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

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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.

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Supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan.

Disclosure of potential conflict of interest: Y. Hitomi, M. Ebisawa, Y. Nakamura, and M. Tamari have applied for a patent in Japan relevant to the *NLRP3* function single nucleotide polymorphism. The rest of the authors have declared that they have no conflict of interest.

Received for publication April 13, 2009; revised July 6, 2009; accepted for publication July 21, 2009.

Available online September 22, 2009.

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0091-6749/$36.00

doi:10.1016/j.jaci.2009.07.044

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			Control subjects, n = 254 (%)
	Total, n = 320 (%)	Anaphylaxis (+), n = 98 (%)	Anaphylaxis (−), n = 222 (%)	
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

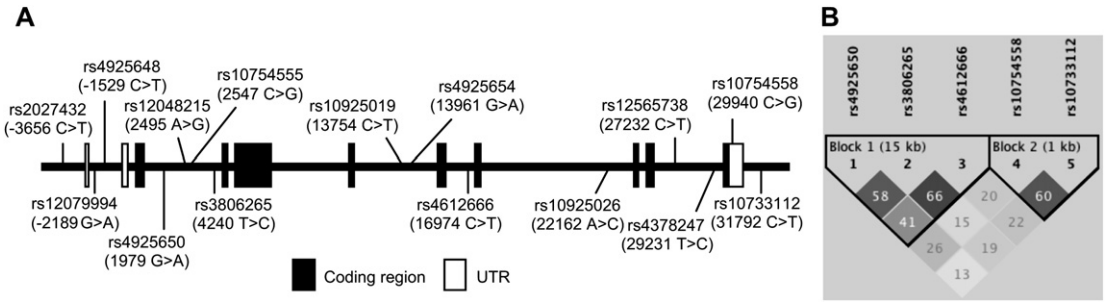

FIG 1. A, Exon–intron structure of the *NLRP3* gene and locations of genotyped tag SNPs. *UTR*, Untranslated region. **B**, LD structures of the 5 significantly associated *NLRP3* SNPs with susceptibility to food-induced anaphylaxis ($P < .005$). LD was calculated by using genotyping data. Pairwise r^2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

			No.		Frequency	
	rs4612666	rs10754558	Case	Control	Case	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			

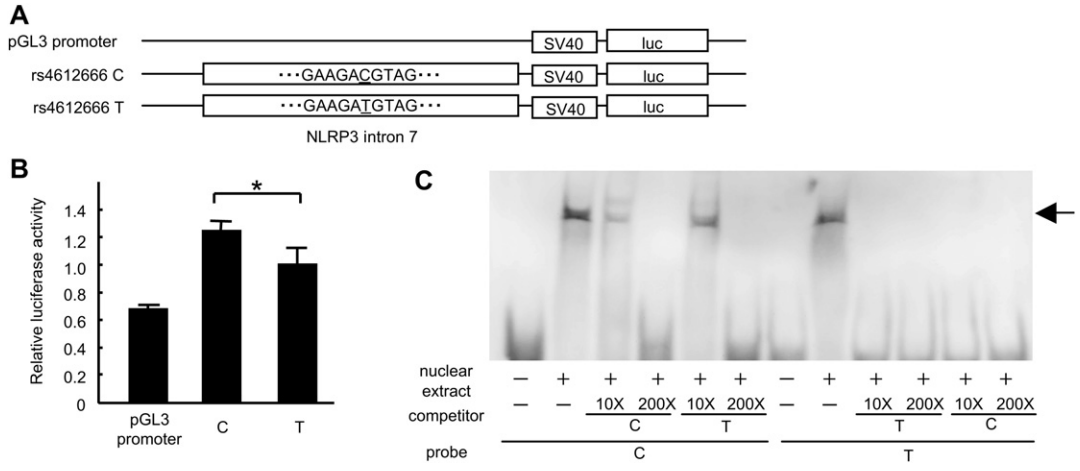

FIG 2. Functional analyses of *NLRP3* rs4612666 using THP-1 cells. **A**, Plasmid constructs used for transfection. **B**, Transcriptional enhancer activities of rs4612666 measured by luciferase *(luc)* activity 24 hours after transfection. Values of relative luciferase activity are shown as means±SDs. **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

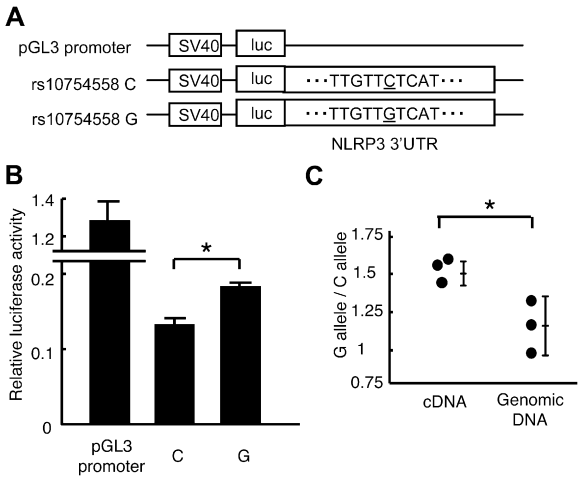

FIG 3. Functional analyses of *NLRP3* rs10754558 using THP-1 cells. **A**, Plasmid constructs used for transfection. *UTR*, Untranslated region. **B**, Effect of the SNP on mRNA stability as measured by luciferase activity. Values of relative luciferase activity are shown as means±SDs. *$P < .0005$, 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.

We thank all the participants in the study. We also thank Dr Akari Suzuki for valuable suggestions and Makiko Shimizu-Terada, Hiroshi Sekiguchi, Nami Kawaraichi, and Aya Jodo-Ito for technical assistance.

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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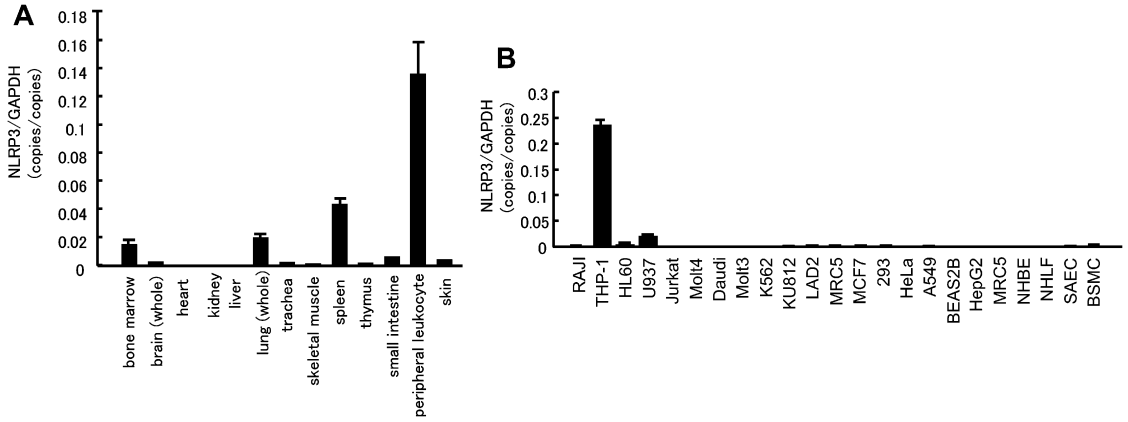

FIG E1. Comparison of relative mRNA expression of *NLRP3* in different tissues **(A)** and cell lines **(B)**. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* transcripts. *NHBE*, Normal human bronchial epithelial cells; *NHLF*, normal human lung fibroblasts; *SAEC*,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

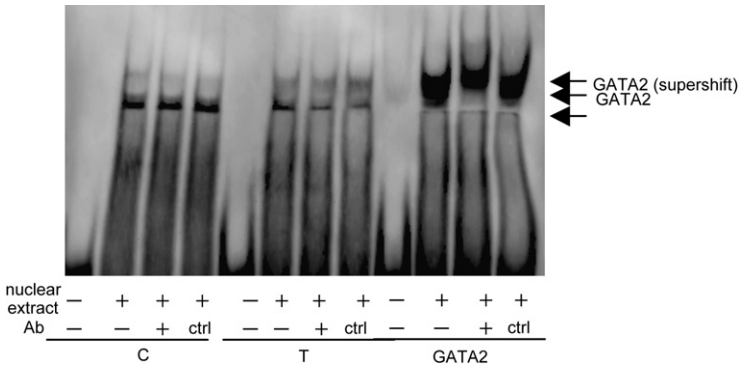

FIG E2. Electrophoretic mobility shift assay of rs4612666. Normal rabbit IgG and a GATA-2 consensus probe were used for control. Two independent experiments were performed with similar results. *Ab*, Antibody; *C*, C allele; *T*, T allele.

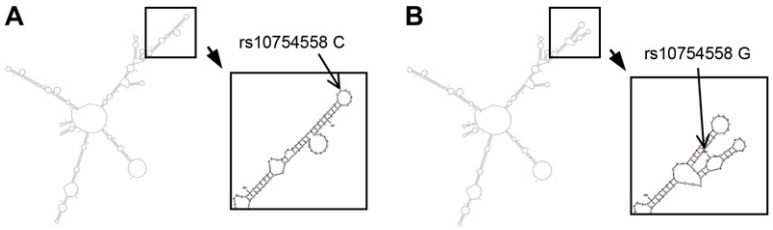

FIG E3. Prediction of the RNA secondary structure. Possible conformations of rs10754558-C **(A)** and rs10754558-G **(B)** and the most stable predicted structure show a dG (Gibbs free energy), which is a free energy increment related to the permissible structural transitions from the unstructured to 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

Characteristics	Patients with bronchial asthma		Control subjects, n = 730 (%)
	AIA (+), n = 79 (%)	AIA (−), n = 470 (%)	
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.