sweden, Italy, UK and Australia, a prior theoretical calculation on genetic case-control studies showed that ignoring ethnicity among non-Hispanic U.S. Caucasians with ancestries from different European countries resulted in bias of less than 1% [55]. Last, the included studies used different genotyping approaches, which may be associated with different genotyping success rates and data quality. However, genotyping errors are expected to be small, and thus the resulting biases are likely to be small.

This study had some advantages. To the best of our knowledge, this is the first meta-analysis on *TLR4* polymorphisms and aggressive PCa, which shows more clinical relevance. All the included studies were reasonably well-designed epidemiological studies. Genotyping was carried out “blind” to the disease status, and assessment of aggressive PCa was carried out “blind” to the genotypes. This study had sufficient power (> 0.95) to detect a potential OR of aggressive PCa associated with a SNP of 1.20. This study presents the best available evidence ~~we have available~~ on the relationship between *TLR4* polymorphisms and risk of aggressive PCa.

In conclusion, this study found that none of the examined *TLR4* SNPs were significantly associated with risk of aggressive PCa under any mode of inheritance. Control selection, different ancestry, small statistical power in some studies, publication bias, gene-gene and gene-environment interactions, different genotyping approaches, and issues of multiple tests may contribute to the inconsistent findings in previous studies. Meta-regression revealed that different study type (candidate-gene studies vs. GWASs) was not a significant source of between-study heterogeneity. Large-scale and well-designed studies using population-based controls and more studies in each ethnic group are needed to confirm our findings.

## Acknowledgement

Thanks to Dr. Elizabeth Platz, who provided detailed information on aggressive PCa from their study [39].

## Supporting Information

**Table S1 Pooled estimated ORs and 95% CIs for the association of *TLR4* SNPs in aggressive PCa risk**

**Table S2 PRISMA checklist**

**Figure S1 Funnel plot of *TLR4* SNPs**

Funnel plot displays the publication bias for each study (indicated as one dot) exploring the relation between *TLR4* SNPs and aggressive prostate cancer. SNPs reported by at least four studies were shown here

## References

1. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. CA Cancer J Clin 63: 11-30.
2. Bostwick DG, Burke HB, Djakiew D, Euling S, Ho SM, et al. (2004) Human prostate cancer risk factors. Cancer 101: 2371-2490.
3. Haqq C, Li R, Khodabakhsh D, Frolov A, Ginzinger D, et al. (2005) Ethnic and racial differences in prostate stromal estrogen receptor alpha. Prostate 65: 101-109.
4. Platz EA, Giovannucci E (2004) The epidemiology of sex steroid hormones and their signaling and metabolic pathways in the etiology of prostate cancer. J Steroid Biochem Mol Biol 92: 237-253.
5. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, et al. (2007) Inflammation in prostate carcinogenesis. Nat Rev Cancer 7: 256-269.
6. Klein EA, Silverman R (2008) Inflammation, infection, and prostate cancer. Curr Opin Urol 18: 315-319.
7. Sutcliffe S, Platz EA (2007) Inflammation in the etiology of prostate cancer: an epidemiologic perspective. Urol Oncol 25: 242-249.
8. Dennis LK, Dawson DV (2002) Meta-analysis of measures of sexual activity and prostate cancer. Epidemiology 13: 72-79.
9. Balistreri CR, Candore G, Lio D, Carruba G (2014) Prostate cancer: from the pathophysiologic implications of some genetic risk factors to translation in personalized cancer treatments. Cancer Gene Ther 21: 2-11.
10. Cheng I, Liu X, Plummer SJ, Krumroy LM, Casey G, et al. (2007) COX2 genetic variation, NSAIDs, and advanced prostate cancer risk. Br J Cancer 97: 557-561.
11. Lee LM, Pan CC, Cheng CJ, Chi CW, Liu TY (2001) Expression of cyclooxygenase-2 in prostate adenocarcinoma and benign prostatic hyperplasia. Anticancer Research 21: 1291-1294.
12. Balistreri CR, Colonna-Romano G, Lio D, Candore G, Caruso C (2009) TLR4 polymorphisms and ageing: implications for the pathophysiology of age-related diseases. J Clin Immunol 29: 406-415.
13. Ferwerda B, McCall MB, Verheijen K, Kullberg BJ, van der Ven AJ, et al. (2008) Functional consequences of toll-like receptor 4 polymorphisms. Molecular Medicine 14: 346-352.
14. El-Omar EM, Ng MT, Hold GL (2008) Polymorphisms in Toll-like receptor genes and risk of cancer. Oncogene 27: 244-252.
15. Hua D, Liu MY, Cheng ZD, Qin XJ, Zhang HM, et al. (2009) Small interfering RNA-directed targeting of Toll-like receptor 4 inhibits human prostate cancer cell invasion, survival, and tumorigenicity. Mol Immunol 46: 2876-2884.
16. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, et al. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282: 2085-2088.
17. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, et al. (2000) Pattern recognition receptors

TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol 1: 398-401.

18. Kropf P, Freudenberg MA, Modolell M, Price HP, Herath S, et al. (2004) Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite Leishmania major. Infection and Immunity 72: 1920-1928.
19. Jing JJ, Li M, Yuan Y (2012) Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms in cancer: a meta-analysis. Gene 499: 237-242.
20. Zhang K, Zhou B, Wang Y, Rao L, Zhang L (2013) The TLR4 gene polymorphisms and susceptibility to cancer: a systematic review and meta-analysis. Eur J Cancer 49: 946-954.
21. Zhu L, Yuan H, Jiang T, Wang R, Ma H, et al. (2013) Association of TLR2 and TLR4 Polymorphisms with Risk of Cancer: A Meta-Analysis. PLoS One 8: e82858.
22. Egger M, Smith GD, Altman DG (2001) Systematic reviews in health care: meta-analysis in context. London: The BMJ Publishing Group.
23. DerSimonian R, Laird N (1986) Meta-analysis in clinical trials. Controlled Clinical Trials 7: 177-188.
24. Higgins JP, Thompson SG (2004) Controlling the risk of spurious findings from meta-regression. Stat Med 23: 1663-1682.
25. Higgins JP, Thompson SG, Deeks JJ, Altman DG (2003) Measuring inconsistency in meta-analyses. BMJ 327: 557-560.
26. Egger M, Davey Smith G, Schneider M, Minder C (1997) Bias in meta-analysis detected by a simple, graphical test. BMJ 315: 629-634.
27. Hong H, Xu L, Liu J, Jones WD, Su Z, et al. (2012) Technical reproducibility of genotyping SNP arrays used in genome-wide association studies. PLoS One 7: e44483.
28. Schumacher FR, Berndt SI, Siddiq A, Jacobs KB, Wang Z, et al. (2011) Genome-wide association study identifies new prostate cancer susceptibility loci. Hum Mol Genet 20: 3867-3875.
29. FitzGerald LM, Kwon EM, Conomos MP, Kolb S, Holt SK, et al. (2011) Genome-wide association study identifies a genetic variant associated with risk for more aggressive prostate cancer. Cancer Epidemiol Biomarkers Prev 20: 1196-1203.
30. Nam RK, Zhang W, Siminovitch K, Shlien A, Kattan MW, et al. (2011) New variants at 10q26 and 15q21 are associated with aggressive prostate cancer in a genome-wide association study from a prostate biopsy screening cohort. Cancer Biol Ther 12: 997-1004.
31. Murabito JM, Rosenberg CL, Finger D, Kreger BE, Levy D, et al. (2007) A genome-wide association study of breast and prostate cancer in the NHLBI's Framingham Heart Study. BMC Med Genet 8 Suppl 1: S6.
32. Lindstrom S, Hunter DJ, Gronberg H, Stattin P, Wiklund F, et al. (2010) Sequence variants in the TLR4 and TLR6-1-10 genes and prostate cancer risk. Results based on pooled analysis from three independent studies. Cancer Epidemiology, Biomarkers and Prevention 19: 873-876.

33. Chen YC, Giovannucci E, Lazarus R, Kraft P, Ketkar S, et al. (2005) Sequence variants of Toll-like receptor 4 and susceptibility to prostate cancer. Cancer Res 65: 11771-11778.
34. Duggan D, Zheng SL, Knowlton M, Benitez D, Dimitrov L, et al. (2007) Two genome-wide association studies of aggressive prostate cancer implicate putative prostate tumor suppressor gene DAB2IP. J Natl Cancer Inst 99: 1836-1844.
35. Yeager M, Orr N, Hayes RB, Jacobs KB, Kraft P, et al. (2007) Genome-wide association study of prostate cancer identifies a second risk locus at 8q24. Nat Genet 39: 645-649.
36. Zheng SL, Augustsson-Balter K, Chang B, Hedelin M, Li L, et al. (2004) Sequence variants of toll-like receptor 4 are associated with prostate cancer risk: results from the CAncer Prostate in Sweden Study. Cancer Res 64: 2918-2922.
37. Cheng I, Plummer SJ, Casey G, Witte JS (2007) Toll-like receptor 4 genetic variation and advanced prostate cancer risk. Cancer Epidemiol Biomarkers Prev 16: 352-355.
38. Breyer JP, McReynolds KM, Yaspan BL, Bradley KM, Dupont WD, et al. (2009) Genetic variants and prostate cancer risk: candidate replication and exploration of viral restriction genes. Cancer Epidemiol Biomarkers Prev 18: 2137-2144.
39. Wang MH, Helzlsouer KJ, Smith MW, Hoffman-Bolton JA, Clipp SL, et al. (2009) Association of IL10 and other immune response- and obesity-related genes with prostate cancer in CLUE II. Prostate 69: 874-885.
40. Balistreri CR, Caruso C, Carruba G, Miceli V, Campisi I, et al. (2010) A pilot study on prostate cancer risk and pro-inflammatory genotypes: pathophysiology and therapeutic implications. Curr Pharm Des 16: 718-724.
41. Shui IM, Stark JR, Penney KL, Schumacher FR, Epstein MM, et al. (2012) Genetic variation in the toll-like receptor 4 and prostate cancer incidence and mortality. Prostate 72: 209-216.
42. Eeles RA, Kote-Jarai Z, Giles GG, Olama AA, Guy M, et al. (2008) Multiple newly identified loci associated with prostate cancer susceptibility. Nature Genetics 40: 316-321.
43. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gotzsche PC, et al. (2009) The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. PLoS Med 6: e1000100.
44. Sakr WA, Grignon DJ, Crissman JD, Heilbrun LK, Cassin BJ, et al. (1994) High grade prostatic intraepithelial neoplasia (HGPIN) and prostatic adenocarcinoma between the ages of 20-69: an autopsy study of 249 cases. In Vivo 8: 439-443.
45. Das D, Wojno K, Imperiale MJ (2008) BK virus as a cofactor in the etiology of prostate cancer in its early stages. J Virol 82: 2705-2714.
46. Cavallaro S, Meiri N, Yi CL, Musco S, Ma W, et al. (1997) Late memory-related genes in the hippocampus revealed by RNA fingerprinting. Proc Natl Acad Sci U S A 94: 9669-9673.

47. Taylor ML, Mainous AG, 3rd, Wells BJ (2005) Prostate cancer and sexually transmitted diseases: a meta-analysis. Fam Med 37: 506-512.
48. DeFranco AL, Locksley RM, Robertson M (2007) Immunity: the immune response in infectious and inflammatory disease. Corby, Northants, UK: Oxford University Press. 387 p.
49. Miller YI, Viriyakosol S, Binder CJ, Feramisco JR, Kirkland TN, et al. (2003) Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. J Biol Chem 278: 1561-1568.
50. Holvoet P, Mertens A, Verhamme P, Bogaerts K, Beyens G, et al. (2001) Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. Arterioscler Thromb Vasc Biol 21: 844-848.
51. Shashkin PN, Jain N, Miller YI, Rissing BA, Huo Y, et al. (2006) Insulin and glucose play a role in foam cell formation and function. Cardiovasc Diabetol 5: 13.
52. Carantoni M, Abbasi F, Warmerdam F, Klebanov M, Wang PW, et al. (1998) Relationship between insulin resistance and partially oxidized LDL particles in healthy, nondiabetic volunteers. Arterioscler Thromb Vasc Biol 18: 762-767.
53. Madoff LC, Kasper DL (2008) Introduction of Infectious Diseases: Host-Pathogen Interactions. In: Fauci AS, Braunwald E, Kasper DL, Hauser SL, Longo DL et al., editors. Harrison's principles of internal medicine. 17 ed. New York: McGraw-Hill.
54. Song J, Kim DY, Kim CS, Kim HJ, Lee DH, et al. (2009) The association between Toll-like receptor 4 (TLR4) polymorphisms and the risk of prostate cancer in Korean men. Cancer Genetics and Cytogenetics 190: 88-92.
55. Wacholder S, Rothman N, Caporaso N (2000) Population stratification in epidemiologic studies of common genetic variants and cancer: quantification of bias. Journal of the National Cancer Institute 92: 1151-1158.

## Figure legends

### Figure 1. Study selection flowchart

Forty studies were reviewed after literature search. Among them, 31 studies were excluded due to duplication, race other than whites, and insufficient data. A total of 9 studies were included for meta-analysis.

### Figure 2. *TLR4* SNPs evaluated in this meta-analysis

This plot was generated by the Locusview program. The highlighted boxed SNPs were *TLR4* polymorphisms explored by at least four studies. The remaining SNPs were those reported by ~~had at least~~ three studies ~~that were~~, discussed in the supplemental data.

### Figure 3. Forest plot examines relationship between *TLR4* SNPs and risk of aggressive prostate cancer

Odds ratios and weights were demonstrated for each individual study and for the pooled analysis, assuming a dominant model. SNPs that were evaluated by at least 4 studies were shown here.

### Figure 4. The role of TLR4 in innate immunity

TLR4 receptors are responsible for the recognition of bacterial lipopolysaccharide (LPS) monomers and partially oxidized LDL (oLDL) on innate immune cells. LPS monomers and oLDL bind to sites on the protein, CD14. CD14 promotes the binding of these ligands to the TLR4-MD-2 complex, which signals the activation of the nuclear factor kappa B (NF-κB) pathway. NF-κB products enter the nucleus and result~~s~~ in transcription followed by the production of cytokines~~,~~ and the activation of multiple inflammatory pathways. This figure was adapted from DeFranco et al. [48].

## Table 1. Characteristics of the study populations that evaluated the relationship between *TLR4* polymorphisms and risk of prostate cancer

| Source, publication year (study year) | Type of study | Country/ ancestry | Aggressive PCa/ control | Control selection | Comments about control selection | Case selection | Definition of aggressive prostate cancer | Outcome assessment "blinded" to genotype | Genotyping procedures | Genotyping quality control |
|---|---|---|---|---|---|---|---|---|---|---|
| Chen et al., 2005<br>(1993-1995) | Candidate gene | U.S. / 97% Caucasians | 260/ 700 | Age- matched controls from prospective cohort | PSA tested in controls | Incident PCa | TNM stage T3b or T4 or N1 or M1 or death due to PCa or Gleason sum ≥ 7 | Yes | MassARRAY system (SEQUENOM)[**] | 100% concordance, > 95% genotyping success |
| Dunggan et al., 2007<br>(2001-2002) | GWAS | Sweden/ Not mentioned | 505/ 507 | Age-matched population controls from the same geographical region | 74% response rate in cases, 52% in controls. No PSA tested in controls. | PCa from cancer registry | TNM stage T3 or T4 or N+ or M+ or grade III or Gleason sum > 7 or PSA > 100 ng/ml | Yes | MassARRAY system (SEQUENOM) [**] | >99 % concordance, >98% genotyping success |
| Yeager et al., 2007<br>(1993-2001) | GWAS | U.S. /White and non-hispanic | 1081/ 1416 | Risk set sampling from a population-based randomized controlled trial | PSA tested in controls | Incident PCa | Gleason sum ≥ 7 or stage ≥ 3 | Yes | Illumina system | >99 % concordance, >99% genotyping success |
| Cheng et al., 2007<br>(2002-2004) | Candidate gene | U.S./ Caucasians | 417/ 417 | From annual medical examinations at the same medical institutions of cases | Hospital-based study. PSA tested in controls | Incident PCa | TNM stage ≥ T2c or Gleason sum ≥ 7 or PSA> 10 ng/ml | Yes | Taqman | 100% concordance, 99.9% genotyping success |
| Eeles et al., 2008<br>(1993-2001) | GWAS | U.K., Australia/ Excluded self-reported "non-white" | 564/ 1894 | Community-based randomized controlled trial/electoral rolls | Controls to be frequency matched to the geographical distribution of the cases. | PCa from cancer registry, urology clinic | Gleason sum ≥ 7 | Yes | Stage 1: Illumina Infinium HumanHap550 array<br>Stage 2: Taqman | >97 % SNPs at a confidence score of ≥ 0.25, 98.8 % concordance |
| Breyer et al.,2009<br>(2002-2008) | Candidate-gene | U.S./ Americans of Northern European decents | 441/ 772 | Age-matched controls from a preventive screening | Hospital-based.<br>PSA tested in controls | Incident PCa | Gleason sum ≥ 7 | Yes | Illumina GoldenGate platform and Taqman | 99.7 % of genotyping success |
| Wang et al., 2009<br>(1992-2002) | Candidate gene | U.S./ White only | 77/ 264 | Age- matched controls from a prospective cohort | No PSA tested in controls | Incident PCa | TNM stage T3 or T4 or N1 or M1 or death due to PCa or Gleason sum ≥ 7 | Not mentioned | Taqman | 93-99 % genotyping success |
| Ballistreri et al., | Candidate | Italy/ | 32/ | Age-matched controls | Hospital-based study. | Prevalent PCa | Gleason sum ≥ 7 | Yes | RFLP-PCR | Not mentioned |

|  |  |  |  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|
| 2010<br>(NA) | gene | European ancestry | 125 | in good health | No clear description on control selection. No PSA tested in controls |  |  |  |  |  |
| Shui et al., 2012<br>(1982-2004) | Candidate gene | U.S./ White | 560/ 1287 | Risk set sampling from a prospective cohort, matched on age and smoking | No PSA tested in controls | Incident PCa | TNM stage T3 or T4, M1 or N1 or death due to PCa or Gleason sum ≥ 7 | Yes | Sequenom iPLEX matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry technology. | 100% concordance, >95% genotyping success |

Abbreviations: PCa, prostate cancer; TNM, the tumor node metastases classification system; PSA, prostate specific antigen; GWAS, genome-wide association study; RLFP-PCR, restriction fragment length polymorphism-polymerase chain reaction

All studies met the following criteria and they were not listed in the table: (1) clear description of laboratory methods, (2) genotyping identical for cases and controls, (3) genotyping blinded to case control status, and (4) specimen came from peripheral blood sample.

**Table 2. Characteristics of included studies**

| | rs2737191 (A/G) | | rs1927914 (A/G) | | rs10759932 (T/C) | | rs1927911 (G/A) | | rs11536879 (A/G) | | rs2149356 (G/T) | | rs4986790 (A/G) | | rs11536889 (A/G) | | rs7873784 (G/C) | | rs1554973 (T/C) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls | MAF case/ control | HWE *P* in controls |
| Chen et al.,2005 | NA | NA | 0.30/ 0.35 | 0.15 | 0.14/ 0.16 | 0.09 | 0.25/ 0.29 | 0.43 | NA | NA | 0.30/ 0.34 | 0.02 | 0.04/ 0.05 | 0.01 | 0.15/ 0.14 | 0.52 | 0.15/ 0.18 | 0.03 | NA | NA |
| Dunggan et al.,2007 | 0.27/ 0.27 | 0.46 | 0.33/ 0.34 | 0.55 | 0.16/ 0.15 | 0.71 | 0.27/ 0.26 | 0.74 | 0.01/ 0.01 | 0.82 | 0.31/ 0.32 | 0.89 | 0.05/ 0.06 | 0.15 | NA | NA | 0.11/ 0.13 | 0.99 | 0.19/ 0.21 | 0.45 |
| Yeager et al.,2007 | 0.28/ 0.29 | 0.88 | 0.32/ 0.32 | 0.94 | NA | NA | NA | NA | 0.04/ 0.04 | 0.83 | NA | NA | 0.06/ 0.05 | 0.59 | NA | NA | NA | NA | 0.24/ 0.23 | 0.11 |
| Cheng et al.,2007 | NA | NA | NA | NA | 0.13/ 0.14 | 0.04 | NA | NA | NA | NA | 0.32/ 0.30 | 0.68 | 0.06/ 0.05 | 0.98 | 0.15/ 0.14 | 0.09 | 0.15/ 0.16 | 0.82 | NA | NA |
| Eeles et al.,2008 | 0.27 /0.29 | 0.76 | 0.33/ 0.33 | 0.79 | NA | NA | NA | NA | 0.05/ 0.04 | 0.71 | NA | NA | 0.05/ 0.06 | 0.74 | NA | NA | NA | NA | 0.26/ 0.26 | 0.86 |
| Breyer et al, 2009 | NA | NA | NA | NA | NA | NA | 0.27/ 0.26 | 0.34 | 0.04/ 0.03 | 0.92 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| Wang et al.,2009 | NA | NA | 0.32/ 0.32 | 0.24 | NA | NA | 0.27/ 0.24 | 0.02 | NA | NA | 0.35/ 0.32 | 0.18 | 0.06/ 0.07 | 0.24 | 0.16/ 0.16 | 0.76 | 0.11/ 0.12 | 0.91 | NA | NA |
| Ballistreri et al.,2010 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | 0/ 0.06 | 0.38 | NA | NA | NA | NA | NA | NA |
| Shui et al.,2012 | 0.26/ 0.26 | 0.08 | NA | NA | 0.13/ 0.13 | 0.03 | NA | NA | NA | NA | 0.30/ 0.30 | 0.06 | NA | NA | 0.16/ 0.14 | 0.55 | 0.14/ 0.14 | 0.20 | 0.25 /0.25 | 0.18 |

SNPs that were evaluated by at least 4 studies were shown here. Abbreviations: MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; NA, not available.

**Table 3. Pooled estimated ORs and 95% CIs for the association of *TLR4* SNPs in aggressive PCa risk**

| | | Random effects model | | Heterogeneity | | | | Random effects model | | Heterogeneity | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Genetic model | OR (95% CI) | *P* | $I^2$ | *P* | | Genetic model | OR (95% CI) | *P* | $I^2$ | *P* |
| rs2737191 | Dominant | 0.96 (0.84-1.11) | 0.61 | 50.8% | 0.11 | rs2149356 | Dominant | 1.01 (0.89-1.14) | 0.90 | 0% | 0.62 |
| | Recessive | 0.86 (0.72-1.03) | 0.40 | 0% | 0.40 | | Recessive | 0.91 (0.73-1.12) | 0.37 | 6% | 0.37 |
| | AG vs. AA | 1.00 (0.83-1.19) | 0.97 | 65.9% | 0.03 | | GT vs. GG | 1.03 (0.91-1.17) | 0.63 | 0% | 0.86 |
| | GG vs. AA | 0.84 (0.84-1.08) | 0.07 | 35.7% | 0.14 | | TT vs. GG | 0.92 (0.72-1.17) | 0.49 | 16.9% | 0.31 |
| | Additive | 0.95 (0.84-1.07) | 0.41 | 30% | 0.18 | | Additive | 0.99 (0.90-1.08) | 0.83 | 0% | 0.69 |
| rs1927914 | Dominant | 0.95 (0.83-1.08) | 0.43 | 0% | 0.82 | rs4986790 | Dominant | 0.98 (0.83-1.16) | 0.82 | 12.2% | 0.34 |
| | Recessive | 0.88 (0.62-1.24) | 0.46 | 52.9% | 0.10 | | Recessive | 1.29 (0.57-2.95) | 0.55 | 0% | 0.81 |
| | AG vs. AA | 0.96 (0.84-1.10) | 0.53 | 0 | 0.86 | | AG vs. AA | 0.98 (0.83-1.16) | 0.81 | 10% | 0.35 |
| | GG vs. AA | 0.87 (0.63-1.21) | 0.41 | 44.9% | 0.14 | | GG vs. AA | 1.28 (0.56-2.93) | 0.59 | 0% | 0.82 |
| | Additive | 0.96 (0.87-1.06) | 0.44 | 1% | 0.42 | | Additive | 1.02 (0.88-1.17) | 0.83 | 0% | 0.62 |
| rs10759932 | Dominant | 0.97 (0.83-1.14) | 0.70 | 19.8% | 0.29 | rs11536889 | Dominant | 1.05 (0.91-1.21) | 0.48 | 0% | 0.49 |
| | Recessive | 1.33 (0.70-2.54) | 0.38 | 44% | 0.15 | | Recessive | 1.25 (0.84-1.86) | 0.26 | 0% | 0.94 |
| | TC vs. TT | 0.94 (0.79-1.14) | 0.54 | 35.7% | 0.20 | | AG vs. AA | 1.03 (0.89-1.20) | 0.66 | 0% | 0.48 |
| | CC vs. TT | 1.31 (0.70-2.46) | 0.40 | 40.9% | 0.17 | | GG vs. AA | 1.27 (0.85-1.89) | 0.24 | 0% | 0.95 |
| | Additive | 0.96 (0.81-1.13) | 0.60 | 20.5% | 0.27 | | Additive | 1.06 (0.94-1.20) | 0.32 | 0% | 0.87 |
| rs1927911 | Dominant | 0.95 (0.83-1.10) | 0.49 | 0% | 0.50 | rs7873784 | Dominant | 0.91 (0.80-1.05) | 0.19 | 0% | 0.85 |
| | Recessive | 1.06 (0.67-1.67) | 0.80 | 56.2% | 0.08 | | Recessive | 1.03 (0.69-1.52) | 0.90 | 0% | 0.56 |
| | GA vs. GG | 0.93 (0.80-1.08) | 0.35 | 0% | 0.44 | | GC vs. GG | 0.91 (0.79-1.04) | 0.17 | 0% | 0.88 |
| | AA vs. GG | 1.03 (0.67-1.61) | 0.88 | 51.1% | 0.11 | | CC vs. GG | 1.00 (0.67-1.48) | 0.99 | 0% | 0.55 |
| | Additive | 0.99 (0.84-1.17) | 0.92 | 23.9% | 0.24 | | Additive | 0.93 (0.83-1.05) | 0.26 | 0% | 0.84 |
| rs11536879 | Dominant | 1.17 (0.96-1.41) | 0.12 | 0% | 0.93 | rs1554973 | Dominant | 0.98 (0.89-1.08) | 0.71 | 0% | 0.69 |
| | Recessive | 0.82 (0.17-3.86) | 0.80 | 0% | 0.80 | | Recessive | 1.01 (0.83-1.24) | 0.91 | 0% | 0.86 |
| | AG vs. AA | 1.18 (0.97-1.43) | 0.10 | 0% | 0.95 | | TC vs. TT | 0.98 (0.88-1.08) | 0.67 | 0% | 0.75 |
| | GG vs. AA | 0.83 (0.18-3.91) | 0.82 | 0% | 0.45 | | CC vs. TT | 1.01 (0.82-1.23) | 0.96 | 0% | 0.83 |
| | Additive | 1.15 (0.95-1.40) | 0.15 | 0% | 0.95 | | Additive | 0.99 (0.92-1.07) | 0.81 | 0% | 0.95 |

SNPs that were evaluated by at least 4 studies were shown here. Abbreviation: OR, odds ratio; CI, confidence interval; PCa, prostate cancer

PONE-D-14-21052
Polymorphisms of an Innate Immune Gene, Toll-Like Receptor 4, and Aggressive Prostate Cancer Risk: A Systematic Review and Meta-analysis
PLOS ONE

**Editor's comment (journal requirements):**

1. We note that you stated "data are available upon request" at submission. Could you please confirm that all data underlying the findings in your study are freely available in the manuscript, supplemental files, or in a public repository? If this is not the case, and your data are available upon request because of an ethical or legal restriction or because you obtained data from a third party, please include the following in your revised cover letter:
a. The reason why your data cannot be made available in the manuscript, the supplemental files, or a public repository;
b. The name(s) of the individual(s) that readers may contact to request the data;
We will make changes to your data availability statement on your behalf, based on the information you provide. For more information about our data policy and acceptable reasons for not making your data fully available, please refer to:
http://www.plosone.org/static/policies#sharing
**Response:**

a. **Some of the details (i.e.: frequencies of variant carriers in cases and controls) used for conducting this meta-analysis was obtained by E-mail request to the original authors of each individual studies. We cannot distribute these original data without their agreement.**
b. **Readers may contact the corresponding authors of each original studies to request the original data.**

2. Thank you for stating the following in the Competing Interests section: "The authors have declared that no competing interests exist"
We note that one or more of the authors are employed by a commercial company "SAIC-Frederick, Inc.".
Please provide amended statements of Competing Interests and Financial disclosure that declare the affiliation(s) to this company, along with any other relevant declarations relating to employment, consultancy, patents, products in development or marketed products etc.

Please confirm that this does not alter your adherence to all PLOS ONE policies on sharing data and materials, as detailed online in our guide for authors http://www.PLOSone.org/static/editorial.action#competing by including the following statement: "This does not alter our adherence to PLOS ONE policies on sharing data and materials." If there are restrictions on sharing of data and/or materials, please state these. Please note that we cannot proceed with consideration of your article until this information has been declared.

This information should be included in your cover letter; we will change the online submission form on your behalf.

Please be assured that it is standard PLOS ONE policy for corresponding authors to declare, on behalf of all authors, all potential competing interests, for the purposes of transparency. PLOS defines a competing interest as anything that interferes with, or could reasonably be perceived as interfering with, the full and objective presentation, peer review, editorial decision-making, or publication of research or non-research articles submitted to one of the journals. Competing interests can be financial or non-financial, professional, or personal. Competing interests can arise in relationship to an organization or another person. Please follow this link to our website for more details on competing interests:
http://www.PLOSone.org/static/editorial.action#competing

**Response: We have confirmed the statement of competing interests with the co-authors. SAIC-Frederick is not a commercial company. It is a sole-customer government contract for the US National Cancer Institute. They are instructed to fill out all forms as NIH researchers and are in turn restricted by the same rules as US government employees. Therefore, all the authors declared that no competing interests exist.**

3. Please ensure that you refer to Supplementary Table 2 in your text as, if accepted, production will need this reference to link the reader to the Table.
**Response: Thank you for your suggestion. Supplementary Table S2 (PRISMA checklist) was referred to in the result part (P10 of the manuscript).**

**Reviewers' comments:**

**Reviewer #1**

1. Is the manuscript technically sound, and do the data support the conclusions?
Reviewer #1: Yes

2. Has the statistical analysis been performed appropriately and rigorously?
Reviewer #1: Yes

3. Does the manuscript adhere to the PLOS Data Policy?
Reviewer #1: Yes

4. Is the manuscript presented in an intelligible fashion and written in standard English?
Reviewer #1: Yes

5. Review Comments to the Author
Reviewer #1: The authors have adequately addressed the concerns raised in the initial review. But I would like to suggest the authors to label and mark the revisions in the revised version of the paper next time to make it more readable. In general, the manuscript was well written, and there are only a few technical points that need to be addressed.

1. Figures 1 and 3 are illegible, and Figure3 is too busy and irregular, it should be more standardized when combining multiple forest plots for a better presentation.

**Response: Thank you for reviewer's suggestion. We have provided a manuscript with track changes this time. Figure 1 and Figure 3 were revised. The figure resolution was increased and the page layout was improved.**

2. In Figure 4, TLR4 should be marked.

**Response: TLR4 was added in the revised Figure 4.**

3. Supplemental figures and tables are not available.

**Response: We have uploaded the supplementary figures and tables during submission. The files are available online.**

4. A full description of the study characteristics has been showed in the table, such as the population, exposure, genotyping methods, comparison group, and outcome, etc. If you do not conduct stratification by such factors, I think it's important to explain the reasons in detail in Discussion section.

**Response: Thank you for reviewer's suggestion. Type of study (candidate gene studies vs. GWASs) was the pre-specified covariate for meta-regression in this analysis. Most of the other study characteristics were very similar across the nine studies (e.g. publication year, ancestry, definition of aggressive prostate cancer). The major between-study differences were the methods of control selection and case selection. For example, using prevalent PCa cases or hospital-based design might introduce selection bias. However, stratification analysis was not performed because the influences of such biases are complex and usually not unidirectional. Another between-study difference is the different genotyping platforms. We did not perform stratification analysis according to this covariate because previous studies revealed high concordance rate across different genotyping platforms [1]. The method part was revised to address this issue.**

**Reviewers' comments:**
**Reviewer #2**

1. Is the manuscript technically sound, and do the data support the conclusions?

Reviewer #2: Partly

2. Has the statistical analysis been performed appropriately and rigorously?
Reviewer #2: N/A

3. Does the manuscript adhere to the PLOS Data Policy?
Reviewer #2: Yes

4. Is the manuscript presented in an intelligible fashion and written in standard English?
Reviewer #2: Yes

5. Review Comments to the Author
Reviewer #2: This article aims to quantify the relationship between TLR4 and the risk of aggressive PCa by meta-analysis of all genetic epidemiologic studies in the literature. The results indicate that none of the examined TLR4 SNPs was associated with risk of aggressive PCa under any inheritance model.
This article is well organized and the methods they employed are appropriate. Most of the concerns from reviewers have been addressed.

The authors attempted to state that there is no significant association via nine studies, my concern is that whether these nine studies are sufficient to draw this conclusion or not. The authors pick out 31 studies from 40 in the literature, and only kept 9 studies. Although some external condition exists, it seems that some information has been lost. Do the authors consider to search over the unpublished results and put them in?

**Response: Thank you for reviewer's advice. To retrieve unpublished studies, we additionally searched the Scopus database. We also searched the abstract books**

**or websites of three major conference proceedings in this field: American Urological Association (AUA), European Association of Urology (EAU), and the Societe Internationale D'Urologie (SIU). However, no new studies met the inclusion criteria. We did not include the process of searching unpublished studies in this meta-analysis because of the questionable validity of grey literature.**

**In this meta-analysis, publication bias was assessed by Funnel plots and the Egger linear regression test. We found no obvious publication bias among the included studies except a borderline significant Egger test *P* value of 0.06 in rs1554973. Therefore, there is little evidence that the results are biased by excluding unpublished studies.**

Reviewer #2: More, it seems there still exist some typos in the paper. I would suggest the authors to carefully read the paper again before next submission.

**Response: Thank you for reviewer's suggestion. We have proof read the manuscript and corrected these mistakes carefully.**

## References

1. Hong H, Xu L, Liu J, Jones WD, Su Z, et al. Technical reproducibility of genotyping SNP arrays used in genome-wide association studies. PLoS One 7: e44483.