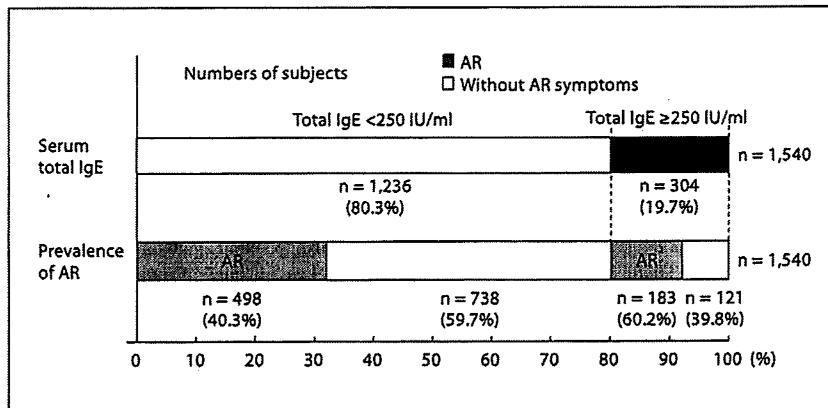

Fig. 4. Serum total IgE levels and prevalence of AR.

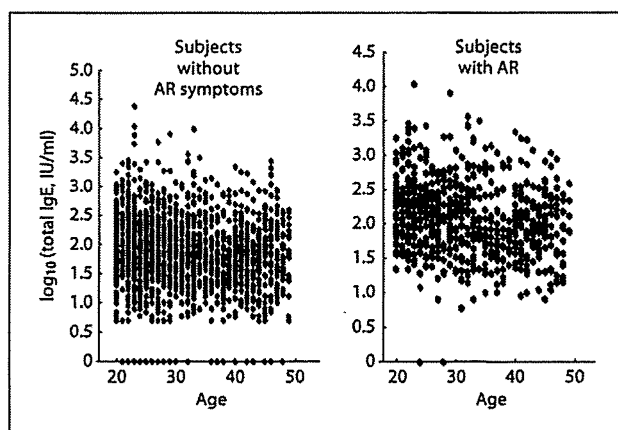

Fig. 5. Age effects on serum total IgE levels in subjects with AR and non-AR.

Total Serum IgE and AR, Sensitization, and Age Effect

There were 304 subjects (19.7%) who had high total IgE levels (≥250 IU/ml), and the prevalence of AR in this group was 60.2% (183 of the 304). However, the prevalence of AR of subjects with normal total IgE (<250 IU/ml) was 40.3% (498 of 1,236) (fig. 4).

The serum total IgE level was analyzed at a quantitative level (fig. 5). The means of $\log_{10}$ [total IgE (IU/ml)] and standard deviations of all 1,540 subjects, subjects without AR and subjects with AR were 1.87 [= $\log_{10}$ (74.1 IU/ml)] ± 0.65, 1.69 [= $\log_{10}$ (49.0 IU/ml)] ± 0.67 and 2.09 [= $\log_{10}$ (123.0 IU/ml)] ± 0.53, respectively.

We investigated the correlation between this level and age using Spearman's rank correlation coefficient (fig. 5). Although we could not find any significant correlation between the serum total IgE level and the age range of the 1,540 subjects, an inverse correlation was found between the total IgE level and age in the AR group ($rs = -0.21$, $p < 0.01$) (fig. 5). Total IgE levels were higher in younger subjects than in older subjects in the AR group. The results of the stepwise logistic regression analysis for positive sensitization to 1 or more of the 7 aeroallergens showed significant effects of total IgE (Wald statistic = 153.5, d.f. = 1, $p < 0.001$) and age (Wald statistic = 9.5, d.f. = 1, $p = 0.002$), but no effect of gender. There was no significant effect of age, gender, or total IgE on AR by logistic regression analysis.

Discussion

Estimates of the latest prevalence provide valuable information to develop effective strategies for the prevention and treatment of disease. We conducted an epidemiologic survey of AR and examined the sensitization rates against 7 aeroallergens by measuring the serum-specific IgE of 1,540 subjects aged between 20 and 49 years in a Japanese population in 2006 and 2007. The population aged between 20 and 49 years represented 38.8% of the population of Japan in 2008 according to current population estimates by the Ministry of Internal Affairs and Communications (http://www.stat.go.jp/english/data). We also examined the role of age effects on the prevalence. In this study, 681 of the 1,540 subjects (44.2%) were diagnosed as having AR. Increases in prevalence of AR and asthma have been reported by studies of relatively large populations in the United States, Great Britain, Australia and New Zealand, with cross-referenc-

es to earlier relevant studies, and the recent AR prevalence in these studies ranged from 23 to 28% [14]. The International Study of Asthma and Allergies in Childhood in 1997 reported that the prevalence of rhinoconjunctivitis varied across centers from 0.8 to 14.9% in 6-to 7-year-olds and from 1.4 to 39.7% in 13- to 14-year-olds [15]. In an Aberdeen population study on 3,537 subjects, the prevalence of hay fever increased significantly from 1994 (13%) to 1999 (15%) [16]. In Japan, Sakurai et al. [9] showed that the prevalence rates of AR, seasonal rhinitis and JCP were 36, 29, and 11%, respectively, and age was a negative risk factor for all allergic conditions. The subjects of the study consisted of 2,307 male railway employees who underwent a health examination from February to May 1995 (mean age, 41.4 years; range, 19–65 years). In the study, AR was determined from self-reported AR or from the seasonal nasal symptoms, and JCP was defined as the presence of cedar-specific IgE positivity among subjects with seasonal rhinitis. The prevalence of AR in this study was 44.2% (681 of the 1,540 subjects), which is higher than in previous reports. However, there was no difference of prevalence between 20 and 49-year-olds. Interestingly, the prevalence of AR in subjects aged 30–39 years was 42.7% in a study conducted in 1995 [9]. These subjects aged 30–39 years in 1995 were 40–49 years old in 2005. The prevalence of AR in this study for subjects from 40 to 49 years of age was 43.9%, and there was no difference in the prevalence between the studies. The prevalence among this age group did not markedly increase during the last 10 years. Further etiological studies in independent populations or those aged less than 20 years and elderly populations are needed to determine the effects of age on the susceptibility to AR.

In the present study, a total of 859 subjects (859/1,540, 55.8%) had no symptoms of AR; however, among them, 392 subjects (392/859, 45.6%) were already sensitized to one or more of the 7 test aeroallergens. It is generally recognized that sensitization to any allergen is an important risk factor for developing allergic diseases; however, those sensitized subjects had no symptoms of AR.

The present study has shown that a total of 167 of 681 subjects with AR (24.5%) were sensitized to JC pollen and not to the other 6 test aeroallergens. Allergen-specific immunotherapy is established as an effective treatment for patients with IgE-mediated reactions, and it has been widely used as a desensitizing therapy for AR [17, 18]. Specific immunotherapy retrospectively reduces new sensitization in monosensitized subjects suffering from AR [19]. Subjects with monoallergen sensitization appear to be good candidates for immunotherapy.

Among the 681 subjects with AR, 451 (66.2%) were sensitized to multiple (two or more) aeroallergens, and 385 (56.5%) were sensitized to dust mites. Although our data strongly indicated an important role of JCP in AR, a significantly higher prevalence of sensitization to dust mites was observed in younger subjects. Dust mites, an indoor allergen, have a predominant impact on asthma, and a recent population-based study has shown that dust mite sensitization is a significant risk factor for developing the disease [20]. Another recent study, a long-term (23-year) follow-up study of university students, has shown that sensitization to pollen leads to an increased risk of developing asthma [21]. A limitation of our study was the lack of longitudinal data. To clarify factors that increased the risk of developing new AR or bronchial asthma, further cohort analyses should be conducted regarding the involvement of the sensitized allergens in airway allergic inflammation.

A recent etiological study in an unselected rural Chinese population tested sensitization to 14 allergens, including 5 aeroallergens (dust mite, cockroach, *Alternaria tenuis*, dog epithelia, and cat hair) by skin prick tests. 2,118 subjects whose ages ranged from 11 to 71 years were tested (43.3% were children between 11 and 17 years old) [22]. The study showed that 41.1% of the children were sensitized to 1 or more aeroallergens, and 36.5% of the adult subjects aged ≥18 years were sensitized [22]. The most common sensitizing aeroallergen in the Chinese study was dust mites (30.6%) [22]. In meta-analyses using data from 12,687 subjects aged 20–44 years in the European Community Respiratory Health Survey conducted in 2002, the highest prevalence of sensitization was found for the house dust mite (20.2%) [23]. In the present study, of the 1,540 subjects, 1,073 (69.7%) were sensitized to at least 1 of the 7 aeroallergens, and 855 (55.5%) and 649 (42.1%) were sensitized to Japanese cedar pollen and dust mites, respectively.

Several limitations of this survey should be mentioned. The survey is likely to be fraught with a certain recruitment bias. In general, individuals affected by a specific disease are more willing and interested in a study. However, only 13 subjects (0.84%) did not agree to participate in this survey whereas 1,540 subjects agreed to assays of serum total IgE and specific IgE for the 7 aeroallergens and to answer the questionnaire in the present study. Hospital workers, nursing and medical students might not be representative of the general population and there might have been a population selection bias with regard to socioeconomic status and higher education. Previous studies in various countries have reported an increased

occurrence of asthma among specific groups of healthcare workers [24–26]. Thus, selection bias might have had an influence on the higher prevalence of sensitization to 1 or more aeroallergens (69.7%) and of AR (44.2%) in our study.

Although a population selection bias might reduce the generalizability of the study, we showed here that the prevalence of AR has increased and that Japanese cedar pollen and dust mites were the predominant allergen sources among the 7 tested allergen sources in the Japanese population. However, further study is needed using larger, more representative samples.

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Associations of functional *NLRP3* polymorphisms with susceptibility to food-induced anaphylaxis and aspirin-induced asthma

Yuki Hitomi, PhD,[a] Motohiro Ebisawa, MD, PhD,[b] Morimitsu Tomikawa, MD,[b] Takanori Imai, MD, PhD,[b] Takatsugu Komata, MD,[b] Tomomitsu Hirota, DDS, PhD,[a] Michishige Harada, PhD,[a] Masafumi Sakashita, MD,[a] Yoichi Suzuki, MD, PhD,[c] Naoki Shimojo, MD, PhD,[d] Yoichi Kohno, MD, PhD,[d] Kimie Fujita, MD, PhD,[e] Akihiko Miyatake, MD, PhD,[f] Satoru Doi, MD, PhD,[g] Tadao Enomoto, MD, PhD,[h] Masami Taniguchi, MD, PhD,[b] Noritaka Higashi, MD, PhD,[b] Yusuke Nakamura, MD, PhD,[i] and Mayumi Tamari, MD, PhD[a] *Kanagawa, Chiba, Shiga, Osaka, Wakayama, and Tokyo, Japan*

Background: NLR family, pyrin domain containing 3 (NLRP3), controls the activity of inflammatory caspase-1 by forming inflammasomes, which leads to cleavage of the procytokines IL-1β and IL-18. Recent studies have shown associations of human *NLRP3* polymorphisms with susceptibility to various inflammatory diseases; however, the association with allergic diseases remains unclear.

Objective: We sought to examine whether *NLRP3* polymorphisms are associated with susceptibility to food allergy, food-induced anaphylaxis, and aspirin-induced asthma (AIA).

Methods: We selected 15 tag single nucleotide polymorphisms (SNPs) of *NLRP3* and conducted association analyses of *NLRP3* using 574 and 1279 samples for food allergy and AIA, respectively. We further performed functional analyses of the susceptible SNPs.

Results: Two *NLRP3* SNPs (rs4612666 and rs10754558) were significantly associated with susceptibility to food-induced anaphylaxis ($P = .00086$ and $P = .00068$, respectively). The *NLRP3* haplotype of the 2 SNPs also showed a significant association ($P = .000098$). We could confirm the association with susceptibility to another hypersensitivity phenotype, AIA (rs4612666, $P = .0096$). Functional analysis revealed that the risk alleles of rs4612666 and rs10754558 increased the enhancer activity of *NLRP3* expression and *NLRP3* mRNA stability, respectively.

Conclusion: Our results indicate that the *NLRP3* SNPs might play an important role in the development of food-induced anaphylaxis and AIA in a gain-of-function manner. Further research on the NLRP3 inflammasome will contribute to the development of novel diagnostic and therapeutic methods for food-induced anaphylaxis and AIA. (J Allergy Clin Immunol 2009;124:779-85.)

Key words: NLR family, pyrin domain containing 3, gene polymorphism, association study, food allergy, hypersensitivity, anaphylaxis, aspirin-induced asthma, enhancer activity, mRNA stability

Food allergy is defined as an adverse immune response to food proteins, and food-induced allergic reactions are responsible for a variety of symptoms involving the skin, gastrointestinal tract, and respiratory tract.[1] Food allergy has increased in the past 10 to 15 years, particularly in industrialized countries.[2] In Japan large-scale morbidity surveys demonstrated that food allergies occur in 5% to 10% of infants and preschool children, which is similar to the rate seen in other industrialized countries.[3-5] Although environmental factors could contribute to the recent increase in food allergies, several family studies have indicated that genetic factors also influence the risk of food allergies.[6,7] Little is known about the specific genes associated with susceptibility to food allergies, and recent studies have shown that polymorphisms of *CD14*[8]; signal transducer and activator of transcription 6 *(STAT6)*[9]; serine peptidase inhibitor, kazal type 5 *(SPINK5)*[10]; and *IL10*[11] are significantly associated with such susceptibility. Anaphylaxis is a life-threatening allergic reaction, and food is one of the most common responsible allergens.[12] In addition to the cutaneous, respiratory, and gastrointestinal symptoms, patients with anaphylaxis can experience cardiovascular symptoms, including hypotension, vascular collapse, and cardiac dysrhythmia, presumably because of massive mast cell mediator release.[13] Double-blind, placebo-controlled food challenge is the gold standard for the diagnosis of food allergy, and strict elimination of the allergenic food is the basic therapy for it.[13,14] Oral desensitization therapy is conducted in some cases to reduce the risk of a critical allergic reaction with accidental ingestion of allergenic food.[15] Although anaphylaxis is a severe side effect of both the food challenge test and oral desensitization therapy,[16] there are no completely reliable methods to estimate the risk for food-induced anaphylaxis.

From [a]the Laboratory for Respiratory Diseases, Center for Genomic Medicine, RIKEN, Kanagawa; [b]the Clinical Research Center for Allergy and Rheumatology, Sagamihara National Hospital, National Hospital Organization; the Departments of [c]Public Health and [d]Pediatrics, Graduate School of Medicine, Chiba University; [e]the School of Human Nursing, the University of Shiga Prefecture; [f]Miyatake Asthma Clinic, Osaka; [g]the Department of Pediatric Allergy, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases; [h]NPO Japan Health Promotion Supporting Network, Wakayama; and [i]the Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo.

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Reprint requests: Mayumi Tamari, MD, PhD, Laboratory for Respiratory Diseases, Center for Genomic Medicine, the Institute of Physical and Chemical Research (RIKEN), 1-7-22 Suehiro, Tsurumi-ku, Yokohama 230-0045 Kanagawa, Japan. E-mail: tamari@src.riken.jp.

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Abbreviations used
AIA: Aspirin-induced asthma
LD: Linkage disequilibrium
NLR: Nucleotide-binding domain, leucine-rich repeat–containing
NLRP3: NLR family, pyrin domain containing 3
OR: Odds ratio
SNP: Single nucleotide polymorphism

Aspirin-induced asthma (AIA) is a common clinical presentation of aspirin hypersensitivity, and this acute reaction is elicited through COX inhibition by nonsteroidal anti-inflammatory drugs.[17] AIA and autoimmune diseases partly share some clinical features and laboratory markers.[18] The natural course of AIA is similar to that of persistent viral infection of the respiratory system, and infectious factors have been shown to play a role in AIA.[17] However, the causative factors for the disease remain elusive.

NLR family, pyrin domain containing 3 (NLRP3; also known as NALP3, CIAS1, PYPAF1, or cryopyrin), is a member of the nucleotide-binding domain, leucine-rich repeat–containing (NLR) family of genes encoding proteins that comprise a nucleotide-binding domain and a leucine-rich repeat domain, and it controls the activity of inflammatory caspase-1 by forming complexes called inflammasomes.[19] Recent studies have shown that tight collaboration between pathogen-associated molecular patterns and damage-associated molecular patterns is needed to start an innate immune response to allergens,[20] and NLRP3 inflammasomes are activated by pathogen-associated molecular patterns, microbial toxins, live bacteria, viruses, and damage-associated molecular patterns.[21] However, the mechanism of activation of the NLRP3 inflammasome is not fully understood. After being activated, NALP3 recruits apoptosis-associated speck-like protein containing a card and procaspase-1, leading to autocatalytic processing and activation of caspase-1. Active caspase-1 catalyzes cleavage of the procytokines IL-1β and IL-18, which are both proinflammatory cytokines involved in the host response to infection and injury.[22] Excessive production of IL-1β and IL-18 is associated with septic shock and autoimmune disorders.[22]

The human *NLRP3* gene is located in 1q44. Previous studies have determined that the nonsynonymous coding substitution of *NLRP3* causes autoinflammatory diseases: V198M, L353P, A439V, and E627G are associated with familial cold autoinflammatory syndrome; R260W, A352V and G569R are associated with Muckle-Wells syndrome; and D303N, F309S and F537S are associated with chronic infantile neurological, cutaneous, and articular syndrome.[19] Furthermore, recent studies have shown that *NLRP3* polymorphisms are significantly associated with susceptibility to common inflammatory diseases, such as Crohn disease,[23] psoriatic juvenile idiopathic arthritis,[24] and essential hypertension.[25] However, the association of *NLRP3* polymorphisms with susceptibility to allergic diseases has not been reported.

To clarify the genetic factors that increase the risk of the hypersensitive phenotype of allergy, we conducted an association study of *NLRP3* polymorphisms with susceptibility to food allergy, food-induced anaphylaxis, and AIA in a Japanese population. Functional effects of the related *NLRP3* variants were also examined.

METHODS

Subjects

Three hundred twenty pediatric patients with food allergies and positive antigen-specific IgE results (CAP-RAST) were recruited at National Sagamihara Hospital, Kanagawa, Japan. We performed food challenge tests for 178 patients with food allergies. The diagnosis of food allergy was made either based on challenge tests or a definitive episode plus confirmation of antigen-specific IgE levels. Two hundred fifty-four nonatopic, nonasthmatic healthy unrelated control children were recruited from an elementary school affiliated with the Education Department of Chiba University, Chiba, Japan. Healthy subjects included in this group had no history of food allergies, asthma, or atopic dermatitis. Detailed information for patients with food allergy and healthy children is shown in Table I. Food-induced anaphylaxis was defined as symptoms evoked in multiple organs. Five hundred forty-nine adult asthmatic patients were recruited from Miyatake Asthma Clinic and the National Sagamihara Hospital.[26,27] Among the 549 patients, 79 were aspirin intolerant, and 470 were aspirin tolerant. All subjects with asthma were given diagnoses according to the criteria of the American Thoracic Society.[28] Detailed information on adult asthmatic patients is provided in Table E1 (available in this article's Online Repository at www.jacionline.org). All were unrelated Japanese subjects and provided written informed consent to participate in the study according to the rules of the Process Committee at the Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN).

Variation screening of *NLRP3*

We carried out screening of single nucleotide polymorphisms (SNPs) with genomic DNA from 12 healthy subjects. A total of 17 overlapping primer sets were designed on the basis of the *NLRP3* genomic sequence available from the National Center for Biotechnology Information (accession no. NM_001079821.1). The complete coding region of *NLRP3*, intron/exon boundaries, and 100 bases of the surrounding intronic sequence and approximately 3 kb of 5′ genomic DNA were resequenced. The PCR product was reacted with BigDye Terminator v3.1 (Applied Biosystems, Foster City, Calif). Sequences were assembled and polymorphisms were identified with the SEQUENCHER program (Gene Codes Corp, Ann Arbor, Mich).

Selection of *NLRP3* polymorphisms for genotyping

Genomic DNA was prepared from peripheral blood samples by using standard protocols. We selected tag SNPs of the exons and introns of *NLRP3* with a minor allele frequency of greater than 10% in the HapMap Japanese data set (http://www.hapmap.org/). Pairwise linkage disequilibrium (LD) was calculated as r^2 values by using the Haploview 4.1 program (http://www.broad.mit.edu/mpg/haploview/). Haplotype frequencies for the 2 most susceptible SNPs were also estimated with the Haploview 4.1 program. Genotyping of SNPs was performed by using the TaqMan allele-specific amplification method (Applied Biosystems).

Real-time quantitative RT-PCR

Total RNA from normal human tissues was purchased from Clontech (Mountain View, Calif), and total RNA from cell lines was extracted with a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). Each RNA was reverse transcribed with Superscript III reverse transcriptase and oligo dT primers (Invitrogen, Carlsbad, Calif). The expression of *NLRP3* transcripts was determined by using real-time quantitative RT-PCR with SYBR Premix Ex Taq (Takara, Shiga, Japan) and specific primers (5′-GGGGTCAT GATGTTCTGTGA-3′ and 5′-CAGGCTTTTCTTCTTGAAGTGTT-3′). In all experiments the amounts of cDNA were standardized by means of quantification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*.

Luciferase assay

Intron 7 and the last exon fragments of *NLRP3* from human genomic DNA were amplified by means of PCR by using specific primers (5′-GCCACTATG

TABLE I. Clinical information about patients with food allergy and control subjects

Characteristics	Patients with food allergy			
	Total, n = 320 (%)	Anaphylaxis (+), n = 98 (%)	Anaphylaxis (−), n = 222 (%)	Control subjects, n = 254 (%)
Age (y)	4.2 ± 3.3	5.4 ± 3.1	3.7 ± 3.3	9.0 ± 1.7
Sex				
Male	233 (72.8)	67 (68.4)	166 (74.8)	124 (48.8)
Female	87 (27.2)	31 (31.6)	56 (25.2)	130 (51.2)
Log serum total IgE (IU/mL)	2.6 ± 0.7	2.8 ± 0.4	2.6 ± 0.7	2.4 ± 1.3
Food allergy phenotype				
Infantile atopic dermatitis	268 (83.8)	76 (77.6)	192 (86.5)	—
Immediate type	294 (91.9)	96 (98.0)	198 (89.2)	—
OAS	12 (3.8)	4 (4.1)	8 (3.6)	—
FEIAn	6 (1.9)	5 (5.1)	1 (0.5)	—
Complications				
Atopic dermatitis	223 (69.7)	64 (65.3)	159 (71.6)	—
Bronchial asthma	108 (33.8)	42 (42.9)	66 (29.7)	—
Allergy rhinitis	47 (14.7)	17 (17.3)	30 (13.5)	—
Allergic conjunctivitis	39 (12.2)	15 (15.3)	24 (10.8)	—

OAS, Oral allergy syndrome; *FEIAn*, food-dependent exercise-induced anaphylaxis.

GAAAACAGCAC-3′ and 5′-AAGGAAGCACCCGTACCTGC-3′ and 5′-GTTGTCTGAAATGTATTTCAATT-3′ and 5′-TTTGAAAAATTTCTAGG TACTCT-3′, respectively). PCR products were subcloned into the reporter gene pGL3-promoter vector (Promega, Madison, Wis). Vector pRL-TK was used to normalize for variations in transfection efficiency. These plasmids were transfected into THP-1 cells by using FuGENE 6 (Roche, Basel, Switzerland). The luciferase activities were determined by using the Dual-Luciferase Reporter Assay system (Promega).

Allele-specific transcript quantification

PBMCs were isolated from 3 healthy donors with heterozygous *NLRP3* rs10754558 by means of density gradient centrifugation. Primary monocytes were sorted with human CD14 microbeads and an autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany), and genomic DNA and total RNA were isolated. We performed allelic expression analyses using the TaqMan assay with SNP genotyping probes. Genomic DNA was used as a control for equal biallelic representation. The ratio of 5-carboxyfluorescein (FAM) intensity to VIC intensity for *NLRP3* was plotted for mixtures of homozygous DNAs at 6 different ratios (3:1, 2:1, 3:2, 2:3, 1:2, and 1:3), with correction based on the signal intensities of heterozygote controls for a standard line. We then measured the allelic ratio for each cDNA and genomic DNA from each subject and calculated the allelic ratio of cDNA and genomic DNA based on the standard line.

Prediction of transcription factor binding sites

The TRANSFAC Professional 10.3 (http://www.biobase.de/pages/) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) Web sites were used to predict putative transcription factor binding sites. RNA secondary structures were predicted by using the mfold Web server (http://mfold.bioinfo.rpi.edu/) setting default parameters and a folding temperature of 37°C.

Electrophoretic mobility shift assays

Nuclear extracts were prepared from THP-1 cells. Extracts were quickly frozen and stored in aliquots at −80°C. Electrophoretic mobility shift assays was performed by using a 2nd Generation DIG Oligonucleotide 3′-end Labeling Kit and 2nd Generation DIG Gel Shift Kit (Roche), according to the manufacturer's instructions. We incubated the nuclear extract with 28-bp double-strand oligonucleotide probes for rs4612666 (C and T) for 30 minutes at room temperature. The oligonucleotide sequences were 5′-GGAGCTGGGAAGACGTAGTATTGGTGGG-3′ for the C allele and 5′-GGAGCTGGGAAGATGTAGTATTGGTGGG-3′ for the T allele, respectively. For the supershift experiments, a rabbit anti-human GATA-2 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) and GATA-2 consensus probe (5′-CACTTGATAACAGAAAGTGATAACTCT-3′) were used.

Statistical analysis

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 allelic frequencies of the polymorphisms between cases and control subjects by using a 2×2 contingency χ^2 test with 1 *df* and calculated odds ratios (ORs) with 95% CIs. Logistic regression analysis was implemented for the susceptibility to anaphylaxis or AIA and genotype to assess the effects of sex (SPSS 14.0 J; SPSS, Inc, Chicago, Ill). The small sample size of this study decreased the ability to detect associations. Power in this study was estimated with the aid of Sample Power 2.0 (SPSS, Inc). If ORs of risk alleles with control group frequencies of 0.05, 0.1, 0.2, and 0.4 were greater than 3.41, 2.61, 2.16, and 1.98, respectively, power exceeded 80% (at $P = .05$) in allelic association tests of food allergy (222 subjects with food allergy without anaphylaxis [control] and 98 subjects with food-induced anaphylaxis). Similarly, in allelic association tests in patients with adult asthma (470 asthmatic patients without AIA [control] and 79 patients with AIA), a power of 80% was ensured if alleles with frequencies of 0.05, 0.1, 0.2, and 0.4 had ORs of greater than 3.41, 2.61, 2.16, and 1.98, respectively. A P value of less than .05 was considered statistically significant. Expression differences between genotypic groups were tested with the Student t test and Mann-Whitney U test.

RESULTS

Association of *NLRP3* SNPs with susceptibility to food-induced anaphylaxis

Thirty-nine polymorphisms with a frequency of greater than 10% in the *NLRP3* region were contained in the public databases at the National Center for Biotechnology Information dbSNP Web site (http://www.ncbi.nlm.nih.gov/SNP/). We selected 15 polymorphisms for association studies using tagger in the Haploview 4.1 program, and these 15 SNPs captured 39 of the 39 alleles with a mean r^2 value of 0.98 ($r^2 > 0.80$; Fig 1, *A*). We next carried out case-control association studies of the 15 SNPs. The control genotypes did not deviate from Hardy-Weinberg equilibrium. Although no significant association was observed between any SNP and food allergy, 7 SNPs showed significant associations with susceptibility to food-induced anaphylaxis (Table II and see Table E2 in this article's Online Repository at www.jacionline.org). Among

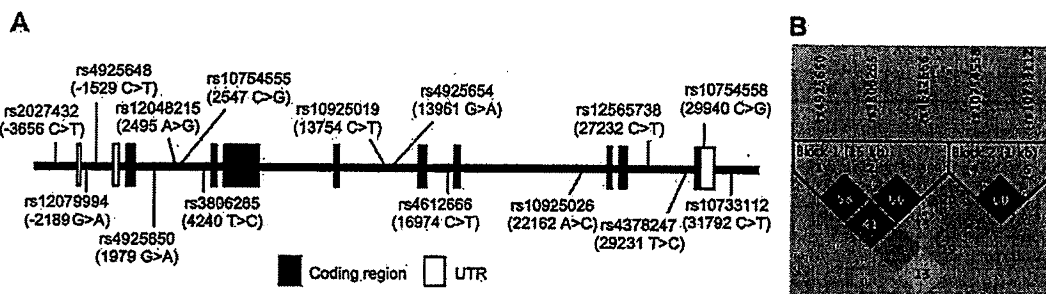

FIG 1. **A,** Exon–intron structure of the *NLRP3* gene and locations of genotyped tag SNPs. *UTR,* Untranslated region. **B,** LD structures of the 5 significantly associated *NLRP3* SNPs with susceptibility to food-induced anaphylaxis ($P < .005$). LD was calculated by using genotyping data. Pairwise r^2 values for all combinations of SNPs are shown in gray scale.

TABLE II. Association between *NLRP3* polymorphisms and susceptibility to food-induced anaphylaxis

			Anaphylaxis (+), n = 98 (%)			Anaphylaxis (−), n = 222 (%)			Frequency (allele 1)				
Allele 1/2	dbSNP ID	Location	1/1	1/2	2/2	1/1	1/2	2/2	(+)	(−)	*P* value	OR	95%CI
−3656 C/T	rs2027432	5′ Flanking	78 (80)	19 (19)	1 (1)	169 (76)	50 (23)	3 (1)	0.89	0.87	NS		
−2189 G/A	rs12079994	Intron 1	78 (80)	18 (18)	2 (2)	147 (67)	61 (28)	10 (5)	0.89	0.81	.021	1.81	1.09–2.99
−1529 C/T	rs4925648	Intron 1	81 (83)	17 (17)	0 (0)	166 (75)	54 (24)	2 (1)	0.91	0.87	NS		
1979 G/A	rs4925650	Intron 3	18 (18)	51 (52)	29 (30)	71 (32)	118 (53)	33 (15)	0.44	0.59	.00091	1.77	1.26–2.49
2495 A/G	rs12048215	Intron 3	58 (59)	39 (40)	1 (1)	123 (55)	85 (38)	14 (6)	0.79	0.75	NS		
2547 C/G	rs10754555	Intron 3	42 (43)	47 (49)	8 (8)	77 (35)	115 (52)	29 (13)	0.68	0.61	NS		
4240 T/C	rs3806265	Intron 3	43 (44)	44 (45)	10 (10)	62 (28)	114 (52)	43 (20)	0.67	0.54	.0029	1.71	1.20–2.43
13754 C/T	rs10925019	Intron 6	54 (55)	39 (40)	5 (5)	109 (49)	92 (41)	21 (10)	0.75	0.70	NS		
13961 G/A	rs4925654	Intron 6	67 (68)	25 (26)	6 (6)	144 (65)	71 (32)	7 (3)	0.81	0.81	NS		
16974 C/T	rs4612666	Intron 7	44 (45)	41 (42)	13 (13)	56 (26)	114 (52)	49 (22)	0.66	0.52	.00086	1.81	1.27–2.56
22162 A/C	rs10925026	Intron 8	27 (28)	49 (50)	22 (22)	83 (38)	111 (51)	26 (12)	0.53	0.63	.013	1.53	1.09–2.16
27232 C/T	rs12565738	Intron 10	81 (83)	16 (16)	1 (1)	176 (79)	43 (19)	3 (1)	0.91	0.89	NS		
29231 T/C	rs4378247	Intron 10	84 (86)	13 (13)	1 (1)	181 (82)	40 (18)	1 (1)	0.92	0.91	NS		
29940 C/G	rs10754558	Exon 11 (3′UTR)	25 (26)	46 (47)	27 (28)	83 (38)	109 (50)	25 (12)	0.49	0.63	.00068	1.80	1.28–2.54
31792 C/T	rs10733112	3′ Flanking	35 (36)	49 (50)	14 (14)	46 (21)	119 (54)	57 (26)	0.61	0.48	.0021	1.71	1.21–2.40

NS, Not significant; *UTR*, untranslated region.

5 food-induced anaphylaxis–susceptible SNPs for which the *P* value was less than .005, 3 (rs4925650, rs3806265, and rs4612666; $r^2 \geq 0.41$) and 2 (rs10754558 and rs10733112, $r^2 \geq 0.60$) were in moderate LD with each other (Fig 1, *B*). Because we could not find any SNP that was in strong LD with the 5 variants by resequencing and searching the dbSNP database, we considered the rs4612666 (P = .00086; OR, 1.81) and rs10754558 (P = .00068; OR, 1.80) variants that were the most susceptible SNPs in each LD block to be associated with the susceptibility to food-induced anaphylaxis. The results of stepwise logistic regression analysis for the susceptibility to anaphylaxis showed significant effects of the genotypes rs4612666 (Wald statistic = 13.38, $df = 1$, $P = .00025$) and rs10754558 (Wald statistic = 6.23, $df = 1$, $P = .013$). There was no significant effect of sex by means of logistic regression analysis. We further divided the subjects with food allergies into 2 groups, those with and without food challenge tests. We found significant associations between the 2 SNPs rs4612666 and rs10754558 and food-induced anaphylaxis in both groups, but a marked effect of food challenges was not observed (data not shown).

We next investigated the effects of *NLRP3* haplotypes with susceptibility to food-induced anaphylaxis. As shown in Table III, the frequency of combination of the most susceptible alleles between LD blocks (rs4612666-C and rs10754558-G) was significantly increased in patients with food-induced anaphylaxis (haplotype_1 vs haplotype_4; P = .000098; OR, 2.21).

An association study of *NLRP3* SNPs with susceptibility to AIA

Approximately 10% of adult asthmatic patients are affected by AIA. As well as food-induced anaphylaxis, patients with AIA show acute life-threatening hypersensitivity symptoms.[29] Therefore we performed an association study between the 5 food-induced anaphylaxis–susceptible *NLRP3* SNPs and susceptibility to AIA. rs4612666 showed a significant association with susceptibility to AIA, and the direction of association was similar to that of food-induced anaphylaxis (see Table E3 in this article's Online Repository at www.jacionline.org). The results of stepwise logistic regression analysis for the susceptibility to AIA showed significant effects of genotype rs4612666 (Wald statistic = 4.34, $df = 1$, $P = .037$). No significant effect of sex was found by means of logistic regression analysis. These results indicated that *NLRP3* SNPs were significantly associated with hypersensitivity, such as food-induced anaphylaxis and AIA.

TABLE III. Haplotype frequency in *NLRP3*

	rs4612666	rs10754558	No. Case	No. Control	Frequency Case	Frequency Control
Haplotype_1	C (susceptible)	G (susceptible)	89	130	0.45	0.30
Haplotype_2	T (nonsusceptible)	G (susceptible)	11	27	0.06	0.06
Haplotype_3	C (susceptible)	C (nonsusceptible)	39	92	0.20	0.21
Haplotype_4	T (nonsusceptible)	C (nonsusceptible)	56	181	0.29	0.42
	P value	OR	95% CI			
Haplotype_1 vs haplotype_4	.000098	2.21	1.48-3.31			

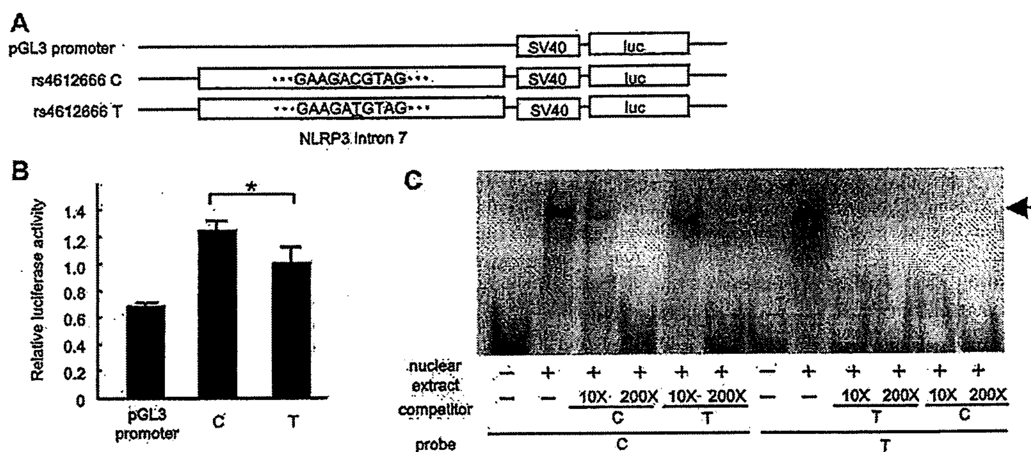

FIG 2. Functional analyses of *NLRP3* rs4612666 using THP-1 cells. **A**, Plasmid constructs used for transfection. **B**, Transcriptional enhancer activities of rs4612666 measured by luciferase *(luc)* activity 24 hours after transfection. Values of relative luciferase activity are shown as means±SDs. *$P < .05$, Student *t* test. **C**, Electrophoretic mobility shift assays of rs4612666. An unlabeled probe was used as a competitor. C, C allele; T, T allele.

Influence of rs4612666 on differential expression of *NLRP3* mRNA

We next conducted functional analysis of the rs4612666 SNP. Because rs4612666 was located in intron 7, we examined whether the genomic region around rs4612666 had enhancer activity. At first, to survey the *NLRP3* mRNA expression levels in cells and tissues, we conducted real-time quantitative RT-PCR. *NLRP3* mRNA was dominantly expressed in peripheral leukocytes (see Fig E1*A*, in this article's Online Repository at www.jacionline.org), and among the cell lines of leukocytes, higher expression of *NLRP3* mRNA was detected in the monocyte cell line THP-1 (see Fig E1*B*). We subsequently performed a luciferase assay to test whether the allelic difference contributed to the efficiency of expression of *NLRP3* mRNA using THP-1 cells. The allele-specific constructs containing the food-induced anaphylaxis risk allele rs4612666 showed 1.2-fold higher transcriptional enhancer activity than the other constructs containing the T allele of rs4612666 (Fig 2, *A* and *B*). These experiments were performed 4 times with similar results. We next searched for nuclear transcription factors that might bind to oligonucleotide sequences containing rs4612666 by using TRANSFAC and TFSEARCH, and the genomic region containing the C allele of rs4612666 was found to create a novel consensus sequence corresponding to the putative binding element of GATA-2. Therefore we examined the allelic differences in the binding of nuclear proteins by using the electrophoretic mobility shift assay. The signal intensity of the DNA-protein complex derived from the C allele was higher than that from the T allele in the presence of THP-1 nuclear extract, and the complex was diminished by excess amounts of a nonlabeled allele-specific competitor probe (Fig 2, *C*). Four independent experiments were performed with similar results. However, the band was not supershifted by the addition of antibodies to GATA-2 in the present study (see Fig E2 in this article's Online Repository at www.jacionline.org).

Contribution of the 3' untranslated region rs10754558 SNP to *NLRP3* mRNA stability

Like the *NLRP3* rs4612666 in intron 7, rs10754558 was significantly associated with susceptibility to food-induced anaphylaxis. Because of the location of rs10754558 in the 3' untranslated region, we further examined whether the risk allele of rs10754558 affected the stability of the *NLRP3* mRNA. RNA secondary structure prediction with the mfold Web server showed that the risk allele contributed to the mRNA stability more than the other allele of rs10754558 (see Fig E3 in this article's Online Repository at www.jacionline.org). First, we performed a luciferase assay using THP-1 cells. The allele-specific construct containing the G allele of rs10754558 showed 1.3-fold higher activity than the other constructs containing the C allele of rs10754558 (Fig 3, *A* and *B*). These experiments were performed 4 times

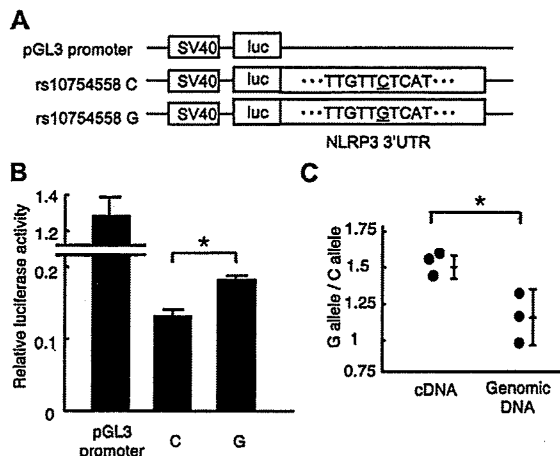

FIG 3. Functional analyses of *NLRP3* rs10754558 using THP-1 cells. **A**, Plasmid constructs used for transfection. *UTR*, Untranslated region. **B**, Effect of the SNP on mRNA stability as measured by luciferase activity. Values of relative luciferase activity are shown as means±SDs. $*P < .0005$, Student *t* test). *C*, C allele; *G*, G allele. **C**, Allele-specific transcript quantification of rs10754558 is shown as means ± SDs. $*P < .05$, Mann-Whitney *U* test.

with similar results. To further investigate the effect of rs10754558 on transcription of mRNA, we performed allele-specific quantitative PCR with a TaqMan probe on human primary monocytes from healthy donors with heterozygous genotypes of rs10754558. In these cells the mean ratio (susceptible vs nonsusceptible allele) was 1.50, which is significantly higher than that of DNA amplicons (ratio = 1.15; $P = .0495$, Mann-Whitney *U* test; Fig 3, *C*). Three independent experiments were performed with similar results. These results indicated that the higher expression of *NLRP3* mRNA was a component of the pathologic mechanisms leading to food-induced anaphylaxis.

DISCUSSION

The common feature of food-induced anaphylaxis and AIA is the immediate hypersensitivity reaction. In the present study we identified significant associations between human *NLRP3* polymorphisms and susceptibility to food-induced anaphylaxis and AIA. Because the 2 *NLRP3* SNPs rs4612666 and rs10754558 were not in strong LD (r^2<0.20), it is possible that the SNPs could contribute susceptibility to food-induced anaphylaxis independently. We further found a significant association between AIA and the rs4612666 variant, and the direction of association was similar to the finding in food-induced anaphylaxis. Functional analyses of the 2 related *NLRP3* polymorphisms showed that both variants influenced higher mRNA expression by altering expression enhancer activity or mRNA stability. These observations suggest that human NLRP3 appears to be involved in the hypersensitive immune reaction in allergy through gain-of-function variants.

Several recent studies have shown that nonsynonymous substitutions of *NLRP3* are associated with rare, severe autoinflammatory diseases, such as familial cold autoinflammatory syndrome, Muckle-Wells syndrome, and chronic infantile neurological, cutaneous, and articular syndrome.[19] However, these nonsynonymous substitutions and polymorphisms were not in LD with either of the SNPs associated with susceptibility to food-induced anaphylaxis and AIA in the present study ($r^2 < 0.20$).

In this study the 2 *NLRP3* SNPs associated with food-induced anaphylaxis did not show any association with susceptibility to food allergy. Food-induced allergic reactions exhibit various symptoms, ranging from localized urticaria to severe life-threatening anaphylaxis. In subjects with anaphylaxis caused by insect stings or food, many recent studies have shown no clear relationship between the levels of allergen-specific IgE and the presence, absence, or severity of the clinical response to the allergen.[16] Different genetic factors might be involved in the diverse immunologic responses to foods, and innate immune activation through NLRP3 inflammasomes sensing food components might be one of the immunologic mechanisms in anaphylaxis.

In the present study we found *NLRP3* polymorphisms that increased the risk of the hypersensitive phenotype of allergy. Murine studies have shown that the NLRP3 inflammasome is essential for the establishment of contact hypersensitivity, in which chemical damage to cells or tissues by a hapten is required for establishment of the allergy.[22,30] Recent reports have demonstrated that uric acid, calcium pyrophosphate dehydrate, silica, and asbestos particles activate the NLRP3 inflammasome.[31] Some components contained in food or food ingredients might become activators of the NLRP3 inflammasome and lead to caspase-1 activation to promote the processing and secretion of proinflammatory inflammatory cytokines. However, further analyses to clarify the relationship between NLRP3 inflammasomes and food components are needed. A recent study has reported that aspirin enhances allergic symptoms in patients with food-dependent exercise-induced anaphylaxis, the symptoms of which are severe allergic reactions, such as shock or hypotension.[32,33] The roles of aspirin and nonsteroidal anti-inflammatory drugs in the NLRP3 inflammasome response also remain unexplored.

NLRP3 is in the NLR family of proteins, and other NLR family genes have been shown to be associated with susceptibility to various inflammatory diseases: polymorphisms of nucleotide-binding oligomerization domain containing 1 *(NOD1)* with asthma[34] and inflammatory bowel disease,[35] nucleotide-binding oligomerization domain containing 2 *(NOD2)* with Crohn disease,[36] and *NLRP1* with vitiligo-associated multiple autoimmune disease.[37] Genetic studies on whether the polymorphisms of other NLR family genes are associated with food allergy, food-induced anaphylaxis, and AIA susceptibility remain to be conducted.

Further investigation of the roles of NALP3 inflammasomes in food-induced anaphylaxis and AIA might contribute to our understanding of the pathophysiology of these severe and potentially life-threatening systemic allergic reactions and to the development of novel diagnostic methods for risk assessment of patients with anaphylaxis or AIA.

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Key messages

- Gain-of-function variants of the *NLRP3* gene are associated with food-induced anaphylaxis and AIA.
- The NLRP3 inflammasome might play an important role in the hypersensitivity phenotype of allergy.

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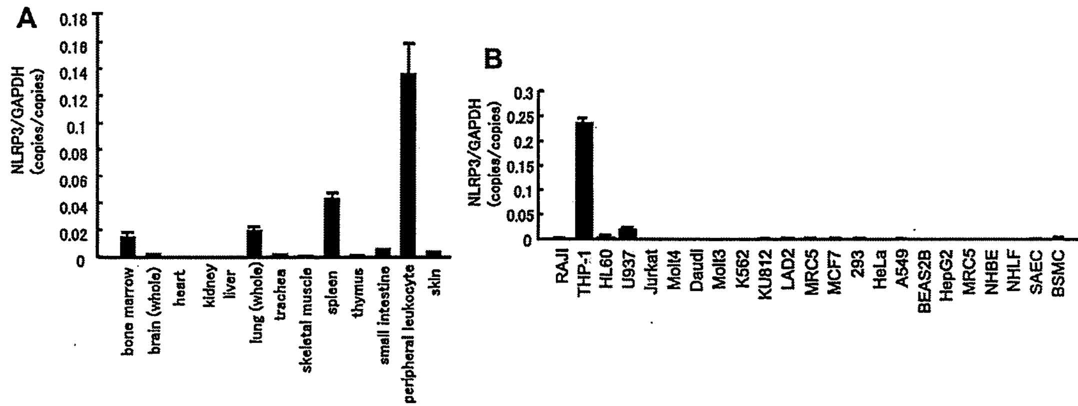

FIG E1. Comparison of relative mRNA expression of *NLRP3* in different tissues (A) and cell lines (B). The results were normalized to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* transcripts. *NHBE,* Normal human bronchial epithelial cells; *NHLF,* normal human lung fibroblasts; *SAEC,* normal human small airway epithelial cells; *BSMC,* bronchial smooth muscle cells. Results are means ± SDs of triplicate assays.

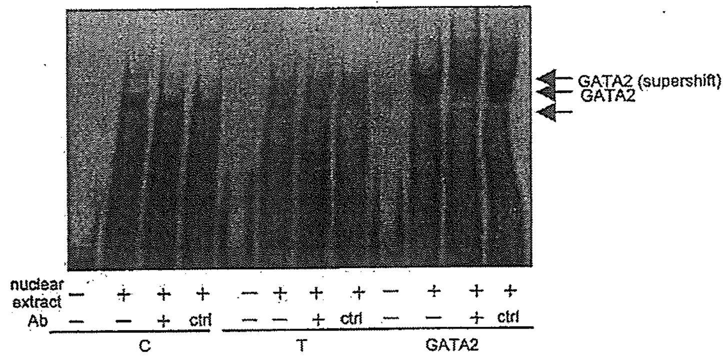

FIG E2. Electrophoretic mobility shift assay of rs4612666. Normal rabbit IgG and a GATA-2 consensus probe were used for control. Two independent experiments were performed with similar results. *Ab*, Antibody; *C*, C allele; *T*, T allele.

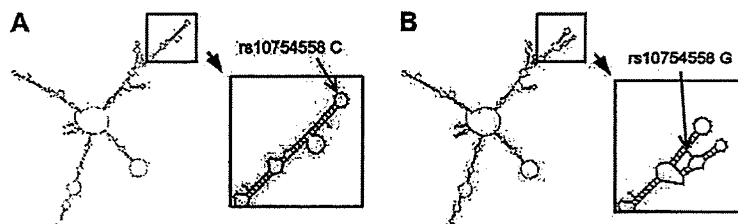

FIG E3. Prediction of the RNA secondary structure. Possible conformations of rs10754558-C (**A**) and rs10754558-G (**B**) and the most stable predicted structure show a dG (Gibbs free energy), which is a free energy increment related to the permissible structural transitions from the unstructured to structured state of −130.3 and −133.1 kcal/mol, respectively. *Arrows* indicate the region in which the rs10754558 SNP is located.

TABLE E1. Clinical information about patients with AIA and control subjects

	Patients with bronchial asthma		
Characteristics	**AIA (+), n = 79 (%)**	**AIA (−), n = 470 (%)**	**Control subjects, n = 730 (%)**
Age (y)	37.7 ± 14.2	37.0 ± 19.8	49.4 ± 14.5
Sex			
Male	25 (31.6)	205 (43.6)	532 (72.9)
Female	54 (68.4)	265 (56.4)	198 (27.1)
Log serum total IgE (IU/mL)	2.3 ± 0.5	2.3 ± 0.6	—

TABLE E2. *NLRP3* polymorphisms and susceptibility to food allergy

			Patients with food allergy, n = 320 (%)			Control subjects, n = 254 (%)			Frequency of allele 1		
Allele 1/2	dbSNP ID	Location	1/1	1/2	2/2	1/1	1/2	2/2	Case	Control	*P* value
−3656 C/T	rs2027432	5′ Flanking	247 (77.2)	69 (21.6)	4 (1.3)	197 (77.6)	53 (20.9)	4 (1.6)	0.88	0.88	NS
−2189 G/A	rs12079994	Intron 1	225 (71.2)	79 (25.0)	12 (3.8)	192 (75.6)	56 (22.0)	6 (2.4)	0.84	0.87	NS
−1529 C/T	rs4925648	Intron 1	247 (77.2)	71 (22.2)	2 (0.6)	193 (76.0)	56 (22.0)	5 (2.0)	0.88	0.87	NS
1979 G/A	rs4925650	Intron 3	89 (27.8)	169 (52.8)	62 (19.4)	68 (26.8)	123 (48.4)	63 (24.8)	0.54	0.51	NS
2495 A/G	rs12048215	Intron 3	181 (56.6)	124 (38.8)	15 (4.7)	148 (58.5)	88 (34.8)	17 (6.7)	0.76	0.76	NS
2547 C/G	rs10754555	Intron 3	119 (37.4)	162 (50.9)	37 (11.6)	101 (39.9)	120 (47.4)	32 (12.6)	0.63	0.64	NS
4240 T/C	rs3806265	Intron 3	105 (33.2)	158 (50.0)	53 (16.8)	95 (37.4)	121 (47.6)	38 (15.0)	0.58	0.61	NS
13754 C/T	rs10925019	Intron 6	163 (50.9)	131 (40.9)	26 (8.1)	145 (57.1)	89 (35.0)	20 (7.9)	0.71	0.75	NS
13961 G/A	rs4925654	Intron 6	211 (65.9)	96 (30.0)	13 (4.1)	154 (60.6)	87 (34.3)	13 (5.1)	0.81	0.78	NS
16974 C/T	rs4612666	Intron 7	100 (31.5)	155 (48.9)	62 (19.6)	95 (37.5)	119 (47.0)	39 (15.4)	0.56	0.61	NS
22162 A/C	rs10925026	Intron 8	110 (34.6)	160 (50.3)	48 (15.1)	100 (39.7)	106 (42.1)	46 (18.3)	0.60	0.61	NS
27232 C/T	rs12565738	Intron 10	257 (80.3)	59 (18.4)	4 (1.3)	197 (78.2)	55 (21.8)	0 (0.0)	0.90	0.89	NS
29231 T/C	rs4378247	Intron 10	265 (82.8)	53 (16.6)	2 (0.6)	206 (81.1)	47 (18.5)	1 (0.4)	0.91	0.90	NS
29940 C/G	rs10754558	Exon 11 (3′UTR)	108 (34.3)	155 (49.2)	52 (16.5)	92 (36.4)	114 (45.1)	47 (18.6)	0.59	0.59	NS
31792 C/T	rs10733112	3′ Flanking	81 (25.3)	168 (52.5)	71 (22.2)	73 (28.7)	118 (46.5)	63 (24.8)	0.52	0.52	NS

NS, Not significant; *UTR*, untranslated region.

TABLE E3. Association between *NLRP3* polymorphisms and susceptibility to AIA

	Allele 1/2	dbSNP ID	Location	AIA (+), n = 79 (%)			AIA (−), n = 470 (%)			Control, n = 730 (%)		
				1/1	1/2	2/2	1/1	1/2	2/2	1/1	1/2	2/2
1	1979 G/A	rs4925650	Intron 3	22 (27.8)	38 (48.1)	19 (24.1)	108 (23.3)	240 (51.8)	115 (24.8)	204 (28.1)	346 (47.6)	177 (24.3)
2	4240 T/C	rs3806265	Intron 3	36 (45.6)	34 (43.0)	9 (11.4)	174 (38.1)	209 (45.7)	74 (16.2)	290 (39.9)	331 (45.5)	106 (14.6)
3	16974 C/T	rs4612666	Intron 7	41 (51.9)	30 (38.0)	8 (10.1)	174 (37.7)	215 (46.6)	72 (15.6)	268 (36.9)	341 (46.9)	118 (16.2)
4	29940 C/G	rs10754558	Exon 11 (3′UTR)	22 (28.2)	35 (44.9)	21 (26.9)	146 (31.4)	231 (49.7)	88 (18.9)	229 (31.5)	360 (49.5)	139 (19.1)
5	31792 C/T	rs10733112	3′ Flanking	32 (41.0)	30 (38.5)	16 (20.5)	136 (29.5)	218 (47.3)	107 (23.2)	211 (29.0)	346 (47.6)	170 (23.4)

	Frequency of allele 1			AIA (+) vs AIA (−)				AIA (+) vs control			
	AIA (+)	AIA (−)	Control	*P* value	OR	95% CI		*P* value	OR	95% CI	
1	0.52	0.49	0.52	NS				NS			
2	0.67	0.61	0.63	NS				NS			
3	0.71	0.61	0.60	.018	1.55	1.08	2.24	.0096	1.60	1.12	2.29
4	0.51	0.56	0.56	NS				NS			
5	0.60	0.53	0.53	NS				NS			

UTR, Untranslated region; *NS*, not significant.

A Functional Polymorphism in *IL-18* Is Associated with Severity of Bronchial Asthma

Michishige Harada[1*], Kazuhiko Obara[2*], Tomomitsu Hirota[1], Tomohiro Yoshimoto[3], Yuki Hitomi[1], Masafumi Sakashita[1], Satoru Doi[4], Akihiko Miyatake[5], Kimie Fujita[6], Tadao Enomoto[7], Masami Taniguchi[8], Noritaka Higashi[8], Yuma Fukutomi[8], Kenji Nakanishi[3], Yusuke Nakamura[9], and Mayumi Tamari[1]

[1]Laboratory for Respiratory Diseases, Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN), Kanagawa; [2]Hitachi Chemical Co., Ltd., Hitachi, Ibaraki; [3]Department of Immunology and Medical Zoology, Hyogo College of Medicine, Hyogo; [4]Department of Pediatric Allergy, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Osaka; [5]Miyatake Asthma Clinic, Osaka; [6]School of Human Nursing, The University of Shiga Prefecture, Shiga; [7]NPO Japan Health Promotion Supporting Network, Wakayama; [8]Clinical Research Center, National Hospital Organization, Sagamihara National Hospital, Kanagawa; and [9]Laboratory of Molecular Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Rationale: IL-18 is a unique cytokine that enhances innate immunity and both Th1- and Th2-driven immune responses. Recent murine and human genetic studies have shown its role in the pathogenesis of asthma.

Objectives: We conducted an association study in a Japanese population to discover variants of *IL-18* that might have an effect on asthma susceptibility and/or progression and conducted functional analyses of the related variants.

Methods: The *IL-18* gene locus was resequenced in 48 human chromosomes. Asthma severity was determined according to the 2002 Global Initiative for Asthma Guidelines. Association and haplotype analyses were performed using 1,172 subjects.

Measurements and Main Results: Although no polymorphisms differed significantly in frequency between the control and adult asthma groups, rs5744247 C>G was significantly associated with the severity of adult asthma (steps 1, 2 vs. steps 3, 4; $P = 0.0034$). We also found a positive association with a haplotype ($P = 0.0026$). By *in vitro* functional analyses, the rs5744247 variant was found to increase enhancer-reporter activity of the *IL-18* gene in bronchial epithelial cells. Expression levels of *IL-18* in response to LPS stimulation in monocytes were significantly greater in subjects homozygous for the susceptibility G allele at rs5744247 C>G. Furthermore, we found a significant correlation between the serum IL-18 level and the genotype of rs5744247 ($P = 0.031$).

Conclusions: Although the association results need to be replicated by other studies, *IL-18* variants are significantly associated with asthma severity, and the rs5744247 variant reflects higher transcriptional activity and higher expression of *IL-18* in LPS-stimulated monocytes and a higher serum IL-18 level.

Keywords: asthma severity; IL-18; LPS; monocytes; genetic polymorphisms

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

IL-18 plays multiple roles in chronic inflammation and in a number of infections and enhances both Th1- and Th2-mediated immune responses. The influence of genetic changes in this crucial cytokine on the etiology of asthma is unclear.

What This Study Adds to the Field

Our results suggest that a functionally relevant IL-18 polymorphism contributes to the disease severity of asthma. The variant affects the level of mRNA expression induced by LPS in human monocytes and correlates with the serum IL-18 level in individuals with asthma. The LPS-induced IL-18 expression was not suppressed by dexamethasone and salmeterol.

Bronchial asthma is a complex disorder caused by a combination of genetic and environmental factors (1, 2). Cytokines recruit and activate immune cells and play an important role in the coordination and persistence of the airway inflammation of asthma (3, 4). IL-18 is produced by both immune and nonimmune cells, such as peripheral blood mononuclear cells and bronchial epithelial cells, and plays multiple roles in chronic inflammation and in a number of infections and enhances both Th1- and Th2-mediated immune responses (5). Although originally discovered as a factor that induced IFN-γ production from Th1 cells (5), IL-18 also has the potential to induce IL-4 and IL-13 production in T cells, natural killer (NK) cells, NK T cells, mast cells, and basophils (6–9).

Th2-type airway inflammation is a characteristic feature of bronchial asthma; however, important roles of IFN-γ in allergic inflammation have been shown in recent reports (10–16). Intranasal administration of an antigen and IL-18 stimulates Th1 cells to induce severe airway inflammation through IFN-γ and IL-13 in a murine model (11, 12). In humans, IFN-γ production by peripheral blood T cells is associated with the alteration of lung function in individuals with chronic stable asthma (13). Overproduction of IFN-γ has been observed in asthma, and the number of IFN-γ–producing $CD8^+$ T cells is related to asthma severity (14). Furthermore, both Th-1 and Th-2 chemokines and cytokines are involved in antigen-induced airway inflammation by segmental allergen bronchoprovocation related to disease severity (15, 16). These findings imply that Th-1 responses together with Th-2 responses cause severe allergic inflammation, and a polymorphism of the *IL-18* gene might be a genetic marker of asthma and disease severity.

Several association studies using polymorphic markers of the *IL-18* gene have been performed to discover genetic compo-

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* These authors contributed equally to this article.

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Correspondence and requests for reprints should be addressed to Mayumi Tamari, M.D., Ph.D., Laboratory for Respiratory Diseases, Center for Genomic Medicine, Institute of Physical and Chemical Research (RIKEN); 1-7-22 Suehiro, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan. E-mail: tamari@src.riken.jp

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nents in the pathogenesis of bronchial asthma (17–21). In this study, we focused on the *IL-18* gene, resequenced the gene regions including introns, performed linkage disequilibrium (LD) mapping, and conducted an association study and functional analyses of the related variants. Increased *IL-18* expression and serum levels in human allergic diseases have been reported (22–25). Here we measured serum IL-18 levels of patients with adult asthma and examined the correlation between the IL-18 level and related genotype.

Some of the results of these studies have been previously reported in the form of an abstract (26).

METHODS

Additional details on methods are provided in the online supplement.

Study Subjects

All subjects with asthma were diagnosed according to the American Thoracic Society criteria as described (27–29). We recruited 453 adults with asthma and recorded the age, sex, serum total IgE level, eosinophil count, lung functions, and clinical severity (Table 1). The clinical severity of adult asthma was classified according to the criteria of the National Institutes of Health/Global Initiative for Asthma 2002 by physicians who were experts in allergic diseases, and was defined by controller medication use at the time of entry into the study (30). Genomic DNA was prepared in accordance with standard protocols.

Screening for Polymorphisms and Genotyping

We resequenced the *IL-18* gene regions in 48 human chromosomes from 24 control subjects (*see* Table E1 in the online supplement). Pairwise LD was calculated as D′ and r^2 by using the Haploview 4.1 program (http://www.broad.mit.edu/mpg/haploview/). The polymorphisms were genotyped by use of the TaqMan system (Applied Biosystems, Foster City, CA).

Cells, Reagents, and Stimulation

Normal human bronchial epithelial cells (NHBE) (n = 4, aged 17 to 58 yr, white male), normal human lung fibroblasts (NHLF) (n = 1, aged 10 yr, white male) and bronchial smooth muscle cells (BSMC) (n = 1, aged 63 yr, white male) were purchased and maintained using Clonetics medium kits (Lonza Walkersville, Inc, Walkersville, MD). Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of healthy Japanese volunteers (aged 32 to 46 yr) by magnetic activated cell sorting according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stimulated with the indicated concentrations of poly(I:C) (InvivoGen, San Diego, CA), LPS (InvivoGen), and macrophage-activating lipopeptide 2 (Alexis, Lausen, Switzerland). PBMCs, $CD4^+$ T cells, and $CD8^+$ T cells were stimulated by plate-bound anti-CD3 monoclonal antibodies (incubated at 1 μg/ml, clone number UCHT1) with soluble anti-CD28 monoclonal antibodies (1 μg/ml, clone number CD28.2). NHBE and monocytes were also cultured with dexamethasone (DEX) (ICN Biomedicals, Costa Mesa, CA) and/or salmeterol (SAL) (TOCRIS Inc., Ellisville, MO).

Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction, ELISA, and Luciferase Assay

The expression of *IL-18* and *IL-6* was determined by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) using SYBR Premix Ex Taq (Takara, Shiga, Japan). Concentrations of IL-18 were measured in duplicate with a human-specific IL-18 ELISA kit (MBL, Nagoya, Japan) (31). Luciferase assays were conducted using pGL3-enhancer vector (Promega, Madison, WI).

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, we compared differences in the allele frequency and genotype distribution of each polymorphism by using a contingency χ^2 test or Fisher exact test. We applied Bonferroni corrections, the multiplication of *P* values by three, the number of Tag single nucleotide polymorphisms (SNP)s. In the association study, corrected *P* values of less than 0.05 were judged to be significant. All tests were two-sided and odds ratios (ORs) with 95% confidence intervals (CIs) were also calculated.

Haplotype frequencies for multiple loci were estimated, and haplotype association tests were performed using Haploview 4.1. Serum total IgE levels, eosinophil counts, FVC (% predicted) and FEV_1 (% predicted) were analyzed as quantitative levels by the Kruskal-Wallis test, Friedman test, or Mann-Whitney *U* test. The Jonckheere-Terpstra trend test was used for *IL-18* genotype–phenotype correlation analyses. Comparison in expression analysis was performed with Student *t* test. A *P* value of less than 0.05 was considered statistically significant.

TABLE 1. BASELINE CHARACTERISTICS AMONG STUDY PARTICIPANTS WITH ASTHMA

Characteristics	Values
No. of Subjects	453
Age, yr, (range), mean ± SD	20–75 (49.7 ± 14.6)
Male (%)	43
Asthma characteristics, mean ± SD	
Serum total IgE, IU/ml	658 ± 1,550
Eosinophils, no./μl	411 ± 387
Asthma severity, n (%)	
Step 1, mild intermittent	11 (2.4)
Step 2, mild persistent	237 (52.3)
Step 3, moderate persistent	122 (26.9)
Step 4, severe persistent	83 (18.3)
Pulmonary function, mean ± SD	
FVC, % predicted	85 ± 20
FEV_1, % predicted	71 ± 21
FEV_1/FVC % ratio	70 ± 12

RESULTS

Fine Mapping of *IL-18* and Identification of *IL-18* Polymorphisms Associated with Asthma Severity

After extensive examination of *IL-18* by direct sequencing, we identified 18 polymorphisms (8 SNPs in the promoter region and 4 SNPs within the transcript) (Table 2, Figure 1). To examine the LD between identified SNPs, pairwise LD coefficients D′ and r^2 were calculated using the Haploview 4.1 program. Because five of the SNPs were quite rare, pairwise LD was measured by D′ and r^2 among the 13 SNPs with minor allele frequencies of greater than 5% (Table 2 and Table E2, Figure E1). One SNP, -9731T>G (rs1946519), was in complete LD (D′ = 1.00 and r^2 = 1.00) with -9682A>C (rs1946518) and in strong LD (D′ = 1.00 and r^2 = 0.84) with -12310C>T (rs2904613). Another SNP, -8963T>G (rs360718), was in complete LD (D′ = 1.00 and r^2 = 1.00) with -11877C>T (rs11214105), -11778G>T (rs1290349), -11608T>C (rs1293344), -9212G>C (rs187238), and -8949C>T (rs360717) and in strong LD (D′ = 1.00 and r^2 = 0.87) with -140C>G (rs360721) and 4861A>C (rs549908). The -380C>G (rs5744247) variant was in strong LD (D′ = 1.00 and r^2 = 0.84) with 1867->insertion C (rs5744252). We selected three tag SNPs, -9731T>G (rs1946519), -8963T>G (rs360718), and -380C>G (rs5744247), for association studies using tagger in the Haploview 4.1 program, and these three SNPs captured 13 of the 13 alleles with a mean r^2 of 0.955 (r^2 > 0.84) (Table E2, Figure E1). We also genotyped all 18 polymorphisms. The 18 SNPs were successfully genotyped in more than 96% of the people studied, and were in Hardy-Weinberg equilibrium (Table E3).

TABLE 2. FREQUENCIES OF POLYMORPHISMS OF THE *IL-18* GENE IN A JAPANESE POPULATION

SNP*	NCBI[†]	Position in the gene structure	mRNA	MAF[‡]
-12561C>T		5′ flanking region		0.02
-12310C>T	rs2904613	5′ flanking region		0.40
-11877C>T	rs11214105	5′ flanking region		0.19
-11778G>T	rs1290349	5′ flanking region		0.19
-11608T>C	rs1293344	5′ flanking region		0.19
-9731T>G[§]	rs1946519	5′ flanking region		0.44
-9682A>C	rs1946518	5′ flanking region		0.44
-9212G>C	rs187238	5′ flanking region		0.19
-8963T>G[§]	rs360718	exon 1	5′UTR	0.19
-8949C>T	rs360717	exon 1	5′UTR	0.19
-380C>G [§]	rs5744247	intron 1		0.37
-140C>G	rs360721	intron 1		0.17
-139A>G	rs4988359	intron 1		0.04
-109A>G	rs12721559	intron 1		0.02
1867-> ins. C	rs5744252	intron 3		0.37
1950G>C	rs1834481	intron 3		0.04
4861A>C	rs549908	exon 4	Ser35Ser	0.17
11641A>G	rs5744292	exon 6	3′UTR	0.04

Definition of abbreviations: dbSNP = single nucleotide polymorphism database; NCBI = National Center for Biotechnology Information; MAF = minor allele frequencies; SNP = single nucleotide polymorphism; UTR = untranslated region.
* Numbering according to the genomic sequence of *IL-18* (NT_033899.7). Position 1 is the A of the initiation codon.
† NCBI, number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/)
‡ MAF in the screening population (n = 24).
§ SNPs were genotyped in this study.

The results for genotype frequencies in the group with asthma and the control group are shown in Table E3. None of the three SNPs tested in this study showed a significant association with adult asthma. We next surveyed associations between the SNPs and disease severity. The distribution of subjects was as follows: step 1, mild intermittent 2.4% (11 individuals); step 2, mild persistent 52.3% (237 individuals); step 3, moderate persistent 26.9% (122 individuals); and step 4, severe persistent 18.3% (83 individuals). Because the step 1 subgroup comprised 11 patients (2.4%) and step 4 subgroup comprised 83 patients (18.3%), we divided the subjects with asthma into two groups, steps 1 and 2 versus steps 3 and 4 by sample size (54.7 vs. 45.3%). The results for genotype frequencies are shown in Table 3 and Table E4. We found a significant association between the -380C>G (rs5744247) genotype and asthma severity (allelic model, P = 0.0034, corrected P = 0.010; OR, 1.49; 95% CI, 1.14–1.94; recessive model, P = 0.012, corrected P = 0.036; OR, 1.79; 95% CI, 1.14–2.74). The -9731T>G (rs1946519) polymorphism also showed a significant association with asthma severity (allelic model, P = 0.0078, corrected P = 0.023; OR, 0.69; 95% CI, 0.53–0.91; dominant model, P = 0.0077, corrected P = 0.023; OR, 0.59; 95% CI, 0.40–0.87). However, the -8963T>G SNP (rs360718) was not associated with asthma severity. Rs 2904613 and rs1946518, in strong LD with rs1946519 (r^2 = 0.84 and 1.0, respectively), and rs5744252, in strong LD with 5744247 (r^2 = 0.84), also showed similar results (Table E4).

In addition, we surveyed associations between the three SNPs and patients with asthma who had high eosinophil counts and high serum IgE levels as quantitative phenotypes. However, we could not find any association between the three SNPs and eosinophil counts or total serum IgE levels.

Associations of *IL-18* Haplotypes with Asthma Severity

We next constructed the haplotypes of the three SNPs and estimated the frequency of each haplotype in the step 1, 2 and step 3, 4 adult asthma groups (Table E5). We identified three common haplotypes covering more than 99% of the population in both groups, and found a positive association with a haplotype of *IL-18* in adult asthma severity (χ^2 = 9.07, P = 0.0026) (haplotype T-T-G [-9731T, -8963T, and -380G] versus others) using the Haploview 4.1 program.

IL-18 mRNA Is Highly Expressed in Bronchial Epithelial Cells and rs5744247 Increases Transcriptional Activity

We next investigated whether *IL-18* mRNA was up-regulated by Toll-like receptor (TLR) ligands in cultured NHBE, NHLF, and BSMC. Although *IL-18* was expressed in NHBE, it was barely expressed in NHLF and BSMC (Figure 2A). Furthermore, *IL-18* mRNA expression in NHBE was not induced by stimulation with LPS, poly(I:C), and macrophage-activating lipopeptide-2 for 4 hours (Figure 2A) or 24 hours (data not shown).

To clarify whether the SNPs in the intronic region associated with asthma severity affected the expression of *IL-18*, we constructed plasmid clones containing genomic DNA fragments corresponding to these SNPs. PCR products were subcloned into the upstream or downstream regions of the luciferase gene in the pGL3-enhancer vector (Figure 2B). We compared enhancer-like effects of sequences containing the intron 1 -380C>G (rs5744247) or intron 3 1867->insertion C (rs5744252) SNPs in NHBE (Figure 2B). The clone containing the susceptible -380G (rs5744247) showed significantly greater transcriptional activity than the other allele, -380C, when the genomic DNA fragments were inserted downstream of the luciferase gene in the vector (Figure 2B). In contrast, the reporter activities of clones of the intron 3 SNP (rs5744252) had no effect on transcriptional activity (Figure 2B).

Inhaled corticosteroids (ICS) are widely used as first-line therapeutic agents in patients with inflammatory lung diseases such as asthma (32), and patients with severe asthma need higher-dose ICS and oral steroids to control their symptoms. In addition, recent studies have shown that the combination of an

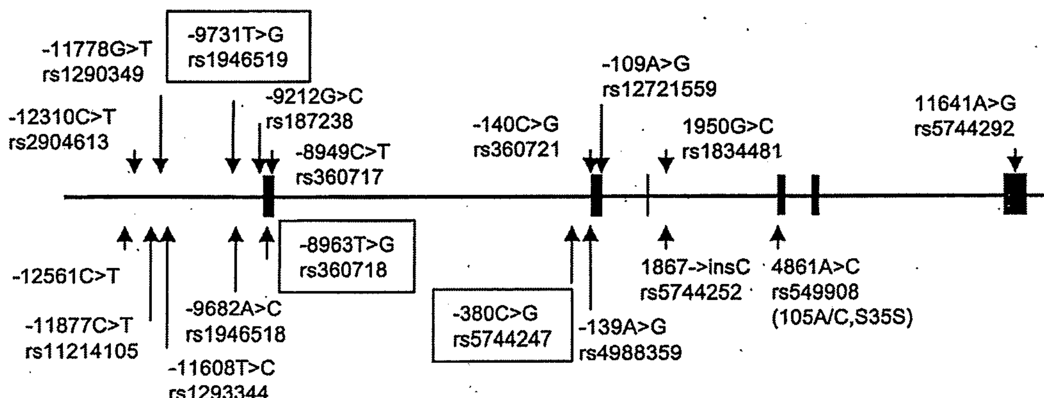

Figure 1. A graphical overview of polymorphisms identified in relation to the exon/intron structure of the human *IL-18* gene. Six exons are shown by black boxes, and positions for polymorphisms are relative to the translation start site (+1). Polymorphisms enclosed within boxes were genotyped in the whole samples.

TABLE 3. GENOTYPE FREQUENCIES FOR *IL-18* SINGLE NUCLEOTIDE POLYMORPHISMS AND ASTHMA SEVERITY

Genotype	Steps 1,2 n = 248 (%)	Steps 3,4 n = 205 (%)	Allele	Steps 1,2 n = 248 (%)	Steps 3,4 n = 205 (%)	P Value and ORs (95% CI)			
						Genotype	Dominant	Recessive	Allelic
rs1946519									
TT	77 (31)	89 (44)	T	268 (55)	259 (63)	0.026	0.0077	0.153	0.0078
TG	114 (47)	81 (40)	G	222 (45)	149 (37)		0.59		0.69
GG	54 (22)	34 (17)					0.40–0.87		0.53–0.91
rs360718									
TT	182 (73)	159 (78)	T	427 (86)	359 (88)	0.266	0.305	0.323	0.515
TG	63 (25)	41 (20)	G	69 (14)	51 (12)				
GG	3 (1)	5 (2)							
rs5744247									
CC	87 (35)	51 (25)	C	294 (59)	200 (50)	0.014	0.024	0.012	0.0034
CG	120 (48)	98 (49)	G	202 (41)	204 (50)		1.60	1.79	1.49
GG	41 (17)	53 (26)					1.06–2.41	1.14–2.74	1.14–1.94

Definition of abbreviations: CI = confidence interval; OR = odds ratio.

inhaled corticosteroid and long-acting β_2-agonist is more efficacious in asthma than either alone (33, 34). Another study has shown synergistic suppression of virus-induced chemokines in airway epithelial cells by combination therapy (35). We next investigated the effects of DEX and SAL on the expression of *IL-18*. We examined effects of DEX and SAL on *IL-18* mRNA expression levels in NHBE. The expression of *IL-18* was not affected by DEX or SAL (Figure 2C).

IL-18 mRNA Is Highly Expressed in NK Cells, Dendritic Cells, and Monocytes and Is Highly Induced by LPS in Human Monocytes

We further investigated whether *IL-18* mRNA was up-regulated by TLR ligands in immune blood cells, using quantitative real-time PCR. *IL-18* mRNA was highly expressed in nonstimulated NK cells, dendritic cells, and monocytes as compared with $CD4^+$ or $CD8^+$ T cells and B cells (Figure 3A). After stimulation, levels of *IL-18* mRNA in monocytes were increased threefold in response to LPS (Figure 3A).

Polymorphism rs5744247 Is Associated with *IL-18* mRNA Expression Level in LPS-Stimulated Monocytes

To investigate whether the -380C>G (rs5744247) polymorphism affected the mRNA levels of *IL-18*, we measured relative mRNA expression and compared it in subjects with different genotypes. We isolated monocytes from PBMCs of a total of nine healthy volunteers, five of whose genotypes were homozygous for -380C and four of whose were homozygous for -380G, and stimulated them with 5 μg/ml LPS. After LPS stimulation, we found significant increases in both *IL-18* (-380C group, $P = 0.0044$; -380G group, $P = 0.011$) and *IL-6* (-380C group, $P = 0.0036$; -380G group, $P = 0.014$) mRNAs within each group by the Friedman test. Monocytes homozygous for the -380G allele exhibited significantly higher expression of *IL-18* mRNA in response to LPS for 1.5, 3, and 6 hours ($P =$ 0.014, 0.014, and 0.033, respectively, by the Mann-Whitney *U* test); however, this genotype effect was not observed for *IL-6* mRNA induction (Figure 3B). A recent study has shown antiinflammatory effects of SAL after inhalation of LPS in humans (36). However, a combination effect of glucocorticoid and SAL on inflammatory cytokines in response to LPS has not been reported. We next investigated the effects of DEX and SAL on the induction of *IL-18* mRNA by LPS in monocytes. Although the addition of DEX with/without SAL to the medium with LPS significantly reduced induction of *IL-6* mRNA, DEX and SAL did not affect the level of induction of *IL-18* mRNA (Figure 3C).

Polymorphism rs5744247 Is Associated with Serum IL-18 Level

To evaluate whether the serum IL-18 protein level correlated with the *IL-18* genotype, we conducted ELISA assays of sera of 88 patients with asthma. Baseline characteristics among serum study participants are shown in Table E6. We compared the distribution of severity between subjects with serological studies and that with genetic studies by the Mann-Whitney *U* test. In the subgroup examined for serum IL-18, asthma tended to be more severe than in the genotyped group ($P = 0.023$; Table E7). The Jonckheere-Terpstra trend test is a nonparametric trend statistic to test for ordered differences among groups assumed to be arranged ordinally. The test is superior to the Kruskal-Wallis procedure when the conjectured ordering of the genotype effects is, indeed, appropriate (37). Serum levels of the IL-18 protein positively correlated with the *IL-18* genotype in the Jonckheere-Terpstra trend test ($P = 0.031$; Figure 4).

DISCUSSION

In this study we identified 18 polymorphisms, conducted LD mapping of the gene region, and found significant associations between polymorphisms and asthma severity. Several genetic studies have already surveyed the genes involved in the IL-18 signaling pathway as candidate genes for asthma. For the *IL-18* receptor and related molecules, a recent study has shown significant replicated associations between polymorphisms in the *IL18R1* gene and asthma, atopic asthma, and bronchial hyperreactivity (38). Another study, in which adult asthma probands aged 18.1–64.7 years were examined, has reported associations of *IL1RL1*, *IL18R1*, and *IL18RAP* gene cluster polymorphisms with asthma and atopy in a Dutch population (21). Several association studies of the *IL-18* gene have also been conducted. Five polymorphisms, rs1946518, rs187238, rs360718, rs360717, and rs360721, located in the promoter and exon 1 region, were screened in a cohort of 228 children with asthma, but no polymorphism showed a significant difference in frequency between the case and control groups (17). The association study did not contain adult subjects with asthma and did not examine associations between the SNPs and asthma severity. Among the five SNPs, we found a significant association between rs1946518 and asthma severity. The recent SAPALDIA Cohort study using a Swiss population (mean ± SD, 41.2 ± 11.4) has shown associations between the *IL-18* promoter variant -137G>C and atopic asthma (19). SNP rs187238 (-137G>C) was not associated with either adult asthma susceptibility or severity in our study. The proportion