

Epistatic Effect of TLR4 and IL4 Genes on the Risk of Asthma in Females

Kati Ådjers^a Jussi Karjalainen^b Tanja Pessi^a Carita Eklund^a
Mikko Hurme^{a,c}

^aDepartment of Microbiology and Immunology, Medical School, University of Tampere, ^bDepartment of Respiratory Medicine, ^cThe Laboratory Centre, Tampere University Hospital, Tampere, Finland

Key Words

Asthma · Atopy · Epistatic effect · IL4 · TLR4

Abstract

Background: Many studies have demonstrated a connection between asthma and T-cell cytokine genes, such as genes coding for interleukin-4 (IL4) and IL-13, which are involved in the regulation of the TH1/TH2 balance. The toll-like receptor 4 (TLR4), the principal receptor for bacterial endotoxin, has attracted attention as a potential risk factor for asthma. We examined whether the polymorphisms of the TLR4 (A/G at +896) and IL4 (C/T at –590) showed an epistatic effect on the risk of asthma or atopy.

Methods: Gene polymorphism analyses and skin prick tests were performed on asthmatic and nonasthmatic adult subjects of a Finnish population-based case-control study. The phenotype studied was persistent asthma. **Results:** The results showed that genotypes of neither the TLR4 SNP at +896 nor IL4 SNP at –590 were separately found to be associated with asthma. However, the female carriers of allele G (i.e. genotype AG or GG) of TLR4 and allele T (genotype CT or TT) of IL4 had a significantly increased risk for asthma. No association of these genes and atopy was found. **Conclusions:** Our results indicate that in females the TLR4 and IL4 genes show an epistatic effect on the risk of asthma. The low

LPS-responsive allele G of TLR4 and high IgE production allele T of IL4 were found to be the predisposing combination. However, there was no epistatic effect on the risk of atopy.

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Introduction

Asthma is recognized as a chronic inflammatory disorder of the lungs resulting in bronchial hyperresponsiveness, and is determined by genetic and environmental factors [1, 2]. Asthma can be divided into two distinct variants: the extrinsic, allergic variant, which occurs in the context of sensitization toward environmental allergens, and the intrinsic, nonallergic variant, with no detectable sensitization [3].

Atopy is characterized by the presence of specific immunoglobulin E (IgE) antibodies to common allergens. The skin prick test (SPT) is used to examine IgE-mediated allergic responses, and the results are generally in line with anamnestic data on atopy [4].

The toll-like receptor 4 (TLR4) is the principal receptor for bacterial endotoxin [5]. It plays an important role in the innate immune response to both gram-negative pathogens and respiratory syncytial virus [6]. Endotoxin binds to membrane-bound TLR4 and initiates an intra-

cellular signaling pathway, which results in the activation of nuclear transcription factors. Polymorphisms in the coding region of the TLR4 gene are partly responsible for variability. A single A/G base exchange at +896 in the TLR4 gene, inducing an amino acid substitution of glycine for asparagine (at amino acid position 299), results in reduction in cell surface expression of TLR4 and subsequent disruption of LPS-mediated signaling [7]. According to the hygiene hypothesis, early exposure to high endotoxin levels protects from the development of asthma and atopy, whereas low doses increase the prevalence of disease [8, 9]. Exposure to endotoxin has attracted attention as a potential risk factor for the development and severity of asthma, and TLR4 gene has been shown to modify the endotoxin effects [10–13]. Transfection of the THP-1 cell line demonstrated that the A/G base exchange at +896 (Asp299Gly mutation) diminished TLR4-mediated signaling, which showed that the gene sequence change in receptor responsible for LPS signaling can alter the responsiveness of the host to microbial agents [13]. Previously, an association of TLR4 SNP at +896 and asthma has been reported in children, allele G being the predisposing allele [14]. However, negative findings about the connection between TLR4 gene polymorphisms and both asthma and atopy have also been reported [15, 16].

Interleukin-4 (IL4), a major anti-inflammatory cytokine produced by activated T cells, mast cells, and basophils, plays a central role in the regulation of B-cell- and T-cell-mediated immune responses, including the induction of IgE synthesis [17, 18]. IL4 exerts its biological effects through binding to the IL4 receptor complex. A variant of the IL4 gene promoter, –590 C/T, has been shown to associate in vitro with IL-4 activity and show higher binding activity to nuclear transcription factors and alteration in an electrophoretic mobility shift assay, allele T being the high producer [19]. In vivo allele T has been associated with high total IgE levels, asthma, and atopy in several studies [20–28].

The results of allelic associations have only partly explained the predisposition to asthma and have sometimes even been contradictory. In the case of a multifactorial disease, the predisposition might better be explained by a combination of alleles than just one allele [29]. Therefore, we evaluated whether the TLR4 and IL4 polymorphisms would show an epistatic effect on the risk of asthma or atopy (determined by skin prick tests). The analyses were made in a population-based sample of adult asthmatic and nonasthmatic subjects.

Table 1. Characteristics of the study group

Variable	Asthmatic patients (n = 245)	Nonasthmatic controls (n = 405)
Age (mean $\pm$ SD), years	59 $\pm$ 11	60 $\pm$ 11
Sex f/m	152/93	254/151
Smoking (non/ex/current)	123/80/42	237/89/79
Atopy ^{1, 2}	137	154
FEV ₁ (mean $\pm$ SD), % predicted	86.3 $\pm$ 25	102.5 $\pm$ 20
Allergic symptoms		
Rhinitis ²	134	111
Eye symptoms ²	104	92
Skin symptoms ³	84	97

¹ One or more positive reactions on SPTs.

² $p < 0.001$ for difference between groups, as calculated with the χ^2 test.

³ $p < 0.004$ for difference between groups, as calculated with the χ^2 test.

Methods

Study Population

A total of 245 asthmatic patients and 405 nonasthmatic controls (mean age 60 years, range 31–89 years) participated in the study. The participants were the subjects of a Finnish population-based case-control study conducted to investigate the risk factors and predictors of the outcome of adult asthma [30]. Approval for the study was obtained from the ethical committee of Tampere University Hospital. Informed consent to participate was obtained from all subjects. The characteristics of the study group are shown in table 1. Inclusion criteria for asthmatic subjects were age over 30 years and entitlement to reimbursement for asthma medication from the Social Insurance Institution of Finland. Entitlement was granted if the criteria for persistent asthma were fulfilled as certified by a chest specialist. Typical history, clinical features and course of asthma had to be documented on the medical certificate. At least one of the following physiological criteria was required for diagnosis: (1) a variation of $\geq 20\%$ in diurnal peak expiratory flow (PEF) recording (reference to maximal value); (2) an increase of $\geq 15\%$ in PEF or forced expiratory volume of 1 s (FEV₁) with a β_2 -agonist; (3) a decrease of $\geq 15\%$ in PEF or FEV₁ in exercise testing. Moreover, a period of at least 6 months of continuing regular use of asthma medication had to have elapsed by the time of the decision. This method of case ascertainment has recently been described in detail and evaluated [31]. One or two controls without asthma or chronic obstructive pulmonary disease were initially selected for each subject using a registry covering the entire population. They were matched for age, sex, and area of residence. No difference was found in the prevalence of other chronic diseases between the patients and the controls. The ethnic origin of patients and controls was the same (Finnish Caucasian).

Allergy Testing

Allergy testing by the skin prick test and TLR4 and IL4 polymorphism testing were carried out on 99.2% of the asthmatics (151 females and 92 males) and 99.0% of nonasthmatic subjects (250 females and 151 males). Atopy was determined by an SPT performed by trained nurses with a panel of 22 allergen extracts selected to cover exposures in both urban and rural environments (ALK A/S, Copenhagen, Denmark). Detailed information on the allergen extracts has been provided elsewhere [30]. The subject was considered SPT positive if at least one allergen showed a wheal with a diameter at least 3 mm larger than that of the negative control.

Polymorphism Analyses

Genomic DNA was extracted from peripheral blood anticoagulated with EDTA with the QIAamp DNA Blood Mini Kit (QIAGEN) according to the manufacturer's instructions. The A/G polymorphism at +896 of the TLR4 gene (rs 4986790) was analyzed by an allele-specific hybridization method (TaqMan). Forward primer was 5'-TGA CCA TTG AAG AAT TCC GAT TAG CA-3', reverse primer 5'-ACA CTC ACC AGG GAA AAT GAA GAA-3', and hybridization probes FAM-5'-CCT CGA TGGTAT TAT T-3' and VIC-5'-TAC CTC GAT GAT ATT ATT-3'. Amplification was performed in an ABI Prism Sequence Detector 7000 (Applied Biosystems, Foster City, Calif., USA) using 100-well plates. The final volume was 5 µl containing 0.8 µl of DNA solution at a concentration of 5 ng/µl, 0.1 µl of each primer (100 pmol/µl), 0.1 µl of each probe (100 pmol/µl), 2.5 µl of TaqMan Universal PCR Master Mix (Perkin-Elmer), and 1.3 µl of distilled water. In every assay, controls for the wild type and mutations were included. The PCR conditions were as follows: initial holding at 50°C for 2 min, denaturation at 95°C for 10 min, followed by 40 cycles of denaturation (95°C for 15 s), annealing, and extension in one step (60°C for 60 s).

For analyses of the C/T polymorphism at -590 of IL4 gene (rs 2243250), the region containing the *Ava* II polymorphic site was amplified by PCR [21]. The PCR reaction mix contained 1 µl of each primer diluted to 20 pmol/µl, 0.5 µl dNTP mix, 5 µl PCR buffer of 200 mM (NH₄)₂SO₄ and 0.1% Tween 20, 10 µl of 25 mM MgCl₂, 0.25 µl Taq polymerase, 1 µl DMSO, 4-µl sample, and 27.25 µl distilled water. Forward primer was 5'-TAA ACT TGG GAG AAC ATG GT-3' and reverse primer 5'-TGG GGA AAG ATA GAG TAA TA-3'. The PCR conditions were 2 min at 94°C, followed by 35 cycles of 40 s at 94°C, 40 s at 58°C and 50 s at 72°C, and last 10 min at 72°C. The digestion was performed over night in a mix containing 12.5 µl of sample, 2.5 µl *Ava* II buffer, 0.25 µl *Ava* II and 9.75 µl distilled water. Fragments were analyzed by electrophoresis on 3.5% MetaPhor agarose with Tris-borate.

Statistical Analyses

The statistical method selected is presented in the Results section as used. When allele distributions were compared in the case of one gene, we used the χ^2 test of Statistica (version Win.5.1.D; StatSoft Inc., Tulsa, Okla., USA) for calculating the p value. In the case of two genes, Fisher's exact test of SPSS (version 11.1; SPSS Inc., Chicago, Ill., USA) was used. The interaction analysis was performed by binary logistic regression analysis (of SPSS), which is a traditional method for finding an interaction between two loci, i.e. studying epistasis [32, 33]. The findings were considered significant in case of a p value less than or equal to 0.05. CIA software (version 1.1, Martin J. Gardner & *British Medical Journal*) was used for the calculations of odds ratios.

Results

For both the asthmatics and the nonasthmatics, the genotype frequencies were 0.83 for AA, 0.16 for AG, and for 0.01 for GG at +896 of the TLR4 gene. At -590 of the IL4 gene the genotype frequencies were 0.41 for CC, 0.45 for CT, and 0.14 for TT in the case of asthmatics and 0.47 for CC, 0.41 for CT, and 0.12 for TT in the case of nonasthmatics. The genotype frequencies of both genes did not differ significantly between the asthmatic and nonasthmatic groups.

For atopic and nonatopic subjects, the genotype frequencies were 0.83 for AA, 0.16 for AG, and 0.01 for GG at +896 of the TLR4 gene. At -590 of the IL4 gene the genotype frequencies were 0.38 for CC, 0.44 for CT, and 0.17 for TT in the case of atopic subjects and 0.50 for CC, 0.40 for CT, and 0.10 for TT in the case of nonatopic subjects. The trend of genotype frequencies of IL4 gene differing between atopic and nonatopic subjects was found, allele T being predisposing to atopy. It was found in both asthmatic ($p = 0.076$, χ^2 test) and nonasthmatic subjects ($p = 0.072$, χ^2 test). The genotype frequencies of TLR4 gene did not differ in these groups.

The subjects were grouped according to their carrier status for TLR4 allele G (genotype AG or GG) and IL4 allele T (CT or TT). The genotype distribution followed the Hardy-Weinberg equilibrium.

There was no association between the TLR4 allele carrier status and asthma, as shown in table 2. The distribution of IL4 allele carriers was not significantly different between the subjects with or without asthma, but a trend was detected in females ($p = 0.073$, χ^2 test). This difference in the distribution was caused by an increase in the frequency of the carriers of allele T in the asthmatic subjects.

As shown in table 2, an epistatic effect between TLR4 and IL4 polymorphisms was found in females ($p = 0.037$, binary logistic regression). In the case of the carriers of allele G of the TLR4 gene, IL4 allele carrier distribution differed significantly between the asthmatic and the nonasthmatic females ($p = 0.011$, Fisher's exact test). For female carriers of allele T of IL4 who were also carriers of allele G of TLR4 gene, the odds ratio was 4.16 (95% CI, 1.41–12.3), when compared to the group where the subjects were the carriers of allele G of TLR4 but noncarriers of allele T of IL4. When compared to all other groups, the odds ratio was 2.26 (95% CI, 1.11–4.59). No significant association between IL4 and asthma was found in the case of the allele A of the TLR4 gene. No

Table 2. Genotype distributions of TLR4 (single nucleotide polymorphism +896), IL4 (single nucleotide polymorphism –590) and combinations of the TLR4 and IL4 genotypes in male and female subjects with and without asthma

Genotype combinations	Asthmatics		Non asthmatics		OR	95% CI	p value ¹	Interaction- analysis ²
	n	%	n	%				
<i>Males</i>								
TLR4 AA	77	83.7	122	80.8	1	–	0.569	0.624
TLR4 AG/GG	15	16.3	29	19.2	0.82	0.41–1.63		
IL4 CC	38	41.3	65	43.0	1	–	0.790	
IL4 CT/TT	54	58.7	86	57.0	1.07	0.64–1.82		
TLR4 AA * IL4 CC	31	40.3	53	43.4	1	–	0.768	
TLR4 AA * IL4 CT/TT	46	59.7	69	56.6	1.14	0.64–2.03		
TLR4 AG/GG * IL4 CC	7	46.7	12	41.4	1	–	0.759	
TLR4 AG/GG * IL4 CT/TT	8	53.3	17	58.6	0.81	0.23–2.83		
<i>Females</i>								
TLR4 AA	125	82.8	212	84.8	1	–	0.592	0.037
TLR4 AG/GG	26	17.2	38	15.2	1.16	0.67–2.00		
IL4 CC	61	40.4	124	49.6	1	–	0.073	
IL4 CT/TT	90	59.6	126	50.4	1.45	0.97–2.19		
TLR4 AA * IL4 CC	54	43.2	101	47.6	1	–	0.497	
TLR4 AA * IL4 CT/TT	71	56.8	111	52.4	1.20	0.77–1.87		
TLR4 AG/GG * IL4 CC	7	26.9	23	60.5	1	–	0.011	
TLR4 AG/GG * IL4 CT/TT	19	73.1	15	39.5	4.16	1.41–12.3		

¹ p values were calculated using the χ^2 test for genotypes of TLR4 and IL4 and Fisher's exact test for the combinations of the TLR4 and IL4 genotypes.

² Interaction analysis was obtained by binary logistic regression analysis.

association of any kind was found in males. No epistatic effect on the risk of atopy was found (data not shown).

Discussion

We evaluated whether TLR4 and IL4 genotype combinations affect asthma or atopy in a population-based sample of asthmatic and nonasthmatic subjects. Genotypes of neither the TLR4 SNP at +896 nor IL4 SNP at –590 were found to be associated with asthma in our subjects, even though an association between asthma and IL4 genotype has previously been shown to exist in some populations [20, 21]. However, some contradictory results exist [26, 29].

The female carriers of allele G (AG or GG) of TLR4 and allele T (CT or TT) of IL4 were shown here to have an increased risk for asthma. There was an interaction between two genes to control a single phenotype, an epistatic effect, because these alleles did not separately affect

the risk of asthma. The alleles of TLR4 and IL4 genes strengthen each other so that a significant increase in the risk of asthma could be found. No epistatic effect could be found in the case of atopic phenotype. However, this finding does not eliminate the possibility of this combination of genes being associated with the severity of atopy, the effect recently found in asthmatics [34].

The epistatic effect was found only in females. The probable reason for this is that there are sex-dependent differences in the body's defense mechanisms in prevalences of inflammatory and autoimmune diseases [35]. Since the frequencies of the alleles do not vary between the sexes, the functions of the alleles or their interactions with other, still unknown genes can be assumed to vary. Previous epidemiologic evidence supports this in asthma: females have been reported to carry a higher risk of developing asthma after puberty [36].

Despite the rareness of the allele G of TLR4 gene (frequency of carriers 0.17) in the population, the epistatic effect described here may account for the different basic mechanisms of asthma and atopy. According to the hy-

giene hypothesis, early exposure to high endotoxin levels protects the individual against the development of asthma and atopy later in life through Th cell differentiation towards Th1 [8]. Bacterial LPS signaling through TLR4 may play an important role in this [7, 37]. A low level of inhaled LPS is necessary to induce Th2 responses (including increased production of IL4) to inhaled antigens, whereas a high level of inhaled LPS is needed to induce Th1 responses [9]. The allele G of TLR4 has previously been found to be responsible for attenuated inflammatory responses and impaired bronchoconstrictor response to inhaled LPS, pointing to its role in inducing Th2 responses [7, 14]. The allele T of IL4 has been associated with high IL4 production [38]. When the allele of TLR4 linked to induced Th2 responses and the high IL4 production allele of IL4 are combined, the risk of asthma increased. From the molecular perspective this epistatic effect on asthma can be speculated to be caused by IL-4 potentiating the signal through TLR4, but the mechanism remains unknown. No effect on atopy could be found, indicating the diverse role of Th1/Th2 differentiation in asthma and atopy. This may be due to the IL4 effects other than induced IgE production, such as mitogenic activity or T cells [39]. These effects also include the tendency of IL4 to enhance the production of other Th2 cy-

tokines, such as IL-3, IL-5, IL-9, IL-13, some of which increase eosinophil growth and mucus production, and induce airway hyperreactivity [40]. It is also possible that IL4 is stored within the bronchial mucosa of intrinsic asthmatics [41]. The mechanism still remains unknown, but it is clear that the mechanisms of Th1/Th2 differentiation are not identical in asthma and atopy.

In summary, we found that TLR4 and IL4 genes show an epistatic effect on the risk of asthma in females. However, this allele combination was found only in 13% of the female asthmatics. This can be seen as an example of the genetic heterogeneity behind complex diseases such as asthma.

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