the surface. IL-5 receptor activation in eosinophils has been shown to stimulate JAK2, STAT1, Lyn and Syk [24]. IL-5 and GM-CSF inhibited the apoptosis of eosinophils *in vitro* and *in vivo*. Both Syk and Lyn are essential signal molecules for the activation of the anti-apoptotic pathway(s) induced by the IL-3/IL-5/GM-CSF receptor subunit in human eosinophils [25]. Thus, one possibility is that Syk expression in the nasal polyps of AR may indicate the activation of Syk by IL-5 receptor to lead to the elongation of eosinophil-survival.

Conversely, the analysis of Syk-knock out mice ($Syk^{-/-}$) demonstrated that the anti-apoptotic effect of IL-5 in cells does not require Syk despite the activation of this tyrosine kinase upon IL-5 receptor ligation [26]. However, Syk is important in activation events (oxidative burst or phagocytosis) induced by Fcγreceptor (FcγR) stimulation [26]. FcγR is found on the surface of eosinophils and plays a critical role in eosinophil activation in cooperation with Syk phosphorylation. Among several FcγRs, allergen-specific IgG1 and IgG3 induces degranulation of eosinophils as inflammatory reaction through FcγRII (CD32) [27]. Also, FcγRII may pivotally regulate both the survival and death of eosinophils, depending on the manner of receptor ligation and β2 integrin involvement [28]. The integrin family of cell adhesion receptors mediates both cell–cell and cell–matrix interaction and plays critical roles in development, inflammation, angiogenesis, migration, metastasis and other important biological processes [29]. The binding of β2 integrin receptors to their ligands (ICAM-1) is critical for firm attachment, spreading and the transendothelial migration of eosinophils [30]. Syk is essential to activate signal transduction cascades initiated by the binding of β2 integrin receptors to their ligands [31]. Thus, Syk expression might suggest that signal transduction from β2 integrin receptors in eosinophils was working to migrate into the nasal polyp. As Syk is regulated by multiple classes of integrins, Syk is deeply associated with the integrin family [32]. Additionally, signaling by integrin and ICAM-1 prolong eosinophil survival [33].

Aggregation of the high affinity IgE receptor (FcεRI) by IgE binding results in the sequential activation of Syk and Lyn on mast cells [34]. Local IgE class switchings and local IgE syntheses were demonstrated in human allergic nasal mucosa [35,36]. IgE itself up-regulates FcεRI, which prevents protease digestion at the cell surface [37]. Recently, nasal polyps have been characterized by a high concentration of IgE in the nasal polyp associated with presence of *Staphylococcus aureus* enterotoxin-specific IgE [38]. These data led us to speculate that Syk-dependent FcεRI signaling is working well in nasal polyps of AR. However, the positive expression of Syk in mast cells was less than eosinophils in this study (Fig. 3D–F). Although eosinophils express FcεRI, most of the protein is confined to the cytoplasm [39]. Our data showed that IgE in the serum was correlated with the Syk score in nasal polyps in AR patients. However, there is little evidence for IgE-dependent function in eosinophils. IgG appears to be more important for eosinophil activation in allergic disease than IgE.

Recently, it was reported that enhanced IFN-α signaling and proinflammatory function were dependent on the tyrosine kinase Syk and on adaptor proteins that activate Syk through immunoreceptor tyrosine activation motifs [40]. IFN-α inhibited IL-5 and GM-CSF genreration of cord blood. IFN-α receptor was found on eosinophils collected from patients with various eosinophilic disorders and inhibited the release of eosinophil granule proteins, such as eosinophil cationic protein, neurotoxin, or IL-5 [41]. The oromucosal administration of IFN-α reduced allergen-specific IgE production and allergen-induced eosinophil recruitment in the absence of detectable toxicity for the treatment of allergic disease [42]. However, our previous study showed that IFN-α was not detected in the nasal lavage from patients with AR and nasal polyps [5] and in the supernatant of nasal polyp-derived fibroblasts (data not shown). There might be the possibility that Syk activation in eosinophils of allergic rhinitis polyp induces suppressive signaling for allergic disease, but this possibility was low.

The delivery of Syk antisense oligodeoxynucleotides (ASO) by aerosol to rat lungs *in vivo* has the potential to reduce Syk expression in infiltrated immune cells and to suppress Ag-pulmonary inflammation [43]. Additionally, the treatment of Syk ASO greatly inhibited the number of eosinophils in the lung parenchyma [44]. They suggested that Syk ASO may be a useful anti-inflammatory agent. Intranasal application of Syk inhibitor R112 improved allergic symptoms of seasonal allergy in a park setting [45]. They suggested that intranasal application of Syk inhibitor become a new treatment of a seasonal allergy.

The degree of Syk expression is not equal to the activity of Syk in cells, while no expression of Syk does not means any activities of Syk in cells. The autophosphorylation and activation of Syk (phosphorylation of adaptor molecule) produce the signal pathway in eosinophils. Constitutive phosphorylated Syk was detected in nasal polyps with a high Syk expression from patients with AR by Western blotting in this study (data not shown). Although further study is necessary to investigate how Syk works in nasal polyps with AR, Syk may be a target molecule for the treatment of nasal polyps with allergy.

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Single nucleotide polymorphism-based genome-wide linkage analysis in Japanese atopic dermatitis families

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Abstract

Background: Atopic dermatitis develops as a result of complex interactions between several genetic and environmental factors. To date, 4 genome-wide linkage studies of atopic dermatitis have been performed in Caucasian populations, however, similar studies have not been done in Asian populations. The aim of this study was to identify chromosome regions linked to atopic dermatitis in a Japanese population.

Methods: We used a high-density, single nucleotide polymorphism genotyping assay, the Illumina BeadArray Linkage Mapping Panel (version 4) comprising 5,861 single nucleotide polymorphisms, to perform a genome-wide linkage analysis of 77 Japanese families with 111 affected sib-pairs with atopic dermatitis.

Results: We found suggestive evidence for linkage with 15q21 (LOD = 2.01, NPL = 2.87, P = .0012) and weak linkage to 1q24 (LOD = 1.26, NPL = 2.44, P = .008).

Conclusion: We report the first genome-wide linkage study of atopic dermatitis in an Asian population, and novel loci on chromosomes 15q21 and 1q24 linked to atopic dermatitis. Identification of novel causative genes for atopic dermatitis will advance our understanding of the pathogenesis of atopic dermatitis.

Background

Atopic dermatitis (ATOD) is a hereditary, pruritic, inflammatory, chronic skin disease that occurs most commonly in early childhood but can persist or start in adulthood. The prevalence of ATOD has been studied in a wide variety of populations [1], and its frequency ranged from 0.73% to 23% of the study populations. The 12-month prevalence value of symptoms of atopic eczema in Japanese children 6 to 7 years of age was 16.9%, the second highest after Sweden [2]. Living in lower, more tropical latitudes, rural areas, and less industrialized regions correlates with a lower prevalence of ATOD[1]. The etiology of ATOD is not fully understood, but atopy, which is characterized by increased levels of immunoglobulin E (IgE) against common environmental allergens, is considered one of the strongest predisposing factors for ATOD.

ATOD is associated with cutaneous hyperresponsiveness to environmental triggers that are innocuous to healthy individuals [3]. In the acute lesions of ATOD, marked perivascular infiltration of inflammatory cells consisting predominantly of lymphocytes and occasional monocyte-macrophages is frequently observed. In chronic lichenified lesions, there are increased numbers of Langerhans' cells and mast cells in the epidermis, and macrophages dominate the dermal mononuclear cell infiltrate [3]. ATOD and its prevalence are often associated with other clinical atopic manifestations, including asthma, allergic rhinitis, rhinoconjunctivitis, and elevated total and/or allergen-specific serum IgE levels. Nearly 80% of children with ATOD develop allergic rhinitis or asthma, suggesting that allergen sensitization through the skin predisposes subjects to respiratory diseases [3].

ATOD is the result of complex interactions between multiple genetic and environmental factors. Sixty-nine percent of patients with ATOD have one or both of parents affected by ATOD [4], and children have a risk of up to 75% of developing the disease when both parents have ATOD [5]. Twin studies have supported the role of a strong genetic contribution with a concordance rate of 0.72–0.86 in monozygotic twins and 0.21–0.23 in dizygotic twins, indicating high heritability of ATOD [5]. Indeed, the heritability of ATOD was estimated at 0.72 by a Norwegian twin study [6].

To identify susceptibility genes for ATOD, 2 approaches can be applied: candidate gene association study, and genome-wide linkage analysis. Several ATOD candidate genes have been identified. The chromosome 5q region harbors many candidate genes for ATOD, including interleukin (IL) -4 (*IL4*), *IL13*, *IL5*, *IL12B*, and serine protease inhibitor Kazal-type 5 (*SPINK5*) [7]. Other candidate genes include high affinity IgE receptor beta chain gene (*FCER1B*), mast cell chymase gene (*CMA1*), and IL4 receptor alpha chain gene (*IL4RA*)[7]. Recent studies have emphasized the importance of skin barrier function in development of ATOD. Two loss-of-function mutations of the filaggrin gene (*FLG*) were found to be associated with ATOD in 2 independent Caucasian populations [8]. For the second approach, genome-wide linkage analysis, and across the entire genome polymorphic DNA markers positioned at specific intervals along each chromosome are screened for linkage to the disease of interest. Because ATOD is a complex disease with a mode of inheritance that does not follow typical Mendelian laws, parametric linkage analysis, which assumes a genetic model, cannot be applied. Therefore, nonparametric methods, such as the affected sib-pairs method, have been used widely to localize susceptibility genes for common diseases such as ATOD. To date, 4 genome-wide linkage studies have been performed in Caucasian populations, but there have been no large studies in other ethnic groups. Evidence for linkage to ATOD was obtained for several chromosomal regions [9-12]. These linkage studies were performed with highly polymorphic microsatellite markers. The recent development of high-throughput genotyping technologies has allowed us to perform genome-wide linkage studies of single-nucleotide polymorphisms (SNPs). The SNP-based genome-wide linkage study has the potential to be as powerful as traditional microsatellite-based analysis and offers good identification of peak locations for further fine-mapping association analyses [13]. In the present study, we performed genome-wide linkage analysis with 77 Japanese families with at least 2 siblings affected with ATOD. This is the first SNP-based whole-genome linkage study in an Asian population.

Methods

Subjects

The probands were patients with ATOD who visited the Dermatology Department of the University Hospital of Tsukuba and dermatology departments of 10 hospitals in Ibaraki, and Dermatology Department of the University Hospital of Kyorin in Tokyo, Japan. A full verbal and written explanation of the study was given to patients and all family members interviewed, and all provided informed consent.

ATOD was diagnosed in subjects according to the criteria of Hanifin and Rajka [14]. Patients all had: pruritus, typical appearance of ATOD, and tendency toward chronic or chronically relapsing dermatitis. The diagnosis of all of the patients that participated in this study was confirmed by a dermatologist.

A total of 77 families (287 individuals and 111 sib-pairs) were included in this study (Table 1). The mean age of the probands and their ATOD-affected siblings was 14 years (range 1–43 years); the mean age of the parents was 45

Table 1: Families' structure included in this study

Parents	2-sibs (No. of sib-pairs)	3-sibs (No. of sib-pairs)	4-sibs (No. of sib-pairs)	total
0	10 (10)	1 (3)	0	
1	15 (15)	0	0	
2	38 (38)	11 (33)	2 (12)	
total	63 (63)	12 (36)	2 (12)	77 (111)

years (32–78 years). The male: female ratio of the children with ATOD was 1:1. This study was approved by the Ethics Committee of the University of Tsukuba.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes or oral brushed cells using standard protocol. The Illumina SNP-based Linkage Panel IV (Illumina, San Diego, Calif.) was used for genotyping. This panel includes 5,861 SNPs distributed evenly across the genome. The average and median intervals between markers are 503 Kb (0.64 cM) and 301 Kb (0.35 cM), respectively. The Illumina markers were typed with the Illumina BeadStation 500G according to the manufacturer's recommendations. Genotyping of SORCS receptor 3 (SORCS3) was done by GoldenGate assay (Illumina) following the manufacturers' instruction.

Statistical analysis

Affected sib pair linkage analysis was performed along the entire length of each chromosome with the MERLIN program developed by Abecasis et al [15]. Both the nonparametric linkage (NPL) Z score and nonparametric log of the odds (LOD) score calculated with the Kong and Cox linear model [16] were extracted from the MERLIN runs and used to generate graphic plots of the genome-wide scan results. Because linkage disequilibrium (LD) between closely spaced SNPs can falsely inflate linkage statistics, we used the SNPLINK program [17], which removes LD from the marker sets in an automated fashion.

Empirical P values were calculated for the NPL Z and LOD scores via simulation. MERLIN was used to generate 10,000 replicates of families identical to those in our sample. Markers with similar allele frequencies were also generated under the assumption of no linkage. Linkage analyses were then performed on these unlinked replicates, and peaks of NPL and LOD scores were recorded for each simulation. Simulation studies of our genome scan suggested that LOD > 3.16 would have been expected to occur only once in every 20 genome scans in the absence of linkage and LOD > 1.98 would have been expected to occur once per genome scans. These values correspond to "significant" and "suggestive" thresholds for genomewide significance, as defined by Lander and Kruglyak [18]. Our study had a power of > 99, 0.70, 0.15, and 0.02 to detect a susceptibility locus of λs = 3, 2, 1.5, and 1.25 for ATOD with a genome-wide significance of lod > 3.16. The GeneFinder program [19] was used to obtain 95% confidence intervals for the locations of linked loci. Transmission disequilibrium test (TDT)[20] and pedigree disequilibrium test (PDT) [21]was performed with unphased program. Bonferroni correction was applied for the correction of the multiple testing.

Tag SNP selection

Tag SNPs were selected with Tagger software [22] implemented in Haploview software [23] with r^2 threshold of 0.8 and allele frequencies of 0.1.

Results

We observed an average minor allele frequency (MAF) of 0.28 and a mean heterozygosity of 0.36 in our Japanese population. These values were identical to those in Asian populations on the datasheet for the Illumina Linkage IV Panel. Among 5,861 SNP genotyped, 151 SNPs were not polymorphic in the Japanese population. The call rate (percentage of successful genotype calls among subjects) was used as a measure of quality. The average call rate was 99.5%, and we excluded 19 SNPs with call rates of less than 90%. The rate of Mendelian inconsistency or impossible recombination identified by the MERLIN program was 0.10% in the families with parents available for genotyping. Because the low heterozygosity of SNPs means that only 37% of genotyping errors will appear as Mendelian inconsistencies[15], the approximate genotyping error rate was estimated to be 0.37%.

Results of the linkage analysis are presented in Figure 1. One region, chromosome 15q21, showed genome-wide suggestive linkage to ATOD (rs2017176, LOD = 2.01, NPL = 2.87, P = .0012), with a 95% CI of 49.4 (rs1147129) - 76.4 (rs2001597) Mb on the basis of simulation studies. Weak evidence in favor of linkage to 1q24 (rs761076 and rs1933075, LOD = 1.26, NPL = 2.44, P = .008) was observed.

Results of TDT and PDT are shown in Table 2. TDT and PDT was family-based test for allelic association. TDT was proposed to test for linkage disequilibrium in family tri-

Table 2: Results of TDT and PDT

rs numbers	Allele	Trans*	Not Trans**	TDT P value (corrected)	PDT P value (corrected)	Gene Name	chr	SNP position
rs717227	T	70	42	0.007818 (1)	0.009023 (1)	CHRM3	1	intron
rs1449504	C	54	21	0.0001059 (0.44)	0.002627 (1)	LRP1B	2	intron
rs1370497	A	35	60	0.009876 (1)	0.006856 (1)	FMNL2	2	intron
rs920891	A	19	36	0.02082 (1)	0.008041 (1)		3	
rs1567058	T	11	30	0.002486 (1)	0.007963 (1)	ARL6	3	Intron
rs1317244	A	48	77	0.009169 (1)	0.007166 (1)	CD200	3	Intron
rs1402276	A	53	30	0.01105 (1)	0.00575 (1)	SCHIP1	3	Intron
rs889319	T	53	80	0.01881 (1)	0.009975 (1)	RAI14	5	Intron
rs40207	T	25	54	0.0009624 (0.99)	0.003361 (1)		5	
rs1552104	T	76	33	0.00002976 (0.15)	0.002873 (1)		5	
rs1498252	A	58	32	0.005775 (1)	0.002604 (1)		6	
rs2894891	A	65	42	0.0256 (1)	0.005798 (1)		6	
rs1158747	T	78	39	0.0002723 (0.78)	0.0008586 (1)	LAMA4	6	Intron
rs169902	A	33	51	0.04865 (1)	0.006104 (1)		7	
rs1202169	A	74	44	0.005487 (1)	0.006487 (1)	ABCB1	7	Intron
rs1419607	A	43	75	0.003033 (1)	0.005659 (1)		7	
rs901592	T	37	70	0.001298 (1)	0.003353 (1)		8	
rs9071	A	36	59	0.01771 (1)	0.007423 (1)	LRRC14	8	3UTR
rs1250288	T	58	36	0.02263 (1)	0.005251 (1)		9	
rs1361800	T	38	15	0.001315 (1)	0.002218 (1)	SORCS3	10	Intron
rs1034178	T	75	42	0.002129 (1)	0.006029 (1)	SORCS3	10	Intron
rs2011505	T	11	28	0.005631 (1)	0.004738 (1)	NCAM1	11	intron
rs3345	T	58	30	0.002604 (1)	0.006276 (1)	HNT	11	intron
rs2034954	A	36	68	0.001559 (1)	0.004317 (1)		12	
rs954108	T	13	28	0.01778 (1)	0.006656 (1)		13	
rs1462256	A	50	79	0.01034 (1)	0.009023 (1)		14	
rs725463	T	65	40	0.01423 (1)	0.00625 (1)		15	
rs1984372	A	58	36	0.02263 (1)	0.008914 (1)		18	
rs1943919	T	37	65	0.005266 (1)	0.008719 (1)		18	
rs2012035	C	30	56	0.004717 (1)	0.009644 (1)		19	
rs542419	T	59	38	0.0323 (1)	0.007665 (1)		19	
rs1477340	T	75	43	0.003033 (1)	0.001527 (1)	NPAS1	19	intron
rs714022	A	50	29	0.01746 (1)	0.001986 (1)	ATXN10	22	intron
rs737822	A	49	26	0.00742 (1)	0.001984 (1)		22	
rs728591	A	42	70	0.007818 (1)	0.004015 (1)		22	

* Number of alleles transmitted to the affected children. ** Number of alleles not transmitted to the affected children. Bonferroni correction was applied for multiple testing

ads, containing two parents and an affected offspring, and PDT is a test for linkage disequilibrium that uses all of the informative data in pedigrees. Table 2 shows 35 SNPs with PDT P values less than 0.01 (uncorrected). However, none of the SNPs reached to a significant association with ATOD after Bonferroni correction. Among 35SNPs showing PDT P value s less than 0.01, 19 SNPs were located in the intergenic region, and most of the SNPs located in intragenic region are intronic SNPs (15 SNPs). We genotyped additional tag SNPs in SORCS3 because two SNPs were associated with ATOD in PDT analysis. Several SNPs were shown to be associated with ATOD (Table 3). One (rs7895087) of the SNPs reached to a significant association with ATOD even after Bonferroni correction, though the number of testing is difficult to determine for this tag SNP association analysis.

Discussion

We performed a genome-wide linkage study using 77 Japanese ATOD-affected families comprising 111 affected sib-pairs (287 individuals) and found 2 candidate linkage regions, on chromosomes 15q21 and 1q24.

This is the first genome-wide linkage study of ATOD in an Asian population, and we did not find much overlap with previously identified linkage regions in Caucasians (Table 4) [9-12]. There are a number of possible reasons for conflicting results in linkage analysis, including differences in ethnic backgrounds, diagnostic criteria, and analytical methods. In the present study, ATOD was diagnosed by dermatologic specialists and followed the criteria of Hanifin and Rajka [14], that was used in previous studies [9-12].

Table 3: Results of TDT and PDT by genotypig tag SNPs in SORCS3

rs number	allele	Trans*	Not trans**	TDT P value (corrected)	PDT P value (corrected)
rs768731	C	47	35	0.1843 (0.98)	0.2878 (0.99)
rs790640	C	21	37	0.03446 (0.50)	0.05183 (0.65)
rs790726	G	47	31	0.06905 (0.76)	0.2346 (0.99)
rs791123	G	71	50	0.05563 (0.68)	0.1291 (0.93)
rs971527	G	44	48	0.6766 (1)	0.5258 (1)
rs1472050	T	42	43	0.9136 (1)	0.9178 (1)
rs1490173	G	60	47	0.2083 (0.99)	0.2522 (0.99)
rs1565415	G	50	50	1 (1)	0.9164 (1)
rs1953071	C	52	45	0.4771 (1)	0.3538 (0.99)
rs2491388	A	38	34	0.6373 (1)	0.5675 (1)
rs3011669	A	35	49	0.1257 (0.93)	0.2793 (0.99)
rs4532962	T	51	55	0.6976 (1)	0.6633 (1)
rs7084834	C	19	27	0.237 (0.99)	0.1829 (0.98)
rs7096635	T	53	37	0.09083 (0.85)	0.05368 (0.66)
rs7895087	G	67	29	8.42E-05 (0.0017)	0.0003507 (0.007)
rs9943297	G	44	60	0.1159 (0.91)	0.2017 (0.98)
rs10509784	C	16	39	0.001635 (0.03)	0.007646 (0.14)
rs10509785	C	40	48	0.3934 (1)	0.4579 (1)
rs10884049	G	62	45	0.09957 (0.88)	0.1014 (0.88)
rs11192320	G	41	18	0.002409 (0.047)	0.007646 (0.14)

* Number of alleles transmitted to the affected children. ** Number of alleles not transmitted to the affected children

Examining SNPs, instead of microsatellite markers for linkage is unlikely to yield different results because it has been reported that SNP-based genome-wide linkage study has the potential to be as powerful as traditional microsatellite-based analysis and offers good identification of specific locations for further fine-mapping association analysis [13]. We performed a genome-wide linkage study with 5861 SNP markers, although previously performed

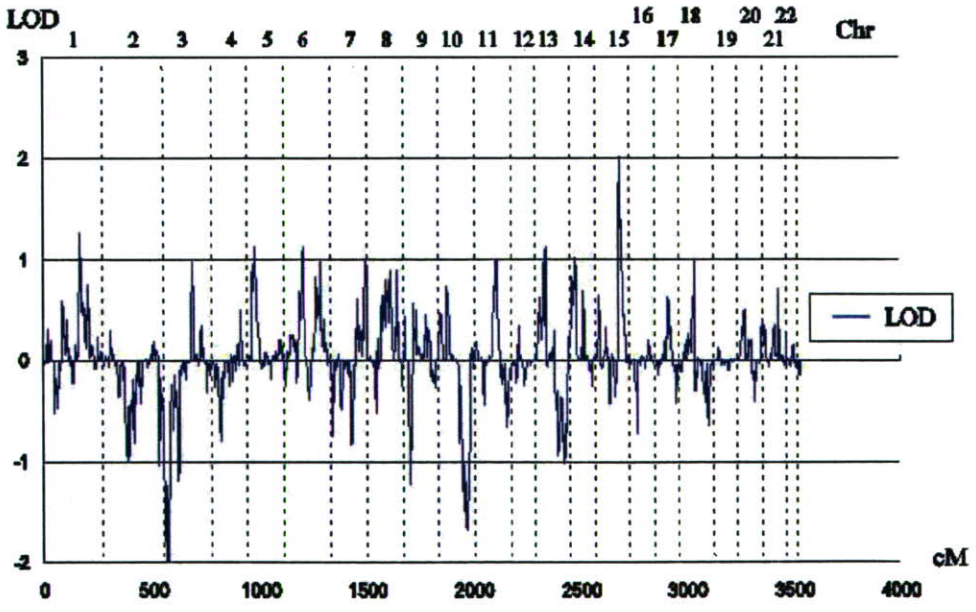

Figure 1
Multipoint nonparametric LOD score of genome-wide scan for ATOD in the Japanese.

Table 4: Whole genome linkage studies for ATOD

Year	Authors	Population	Genotyping	No. of markers	No. of families No. of ASP*	Phenotype	Linkage regions
2007	Present study	Japanese	SNPs	5861	77	ATOD	1q24
					111		15q21
2004	Haagerup	Danish	Microsatellite	446	23	ATOD and specific IgE	3q26-3q24
					N/A		4q15-4q14
							18q11-18q12
2002	Bradley	Swedish	Microsatellite	367	109	ATOD	3p24-22
					206	ATOD and specific IgE	18q21
						ATOD and severity score	3q14
							13a14
							15q14
							17q21
2001	Cookson	British	Microsatellite	385	148	ATOD	1q21
					213		17q25
						ATOD and asthma	20p
2000	Lee	European	Microsatellite	380	199	ATOD	3q21
					N/A		

*ASP; affected sib-pairs

ATOD genoeme-wide scans have been done with smaller numbers of microsatellite markers. SNPs are distributed more abundantly and uniformly along the human genome than are microsatellite markers are more reliably typed, and require a smaller sample of DNA. Genome-wide linkage mapping of genes with fixed SNP panels, such as our Golden Gate assay, is a cost-effective and time-saving technology [24]. Several recent studies have found that SNP panels provide higher data quality, more accurate genotyping results and higher information content, and they may also have higher power to detect linkage than do traditionally used panels of microsatellite markers [25,26]. Because LD between closely spaced SNPs can falsely inflate linkage statistics, we remove LD from the marker sets in an automated fashion.

The 15q21 linkage region has not been reported previously as a region associated with ATOD. However, linkage of 15q21 to several other inflammatory diseases including osteoarthritis [8] and macular degeneration [27], has been reported. The 15q21 region contains candidate genes for ATOD such as Mothers against decapentaplegic homolog of 3 (SMAD3). SMAD proteins are involved in biologic responses to TGF-beta and related ligands. Smad3-knockout mice show accelerated cutaneous wound healing with complete reepithelialization, and Smad3-deficient keratinocytes show altered patterns of growth and migration [28].

The 1q24 linkage region includes candidate genes such as T-cell receptor zeta chain isoform 2 precursor (*CD3Z*) and chemokine ligand 2 (*XCL2*). CD3Z plays an important role to recognize the coupling antigen to several intracellur signal-transduction pathways [29]. Antigen recognition is one of the most important events in the pathology of ATOD, especially in the memory T cells that encounter their specific antigen, generating an allergen response, which then activates leukocytes leading to production of several cytokines and atopic skin inflammation [3]. Chemokines have fundamental roles in regulation of several types of T cells, development, homeostasis, and function of the immune systems, especially in leukocyte trafficking. During the multistep process of leukocyte trafficking, chemokine ligand-receptor interactions mediate the firm adhesion of leukocytes to the endothelium and initiate transendothelial migration from the blood vessel into perivascular pockets [30]. From perivascular spaces, matrix-bound sustained chemokine gradients direct skin-infiltrating leukocyte subsets to subepidermal or intraepidermal locations. In ATOD regions, that caused by chemokines recruit pathogenic leukocytes to skin in response to mechanical injury such as scratching [31].

Our linkage region on chromosome 1 was located near 1q21, which was previously reported as a linkage region in a British population [10]. It was reported that the skin barrier is impaired in patients with ATOD [32], and recent studies showed that loss-of-function mutations in *FLG* on 1q21 were associated with ATOD in 2 independent populations [8]. FLG is involved in aggregation of the keratin cytoskeleton, which causes collapse of granular cells into flattened anuclear squames. The condensed cytoskeleton is crosslinked by transglutaminases during formation of the cornified cell envelope, the outermost barrier layer of

the skin [33], which prevents water loss and impedes the entry of allergens and infectious agents. 1q21, which has been linked to both ATOD and psoriasis [10], houses a cluster of genes known as the epidermal differentiation complex that encode proteins involved in keratinocyte terminal differentiation [34]. Several genes in this region have been reported to be associated with skin diseases such as psoriasis [35]. Because it is possible that the true disease susceptibility gene is located further away from the actual linkage peak, the 1q21 region may include one or more ATOD susceptibility genes for our Japanese population.

Several candidate genes for ATOD were identified by PDT analysis (Table 3). CD200 and its receptor CD200R are both type I membrane glycoproteins that contain two immunoglobulin-like domains. CD200-CD200R interaction has been shown to be important for regulation of the macrophage lineage. In CD200-deficient mice, there were increased numbers of macrophages in the spleen and the mesenteric lymph nodes, and these macrophages show increased activation [36]. In chronic lichenified lesions of ATOD skin, there is an increased number of Langerhans' cells in the epidermis, and macrophages dominate the dermal mononuclear cell infiltrate, and macrophages are important source of cytokines that cause inflammation of the skin [37]. Another candidate is laminin alpha 4 chain (LAMA4). Laminins are a large family of heterotrimeric extracellular matrix glycoproteins in the basement membrane that promote cell adhesion, migration, differentiation, proliferation, and angiogenesis. Lama4-deficient mice showed deterioration of microvessel growth [38], and LAMA4 are located in the basement membrane zone of capillary vessels and in an area adjacent to fibroblast-like cells [39]. SORCS3 is one of the VSP10 domain-containing receptor, that shares the greatest homology with SORCS1. The function of SORCS3 remains unclear, but several SNPs in *SORCS3* showed association with ATOD by PDT analysis (Tables 2 and 3). Although not in the linkage region, the results of family-based association study suggest that these genes may be associated with the pathogenesis of ATOD.

In conclusion, we performed the first genome-wide linkage study for ATOD in an Asian population, and identified 2 linkage regions, one on 15q21 and one on 1q24. A recent review suggested that there was no substantial overlap between the genetic architecture of ATOD and that of other atopic diseases, such as asthma, but there is a greater degree of similarity between ATOD and psoriasis [7]. Our linkage region on 15q21 overlaps with regions linked to other inflammatory diseases, suggesting that common inflammatory genes may be located in this region. The results of our genome-wide linkage study may lead to identification of novel genes for ATOD, which would improve our understanding of the pathogenesis of ATOD.

Conclusion

We report the first genome-wide linkage analysis for ATOD in an Asian population and identified novel loci on chromosomes 15q21 and 1q24 linked to ATOD. The results of our genome-wide linkage study may lead to identification of novel genes for ATOD, which would improve our understanding of the pathogenesis of ATOD.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

HE carried out molecular genetic study, participated in the study design and coordination and wrote the draft of the manuscript. SI, TT, KH, MI, TK, TA, YS, MK, MT, TS, and FO carried out molecular genetic studies. EN and TA participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

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An association study of asthma and related phenotypes with polymorphisms in negative regulator molecules of the TLR signaling pathway

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Abstract Although associations between endotoxin exposure or respiratory infection and asthma have been recognized, the genetic effects in these conditions are unclear. Toll-like receptors (TLRs) play an essential role in innate host defense and in the control of adaptive immune responses. IL-1R-associated kinase-M (IRAK-M) and single immunoglobulin IL-1R-related molecule (SIGIRR) negatively regulate TLR-signaling pathways. To investigate whether polymorphisms in these genes were associated with asthma or asthma-related phenotypes, we screened these genes for polymorphisms by direct sequencing of 24 asthmatics and identified 19 variants in *IRAK-M* and 12 variants in *SIGIRR*. We next conducted linkage disequilibrium mapping of the genes, and examined the association of polymorphisms and haplotypes using 391 child patients with asthma, 462 adult patients with asthma, and 639 controls. None of the alleles or haplotypes of *IRAK-M* and *SIGIRR* were associated with asthma susceptibility or asthma-related phenotype. Our results indicate that polymorphisms in *IRAK-M* and *SIGIRR* are not likely to be associated with the development of asthma in the Japanese population.

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Introduction

Toll-like receptors (TLRs) are pattern-recognition receptors (PRRs) that play an essential role in activation of the innate immune system, which in turn activates adaptive immunity (Medzhitov 2001; Akira and Takeda 2004). The role of TLR proteins in asthma has been intensively studied (Basu and Fenton 2004).

TLR2 and TLR4 ligands activate IL-1R-associated kinase (IRAKs) and TNF receptor-associated factor (TRAF) 6 and induce expression of inflammatory cytokines, and IRAK-M prevents the dissociation of the IRAK1–IRAK4 complex from myeloid differentiation primary response gene (MyD) 88, thereby inhibiting the TLR signaling pathway (Kobayashi et al. 2002; Janssens and Beyaert 2003). IRAK-M regulates TLR signaling and innate immune homeostasis, because IRAK-M-deficient macrophages produce enhanced amounts of inflammatory cytokines on TLR stimulation and bacterial challenge, and increased inflammatory responses to bacterial infection are observed in IRAK-M-deficient mice (Kobayashi et al. 2002). Reduced endotoxin tolerance in IRAK-M-deficient cells has also been reported (Kobayashi et al. 2002). Furthermore, *IRAK-M* is located in 12q14.2, one of the most consistently replicated regions linked to asthma in diverse populations, wherein Yokouchi et al. (2000) mapped a locus linked to mite-sensitive atopic asthma susceptibility in the Japanese population by sib-pair analysis.

Single immunoglobulin IL-1R-related (SIGIRR) molecules, membrane-bound molecules that contain a TIR domain, have recently been shown to be involved in the negative regulation of TLR signaling (Wald et al. 2003). After TLR stimulation, SIGIRR interact transiently with TLR4, TRAK1, and TRAF6. SIGIRR are highly expressed in many epithelial cell lines, but not expressed in primary macrophages, fibroblasts, and endothelial cells (Wald et al. 2003). The high expression of SIGIRR in epithelial cells indicates they may regulate the immune response in cells that are continually exposed to microorganisms, for example lung epithelial cells. Furthermore, SIGIRR-deficient mice were found to be highly sensitive to LPS-induced endotoxic shock (Wald et al. 2003). SIGIRR therefore act as an inhibitory factor in TLR signaling, which may be essential for regulating the detrimental effects of innate immunity, as occurs in chronic inflammation.

To investigate the relationship between the genetic variants of these three genes and asthma or asthma-related phenotypes, we searched the genes for isolated polymorphisms, performed linkage disequilibrium (LD) mapping, and conducted a genetic association study with regard to the LD pattern.

Materials and methods

Subjects

All patients with asthma were diagnosed according to the criteria of the National Institutes of Health (National Heart, Lung, and Blood Institute, National Institutes of Health 1991). Improvement in their FEV_1 measurement was at least 12% in childhood asthma and 20% in adult asthma after β_2-agonist inhalation (National Heart, Lung, and Blood Institute 1991; Hasegawa et al. 2004; Kamada et al. 2004). Diagnosis of atopic asthma was based on one or more positive skin-scratch-test responses to a range of seven common aeroallergens in the presence of a positive histamine control and a negative vehicle control. The seven aeroallergens were house dust, *Felis domesticus* dander (Feld), *Canis familiaris* dander, *Dactylis glomerata*, Ambrosia, *Cryptomeria japonica*, and *Alternaria alternata*. We recruited 391 children with asthma (mean age 9.3, 4–15 years; male:female ratio = 1.43:1.0; mite RAST positive 81.6%; atopic asthma 92%) and 462 adults with asthma (mean age 50.1, 20–75 years; male:female ratio = 1.0:1.35; atopic asthma 91.2%). For children with asthma, we recorded their age, sex, mite-specific IgE positive status, serum total IgE level, eosinophil count, clinical severity, and incidence of atopic dermatitis. Specific IgE was considered positive when values exceeded 0.35 U mL^{-1} (RAST score ≥1). The severity of childhood asthma was defined according to the amount of therapy required to control symptoms at the time of entry into the study. The grades were: grade 1, β stimulants only; grade 2, sodium cromoglycate and/or theophylline; grade 3, inhaled beclomethasone, 400 μg day^{-1} or less; grade 4, inhaled beclomethasone of more than 400 μg day^{-1}. All subjects with atopic dermatitis were diagnosed by dermatology specialists. For adults with asthma, we recorded their age, sex, serum total IgE level, eosinophil count, and clinical severity. The severity of adult asthma was classified according to the system of the National Heart, Lung, and Blood Institute (1997). The serum IgE levels was $\log_{10}$-transformed before analyses. The means of $\log_{10}$[total IgE (tIgE) (IU mL^{-1})] of patients were 2.63 [$=\log_{10}$(426.6 IU mL^{-1})] in childhood asthma and 2.34 [$=\log_{10}$(218.8 IU mL^{-1})] in adult asthma. In this study, "high IgE" and "high eosinophil count" levels were defined as those values in the 75th percentile or higher for total IgE and eosinophil count (%). The 75th percentile values of $\log_{10}$(tIgE) in patients were 3.06 [$=\log_{10}$(1,148 IU mL^{-1})] in childhood asthma and 2.71 [$=\log_{10}$(512.9 IU mL^{-1})] in adult asthma. The 75th percentile values of eosinophils in patients were 9.9 (%) in childhood asthma and 8.0 (%) in adult asthma. A total of 639 healthy

Table 1 Primers for the SNPs survey in this study

IRAK-M	F1	GCA GGC CTT TCT GAT TGC TT	R1	CAG AAA AGA CAC CAA ATC AGC
	F2	AGA AGT AAT GAC ACC GCT AG	R2	TAC ATT GCG AAC CCA GTG AG
	F3	CAG AAG GCA GGT GAA TAT ATT C	R3	AGA CAA AGG GAA GAA TTA GGC
	F4	CAA CTA CTT ATG TTT TAA GTG AAC	R4	CAG TGC AAC AGA GTG CAA CC
	F5	ACT TTG ACT GAC TAT GAC ATT G	R5	TCA GAT CTA GTG GCA AAG ACT
	F6	GTA GAG CAA TGC TGA AGG TC	R6	GCT AAG AAG GAA CAT CAC CAT
	F7	CTC TGT GGA ATG GTG GGA AC	R7	TGA CCC TCT TTA ACA AAG TCC
	F8	CTA GCT GTC ATG GGA TTG TC	R8	GAC TCT CAG ACT CAG GAG TG
	F9	TGG AAA GCA AAT CTG TGT CTG	R9	CTG TGT CAC GCT ATG GTG A
	F10	AGC AGA AGG AAA CCC ATC TG	R10	CAC CTA ACC TAC CGA ACA TC
	F11	AGA ATG TTC TCC AGT TCA TGG	R11	AGA GAT AGG TGC CAG GA TGA
	F12	GAC TCA TTG ATT TCC TGT TAG C	R12	AAT ATT CCC TGC AAA CTG CTC
	F13	AAA TAA AGG GCG TTA GCT AAT C	R13	CTC TTG GCA TTG CTT ATG GAG
	F14	TAT ATA GTT CCA TCC CAG GAC	R14	CTT GAC CAG CCA TTT TCT CAG
SIGIRR	F1	TAA TCT CTC GGA TCT CAG GC	R1	TGA GGC CTT ACT CGA CAG TA
	F2	TCA TTG CCA ATG GGA TGG TC	R2	TCA GGA GTT CAG AGG GCA TT
	F3	TCT TCC ACA CCA AGG ACT TC	R3	TCA CCC AGA GTT CAA GTC AG
	F4	CAG GAA TCC CCT GTA TGT TC	R4	ATC TCT TCC CTT TCC TCC AG
	F5	TCC AGT TTT CCA TGG GCT TC	R5	TTC GCC CAC TTT CCT CCC TT
	F6	AGG TGA TCC TGG ACT TGA TG	R6	TTA CAT CAG GGT GAT GAG CC
	F7	GCT CAT GAG GGT CAG TAA AG	R7	GAA GAG AGA GGA CAC AGT GG
	F8	TGG ACA GAC ATG GTG TGA CT	R8	AAG CCA AGA GAA GTG ACC TG
	F9	TCT GAA TGA ACA CCG ACC AG	R9	TAA CCA TCT CCC ACG TGC AC
	F10	ATG GGG AGG TGG AGA TAA AC	R10	AGG TGA TGA AGA TGG GTC TG
	F11	TCA TCG TGG TGC TTT CGG AC	R11	AAG AGT CCT CAA CAC CTG GA
	F12	ACA AGG ACC CCA TGC TGA TT	R12	AAG CCG AAT CCG AAA CCT TC

individuals who had neither respiratory symptoms nor a history of asthma-related diseases (mean age 43.5, 20–75 years; male:female ratio = 2.67:1.0) were recruited by physicians' interviews about whether they had been diagnosed with asthma and/or atopy. Genomic DNAs were prepared in accordance with standard procedures. All individuals were Japanese and gave written informed consent to participate in the study in accord with the rules of the process committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN).

Genotyping

To identify SNPs in the human *IRAK-M* and *SIGIRR* genes, we sequenced all exons, including a minimum of 200 bases of the flanking intronic sequence, 2 kb of the 5′ flanking region, and a 2 kb continuous 3′ flanking region of the last exon except for regions of interspersed repeats from 24 asthmatic subjects (12 unrelated children and 12 adults). Primer sets were designed on the basis of genomic sequences from the GenBank database (Table 1). The sequences were analyzed and polymorphisms identified using SEQUENCHER software (Gene Codes Corporation, Ann Arbor, MI, USA). Genotyping of polymorphisms was performed by using the Invader assay or the TaqMan allele-specific amplification (TaqMan-ASA) method or PCR restriction fragment length polymorphism (PCR-RFLP) analysis as described (Hasegawa et al. 2004; Kamada et al. 2004). For the −1464A > G, 21927A > T, 22149G > A, 48837A > G, and 54406C > T polymorphisms in *IRAK-M*, genotyping was performed by the Invader method (Ohnishi et al. 2001). For the −1195C > T and 39384A > del polymorphisms in *IRAK-M* and the −10137C > T, −8778C > T, and 1523T > G polymorphisms in *SIGIRR*, genotyping was performed by the TaqMan method.

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy–Weinberg equilibrium using a χ^2 goodness-of-fit test at each locus. To test the association between each gene and childhood or adult asthma, we compared differences in allele frequency and genotype distribution of each polymorphism between case and control subjects by using a contingency chi-square test with one degree of freedom (DF). Odds ratios (ORs) with 95 percent confidence intervals (95% CI) were also calculated.

In the association study between a single SNP and an asthma-related phenotype, we performed many statistical tests; therefore, inflation of the false-positive results (type-1 error) is a concern. In this study, we consider those results to be hypothesis-generating, and only results with P values of less than 0.01 are shown here to minimize type-1 errors.

Pairwise LD was calculated as $|D'|$ and r^2 by using the SNP Alyze statistical package (Dynacom, Chiba, Japan) as described by Nakajima et al. (2002). Haplotype frequencies for multiple loci were estimated using the expectation-maximization method with SNP Alyze software (Nakajima et al. 2002). Those frequencies in cases and controls were evaluated both by the whole

Table 2 Polymorphisms in the *IRAK-M* and *SIGIRR* genes

Name	SNP[a]	Location	Nucleotide	Position[b]	Amino acid	Minor allele frequency (%)	JSNP ID IMS-JST	NCBI dbSNP
IRAK-M	SNP 1	5′ genome	T > C	−1494		14	168748	rs1732888
	SNP 2[a]	5′ genome	A > G	−1464		14	168749	rs1732887
	SNP 3[a]	5′ genome	C > T	−1195		30	168750	rs2701653
	SNP 4	Intron 1	T > A	14340		27		rs1185630
	SNP 5	Intron 2	C > T	14622		22		rs1882200
	SNP 6	Intron 2	C > T	14785		2		
	SNP 7	Intron 2	T > C	15608		19	040604	rs2289134
	SNP 8	Intron 3	C > T	20320		20		rs11465955
	SNP 9	Intron 3	T > A	20780		2		
	SNP 10	Intron 4	G > A	21076		2		
	SNP 11	Intron 4	A > del	21141		27		rs3830660
	SNP 12[a]	Intron 4	A > T	21927		21	046869	rs2293657
	SNP 13	Intron 4	G > C	22000		2		
	SNP 14[a]	Exon 5	G > A	22149	V147I	25	046868	rs1152888
	SNP 15	Intron 8	T > C	39263		2		
	SNP 16[a]	Intron 8	A > del	39384		48		rs10716217
	SNP 17[a]	Intron 8	A > G	48837		10	138735	rs3782347
	SNP 18[a]	Intron 8	C > T	54406		41	138737	rs3782348
	SNP 19	Intron 9	A > G	55583		2		
SIGIRR	SNP 1[a]	5′ genome	C > T	−10137		10		
	SNP 2[a]	5′ genome	C > T	−8778		27		
	SNP 3	Intron 1	G > A	−259		4		rs11246149
	SNP 4	Intron 2	T > C	60		29		rs4074794
	SNP 5	Intron 2	T > C	283		29		rs4076104
	SNP 6	Intron 2	C > T	785		2		
	SNP 7	Intron 3	G > A	1320		2		
	SNP 8[a]	Intron 3	T > G	1523		29		rs7396562
	SNP 9	Exon 5	C > G	1921	P115R	2		
	SNP 10	Exon 8	C > T	2921	P256P	2		
	SNP 11	Intron 8	C > G	3195		2		
	SNP 12	Intron 8	A > G	3260		29		rs10902159

Position 1 is the A of the initiation codon
[a]SNPs were genotyped in this study
[b]Numbering according to the genomic sequence of *IRAK-M* (NT_029419.10) and *SIGIRR* (AC138230.5)

distribution with Fisher's exact test and by χ^2 tests of one haplotype against others (haplotype-wise test).

Results

Polymorphisms in the *IRAK-M* and *SIGIRR* genes

We performed screening of polymorphisms with genomic DNA from 24 randomly selected asthmatic individuals. After extensive examination of *IRAK-M* and *SIGIRR* by direct sequencing, we identified 19 polymorphisms in *IRAK-M* and 12 SNPs *in SIGIRR* (Table 2). Eighteen polymorphisms were contained in the two available public databases; NCBI dbSNP (http://www.ncbi.nlm nih.gov/SNP/) and IMS-JST JSNP DATABASE (http://www.snp.ims.u-tokyo.ac.jp/). Non-synonymous substitutions were located in *IRAK-M* (Val147Ile) and *SIGIRR* (Pro115Arg). IRAK molecules consist of two major functional domains, death domain and kinase domain. SNP14 V147I did not locate in these functional domains. SIGIRR contains the immunoglobulin (Ig) domain and Toll and interleukin-1 receptor (TIR) domain, and the SNP9 P115R located in the Ig domain. To examine the LD between identified SNPs, pairwise LD coefficients D' and r^2 were calculated using the SNP Alyze program. Because most of the SNPs were quite rare, pairwise LD was measured by $|D'|$?and r^2 among the SNPs with a frequency of greater than 5%. Results for the molecules are shown in Tables 3 and 4. The LD pattern in four different ethnic populations is available on the website http://www.hapmap.org. The LD pattern using HapMap data of *IRAK-M* SNPs identified in this study is shown in a table in the supplementary material. The LD pattern in the Japanese was significantly different from that in the Yoruba, and almost the same as that in the Chinese. In the *SIGIRR* gene, SNPs identified in this study are not contained in HapMap database. In *IRAK-M* gene, SNP1 was in complete LD ($D' = 1.00$ and $r^2 = 1.00$) with SNP2. SNP12 was in complete LD with SNP4 and SNP5, and was in strong LD ($D' = 1.00$ and $r^2 = 0.87$) with SNP7 and SNP8. In the *SIGIRR* gene, SNP8 was in complete LD with SNP4, SNP5, and SNP11. We finally selected ten polymorphisms for association studies. In addition, we searched the putative transcription factor binding site using TFSEARCH (http://www.mbs.cbrc.jp/research/db/TFSEARCH.html) (Heinemeyer et al. 1998). We found that SNP3 AAACAA(C > T) in the *IRAK-M* gene contains putative SRY binding site with higher probability (96.4 vs. 90.0%, respectively).

Table 3 Pairwise linkage disequilibrium for all possible two-way comparisons among 13 SNPs in *IRAK-M*

	SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	SNP 7	SNP 8	SNP 11	SNP 12	SNP 14	SNP 16	SNP 17	SNP 18
SNP 1	D'	1.00	1.00	1.00	0.74	0.04	0.47	1.00	0.74	1.00	1.00	0.02	1.00
	r^2	1.00	0.07	0.07	0.02	0.00	0.01	0.06	0.02	0.06	0.14	0.00	0.09
SNP 2		D'	1.00	1.00	0.74	0.04	0.47	1.00	0.74	1.00	1.00	0.02	1.00
		r^2	0.07	0.07	0.02	0.00	0.01	0.06	0.02	0.06	0.14	0.00	0.09
SNP 3			D'	0.87	1.00	1.00	1.00	0.87	1.00	0.87	0.64	1.00	0.54
			r^2	0.68	0.11	0.11	0.10	0.60	0.11	0.61	0.20	0.05	0.20
SNP 4				D'	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.48
				r^2	0.08	0.08	0.10	1.00	0.08	1.00	0.45	0.03	0.15
SNP 5					D'	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.15
					r^2	0.86	0.86	0.11	1.00	0.10	0.30	0.39	0.01
SNP 7						D'	1.00	1.00	1.00	1.00	1.00	1.00	0.10
						r^2	0.71	0.11	0.86	0.09	0.24	0.45	0.00
SNP 8							D'	1.00	1.00	1.00	1.00	1.00	0.23
							r^2	0.09	0.87	0.09	0.29	0.32	0.02
SNP 11								D'	1.00	1.00	1.00	1.00	0.47
								r^2	0.10	1.00	0.38	0.05	0.13
SNP 12									D'	1.00	1.00	1.00	0.14
									r^2	0.09	0.29	0.39	0.01
SNP 14										D'	1.00	1.00	0.45
										r^2	0.36	0.04	0.11
SNP 16											D'	1.00	0.57
											r^2	0.11	0.23
SNP 17												D'	1.00
												r^2	0.07

Association of each SNP with asthma and asthma related-phenotypes

Ten SNPs were genotyped in 391 patients with childhood asthma, 462 patients with adult asthma, and 639 controls. All genotype results of the SNPs in the control samples were in Hardy–Weinberg equilibrium. The results of allele frequencies in the asthmatic and control groups are shown in Table 5. None of the SNPs tested in this study had significant association with adult or childhood asthma.

In addition, we surveyed associations between SNPs of those two genes and asthmatic patients with a high eosinophil count, a high serum IgE level, disease severity, atopic asthma, and child asthmatic patients with atopic dermatitis. There was no association between any SNP of *IRAK-M* and *SIGIRR* genes and asthma-related phenotype.

Power in this study was estimated with the aid of SamplePower 2.0 (SPSS, Chicago, IL, USA). If ORs of risk alleles with control group frequencies of 0.05, 0.1, 0.2, and 0.4 were more than 1.86, 1.60, 1.44, and 1.37, respectively, power exceeded 80% (at $P=0.01$) in allelic association tests of childhood asthma (639 controls and 391 patients). Similarly, in allelic association tests in adult asthma (639 controls and 462 patients), power of 80% was assured if alleles with frequencies of 0.05, 0.1, 0.2, and 0.4 had ORs of more than 1.81, 1.56, 1.42, and 1.35, respectively.

Association between haplotypes of the *IRAK-M* and *SIGIRR* genes and asthma

We next constructed the haplotypes of those three genes and estimated the frequency of each haplotype in the control, childhood asthma, and adult asthma groups (Table 6). The frequency pattern of the haplotypes of *IRAK-M* and *SIGIRR* did not differ between the control and asthma groups.

Table 4 Pairwise linkage disequilibrium for all possible two-way comparisons among six SNPs in *SIGIRR*

	SNP 1	SNP 2	SNP 4	SNP5	SNP 8	SNP 12
SNP 1	D'	1.00	1.00	1.00	1.00	1.00
	r^2	0.04	0.05	0.05	0.05	0.05
SNP 2		D'	0.77	0.77	0.77	0.77
		r^2	0.53	0.53	0.53	0.53
SNP 4			D'	1.00	1.00	1.00
			r^2	1.00	1.00	1.00
SNP 5				D'	1.00	1.00
				r^2	1.00	1.00
SNP 8					D'	1.00
					r^2	1.00

Table 5 Genotype frequencies for *IRAK-M* and *SIGIRR* SNPs and asthma susceptibility

Gene	SNP location	Control ($n=639$)			Child BA ($n=391$)			Adult BA ($n=462$)			Child BA			Adult BA		
		1	2	3	1	2	3	1	2	3	P^a	P^b	P^c	P^a	P^b	P^c
IRAK-M	SNP 2	0.81	0.18	0.01	0.82	0.17	0.01	0.80	0.19	0.01	NS	NS	NS	NS	NS	NS
	SNP 3	0.33	0.51	0.16	0.30	0.55	0.15	0.30	0.50	0.20	NS	NS	NS	NS	NS	NS
	SNP 12	0.76	0.22	0.02	0.72	0.26	0.02	0.78	0.21	0.01	NS	NS	NS	NS	NS	NS
	SNP 14	0.35	0.50	0.15	0.33	0.52	0.14	0.33	0.48	0.19	NS	NS	NS	NS	NS	NS
	SNP 16	0.30	0.48	0.21	0.31	0.49	0.20	0.32	0.46	0.22	NS	NS	NS	NS	NS	NS
	SNP 17	0.92	0.07	0.00	0.93	0.07	0.00	0.93	0.07	0.00	NS	NS	NS	NS	NS	NS
	SNP 18	0.38	0.46	0.16	0.35	0.51	0.15	0.36	0.48	0.16	NS	NS	NS	NS	NS	NS
SIGIRR	SNP 1	0.86	0.13	0.01	0.86	0.13	0.01	0.85	0.14	0.00	NS	NS	NS	NS	NS	NS
	SNP 2	0.61	0.35	0.05	0.59	0.35	0.05	0.60	0.34	0.05	NS	NS	NS	NS	NS	NS
	SNP 8	0.44	0.44	0.12	0.45	0.44	0.11	0.44	0.45	0.11	NS	NS	NS	NS	NS	NS

NS Not significant
[a]Dominant model
[b]Recessive model
[c]Allele frequency

Discussion

Recent studies have shown that the immune response induced by an endotoxin could play an important role in the initiation or prevention of asthma (Braun-Fahrlander et al. 2002; Gereda et al. 2000; Gehring et al. 2002). Immunization with an antigen in the context of TLR2 ligands can result in experimental asthma (Redecke et al. 2004) and genetic variation in *TLR2* is a major factor in the susceptibility to asthma of children of farmers (Eder et al. 2004). Although no association was observed between *TLR4* polymorphism and the risk of asthma (Raby et al. 2002), several reports have shown that *TLR4* gene variants modify endotoxin effects on asthma and relate to the severity of asthma (Yang et al. 2004). Given these studies, the TLR signaling pathway seems to be a possible inducer of the immune deviation that affects asthma susceptibility. We identified polymorphisms in *IRAK-M* and *SIGIRR*, and performed case-control and case-only association studies and haplotype analyses using clinically characterized asthma patients. In this study, no significant association between the tested SNPs in *IRAK-M* or *SIGIRR* and asthma or any asthma-related phenotype was found. In this study, if the allelic OR was more than 1.86 with a risk allele frequency of 0.05 in the control group, power exceeded 80% in association tests of childhood asthma. Similarly, power of 80% was assured in association tests of adult asthma if the allelic OR was greater than 1.81 with a control group risk allele frequency of 0.05. It is possible that rare variants are associated with the development of asthma. We screened a minimum of 200 bases of the flanking intronic sequence, 2 kb of the 5′ flanking region, and a 2 kb continuous 3′ flanking region to the last exon, although other SNPs in unsequenced regions might be associated with asthma or its related phenotypes. On the other hand, a gene–environment interaction might affect these results. Recent studies have shown that exposure to germs early in life

Table 6 Haplotype frequencies of polymorphisms of the *IRAK-M* and *SIGIRR* genes

	Haplotype no.	SNP position							Child BA ($n=391$)	Adult BA ($n=462$)	Controls ($n=639$)
IRAK-M		SNP 1	SNP 3	SNP 12	SNP 14	SNP 16	SNP 17	SNP 18			
	1	A	C	A	G	del	A	C	0.31	0.30	0.32
	2	A	T	A	A	A	A	T	0.30	0.30	0.30
	3	G	C	A	G	del	A	C	0.07	0.09	0.09
	4	A	T	A	A	A	A	C	0.09	0.10	0.08
	5	A	C	T	G	A	A	T	0.06	0.05	0.06
	6	A	C	T	G	A	G	C	0.03	0.03	0.04
	7	A	C	T	G	A	A	C	0.05	0.03	0.03
	8	A	T	A	G	del	A	C	0.03	0.03	0.02
	9	A	C	A	G	del	A	T	0.02	0.02	0.01
	Others								0.04	0.05	0.04
SIGIRR	Haplotype no.	SNP 1	SNP 2	SNP 8							
	1	C	C	T					0.59	0.58	0.57
	2	C	T	G					0.17	0.17	0.17
	3	C	C	G					0.12	0.13	0.13
	4	C	T	T					0.05	0.04	0.05
	5	T	C	T					0.04	0.03	0.04
	Others								0.03	0.04	0.03

may facilitate the development of an immune system that is appropriately balanced with respect to Th1 and Th2 cells (Braun-Fahrlander et al. 2002; Gereda et al. 2000; Gehring et al. 2002). TLRs contact the environment, and play a crucial role in host defense against infection (Medzhitov 2001; Akira and Takeda 2004). Subjects carrying wild-type *TLR4* genotypes have an increased risk of asthma with greater endotoxin exposure but there is no such effect in subjects with variant genotypes (Werner et al. 2003). Eder et al. (2004) showed that the *TLR2* gene is a major factor in the susceptibility of children of European farmers to asthma. We recruited subjects from the Osaka area, an urban area in Japan. A genetic variation in *IRAK-M* and *SIGIRR* might be one determinant of susceptibility to asthma in a farming environment. In addition, epistatic interactions may affect the results.

Innate immunity plays a major role in host defense during the early stages of infection, and differences in population history have produced unique patterns of SNP allele frequencies, LD, and haplotypes when ethnic groups are compared (Lazarus et al. 2002). Analysis of genetic variation in 16 innate immunity genes of African Americans, European Americans, Hispanic Americans, and Asthmatic Europeans has revealed higher haplotype diversity among the African Americans (Lazarus et al. 2002). In the *IRAK-M* gene, the LD pattern in Japanese was significantly different from that in Yoruba and was almost the same as that in Chinese.

The function of TLRs in various human diseases has been investigated, and these studies have shown that TLR function affects several diseases such as sepsis, immunodeficiencies, and atherosclerosis (Cook et al. 2004). Mice deficient in SIGIRR have a very similar phenotype to that of IRAK-M-deficient mice in terms of LPS hyperresponsiveness (Wald et al. 2003). It is possible that *SIGIRR* or *IRAK-M* polymorphisms contribute to the etiology of other diseases, for example bacterial infections. In this, we newly identified a non-synonymous substitution in *SIGIRR* (Pro115Arg). The variants, *IRAK-M* (Val147Ile) and *SIGIRR* (Pro115Arg), might be associated with the etiology of another disease, by alteration of protein function.

In this study we found that the region containing SNP3 in the *IRAK-M* gene is more likely to contain a putative SRY-binding site. The sex-determining region on the Y chromosome (*SRY*) is a master gene that initiates testis differentiation in mammals. *SRY* and *SOX* (for "*SRY*-like HMG-box-containing") belong to the same family, which contain an "HMG box", a protein domain that binds to DNA at a target sequence (Marshall Graves 2002). In previous studies, Sox-4 seemed crucial for B-lymphopoiesis and thymocyte development (Smith and Sigvardsson 2004), but the relationship between the transcription factor SRY and development of immune cells remained unclear.

Although we could not find any significant association between the tested polymorphisms and asthma susceptibility or asthma-related phenotype, our findings will be helpful for choosing SNPs for further association and functional studies of other diseases. Examinations on other molecules in the TLR signaling pathway are needed to clarify the pathogenesis of asthma.

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Collaborative Action of NF-κB and p38 MAPK Is Involved in CpG DNA-Induced IFN-α and Chemokine Production in Human Plasmacytoid Dendritic Cells[1]

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CpG DNA induces plasmacytoid dendritic cells (pDC) to produce type I IFN and chemokines. However, it has not been fully elucidated how the TLR9 signaling pathway is linked to these gene expressions. We examined the mechanisms involving the TLR9 and type I IFN signaling pathways, in relation to CpG DNA-induced IFN-α, IFN regulatory factor (IRF)-7, and chemokines CXCL10 and CCL3 in human pDC. In pDC, NF-κB subunits p65 and p50 were constitutively activated. pDC also constitutively expressed IRF-7 and CCL3, and the gene expressions seemed to be regulated by NF-κB. CpG DNA enhanced the NF-κB p65/p50 activity, which collaborated with p38 MAPK to up-regulate the expressions of IRF-7, CXCL10, and CCL3 in a manner independent of type I IFN signaling. We then examined the pathway through which IFN-α is expressed. Type I IFN induced the expression of IRF-7, but not of IFN-α, in a NF-κB-independent way. CpG DNA enabled the type I IFN-treated pDC to express IFN-α in the presence of NF-κB/p38 MAPK inhibitor, and chloroquine abrogated this effect. With CpG DNA, IRF-7, both constitutively and newly expressed, moved to the nuclei independently of NF-κB/p38 MAPK. These findings suggest that, in CpG DNA-stimulated human pDC, the induction of IRF-7, CXCL10, and CCL3 is mediated by the NF-κB/p38 MAPK pathway, and that IRF-7 is activated upstream of the activation of NF-κB/p38 MAPK in chloroquine-sensitive regulatory machinery, thereby leading to the expression of IFN-α. *The Journal of Immunology,* **2006, 177: 4841–4852.**

Dendritic cells (DC)[3] consist of a heterogeneous population of APC that regulates immune responses. They are characterized by surface markers and cytokines induced in response to inflammatory stimuli, including the ligands for TLRs (1), which recognize distinct families of pathogenic products. Among DC, plasmacytoid DCs (pDC) are a unique population exhibiting plasmacytoid morphology (2). The biological distinction of pDC is that they produce a large amount of IFN-α through the ligation of TLR9 (3) with bacterial DNA or its synthetic counterpart, the so-called oligoDNA containing unmethylated CpG motifs (CpG DNA) (4–6). They also produce chemokines (7–9); through the interplay with the IFN-α, pDC participate not only in innate immunity but also in adaptive immunity (2).

Due to the identification of pDC and the discovery of microbial inducers of IFN-α, IFN-α has recently been recognized as a multifunctional cytokine (2, 10). IFN-α, as a member of the type I IFN-family, deliver signals to the classical type I IFN pathway via IFN-αβ receptor, and consequently the genes whose promoters carry IFN-stimulated response elements (ISRE) are transcribed. IFN regulatory factor (IRF) 7 is one of the genes up-regulated by type I IFN (11–13). In pDC, however, IRF-7 is constitutively expressed (14–17). Its association with MyD88 and TNF receptor-associated factor 6 (TRAF6) has recently been reported to be a prerequisite for the activation of the IFN promoter (18, 19).

Following ligation of TLR9, the adaptor MyD88 recruits signaling mediators to activate NF-κB (20). Therefore, most of the chemokines whose gene expression requires the activation of NF-κB could be induced through the TLR9 signaling in pDC. However, to our knowledge, there has not yet been any report that referred to the integral participation of NF-κB in the expression of IFN-α and chemokines in CpG DNA-stimulated human pDC. Recently, it has been demonstrated that, besides ISRE, the human IRF-7 gene promoter has a NF-κB binding site (21), and the activation of NF-κB is required for EBV latent membrane protein 1- and TNF-α-induced IRF-7 expression (21, 22). Then, we speculated that, in the end-point of TLR9 signaling, the activation of NF-κB could be involved in the up-regulation of the gene expression of IRF-7, subsequently of IFN-α, and that of other NF-κB-dependent chemokines in human pDC.

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[3] Abbreviations used in this paper: DC, dendritic cell; pDC, plasmacytoid DC; ISRE, IFN-stimulated response element; IRF, IFN regulatory factor; TRAF6, TNF receptor-associated factor 6; ODN, oligonucleotide; Act D, actinomycin D; CHX, cycloheximide; PDTC, pyrrolidinedithiocarbamate; CAPE, caffeic acid phenethyl ester; DEX, dexamethasone; PMX, polymixin B; BDCA, blood DC Ag; SR, scavenger receptor.

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