

Association of polymorphisms of toll-like receptor 4 with a reduced prevalence of hay fever and atopy

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Background: The response to innate immune stimuli seems to be critical to conditioning adaptive immunity. Early exposure to endotoxin initiates immune responses that have been shown to alter the risk of asthma and allergic diseases. The toll-like receptor 4 (*TLR4*) gene encodes the principal innate immunity receptor in humans for bacterial endotoxin. Polymorphisms in the *TLR4* gene may regulate the effects of endotoxin exposure and could play a role in the development of asthma and atopy-related phenotypes.

Objective: To investigate the association between *TLR4* polymorphisms and allergic phenotypes in nonsmokers.

Methods: The data from 915 nonsmoking students were available for the study. The *TLR4* 299 and 399 polymorphisms were genotyped using mouthwash samples. The *TLR4* 299 and 399 polymorphisms were grouped together to define the *TLR4* polymorphic group. Skin prick tests were conducted in a subgroup of healthy participants. A brief questionnaire was administered to determine demographic characteristics and chronic health conditions.

Results: The prevalence of hay fever was 0% in the *TLR4* polymorphic group and 7.5% in the wild-type group ($P = .01$). After controlling for age group and sex using logistic regression, the odds of having hay fever were reduced by 88% ($P = .009$) in the *TLR4* polymorphic group compared with the wild-type group. In a subgroup analysis, the association between *TLR4* polymorphisms and atopy was only observed among females.

Conclusions: To our knowledge, this study is the first to report an association between *TLR4* polymorphisms and atopy-related phenotypes in a nonsmoking population. Further investigation of the role of *TLR4* polymorphisms in asthma and atopy-related phenotypes is warranted.

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INTRODUCTION

The toll-like receptor 4 (*TLR4*) gene encodes the principal innate immunity receptor in humans for lipopolysaccharide (LPS), also known as endotoxin. Recognition of LPS by this receptor and other receptors, including CD14, leads to release of proinflammatory cytokines, including tumor necrosis factor (TNF) α , interleukin (IL) 1 β , and IL-6.¹ In addition, *TLR4* provides an innate immune response to respiratory syncytial virus.² Two cosegregating single-nucleotide polymorphisms at amino acid positions 299 (Asp299Gly or A896G) and 399 (Thr399Ile or C11996T) of the *TLR4* gene have been asso-

ciated with reduction in the inflammatory responses to LPS in humans.³ The prevalence of the Asp299Gly polymorphism in the human *TLR4* gene has been reported to be approximately 10% in white populations.⁴

Several studies^{5–13} have investigated the association between the *TLR4* 299 polymorphism and asthma and allergy during the past decade. In a recent study¹⁴ to quantify messenger RNA (mRNA) and protein expression of *TLR2*, *TLR3*, and *TLR4* in nasal mucosa of patients with seasonal allergic rhinitis before and after challenges with relevant pollens identified from skin prick test results, protein expression for all 3 TLRs was demonstrated to increase in patients with rhinitis after the challenge. This study raises the possibility that TLR polymorphisms may have a role in the development of allergic airway inflammation.¹⁴ Furthermore, murine studies have recently highlighted the important role of innate immune stimulation through *TLR4* and the development of an allergic airway response.¹⁵ To further explore this possibility, we investigated the association between *TLR4* 299 and 399 polymorphisms and self-reported hay fever in a cohort of nonsmoking university students and also investigated the association between *TLR4* 299 and/or 399 polymorphisms and atopy as measured by skin prick testing in a subgroup of nonsmoking healthy subjects with and without

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TLR4 299 or 399 polymorphisms, chosen for a future swine exposure study from the larger group of students.

METHODS

Recruitment

Study participants were volunteers from the University of Saskatchewan, Saskatoon, student body. Advertisements were placed in campus buildings to recruit interested students who were nonsmokers and willing to provide a mouthwash sample for genetic material and complete a brief screening questionnaire. Students were informed that based on the responses to the questionnaire, they might be invited back to undergo a series of supplementary pulmonary function and allergy skin prick tests. These clinical results were used to determine if they would be invited to participate in a subsequent study involving exposure in a swine-rearing facility, the results of which will be published separately from this report. Students were paid an honorarium for participation in each portion of the study. The study was approved by the Biomedical Research Ethics Board of the University of Saskatchewan.

Preexisting respiratory health conditions were ascertained from responses to a questionnaire. Students were asked: "Have you ever had the following (please circle as many as apply)?" Chronic bronchitis, pneumonia, hay fever, and known allergy were listed as possible responses. Asthma and wheeze apart from the common cold were evaluated from the response to the questions: "Has a doctor told you that you have asthma?" and "Does your chest sound wheezy or whistling occasionally apart from colds?" In total, 972 subjects completed the brief questionnaire and provided a mouthwash sample for genetic analysis. Of these subjects, 57 either reported that they currently smoked or did not complete the question on smoking and were excluded.

Study Sample for the Initial Analysis

Of the 915 remaining subjects, *TLR4* 299 genotypes were determined in 846 subjects and *TLR4* 399 genotypes were determined in 879 subjects. Our study was restricted to 842 subjects who had genotyping for both *TLR4* 299 and *TLR4* 399. There were no significant differences in age ($P = .30$), sex ($P = .40$), allergy ($P = .30$), hay fever ($P = .10$), asthma ($P = .40$), ever wheeze ($P = .60$), bronchitis ($P = .90$), and pneumonia ($P = .70$) between subjects who were included ($n = 842$) and excluded ($n = 73$) in the analysis.

Study Sample for the Subgroup Analysis

After excluding the subjects with a self-report of hay fever, allergy, current wheeze, or asthma and without any history of visiting a swine barn for more than a few hours, 41 subjects with *TLR4* 299 and/or *TLR4* 399 polymorphisms were identified for skin prick tests. In addition, a group of 67 subjects without either of these polymorphisms who were matched on exact age and sex to the 41 subjects with the polymorphism were also invited for skin prick tests. This selection was performed to ensure that there would be an equal number of

healthy nonatopic subjects available from the wild-type and polymorphic groups for a future swine barn exposure study. A single isolated polymorphism each was observed in *TLR4* 299 and *TLR4* 399 polymorphisms.

DNA Isolation and Genotyping

Study participants were instructed to swish 20 mL of mouthwash (Scope; Procter & Gamble Inc, Toronto, Ontario) vigorously around their mouth for 30 seconds before spitting into a sterile 50-mL bar-coded tube. Genomic DNA was extracted from oral epithelial cells in the mouthwash sample using a robot (Gentra Autopure; Gentra Systems, Minneapolis, Minnesota). Working dilutions of DNA at a concentration of 10 ng per microtiter in RNase- or DNase-free purified water were prepared for subsequent genotyping assays and sequencing. Allelic discrimination assays (TaqMan; Applied Biosystems, Foster City, California) for the human *TLR4* A299G and T399I polymorphisms were designed using software (Primer Express; Applied Biosystems), and polymerase chain reactions (PCRs) were run on thermal cyclers (MJ Tetrad; Bio-Rad Laboratories, Hercules, California). Genotypes (TaqMan; Applied Biosystems) were determined by postamplification plate reading on a detection system (ABI Prism 7900 Sequence Detection System; Applied Biosystems). Human subject DNA samples that did not genotype well in 2 of the assays were submitted for direct sequencing of the amplicon by fluorescent dye termination sequencing on a capillary sequencer (ABI 3730). Plasmids containing inserts with the human *TLR4* 299 and 399 polymorphisms serve as "known genotype" positive control DNA. Plasmids diluted to a concentration of 20 fg/ μ L serve as excellent genotyping controls and substitute well for genomic DNA at the standard working dilution of 10 ng/ μ L in most PCR-based assays. These plasmids are a renewable and consistent assay standard. No template control wells are included on each plate as negative controls. Once optimized, each new assay is validated by direct DNA sequencing of PCR products. At least 10% of the samples from each cohort are regenotyped as an ongoing quality control measure.

Skin Prick Testing

Allergy skin prick tests consisting of a battery of 25 allergens and control saline and histamine were conducted. The items included in the skin prick tests in addition to saline and histamine were aspergillus, *Helminthosporium*, *Cladosporium*, *Alternaria*, egg, milk, peanut, shellfish, house dust mite, house dust, grain dust, wheat dust, feathers, cat dander, dog dander, cattle dander, horse dander, box elder, birch, poplar, willow, mixed tree, mixed grass, mixed weeds, and ragweed (Western Allergy Services Ltd, Burnaby, British Columbia). Subjects were considered to have a positive test result for atopy if there was 1 or more skin prick test results with a raised wheal of greater than 3 mm compared with the saline control.¹⁶ The nurse-technician conducting the skin prick tests was blind to the *TLR4* genotype of the subjects.

None of the subjects in this subgroup had a positive response to nonallergic triggers in the skin tests.

Statistical Analysis

The linkage disequilibrium coefficients D' and r^2 were calculated to determine cosegregation using a linkage disequilibrium analyzer.¹⁷ Differences in continuous and categorical variables were examined by 2 independent-sample t tests and χ^2 tests, respectively. Standard logistic regression analysis was conducted to determine the association between atopy and TLR4 polymorphisms. Because the frequency of hay fever was 0 or smaller for the TLR4 polymorphic groups, exact logistic regression analysis was used to determine the odds ratio and 95% confidence interval.^{18,19}

RESULTS

For TLR4 299, the frequency of the wild type (Asp299) was 762 of 842 (90.5%) and the frequencies of Asp299Gly variants were 76 of 842 (9.0%) for heterozygous and 4 of 842 (0.5%) for homozygous subjects. For TLR4 399, the frequency of the wild type (Thr399) was 766 of 842 (91.0%) and the frequencies of Thr399Ile variants were 73 of 842 (8.7%) for heterozygous and 3 of 842 (0.4%) for homozygous subjects (percentages do not total 100 because of rounding). The Hardy-Weinberg equilibrium was met for distributions of TLR4 299 ($\chi^2_1 = 1.92$, $P = .17$) and TLR4 399 ($\chi^2_1 = 0.78$, $P = .38$) wild type and variants. A strong cosegregation was observed between the 2 polymorphisms ($D' = 0.97$, $r^2 = 0.89$, $P < .001$). The heterozygous and homozygous variants were combined to define TLR4 299 and TLR4 399 polymorphisms. Noncosegregating polymorphisms were observed in 6 subjects for TLR4 299 and in 2 subjects for TLR4 399. The mean age for the TLR4 polymorphic group (TLR4 299 and/or TLR4 399) was slightly lower than that for the wild-type group (Table 1). Reflecting the higher prevalence of male participants, there were more males than females in the wild-type and polymorphic groups (Table 1).

Table 1. Demographic and Lifestyle Characteristics of the TLR4 Wild-Type and Polymorphic Groups^a

Characteristic	Wild-type group (n = 760)	Polymorphic group (n = 82)
Age, y ^b		
≤20.3	49.4	51.9
>20.3	50.5	48.1
Sex		
Male	55.1	52.4
Female	44.9	47.6

Abbreviation: TLR, toll-like receptor.

^a Data are given as percentage of subjects in each group. Percentages may not total 100 because of rounding.

^b Data were missing for 10 subjects. For computational feasibility in exact logistic regression, age was categorized into a binary variable at the median (20.3 years). The mean (SD) age was 21.3 (3.3) years for the wild-type group and 20.4 (2.1) years for the polymorphic group ($P = .02$).

The self-reported prevalence of current and chronic health conditions is given in Table 2 for the TLR4 wild-type and polymorphic groups. There were no significant differences between wild-type and polymorphic groups in all the health conditions, except for self-reported hay fever.

Hay fever was significantly ($P < .001$) more prevalent among subjects with asthma (16.0%) than among those without asthma (5.1%). Similar differences were also observed in the prevalence of hay fever in subjects with and without current wheeze (20.0% vs 6.4%; $P = .01$), wheeze apart from cold (17.1% vs 5.3%; $P < .001$), bronchitis (15.2% vs 6.4%; $P = .07$), and self-reported allergy (15.1% vs 4.4%; $P < .001$). However, the TLR4 polymorphism was not associated with either wheezing or asthma (Table 2).

After controlling for age and sex using exact logistic regression, the TLR4 polymorphic group demonstrated a significant reduction in the odds of having hay fever (Table 3). The odds of having hay fever were reduced by 88% in the TLR4 polymorphic group compared with the wild-type group.

The relationship between TLR4 polymorphisms and atopy was investigated in a subgroup of 108 healthy subjects on whom skin prick tests were conducted. The prevalence of atopy was lower in the polymorphic group compared with the wild-type group, but the difference was not statistically significant (Table 4). The prevalence of atopy for the TLR4 polymorphic and wild-type groups is shown for males and females in Figure 1. In females, significant differences were observed in the prevalence of atopy between wild-type and polymorphic groups ($P = .02$). These significant differences were not observed among males (Fig 1). After controlling for age group, the TLR4 polymorphic group gave a 79% reduction in the odds of having atopy in females, whereas no significant association was observed in males (Table 5).

Table 2. Prevalence of Chronic Conditions in the TLR4 Wild-Type and Polymorphic Groups

Symptom or disease	Wild-type group (n = 760) ^a	Polymorphic group (n = 82) ^a	P value ^b
Current wheeze	21 (2.8)	4 (4.0)	.28
Ever wheeze ^c	104 (14.2)	13 (16.5)	.59
Ever asthma	115 (15.2)	16 (19.5)	.30
Chronic bronchitis	31 (4.1)	2 (2.4)	.47
Pneumonia	75 (9.9)	7 (8.5)	.70
Hay fever	57 (7.5)	0	.01
Self-reported allergy	164 (21.6)	16 (19.5)	.66

Abbreviation: TLR, toll-like receptor.

^a Data are given as number (percentage) of each group. Some percentages are "off" because the denominators used are different from those given at the top of the columns because of missing data.

^b χ^2 Tests for comparison between the wild-type and polymorphic groups.

^c Wheeze apart from cold.

Table 3. Data for the Association Between Hay Fever and TLR4 Polymorphisms^a

Characteristic	OR (95% CI)	P value ^b
Age, y		
≤21.3	1 (Reference)	.02
>21.3	2.12 (1.13–4.11)	
Sex		
Male	1 (Reference)	.71
Female	1.16 (0.63–2.13)	
TLR4		
Wild type	1 (Reference)	.009
Polymorphic	0.12 ^c	

Abbreviations: CI, confidence interval; OR, odds ratio; TLR, toll-like receptor.

^a Results are from exact logistic regression.

^b Value obtained from the exact logistic regression.

^c Conditional maximum likelihood and 95% confidence intervals could not be obtained because of 0 frequency. The median unbiased estimate is reported for the OR.

Table 4. Demographic Characteristics of TLR4 Wild-Type and Polymorphic Subgroups^a

Characteristic	Wild-type group (n = 67)	Polymorphic group (n = 41)
Age, mean (SD), y	21.5 (3.3)	20.3 (2.2) ^b
Sex		
Male	37 (55.2)	23 (56.1)
Female	30 (44.8)	18 (43.9)
Atopy	31 (46.3)	13 (31.7) ^c

Abbreviation: TLR, toll-like receptor.

^a Data are given as number (percentage) of each group unless otherwise indicated.

^b P = .03.

^c P = .14.

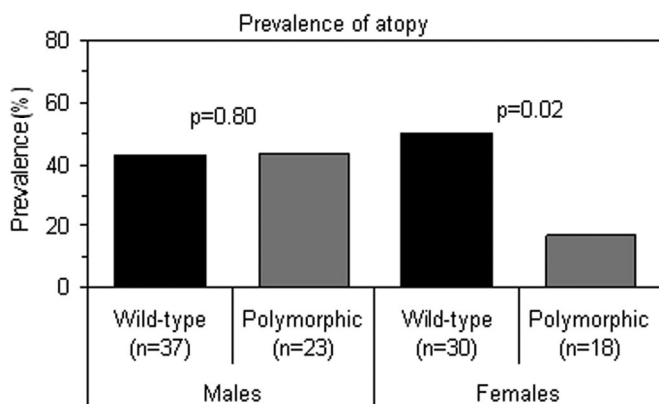


Figure 1. The prevalence of atopy for toll-like receptor 4 polymorphic and wild-type groups is shown for males and females. P values indicate the significance for the difference between wild-type and polymorphic groups.

DISCUSSION

In this study of nonsmoking female and male university students, significant reductions in the risk of hay fever were

Table 5. Association Between Atopy and TLR4 Polymorphisms^a

Characteristic	OR (95% CI)	P value
Age	1.05 (0.93–1.21)	.38
Males		
Wild type	1 (Reference)	.70
Polymorphic	1.37 (0.51–3.62)	
Females		
Wild type	1 (Reference)	.04
Polymorphic	0.21 (0.05–0.90)	

Abbreviations: CI, confidence interval; OR, odds ratio; TLR, toll-like receptor.

^a Results are from logistic regression analysis. The interaction between sex and TLR4 polymorphism was borderline significant (P = .07).

found in the TLR4 polymorphic group. In a subgroup analysis of nonsmoking healthy subjects who underwent skin prick tests, female subjects with TLR4 polymorphisms had a significant reduction in the risk for atopy compared with those in the TLR4 wild-type group. Furthermore, our results provide further evidence of the relationship between the innate and adaptive immune systems and their contribution to human health and disease.

In a study¹⁴ investigating the role of TLR2, TLR3, and TLR4 in allergic rhinitis, mRNA and protein expression were examined in healthy controls and in patients with seasonal rhinitis before and after the pollen seasons and before and after challenges with birch and/or grass pollens. Significant increases in protein expression were observed after allergen challenge in all 3 receptors, but a significant increase in mRNA was observed in TLR4 during the pollen season, raising the possibility that a TLR4 polymorphism may be associated with rhinitis. In a study¹⁰ conducted in Sweden among 115 children aged 8 to 14 years, no significant association was observed between the TLR4 299 polymorphism and allergic conjunctivitis, which was defined as rhinitis and conjunctivitis appearing at least twice after repeated exposure to a particular allergen. In this study, 11 (9.6%) of 115 children carried a TLR4 polymorphism, and allergic conjunctivitis was present only in 4 of the 11 children. Thus, the reported lack of association between the TLR4 299 polymorphism and allergic conjunctivitis might have been because of the small sample size in the study.¹⁰ In contrast, our study considered only hay fever (or rhinitis), which was based on a self-report to the question, "Have you ever had hay fever?" and did not consider conjunctivitis. In a family-based study¹³ of parents and offspring investigating the association between TLR4 polymorphism and atopic eczema, no association was found between the TLR4 299 polymorphism and rhinitis; however, the sample size was too small to select children based on rhinitis.

The TLR4 receptor provides innate immune response to LPSs¹ and respiratory syncytial virus.² The TLR4 299 and 399 polymorphisms have been shown to produce a blunted respiratory response to low-level inhaled endotoxin expo-

sure.²⁰ The TLR4 polymorphisms were associated with increased risk of infections in a surgical setting.²¹ In this study, CD14 polymorphisms were not associated with increased risk of infections.²¹ Septic patients with the TLR4 299 polymorphism had a higher prevalence of gram-negative infections compared with patients who had the wild type.²² These studies raise the possibility that subjects with TLR4 299 and 399 polymorphisms are at increased risk of developing gram-negative and respiratory syncytial virus infections during their early childhood. These infections may, in turn, increase T_H1 immune responses during the maturing period of the immune system and could reduce T_H2 immune response to allergens, thereby reducing the risk of development of future allergic diseases, including hay fever.²³ Our data suggest that an intact innate immune system is needed to trigger adaptive immunity and that without an intact innate immune system, one is at lower risk of adaptive immune conditions, such as hay fever.

In our study, there was no significant association between TLR4 polymorphisms and self-reported asthma. Similar results were observed in studies conducted in the United States and Canada,⁷ Japan,⁸ and the United Kingdom,⁹ whereas children with the TLR4 299 polymorphism had an increased risk of asthma in studies conducted in Sweden.¹⁰ In a study from Finland,¹² only female subjects with the TLR4 299 polymorphism had an increased risk of asthma. In a study from Turkey,¹¹ the TLR4 299 polymorphism was significantly associated with asthma in children with atopy.

In the subgroup analysis conducted in our study, reduced risk of atopy, as measured by skin prick testing, was observed only in female subjects with TLR4 polymorphisms. In a study from the United States,²⁴ venous blood samples from 56 men and 26 women were incubated with LPS; women produced fewer LPS-induced anti-inflammatory cytokines (TNF- α and IL-1 β) compared with men, and these differences persisted when controlling for TLR4 299, TNF- α , IL-1 β , and IL-6 polymorphisms. Improved outcomes after injury or infection in female sex was observed in several studies based on animal models of surgical trauma and hemorrhage.²⁵ In these studies,^{26,27} male sex hormones, including testosterone, have been shown to be associated with deleterious effects, whereas estrogen, a female hormone, seemed to be protective. The results from the subgroup analysis in our study are not generalizable but are useful for generating a hypothesis for future studies.

There were some possible limitations in the study. In the main study, the presence of hay fever was determined from a self-report of participants and was not confirmed by a physician. In our study, 73 participants were not included in the analysis. However, there were no significant differences observed in health outcomes and independent variables between those who underwent genotyping and those who did not undergo genotyping.

In conclusion, to our knowledge, we are the first to report an association between TLR4 polymorphisms and atopy-related phenotypes in a nonsmoking population. Further in-

vestigation into the role of TLR4 polymorphisms in asthma and atopy-related phenotypes is warranted in other study populations.

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