

An association study of asthma and total serum immunoglobulin E levels for Toll-like receptor polymorphisms in a Japanese population

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Summary

Background The prevalence of atopic diseases has been increasing in developed countries. This could be explained by the hygiene hypothesis, which states that exposure to specific infections or endotoxins during infancy drives the maturing immune system towards a Th1 phenotype and away from the Th2 phenotype, which is associated with allergic diseases. Toll-like receptors (TLRs) play important roles in the signalling of many pathogen-related molecules and endogenous proteins associated with immune activation.

Objective The aim of the present study was to investigate whether polymorphisms in genes encoding TLRs are associated with asthma or total serum IgE levels.

Methods We screened the 5' flanking and coding regions of the *TLR2*, *TLR3*, *TLR4*, and *TLR9* genes for polymorphisms by direct sequencing of DNA from 32 asthmatics, and analysed the effect of the polymorphisms on the development of atopic asthma and on total serum IgE levels.

Results We identified 16 variants in *TLRs*. The transmission disequilibrium test of the families revealed that none of the alleles or haplotypes were associated with asthma or total IgE levels ($P > 0.05$). However, we found an insertion/deletion polymorphism in the 5' untranslated region of *TLR2*, and an expression construct containing the deletion allele showed lower luciferase activity than the wild-type alleles, suggesting that the deletion allele has reduced transcriptional activity.

Conclusion Our results indicate that polymorphisms in *TLRs* are not likely to be associated with the development of atopy-related phenotypes in a Japanese population.

Keywords asthma, IgE, polymorphism, TDT, Toll-like receptor

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Introduction

Atopic diseases such as asthma, atopic eczema, and allergic rhinitis are major causes of morbidity in developed countries and have been increasing in frequency [1, 2]. The recent increase in atopic diseases could be explained by the hygiene hypothesis, which is based on the theory that exposure to specific infections or endotoxins during infancy drives the maturing immune system towards the Th1 phenotype, and away from the Th2 phenotype associated with allergic diseases [3]. Innate immune responses are triggered through the binding of bacterial lipopolysaccharide (LPS) and other bacterial components to receptors such as CD14 and Toll-like receptors (TLRs). Toll is essential in activating anti-fungal defence mechanisms in *Drosophila* [4]. In humans, TLRs play important roles in the signalling by many pathogen-related molecules and endogenous proteins associated with immune activation. To date, 10 TLRs have been identified [5, 6]. *TLR4* is the principal receptor for LPS, with CD14 being required as an accessory molecule for signalling. *TLR4* also

mediates innate immune responses to respiratory syncytial virus [7], which is a common cause of bronchiolitis in infancy and is reported to be a risk factor for the development of atopy and asthma [8]. Arbour et al. [9] showed that the common Asp299Gly and Thr399Ile polymorphisms in *TLR4* are associated with differences in LPS responsiveness in humans, suggesting that genetic variations influence host responses to environmental stresses. The Asp299Gly polymorphism has been studied in a variety of diseases and is associated with a decreased risk of atherosclerosis [10] and an increased risk of premature birth [11], but not with the development of asthma or asthma/atopy-related traits [12].

Unmethylated CpG dinucleotides in bacterial DNA activate a subset of dendritic cells to promote Th1-type immune responses [13]. The cellular response to these CpG DNAs is mediated by *TLR9* [14], and a recent report showed a marginally significant association between the –1237C/T polymorphism in *TLR9* and asthma in European Americans [15].

TLR2 signals in response to a wide range of lipoproteins, including those from Gram-positive and mycobacterial cell walls [16–18], whereas *TLR3* is involved in responses to double-stranded viral RNAs [19]. However, no genetic variations in *TLR2* or *TLR3* appear to be associated with diseases. *TLR3* is located on chromosome 4q35,

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which shows linkage to atopic asthma in our genome-wide search [20].

The aim of the present study was to further clarify the role of TLRs in the development of asthma. We screened for polymorphisms in the 5' flanking and coding regions of the *TLR2*, *TLR3*, *TLR4*, and *TLR9* genes, and examined possible associations of these polymorphisms with atopic asthma in Japanese families identified through asthmatic children.

Materials and methods

Subjects

Probands were asthmatic children who visited the Paediatric Allergy Clinic of the University Hospital of Tsukuba. A full verbal and written explanation of the study was given to all family members interviewed, and 137 families (466 members) gave informed consent and participated in this study. The mean age of the probands and their siblings was 10.6 years (range 3–29 years); the mean age of the parents was 40 years (range 26–72 years). Each family member was questioned regarding allergic symptoms and underwent a physical examination performed by a participating paediatrician. Asthma was diagnosed in subjects according to the criteria of the National Institutes of Health, USA, with minor modifications [21]. Patients had to satisfy the following two criteria: (1) two or more episodes of wheezing and shortness of breath during the past year and (2) reversibility of the wheezing and dyspnea, either spontaneously or by bronchodilator treatment. Methacholine challenge was not carried out because of the low ages of the asthmatic patients in the families; however, the diagnoses of asthma in this population were confirmed clinically by participating physicians or paediatricians who had treated the children for more than 2 years. Because wheezing is often associated with viral

respiratory infection in young children, we excluded children younger than 3 years old [22]. The young adult patients included in this study had suffered from chronic asthma since childhood. Total serum IgE levels and IgE levels specific for *Dermatophagoides farinae* (Df) were determined with the Pharmacia CAP System (Uppsala, Sweden). To achieve homogeneity of the asthmatic patients, we selected families having more than two asthmatic children with Df RAST scores ≥ 3 . The families examined in this study are in part the same ones that participated in our genome-wide study [20].

For the case-control study, 133 child-onset asthmatic subjects with Df RAST scores ≥ 3 were recruited. The control subjects consisted of 190 unrelated Japanese (108 men and 80 women) individuals aged 29–75 years (mean = 46.9 years). They were anonymous, healthy volunteers and were not evaluated by paediatricians. This study was approved by the Committee of Ethics of the University of Tsukuba.

Molecular method DNA was extracted from peripheral blood leukocytes. All exons, exon-intron junctions, and the 5' flanking region of the *TLR2*, *TLR3*, *TLR4*, and *TLR9* genes were amplified from the genomic DNAs of 32 asthmatics. The genomic sequences are based on GenBank reference sequence NM_003264, AF424049, and AC013303.5 for *TLR2*; NM_003265 and AC104070.3 for *TLR3*; NM_003266 and AL160272 for *TLR4*; and NM_017442 and AC097637.2 for *TLR9*. Asthmatics for the mutation study were selected randomly from the probands described above.

The regions of interests were amplified by PCR, and amplified products were subjected to direct sequencing with the Big Dye Terminator kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 DNA Sequencer (Applied Biosystems).

The mutations and genotyping methods are described in Table 1. Genotyping of each subject was performed by either restriction fragment length polymorphism (RFLP) or the

Table 1. Genotyping methods of each polymorphism

Gene	Polymorphisms	Methods	Primers	Enzyme
<i>TLR2</i>	c. –196 to –174ins/del	Direct sequence	5'-ctcggaggcagcgagaaa 5'-ctgggccgtgcaaagaag	
	c. –191G/A c. 597T/C	Direct sequence AcycloPrime	The same as C. –196 to –174del 5'-ggttcaagcccttctctct 5'-caaacattccacggaacttg *5'-catatgaaggatcagatgacttac	
	c. 1350T/C	AcycloPrime	5'-tgcctgaaactgtcagtgg 5'-aagagggaggcatctggttag *5'-ttgggaatgcagcctgttac	
<i>TLR3</i>	–7C/A	RFLP	5'-gcatttgaaagccatctgct 5'-aagttggcggctggtatct	<i>Mbo</i> II
	IVS3 +71C/A	RFLP	5'-gctggaaaatctccaagagc 5'-gaggctagagagcattacattcat	<i>Nla</i> III
	Leu412Phe	RFLP	5'-ggctaaaatgtttggagcac 5'-tgagattttatcttggttagctga	<i>Dde</i> I
	c. 1377C/T	RFLP	5'-ccaggcataaaaagcaaatag 5'-ggaccaaggcaaaaggagttc	<i>Taq</i> I
<i>TLR9</i>	c. 1635G/A	RFLP	5'-aagctggacctctaccacga 5'-ttggctgtggatgtttgt	<i>Acl</i> I

*AcycloPrime SNP primer.

AcycloPrime method. AcycloPrime™ (Perkin-Elmer, Wellesley, MA, USA) was performed following the manufacturer's instructions. To remove primers and dNTPs from each PCR product, 0.2 µL of PCR Clean-Up Reagent and 1.8 µL of PCR Clean-Up Dilution Buffer was added to 5 µL of each PCR product, incubated at 37 °C for 60 min, and then incubated at 85 °C for 15 min to inactivate the enzyme. The AcycloPrime™ reaction cocktail containing 2 µL 10 × reaction buffer, 1 µL Acyclo Terminator, 0.5 µL SNP primer (10 µM), 0.05 µL Acyclo Polymerase, and 9.45 µL distilled water was added to 7 µL of processed PCR product. After an initial denaturation at 95 °C for 2 min, 30 cycles of 95 °C for 15 s and 52.5 °C for 30 s were performed on a thermal cycler. Fluorescence polarization was then measured by ARVO (Perkin-Elmer). The accuracy of genotyping was confirmed by the direct sequencing of 48 randomly selected asthmatic children.

Cell culture THP-1 cells (Cell Resource Center for Biomedical Research, Tohoku University, Miyagi, Japan) were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) with 10% heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich). Cells were grown at 37 °C with 5% carbon dioxide in a humidified incubator.

Plasmid construction A fragment of *TLR2* from nucleotides –249 to +110 relative to the transcription initiation site was amplified with primers 5'-GAAGATCTTCCTTCTTC GACCCAGCCTTG-3' and 5'-GAAGATCTTCCTGGGA GAACTCCGAGCAG-3' from the DNA of a subject homozygous for c. –196 to –174del allele and one homozygous for the wild-type allele. This region includes maximal expression of a reporter gene in THP-1 cells [23]. A 5'-gaagatcttc-3' adapter was added to the 5' ends of the primers to create a *Bs*III restriction site. PCR products were digested with *Bs*III overnight at 37 °C then subcloned into *Bs*III-digested pGL3-Basic Vector (Promega, Madison, WI).

Transfection and luciferase assay THP-1 cells were resuspended in culture medium supplemented with 10% FCS. The cells (1.2×10^7 cells in 0.6 mL) were co-transfected with 15 µg of the test construct and 0.8 µg of pRL-TK (Promega) by electroporation with a Gene Pulser II (Bio-Rad, Hercules, CA) set at 250 V and 950 µF. The cells were incubated in 5 mL of RPMI 1640 supplemented with 10% FCS for 5 h. After the incubation, the cells were washed and lysed in 100 µL of passive lysis buffer (Promega), and 20 µL of lysate was used for luciferase assay with the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activity was normalized to Renilla luciferase activity, which was assayed simultaneously. The value of reporter activity is the mean of three independent values.

Statistics Deviation from Hardy–Weinberg expectancy was examined with the χ^2 test. The transmission disequilibrium test (TDT) was performed with the ASPEX program [24]. Haplotype TDT was analysed with the TRANSMIT program [25]. Total serum IgE levels were log transformed, and a quantitative TDT test was performed with the QTDT program [26]. Linkage disequilibrium between polymorphisms was calculated by GOLD [27]. Statistical significance was defined at the 5% level. The statistical power of TDT for

detecting a polymorphism that directly affected risk was calculated according to the method proposed by Risch and Merikangas [28]. The significance of differences in the allele frequencies between case and control groups was determined by the χ^2 test.

SNP information for allele frequencies of other ethnic groups was retrieved on January 22, 2003 from the Innate Immunity PGA, NHLBI Program in Genomic Applications. URL: <http://innateimmunity.net/>. Differences in allele frequencies between Japanese and other ethnic groups were calculated by Fisher's exact test.

Results

Polymorphisms and TDT

We identified four variants in *TLR2* (c. –196 to –174del, c. –191G>A, c. 597T>C, and c. 1350T>C), four variants in *TLR3* (–7C>A, IVS3 +71C>A, Leu412Phe, and c. 1377C>T), five variants in *TLR4* (c. –306G>C, IVS1 –66C>G, c. –92C>T, IVS2+21G>T, and 33S>R), and three variants in *TLR9* (IVS1 –44A>G, c. 1635G>A, and c. 2901G>C) by the sequencing of DNA from 32 asthmatics. IVS1 –44A>G and c. 1635G>A correspond to the 1174A>G and 2848G>A variants, respectively, reported by Lazarus et al. [15]. The –1237T>C polymorphism, which showed marginally significant association with asthma in European Americans [15], was not detected in this series of 32 Japanese asthmatics. Because *TLR9* IVS1 –44A>G and *TLR9* c.1635G>A were in nearly complete linkage disequilibrium, we genotyped only the *TLR9* c. 1635G/A polymorphism in family members. All variants of *TLR4* and *TLR9* c. 2901G>C occurred in less than 4% of asthmatics; therefore, we did not genotype these variants in the families. We did not detect the *TLR4* 299Gly or 399Ile alleles in 32 asthmatics. Allele frequencies of different ethnic groups are shown in Table 2. Most of the *TLR2* and *TLR3* allele frequencies for African Americans and the *TLR2* c. 1350C/T allele frequency for Hispanic Americans were significantly different from those in the Japanese. The linkage disequilibrium among polymorphisms is shown in Table 3. A significant linkage disequilibrium was observed among polymorphisms in *TLR2* and among those in *TLR3*.

TDT revealed that no *TLR2*, *TLR3*, or *TLR9* allele was transmitted preferentially to asthma-affected children and that none of these polymorphisms was associated with total serum IgE level ($P>0.05$, Table 4). In haplotype TDT, no haplotype of *TLRs* was transmitted preferentially to asthmatic offspring (Table 5).

The statistical power of TDT for the *TLR2*, *TLR3*, and *TLR9* alleles, except for *TLR2* c. –191A allele, was more than 0.9 at the α level of 0.05 if the relative risk for asthma in those persons carrying a putative risk allele is 1.7 compared with that in persons without the allele. The statistical power of TDT for *TLR2* c. –191A was 0.57.

The results of the case–control study are shown in Table 6. Genotypic distributions did not deviate significantly from Hardy–Weinberg equilibrium expectations in either group ($P>0.05$). No significant differences in the allele and

Table 2. Allele frequencies of *TLRs* polymorphisms in different ethnic groups

Gene	Allele	*African American	*European American	*Hispanic American	†Japanese	dbSNP No
<i>TLR2</i>	c. – 196 to – 174del	-	-	-	0.34	
	c. – 191A	-	-	-	0.074	
	c. 597C	0.646‡	0.30	0.27	0.34	rs3804099
	c. 1350C	0.083‡	0.17	0.062‡	0.32	rs3804100
<i>TLR3</i>	– 7A	0.19	0.13	0.19	0.3	rs3775296
	IVS3+71A	0.104§	0.13	0.19	0.29	
	Phe412	0.042‡	0.28	0.21	0.32	rs3775291
	c. 1377T	-	-	-	0.4	rs3775290
<i>TLR9</i>	– 1237C	0.25‡	0.15§	0.04	<0.02	
	IVS1 – 44A	0.44	0.44	0.4	0.43	rs352139
	c. 1635G	0.67	0.44	0.4	0.44	rs352140

*Allele frequencies of these ethnic groups were retrieved from the Innate Immunity PGA, NHLBI Program in Genomic Applications. URL: <http://innateimmunity.net/data/homology>. †Allele frequencies were based on parents in asthmatic families. ‡ $P < 0.001$. § $P < 0.01$.

Table 3. Linkage disequilibrium between all pairs of loci (D'')

		c. – 191G/A	c. 597T/C	c. 1350T/C
<i>TLR2</i>	c. – 196 to – 174Ins/del	0.92*	0.68*	0.67*
	c. – 191G/A		0.76**	0.59**
	c. 597T/C		0.83*	
<i>TLR3</i>	IVS3+71C/A		Leu412Phe	c. 1377C/T
	– 7C/A	0.84*	0.85*	0.87*
	IVS3 + 71C/A		0.85*	0.84*
	Leu412Phe			0.91*

* $P < 0.0001$. ** $P < 0.001$.

Table 4. TDT results with alleles at polymorphic sites

Gene	Allele	Transmitted	Not transmitted	P^*	$\log_{10} \text{IgE}$ P^{**}
<i>TLR2</i>	c. – 196 to – 174del	70	76	0.7	0.15
	c. – 191A	20	19	1	0.84
	c. 597C	74	69	0.76	0.6
	c. 1350C	73	70	0.76	0.58
<i>TLR3</i>	– 7A	64	66	0.79	0.64
	IVS3+71A	80	67	0.35	0.17
	Phe412	75	57	0.089	0.92
	c. 1377T	88	83	0.77	0.4
<i>TLR9</i>	c. 1635A	84	75	0.53	0.093

Allele frequencies were based on asthmatic probands in the families. * P values were calculated by the ASPEX program. ** P values were calculated by the QTD program.

genotype distributions were observed between the patient and control groups ($P > 0.05$).

Reporter assay

We performed a reporter assay to determine whether there was a functional difference between the *TLR2* c. – 196 to – 174del and wild-type alleles. The *TLR2* c. – 196 to – 174ins/del polymorphism is located in the 5' untranslated

region (UTR) of exon 1. We generated luciferase reporter gene constructs that contained the region of *TLR2* from nucleotides – 249 to +110 relative to the transcription initiation site and differed only at c. – 196 to – 174 (wild-type vs. deletion type). The activity of each construct was analysed in THP-1 (monocytic leukaemia cell line). Firefly luciferase activity normalized to Renilla luciferase activity is shown in Fig. 1. Constructs with c. – 196 to – 174 del showed reduced luciferase activity compared with the wild-type constructs.

Discussion

In the present study, we identified polymorphisms in *TLR2*, *TLR3*, *TLR4* and *TLR9*, and these polymorphisms do not appear to be associated with the development of asthma or total serum IgE levels.

The allele frequencies of *TLR2* and *TLR3* in Japanese were significantly different from those in African Americans and the degree of differences in allele frequencies are less prominent than those from Hispanic and European Americans.

Environmental exposure to microbial products, as measured by the endotoxin levels in mattress dust, is associated with decreased childhood wheezing, atopic asthma, and atopic sensitization [29]. The Asp to Gly change at position 299 (Asp299Gly) in *TLR4* alters the ability of the host to respond to LPS. This variant is reported to be associated with various diseases, including atherosclerosis [10], premature birth [11], and Gram-negative infection [30]. However, we did not detect the Gly299 variant in 32 Japanese asthmatics. The *TLR4* variants that we detected in this study occurred at less than 4% frequencies. Raby et al. [12] examined relations between *TLR4* polymorphisms, including Asp299Gly, and asthma and asthma-associated traits in two independent sets of families, but they found no evidence that *TLR4* variants contribute to asthma susceptibility. Because we did not detect the Gly299 variant in our population, it is unlikely that Asp299Gly contributes to asthma susceptibility in the Japanese population. However, our results for *TLR4* variants are limited because of the low allele frequencies. Therefore,

Table 5. Haplotype transmission test

Gene	Haplotype	Estimated haplotype frequency	Observed transmissions	Expected transmissions	P*
<i>TLR2</i>	ins/c. – 191G/c. 597T/c. 1350T	0.5	209.7	205.0	0.53
	del/c. – 191G/c. 597C/c. 1350C	0.25	89.0	90.0	0.87
	ins/c. – 191A/c. 597T/c. 1350T	0.06	21.1	21.9	0.81
	del/c. – 191G/c. 597T/c. 1350T	0.05	13.6	16.5	0.34
<i>TLR3</i>	– 7C/IVS3 + 71C/Phe412/c. 1377C	0.31	131.8	120.4	0.17
	– 7C/IVS3 + 71C/Leu412/c. 1377C	0.30	105.5	116.9	0.18
	– 7A/IVS3 + 71A/Leu412/c. 1377T	0.28	112.0	108.9	0.66
	– 7C/IVS3 + 71C/Leu412/c. 1377T	0.10	35.6	35.4	0.96

Haplotypes with allele frequency > 0.05 are shown. Ins/del in *TLR2* corresponds to c. – 196 to – 174ins/del.

*1 000 000 bootstrap samples; global $P = 0.72$ for *TLR2* and $P = 0.27$ for *TLR3*.

Table 6. Case-control results with alleles at polymorphic sites

Gene	Polymorphism	Population	Genotype count (frequency)			P		P
<i>TLR2</i>	c. – 196 to – 174del		del/del	ins/del	ins/ins		del	ins
		Asthma	24 (0.18)	52 (0.39)	57 (0.43)		100 (0.38)	166 (0.62)
	c. – 191G/A	Controls	21 (0.11)	75 (0.40)	91 (0.49)	0.21	117 (0.31)	257 (0.69)
			GG	GA	AA		G	A
	c. 597T/C	Asthma	118 (0.89)	14 (0.11)	1 (0.01)		250 (0.94)	16 (0.06)
		Controls	161 (0.87)	23 (0.12)	2 (0.01)	0.84	345 (0.93)	27 (0.07)
	c. 1350T/C		CC	CT	TT		C	T
		Asthma	12 (0.09)	62 (0.47)	59 (0.44)		86 (0.32)	180 (0.68)
		Controls	17 (0.09)	78 (0.41)	93 (0.49)	0.64	112 (0.30)	264 (0.70)
			CC	CT	TT		C	T
		Asthma	10 (0.07)	60 (0.45)	64 (0.48)		80 (0.30)	188 (0.70)
		Controls	16 (0.08)	76 (0.40)	98 (0.52)	0.69	108 (0.28)	272 (0.72)
<i>TLR3</i>	– 7A		CC	CA	AA		C	A
		Asthma	72 (0.53)	51 (0.38)	12 (0.09)		195 (0.72)	75 (0.28)
		Controls	94 (0.50)	81 (0.43)	14 (0.07)	0.63	269 (0.71)	109 (0.29)
	IVS3 + 71C/A		CC	CA	AA		C	A
		Asthma	74 (0.55)	47 (0.35)	13 (0.10)		195 (0.73)	73 (0.27)
		Controls	94 (0.50)	85 (0.45)	9 (0.05)	0.08	273 (0.73)	103 (0.27)
	Leu412Phe		Leu/Leu	Leu/Phe	Phe/Phe		Leu	Phe
		Asthma	71 (0.53)	56 (0.41)	8 (0.06)		198 (0.73)	72 (0.27)
	c. 1377C/T	Controls	109 (0.57)	71 (0.37)	10 (0.05)	0.69	289 (0.76)	91 (0.24)
			CC	CT	TT		C	T
		Asthma	52 (0.39)	59 (0.44)	22 (0.17)		163 (0.61)	103 (0.39)
		Controls	69 (0.37)	92 (0.49)	28 (0.15)	0.74	230 (0.61)	148 (0.39)
<i>TLR9</i>	c. 1635G/A		AA	GA	GG		A	G
		Asthma	45 (0.34)	58 (0.44)	29 (0.22)		148 (0.56)	116 (0.44)
		Controls	55(0.29)	98 (0.52)	36 (0.19)	0.38	208 (0.55)	170 (0.45)

we could not exclude the possibility that rare variants detected in this study may contribute to susceptibility for asthma or to total serum IgE levels.

We detected common polymorphisms in *TLR2*, *TLR3*, and *TLR9*, but none of the variants or haplotypes were associated with asthma or total serum IgE levels. The power to detect associations of these polymorphisms was greater than 90%, except for the c. – 191A allele in *TLR2*. Because some haplotypes, such as del/c. – 191G/c. 597T/c. 1350T and ins/c. – 191C/c. 597T/c. 1350T in *TLR2*, occurred at low frequencies, it is possible that these rare variants or haplotypes play a role in the development of asthma.

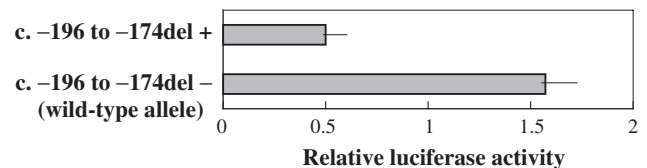


Fig. 1. Relative luciferase activities expressed in THP-1 cells transfected with pGL3 vectors containing *TLR2* c. – 196 to – 174 deletion or wild-type sequences. Firefly luciferase gene activity from each experimental construct was normalized to Renilla luciferase activity from co-transfected pRL-TK plasmid, and expressed as relative luciferase activity. Bars indicate the mean value of three independent experiments, and standard deviation is indicated by error bars.

Lazarus et al. [15] sequenced regions of *TLR9* in DNAs from three different ethnic groups, African Americans, Caucasians, and Hispanic Americans, and performed case-control studies of asthma, deep venous thrombosis, and myocardial infarction. They found an increased risk for asthma with the *TLR9* -1237C allele (odds ratio 1.85) in European Americans, but did not detect other significant associations with *TLR9* polymorphisms. Because we did not detect the *TLR9* -1237C allele in 32 Japanese asthmatics, it is unlikely that *TLR9* -1237C plays a role in the development of asthma in the Japanese population.

The functional significance of a variant in a gene may only be evident in a specific setting of additional variants in the same or different genes, as reported for IL-4 receptor alpha gene polymorphisms [31]. Our data, however, indicated that none of the common haplotypes of *TLR2* or *TLR3* are associated with asthma susceptibility or total serum IgE levels.

We found an insertion/deletion polymorphism in the 5' UTR of *TLR2*, and a construct containing a deletion allele showed lower luciferase activity in comparison with a construct carrying the wild-type allele, suggesting reduced transcriptional activity of deletion allele. 5' UTR is important in the transcriptional and translational regulation of genes [32, 33]. *TLR2* signals in response to a wide range of lipopeptides, and *TLR2* deficiency results in susceptibility to *Staphylococcus aureus* infection [34] and *Streptococcus pneumoniae* meningitis [35]. Although our TDT analysis in the Japanese showed no association between asthma susceptibility and the *TLRs* polymorphisms, it is possible that the *TLR2* c. -196 to -174 insertion/deletion polymorphisms contribute to susceptibility to Gram-positive or Gram-negative bacterial infections. Further studies are needed to evaluate the possible associations of asthma and infectious disease with polymorphisms in *TLRs*.

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