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ORIGINAL ARTICLE

Association of the RIP2 Gene with Childhood Atopic Asthma

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ABSTRACT

Background: Receptor-interacting protein (RIP)-2 is a serine/threonine kinase containing a caspase recruitment domain (CARD) that is involved in the Toll-like receptor-signaling pathway. Although associations between endotoxin exposure or respiratory infection and asthma have been recognized, the genetic influences in these conditions are unclear. The aim of our study was to examine whether polymorphisms or haplotypes in RIP2 were associated with childhood atopic asthma in a Japanese population.

Methods: We screened the RIP2 gene for polymorphisms by direct sequencing and characterized the linkage disequilibrium (LD) mapping of the gene. Seven variants were genotyped in childhood atopic asthma (n = 300) and normal controls (n = 637) . We conducted case-control and case-only association studies between the variants and asthma-related phenotypes. Haplotype association analyses were also performed.

Results: A total of 31 variants were identified and none of the alleles or haplotypes of RIP2 were associated with asthma susceptibility. In the case-only study, an association between an RIP2 promoter polymorphism and childhood severe asthma (P=0.0032;odds ratio (OR) 3.37, 95% confidence interval (CI) 1.45−7.87) was observed.

Conclusions: Although polymorphisms in RIP2 are not likely to be associated with the development of asthma, the genetic variants might contribute to asthma severity in the Japanese population.

KEY WORDS

asthma, linkage disequilibrium, receptor-interacting protein 2 (RIP2), single nucleotide polymorphism, Toll-like receptors

INTRODUCTION

Asthma is defined as a chronic inflammatory lung disease characterized by airway hyperreactivity and mucus hypersecretion that results in intermittent airway obstruction.[1] Clinical and experimental evidence suggest that respiratory infections play an important role in the development of asthma.[2,3] Toll-like receptors (TLRs) play an essential role in activation of the innate immune system, which in turn activates adaptive immunity .[4,5] The association between polymorphisms in TLR genes and asthma has been intensively studied.[6,7] Immunization with an antigen in the context of TLR2 ligands can result in experimental asthma[8] and genetic variation in TLR2 is a major factor in the susceptibility to asthma in children of farmers.[9] Viral infections are thought to be an important factor influencing both the development and the se-

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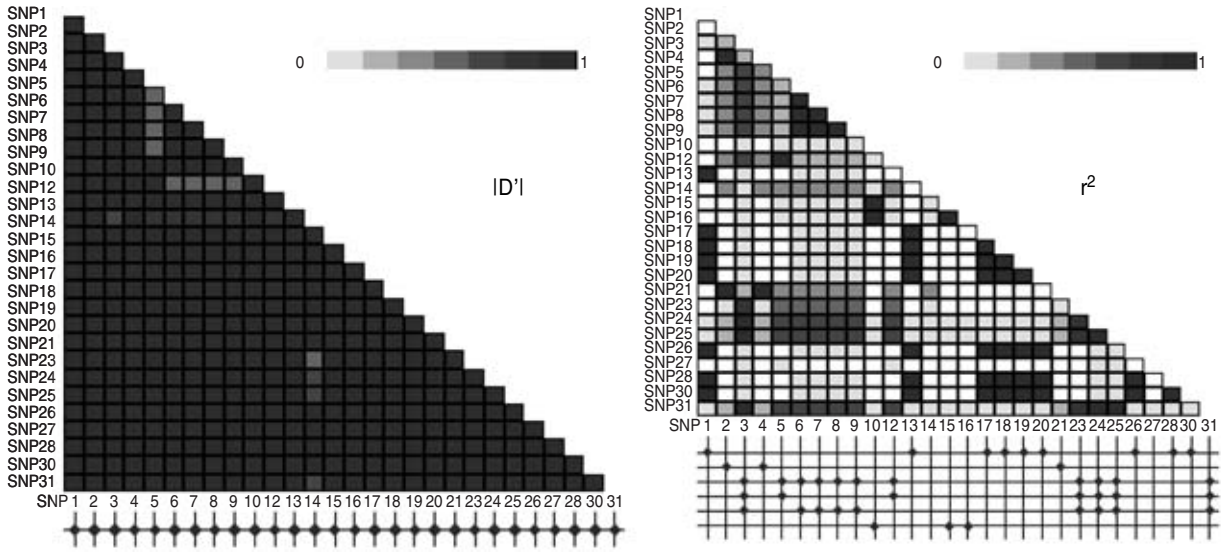

Fig. 1 Genomic structure and linkage disequilibrium at RIP2. Pairwise linkage disequilibrium (LD) was measured by |D'| and r^2 among the 28 SNPs studied in 12 sequenced samples.

verity of asthma.[10,11] TLR3 functions as a receptor for double-stranded RNA (dsRNA), a molecular pattern produced by most viruses at some point in the infection cycle, and dsRNA is a potent inducer of interferon and an activator of the interferon-induced antiviral molecules.[12,13] TLR4 is an essential receptor for the recognition of endotoxin derived from gram-negative bacteria.[4,5] Recent studies showed that the immune response induced by endotoxin could play an important role in the initiation or prevention of asthma.[14,15] Although no association was observed between TLR 4 polymorphism and the risk of asthma,[16] Yang *et al.*[17] have shown that TLR4 gene variants modify endotoxin effects on asthma and relate to the severity of asthma. RIP2 is required for production of the inflammatory cytokines through TLR-2, -3, and -4 signaling.[18]

Many cytokines are involved in the development of the chronic inflammation of asthma.[19] In an animal model, requirements for RIP2 in multiple pathways regulating immune and inflammatory responses were reported. RIP2 deficiency results in impaired interferon (IFN)-γ production in Th1 and natural killer cells due to a defective interleukin (IL)-12-induced signal transducer and activator of transcription (Stat) 4 activation.[20] It is possible that the polymorphisms of RIP 2 result in inappropriate inflammatory responses through altered IFN-γ production.

Given the contributions of respiratory infections and cytokines in asthma, it seems probable that deregulation of the normal RIP2 function could promote disease. To test whether variants of RIP2 were related to asthma, we first carried out linkage disequilibrium mapping of the gene, and conducted an association study and haplotype analyses with regard to the LD pattern.

METHODS

STUDY SUBJECTS

All subjects 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)[21] and demonstrated at least 12% improvement in their FEV_1 measurement after β_2-agonist inhalation. The diagnosis of atopic asthma was based on one or more positive skin scratch test responses to 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. Peripheral blood was obtained from each of the 300 pediatric atopic asthma outpatients at the Osaka Prefectural Habikino Hospital and National Sagamihara Hospital (mean age 9.9, 4–15 years;male:female ratio=1.53:1.0; Dermatophagiodes pteronyssinus or Dermatophagiodes farinae RAST positive 89.0%). The serum IgE level was $\log_{10}$-transformed before analysis. The mean of $\log_{10}$[total IgE (tIgE) (IU/ml)] of patients with childhood atopic asthma was 2.68 [= $\log_{10}$(479 IU/ml)].In this study,"high IgE"levels were defined as those values in the 75th percentile or higher for total IgE and the severity of asthma was defined according to the degree of therapy required to control symptoms at the time of entry into the study. The grades were as follows:grade 1, β agonists only;grade 2, sodium cromoglycate and/or theophylline;grade 3, inhaled beclomethasone, 400 μg/day or less;grade 4,

Table 1 Polymorphisms in RIP2 Genes

SNP†	Location	Nucleotide	Position‡	Amino acid	Minor allele frequency (%)	JSNP ID§ IMS-JST	NCBI¶ dbSNP
SNP 1†	5'genome	T/G	-980		0.08	047071	2293807
SNP 2†	5'genome	G/A	-933		0.17	047072	2293808
SNP 3	5'genome	wt/GCCTCTGGC	-491		0.42		5893110
SNP 4	exon 1	C/T	26	Ala9Ala	0.17	047073	2293809
SNP 5	intron 1	A/G	304		0.33		
SNP 6	intron 1	A/G	2170		0.33	020994	2267540
SNP 7	intron 1	wt/insA	2174		0.33	083036	3831711
SNP 8	intron 1	C/T	2212		0.33	020995	402886
SNP 9†	intron 1	G/A	2377		0.29	020996	39500
SNP 10†	intron 1	T/C	2478		0.08	020997	39501
SNP 11	intron 2	T/A	7170		0.04		
SNP 12	intron 2	G/A	7222		0.33	081084	431264
SNP 13	intron 3	A/C	11644		0.08		16900484
SNP 14†	intron 3	C/T	11665		0.29	119302	40377
SNP 15	intron 4	C/T	13458		0.08		
SNP 16	exon 6	T/C	14691	Ile259Thr	0.08		
SNP 17	intron 7	A/G	25916		0.08		
SNP 18	intron 7	T/C	25920		0.08		
SNP 19	intron 9	A/T	28685		0.08		
SNP 20	intron 9	wt/insC	28689		0.08		
SNP 21	intron 9	A/G	28711		0.17		11995005
SNP 22	intron 9	wt/insT	31179		0.04		
SNP 23†	intron 10	C/A	31684		0.46	081085	40247
SNP 24†	intron 10	A/G	31757		0.42		40453
SNP 25	intron 10	A/G	31811		0.42		400411
SNP 26	exon 11	A/G	32203	Leu491Leu	0.08		
SNP 27	exon 11	A/G	32707		0.08		16900627
SNP 28	exon 11	AAAC/del	32880		0.08		
SNP 29	3'genome	G/A	33541		0.04		16900640
SNP 30	3'genome	AT/del	33562		0.08		
SNP 31	3'genome	G/A	33877		0.42		39509

† SNPs were genotyped in this study.

‡ Numbering according to the genomic sequence of RIP2 (AF117829).

Position 1 is the A of the initiation codon.

§ JSNP ID, Number from the Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/)

¶ NCBI, Number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/)

inhaled beclomethasone of more than 400 μg/day.[22] A total of 637 healthy individuals who had neither respiratory symptoms nor a history of asthma-related diseases (mean age 47.2, 20–75 years;male:female ratio = 2.82:1.0) were recruited by physicians' interviews pertaining to whether they had been diagnosed with asthma and/or atopy. All individuals were Japanese and gave written informed consent to participate in the study (or, for individuals less than 16 years old, their parents gave consent),according to the rules of the process committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN).

SCREENING FOR POLYMORPHISMS AND GENOTYPING

To identify polymorphisms in the human RIP2 gene, we sequenced all eleven exons, including a minimum of 100 bases of the flanking intronic sequence, 1 kb of the 5' flanking region, and a 0.2 kb continuous 3' flanking region of the 11th exon from 12 control subjects. Fifteen primer sets were designed on the basis of the RIP2 genomic sequence from the GenBank database (accession number AF117829). Those sequences were analyzed and polymorphisms were identified using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI, USA). For the -980T/G, -933 G/A, 2377A/G, 2478T/C and 11665C/T

Table 2 Genotype Frequencies for RIP2 SNPs and Childhood Atopic Asthma Susceptibility

Gene	SNP	Control ($n = 637$)			Atopic asthma ($n = 300$)					
	Location	1	2	3	1	2	3	†$P =$	‡$P =$	§$P =$
RIP2	SNP 1	0.910	0.090	0.000	0.915	0.081	0.003	NS	NS	NS
	SNP 2	0.650	0.309	0.041	0.644	0.312	0.044	NS	NS	NS
	SNP 9	0.379	0.463	0.158	0.349	0.490	0.161	NS	NS	NS
	SNP 10	0.896	0.101	0.003	0.906	0.094	0.000	NS	NS	NS
	SNP 14	0.425	0.455	0.120	0.385	0.498	0.117	NS	NS	NS
	SNP 23	0.332	0.485	0.183	0.319	0.490	0.191	NS	NS	NS
	SNP 24	0.373	0.462	0.165	0.364	0.476	0.160	NS	NS	NS

NS, Not significant.
† Dominant model.
‡ Recessive model.
§ Codominant model.

Table 3 Association between SNPs and Asthma-related Phenotypes

RIP2 SNP1 (-980 T/G)		TT	TG	GG	OR (95% CI)	χ^2 (df = 1)	*P*
Severity = 1, 2, 3	233	0.940	0.056	0.004	3.37 (1.45−7.87)†	8.69	0.0032
Severity = 4	62	0.823	0.177	0.000			

† TT vs TG+GG.

Table 4 Haplotype Frequencies of Polymorphisms of the RIP2 Genes

	SNP position							Child asthma ($n = 300$)	Controls ($n = 637$)
	SNP 1	SNP 2	SNP 9	SNP 10	SNP 14	SNP 23	SNP 24		
1	T	A	G	T	T	A	A	0.57	0.58
2	T	G	A	T	C	C	G	0.20	0.20
3	T	A	A	T	C	C	G	0.11	0.10
4	G	A	A	T	T	C	G	0.04	0.04
5	T	A	A	C	C	C	G	0.04	0.05
6	T	A	G	T	T	C	A	0.03	0.03
Others								0.00	0.00

polymorphisms, genotyping was performed by Invader assay as described.[23] The polymorphisms 31684C/A and 31757A/G were genotyped by use of the TaqMan system (Applied Biosystems, Foster City, CA, USA).

STATISTICAL ANALYSIS

Pairwise linkage disequilibrium (LD) was calculated as |D'| and r^2 by using the SNP Alyze statistical package (Dynacom, Chiba, Japan) as described.[24] We calculated allele frequencies and tested agreement with Hardy-Weinberg equilibrium using a χ^2 goodness of fit test at each locus. We then compared differences in allele frequencies and the genotype distribution of each polymorphism between case and control subjects by using a 2×2 contingency χ^2 test with one degree of freedom, and calculated odds ratios (ORs) with 95 percent confidence intervals (95% CI).

Haplotype frequencies for multiple loci were estimated using the expectation-maximization method with SNP Alyze software.[24] Haplotype frequencies in cases and controls were evaluated both by the whole distribution with Fisher's exact test and by χ^2 tests of one haplotype against others (haplotype-wise test).[22] We also examined haplotype frequencies and haplotype association using haploview software (http://www.broad.mit.edu/mpg/haploview/index.php).

Because SNPs in each gene were in linkage disequilibrium (Fig. 1) and asthma-related phenotypes were significantly related, the simple multiplication of *P*-values by the number of SNPs or phenotypes tested is too conservative and the appropriate value for the correction is not evident. Thus, to deal with the multiple comparisons, we did not apply Bonferroni corrections but rather set the significant *P*-values at 0.01 rather than 0.05.[22]

RESULTS

We carried out screening of polymorphisms with genomic DNA from 12 randomly selected control individuals. After extensive examination of RIP2 by direct sequencing, we identified 25 single nucleotide polymorphisms (SNPs) and six insertion-deletion polymorphisms in RIP2 (Table 1). Nineteen polymorphisms were contained in the two available public databases:NCBI dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and IMS-JST JSNP DATABASE (http://snp.ims.u-tokyo.ac.jp/). A non-synonymous substitution was found in RIP2 (Ile259Thr). To examine the linkage disequilibrium (LD) between identified SNPs, pairwise LD coefficients |D'| and r^2 were calculated using the SNPAlyse program as described. Rare SNPs with minor allele frequencies of 4% were not included in the analysis. Most of these SNPs were located in one extended block of LD and some were in tight LD (Fig. 1) .We selected five SNPs with a frequency >0.15 with regard to the LD pattern across the gene. Furthermore, we genotyped a promoter SNP, -980 T/G, and intronic SNP, 2478 T/C, that were in strong linkage disequilibrium with another non-synonymous variant, 14691 T/C (Ile 259 Thr) (|D'| = 1.00 and r^2 = 1.00).

All of these loci were in Hardy-Weinberg equilibrium in the control group. The allele frequency of each selected polymorphism was compared between the patients and the normal controls by the χ^2 test using codominant, dominant, and recessive models. The results of allele frequencies in the asthma group and control group are shown in Table 2. There was no significant association between any SNP of RIP2 and childhood atopic asthma patients (Table 2).

In the case-only study, associations between asthma-related phenotypes and SNPs of the RIP2 gene were also investigated. We found a significant association between SNP1 and the most severe cases of childhood atopic asthma (OR = 3.37, 95%CI = 1.45–7.87, χ^2 = 8.69, P = 0.0032) (Table 3). There was no significant association between any SNP of the RIP2 gene and asthma patients who had a high (>10%) eosinophil count and high serum IgE level.

We further analyzed the haplotype structure using seven SNPs (SNP1, SNP3, SNP9, SNP10, SNP13, SNP21 and SNP22) and compared the frequencies of the haplotypes between controls and childhood atopic asthma (Table 4). The estimated frequency pattern of the haplotype using SNP Alyze or haploview software did not differ between the control and asthma groups.

DISCUSSION

In this study, we identified polymorphisms in RIP2, and performed case-control and case-only association studies as well as haplotype analyses using clinically characterized asthma patients. We found SNP 14691 T/C (Ile259Thr),resulting in non-synonymous amino acid substitution and a variant located in the predicted serine threonine kinase domain of RIP2. Kobayashi *et al.*[18] demonstrated reduced inflammatory cytokine production in response to the ligands for TLR2, TLR3, TLR4 and TLR9 using RIP2-deficient mice. 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.[6] Although we could not find any significant association between the tested SNPs and asthma susceptibility, the 259Thr variant might be associated with the etiology of another disease through alteration of protein function. Further studies are needed to evaluate the effect of variant 259Thr on the kinase activity of RIP2.

In this study, an association was found between a promoter SNP of the RIP2 gene and the most severe cases of childhood atopic asthma. Regarding the TLR4 gene, the Asp299Gly variant is associated with hyporesponsiveness to inhaled endotoxin challenge, reduced density of TLR4 in airway epithelium, and reduced production of inflammatory cytokines in response to endotoxin.[25] Yang *et al.*[17] found that the atopy severity score (based on the size and number of positive skin-prick tests and specific IgE) was higher in subjects with allele 299Gly than in those without allele 299Gly. RIP2-deficient mice were completely resistant to a lethal dose of lipopolysaccharide (LPS),and macrophages from those mice showed impaired NF-κB and p38 mitogen-activated protein kinase (MAPK) activation and reduced cytokine production in response to LPS stimulation.[20] Lu *et al.*[26] demonstrated that upon LPS stimulation, RIP2 is transiently recruited to the TLR4 receptor complex and associates with interleukin-1 receptor-associated kinase (IRAK) 1 and TNF receptor-associated factor (TRAF) 6. They also found that RIP2 kinase activity is transiently induced by LPS treatment.[26] While RIP2 functions as an adaptor molecule in LPS signaling, it is possible that the polymorphisms of RIP2 involved in asthma severity affect TLR4 signaling.

Furthermore, RIP2 mediates cytokine-induced IFN-γ production in Th1 and NK cells.[20] IFN-γ has diverse immunoregulatory effects on various cells and exerts an inhibitory effect on Th2 cells. Patients with severe asthma have significantly reduced IFN-γ production in response to house dust mite allergen relative to control subjects or subjects with resolved asthma.[27] Furthermore, there is reduced production of IFN-γ by T cells in asthma subjects and this correlates with the disease severity.[28] Leung *et al.*[29] reported reduced IFN-γ in corticosteroid resistant patients. Given the role of RIP2, genetic variants in RIP2 might contribute to asthma severity by affecting IFNγ.

We did not analyze the functional effects of the promoter polymorphisms found in this study, and the

polymorphisms SNP12, 16, 17, 18, 19, 24, 26, and 27, that were in linkage disequilibrium with the promoter variant, were not examined either. Recent reports have shown a significant association between promoter and intronic polymorphisms of a gene and a disease.[30,31] Clarifying the alterations of gene functions as the result of polymorphisms is necessary to further validate the involvement of the RIP2 gene in the pathogenesis of asthma.

Although we could not find any association between polymorphisms in RIP2 and the development of asthma, further studies on other molecules in the TLR signaling pathway are needed to clarify the pathogenesis of asthma.

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