Original Article
Allergy Asthma Immunol Res. 2011 April;3(2):123-127.
doi: 10.4168/aair.2011.3.2.123
pISSN 2092-7355 • eISSN 2092-7363

AAIR
Allergy, Asthma & Immunology Research

Role of *Toll-like Receptor 3* Variants in Aspirin-Exacerbated Respiratory Disease

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Purpose: Although the mechanism of virus-induced, aspirin-exacerbated respiratory disease (AERD) is not known fully, direct activation of viral components through Toll-like receptor 3 (TLR3) has been suggested. TLR3 recognizes double-stranded RNA (dsRNA), and activates nuclear factor-κB and increases interferon-γ, which signals other cells to induce airway inflammation in asthma. Considering the association of *TLR3* in viral infections and AERD, we investigated whether promoter and non-synonymous variants of *TLR3* were associated with AERD. **Methods:** The three study groups, 203 with AERD, 254 with aspirin-tolerant asthma (ATA), and 274 normal healthy controls (NC) were recruited from Ajou University Hospital, Korea. Two polymorphisms, -299698G>T and 293391G>A [Leu412Phe], were genotyped using primer extension methods. **Results:** Genetic associations were examined between two genetic polymorphisms of TLR3 (-299698G>T and 293391G>A [Leu412Phe]) in the three study groups. AERD patients that carried the GG genotype of 293391G>A showed a significantly lower frequency compared with ATA in both co-dominant (P=0.025) and dominant models (P=0.036). Similarly, in the minor allele frequency, the A allele was significantly higher (P=0.023) in AERD compared with ATA for this polymorphism. AERD patients who carried HT2 [GA] showed a significantly higher frequency than other haplotypes in co-dominant (P=0.02) and recessive (P=0.026) models. **Conclusions:** Our findings suggest that the -299698G>T and 293391G>A [Leu412Phe] polymorphisms of the *TLR3* gene are associated with the AERD phenotype.

Key Words: *TLR3* polymorphism; aspirin sensitivity; asthma; viral infection

INTRODUCTION

Aspirin-exacerbated respiratory disease (AERD) presents severe clinical symptoms and is frequently associated with chronic eosinophilic rhinosinusitis and nasal polyposis. Increased cysteinyl leukotrienes are potent pro-inflammatory mediators and bronchoconstrictors in AERD pathogenesis. Szczeklik[1] has hypothesized that AERD develops as the result of chronic viral infection. Recently, it has been shown that eosinophils activated via Toll-like receptor 3 (TLR3) might be more able to recruit leukocytes to sites of inflammation, and that eosinophils might function as a link between viral infection and exacerbation of allergic disease.[2] Viral respiratory infections have been suggested to contribute to allergic sensitization and development of asthma, and in subjects with already established asthma, they are known to exacerbate the allergic disease.[3] Aspirin hypersensitivity is diminished in some AERD patients during acyclovir treatment of herpes simplex infection.[4] Moreover, elevated levels of IgG4, derived from chronic antigenic stimulation of viral origin, have been noted in AERD patients.[5] In another study, exacerbation of AERD with airway infection of respiratory syncytial virus was reported.[6]

Although there are a few published studies on the association between viral infection and AERD, no clear mechanism(s) behind virus-induced AERD has been described. One possible explanation is direct activation by viral components through the TLRs. The TLR family comprises TLR1–TLR10, each of which recognizes a conserved microbial structure and then initiates a host defense response.[7,8] The virus-recognizing TLRs are TLR3, TLR7/8, and TLR9, which respond to viral double-stranded RNA (dsRNA), single-stranded RNA, and CpG–DNA, respectively.[8-11]

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Received: July 2, 2010; Accepted: August 3, 2010
• There are no financial or other issues that might lead to conflict of interest.

TLR3 recognizes dsRNA, activates nuclear factor (NF)-κB, and increases interferon (IFN)-γ, which is a signal to other cells and increases antiviral defenses. Mutation of the *TLR3* gene is responsible for susceptibility to viral infection and may induce asthma.[12] As functional deterioration of TLR3 can predispose individuals to increased susceptibility to viral infections, the detection of *TLR3* polymorphisms could be informative for risk assessment in AERD susceptibility. Moreover, no report is available on the genetic loci of *TLR3* in subjects with AERD; however, polymorphisms in the *TLR3* gene have been correlated with disease susceptibility, including to sepsis, immunodeficiency, atherosclerosis, Stevens–Johnson syndrome (SJS), and asthma.[13–15]

Genetic variations in the *TLR3* non-synonymous variant (Leu412Phe) have been correlated with reduced ability to control viral infection. The frequency of this coding single nucleotide polymorphism (SNP) was higher in asthmatics (41%) compared with European Americans (26.97%), Asians (30.1%), and the overall population (20%), indicating that the link between this SNP and asthma phenotypes is induced by viral infection.[12]

Given the association between *TLR3* and viral infection, and the pathogenic mechanisms for viral-induced exacerbation of AERD, we examined the possibility of a genetic predisposition to AERD.

MATERIALS AND METHODS

Study subjects and phenotyping

The case–control study was performed in three groups of patients: 203 with AERD, 254 with aspirin-tolerant asthma (ATA), and 274 normal controls (NCs), who were recruited from Ajou University Hospital, Suwon, South Korea. The diagnosis of AERD was based on a positive response to a lysine-acetyl salicylic acid (ASA) bronchoprovocative test, which was performed on all study subjects according to a previously described method.[16] Changes in FEV1 were followed up for 7 hour after the final aspirin challenge. The ASA-induced change in FEV1 was calculated as the percentage of post-challenge FEV1 relative to the pre-challenge FEV1, and was defined as positive if the FEV1 level declined by >20% from baseline.

Methacholine bronchial challenge tests were performed as described previously.[17] NCs were chosen from the general population using a screening questionnaire, which had to indicate no history of respiratory symptoms or aspirin hypersensitivity. All NC subjects also exhibited an FEV1 >80% of the predicted value, provocation concentration (PC20) of methacholine >25 mg/mL, and normal findings on simple chest radiograms. Atopy was defined as one or more positive reactions on a skin prick test using 12 common aeroallergens (Bencard, Brendford, UK), with histamine and saline controls. To measure specific IgE, venous blood was collected from the antecubital vein, allowed to clot for 1–3 hour at 4°C, and centrifuged (1,500 g, 10 minutes, 4°C). The serum was aspirated, separated, and stored in aliquots at -20°C until analysis. Serum total IgE was measured using the UniCAP system (Phadia, Valinge, Sweden), according to the manufacturer's instructions. The threshold cutoff value for a specific IgE level was 0.35 kU/L, as measured by UniCAP. The presence of rhinosinusitis and nasal polyps was determined using paranasal sinus X-ray and rhinoscopy.

Informed consent was obtained from all subjects. The Institutional Review Board of Ajou University Hospital approved the study.

SNP identification and genotyping

Forty healthy Korean volunteers were used for SNP identification. Genomic DNA was prepared from peripheral blood samples using the Puregene DNA purification kit (Gentra, Minneapolis, MN, USA), according to the manufacturer's protocol. Our objective was to screen promoter, 5'-UTR, and non-synonymous coding SNPs. We selected upstream of exon 1 and exon 2 in the promoter and 5'-UTR SNP screening. The other SNP located in the exon 4 coding region was non-synonymous; it was chosen based on functional importance, as reported previously.[12] Direct sequencing was performed in the *TLR3* region upstream of exons 1 and 2, and at the exon 4 coding region to examine SNPs using the ABI Prism 3100 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Two polymorphisms of *TLR3* (-299698 G>T) (rs3775296) in the 5'-UTR and 293391G>A [Leu412Phe]) (rs3775291) in the coding exon were screened and genotyped using a primer extension method and the SNAPshot ddNTP primer extension kit (Applied Biosystems). No SNP was found upstream of exon 1. The genotyped sample size was based on our enrolled sample for the three study groups, AERD, ATA, and NC.

Statistical analyses

The χ^2 test was used to detect a significant departure in genotype frequency from Hardy–Weinberg equilibrium at each SNP. Differences in the mean values of phenotypic characteristics among AERD patients were compared using the *t* test. Differences in genotype frequency between the two groups were examined using the χ^2 test, and three logical regression models (co-dominant, dominant and recessive) were used after accounting for age and gender covariates. Pair-wise linkage disequilibrium between SNP loci was measured using the absolute value of Lewontin's D' and r^2. The level of statistical significance was set at $P<0.05$.

RESULTS

Clinical characteristics of the study subjects

The clinical characteristics of the study subjects are presented in Table 1. The frequency of atopy was significantly lower in the AERD group than ATA ($P=0.009$) and NC ($P<0.001$) groups. The AERD patients had a significantly higher frequency of rhinosinusitis, compared with the ATA ($P=0.04$) and NC ($P<0.001$)

groups. The incidence of paranasal sinusitis (P<0.001) and nasal polyps (P<0.001) was significantly higher in the AERD than the ATA group. Serum total IgE was significantly higher in the AERD than the NC group (P<0.001). The PC20 methacholine level was significantly lower in the AERD compared with the ATA group (P<0.001).

Allele and genotype frequencies of the *TLR3* gene with the AERD phenotype

Genetic associations were examined between two genetic polymorphisms of *TLR3* (299698G>T and -293391G>A) in the three study groups. The genotype distribution of the two polymorphisms did not depart significantly from Hardy–Weinberg equilibrium (P>0.05).

The AERD patients who carried the GG genotype of -293391 G>A showed a significantly lower frequency than the ATA group in co-dominant (P=0.025) and dominant (P=0.036) models. Similarly, the minor allele frequency 'A' allele was significantly higher in the AERD versus the ATA group (P=0.023) for this polymorphism. AERD patients who carried the GT and TT genotype of 299698G>T showed significantly lower frequencies than in the ATA group (P=0.048) in the recessive model of analysis (Table 2). However, no significant difference was obtained in the case of genotype frequencies between AERD and NC, and ATA and NC of the TLR3 299698G>T or -293391G>A genetic polymorphisms. The statistical power for a significant result in AERD versus ATA for Leu412Phe (293391G>A) was 41%.

Haplotype frequency of the *TLR3* gene with the AERD phenotype

Two polymorphisms in the *TLR3* gene were in linkage disequilibrium (D'=1 and r^2=0.111), and three common haplotypes, HT1 [GG], HT2 [GA] and HT3 [TG] were constructed using the Haploview program. Significant differences were observed in

Table 1. Clinical characteristics of the AERD study subjects

Characteristics	AERD (n=203)	ATA (n=254)	NC (n=274)	*P* value*		
				AERD vs. ATA	ATA vs. NC	AERD vs. NC
Age (yr)	43.9±13	43.1±15.2	32.1±12.7	0.546	**<0.001**	**<0.001**
Gender (male, %)	72 (35.5)	112 (44.1)	108/273 (39.6)	0.062	0.292	0.362
Atopy (+, %)	94 (52.8)	115 (66.5)	27/165 (16.4)	**0.009**	**<0.001**	**<0.001**
Asthma duration (yr)	6.3±5.8	5.2±6.1	NA	0.143	NA	NA
FEV1 (%)	80.5±23.2	88.7±20.5	98.7±7/9	**0.001**	**0.002**	**<0.001**
PC20, methacholine (mg/mL)	4.1±7.2	7.8±9.4	NA	**<0.001**	NA	NA
Serum total IgE (IU/mL)	340.5±441.7	401.4±639.8	105.2±178	0.369	**<0.001**	**<0.001**
Rhinosinusitis (+, %)	114 (85.7)	132 (76.3)	3 (33.3)	**0.040**	**0.004**	**<0.001**
Paranasal sinusitis (+, %)	74 (57.4)	12 (7.0)	NA	**<0.001**	NA	NA
Nasal polyp (+, %)	56 (44.4)	9 (5.3)	NA	**<0.001**	NA	NA
ECP (μg/mL)	20.9±20	24.5±25.9	NA	0.398	NA	NA

*P values were determined using Pearson's χ^2 test for categorical variables and the t test for continuous variables. Values in bold indicate a significant P value.
AERD, aspirin-exacerbated respiratory disease; ATA, aspirin-tolerant asthma; NC, normal control; NA, not applicable; FEV1, forced expiratory volume; ECP, eosinophil cationic protein.

Table 2. Allele and genotype frequencies of *TLR3* gene

SNP loci	Genotype	AERD (n=203)	ATA (n=254)	NC (n=274)	*P* value*		
					AERD vs. ATA	AERD vs. NC	ATA vs. NC
-299698G>T	GG	132 (65.00%)	142 (55.90%)	162 (59.10%)	0.082	0.418	0.438
(rs 3775296)	GT	62 (30.50%)	99 (39.00%)	92 (33.60%)	0.806	0.289	0.306
	TT	9 (4.40%)	13 (5.10%)	20 (7.30%)	**0.048**	0.624	0.149
	q	0.197	0.246	0.241	0.083	0.403	0.437
293391G>A	GG	96 (47.30%)	141 (55.50%)	145 (52.90%)	**0.025**	0.366	0.812
(rs3775291)	GA	83 (40.90%)	97 (38.20%)	116 (42.30%)	0.036	0.118	0.873
	AA	24 (11.80%)	16 (6.30%)	13 (4.70%)	0.087	0.759	0.72
	q	0.323	0.254	0.259	**0.023**	0.375	0.818

*Each P value was calculated with co-dominant, dominant, and recessive models. Logistic regression analysis was applied to control for age and gender as covariables q; minor allele frequency. Values in bold indicate significant P values.
AERD, aspirin-exacerbated respiratory disease; ATA, aspirin-tolerant asthma; NC, normal control.

Table 3. Haplotype analysis of *TLR3* -299698G>T and *TLR3* 293391G>A in study subjects

		AERD (n=203)	ATA (n=253)	NC (n=274)	*P* value*		
					AERD vs. ATA	AERD vs. NC	ATA vs. NC
HT1[GG]	ht1/ht1	48 (23.60%)	64 (25.30%)	69 (25.20%)	0.477	0.917	0.732
	ht1/-	99 (48.80%)	127 (50.20%)	136 (49.60%)	0.469	0.843	0.898
	-/-	56 (27.60%)	62 (24.50%)	69 (25.20%)	0.662	0.977	0.666
HT2[GA]	ht2/ht2	24 (11.80%)	15 (5.90%)	13 (4.7%)	**0.020**	0.366	0.731
	ht2/-	83 (40.90%)	97 (38.30%)	116 (42.30%)	0.079	0.759	0.685
	-/-	96 (47.30%)	141 (55.70%)	145 (52.90%)	**0.026**	0.118	0.989
HT3[TG]	ht3/ht3	9 (4.40%)	13 (5.10%)	20 (7.30%)	0.088	0.418	0.459
	ht3/-	62 (30.50%)	98 (38.70%)	92 (33.60%)	0.053	0.624	0.163
	-/-	132 (65.00%)	142 (56.10%)	162 (59.10%)	0.798	0.289	0.312

*Each *P* value was calculated with co-dominant, dominant, and recessive models. Values in bold indicate significant *P* values.
AERD, aspirin-exacerbated respiratory disease; ATA, aspirin-tolerant asthma; NC, normal control.

the haplotype frequencies among the three groups. The AERD patients who carried homozygous HT2 [GA] showed a significantly higher frequency than other haplotypes in co-dominant (P=0.02) and recessive (P=0.026) models (Table 3).

DISCUSSION

We evaluated the associations of two genetic polymorphisms of *TLR3* in the three study groups, AERD, ATA, and NC, in a Korean population. To our knowledge, this is the first reported study to investigate the genetic polymorphisms of the 5'-UTR of *TLR3*, -299698G>T, and the coding region, Leu412Phe (293391G>A), in AERD patients.

Our genotype, allele, and haplotype results suggest that polymorphism in the *TLR3* gene is associated with AERD in the Korean population. However, we did not find any clinical evidence that patients with AERD were more prone than those with ATA to viral infection or virus-induced asthma exacerbation. A previous study has shown that there was no association of *TLR3* -299698G>T and Leu412Phe (293391G>A) SNPs with asthma and total serum IgE level in a Japanese population.[18] However, -299698G>T was found to be strongly associated with SJS in the same population,[12] which raises the possibility of a link between *TLR3* gene polymorphisms and viral infections. The suggested mechanism is that specific cytotoxic lymphocytes are produced in response to virus infection. The activity of these lymphocytes is suppressed by prostaglandin E2 (PGE2), which is produced by pulmonary alveolar macrophages. If PGE2 removal occurs, cytotoxic reactions are preceded by COX inhibitors and cytotoxic-lymphocyte-mediated attack and destruction of virus-affected cells in the respiratory tract. Reactive oxygen species, toxic metabolites, and mediators released then precipitate asthma attacks.[1,19] Overproduction of cysteinyl leukotrienes (cys-LTs) is unique to mast cells in AERD patients, and is particularly sensitive to suppression by PGE2. Consequently, reduction of PGE2 production, by aspirin, removes this endogenous control of cys-LT overproduction, which can result in asthma exacerbation.[20] These findings suggest that *TLR3* polymorphisms may be associated with the AERD phenotype in the Korean population.

Significantly higher minor allele frequency in AERD versus ATA indicated that the "A" allele of 293391G>A (Leu412Phe) might be involved in susceptibility to AERD pathogenesis. The change in amino acid from Leu to Phe results in functional deterioration of TLR3 and predisposes individuals to increased susceptibility to viral infections, which could be a potential cause of viral-induced AERD. A functional requirement for TLR3 in the host response against rhinovirus infection has also been proposed. TLR3 is expressed in dendritic cells and airway epithelial cells. TLR3 signaling activates the transcription of NF-κB and interferon regulatory factor 1 (IRF-3). IRF-3 induces expression of type 1 interferons that contribute to antiviral effects via activation of other genes. The non-synonymous SNP of *TLR3* has been previously tested for alteration of the function of TLR3, and its detrimental effect on TLR3 activity has been confirmed. TLR3-dependent activation of a firefly luciferase reporter driven by NF-κB has reduced reporter activity of NF-κB promoter by 30%.[12] Additionally, the frequency of the Phe allele of *TLR3* in viral infection and patients with sub-acute sclerosing panencephalitis is significantly higher than in controls in Japan.[21] This could be due to destabilization of the solenoid structure by 412Phe, and might disrupt potential glycosylation of the neighboring residue, Asn413, which has been shown to have N-acetylglucosamine residues attached.[20] The 412Phe allele of *TLR3* gene shows reduced interferon production in response to synthetic dsRNA,[12] as shown by reporter assay-dependent TLR3 activation. L412F mutations show decreased cell surface abundance of these TLR3 proteins, by flow cytometry analysis, and might affect the normal dimerization of TLR3 by improper targeting of the protein. Thus, *TLR3* polymorphism might contribute to the induction of frequent viral infections in relation to

TLR3 instability, or augmentation of inflammatory responses within cells.[12,21,23] More specifically, the 'A' allele of 293391G>A [Leu412Phe] might be involved in susceptibility to TLR3-mediated viral infections, which could contribute to the development and exacerbation of asthmatic symptoms in AERD patients. Despite limitations with regard to sample size and statistical power, this genetic polymorphism may provide useful information for those investigating the mechanism of AERD associated with viral infection. Further study is needed in larger cohorts to obtain a better understanding of the mechanism of AERD associated with viral infection.

In conclusion, our findings suggest that the *TLR3* -299698G>T and 293391G>A [Leu412Phe] polymorphisms of the *TLR3* gene are associated with the AERD phenotype. More specifically, the 'A' allele [412Phe] of non-synonymous SNP 293391G>A [Leu412Phe] may be associated with TLR3-mediated viral infection in AERD patients.

ACKNOWLEDGMENTS

This study was supported by a grant from the Korea Health 21 R&D Project of the Ministry of Health & Welfare, Republic of Korea (A030001).

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