

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 ΔG (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

Allele 1/2	dbSNP ID	Location	Patients with food allergy, n = 320 (%)			Control subjects, n = 254 (%)			Frequency of allele 1		P value
			1/1	1/2	2/2	1/1	1/2	2/2	Case	Control	
-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.

Association study of the *C3* gene with adult and childhood asthma

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Abstract Bronchial asthma (BA) is a multifactorial disorder, the development of which is affected by both environmental and genetic factors. The complement system plays an important role in immunological response against invading microorganisms. It has been shown that complement-C3-deficient mice have reduced inflammation of asthmatic airways. Previously, we reported the association of four single nuclear proteins (SNPs) in the exons of the *C3* gene with childhood and adult BA. The *C3* gene, however, is a large gene, and functional SNPs associated with susceptibility to BA have not yet been identified. We analyzed

26 SNPs in the *C3* gene and its promoter region to narrow down the regions showing association with childhood and adult BA. Childhood and adult atopic BA patients and healthy child and adult controls were recruited from urban cities in Japan and genotyped. In SNP analysis, an SNP (SNP24, rs11569562) located in intron 31 of the *C3* gene was associated with adult BA [corrected P (P_{cor}) = 0.030]. In linkage disequilibrium (LD) block 4 spanning exons 24–41, the frequency of the CCC haplotype in adult BA was significantly higher than that in adult controls (P_{cor} = 0.038). Neither the SNP nor the haplotype showing association with adult BA demonstrated a significant association with serum total immunoglobulin E (IgE) level in BA patients and controls. Our results suggest that LD block 4 confers susceptibility to adult BA with mechanisms relevant to the effector phase of allergic inflammation.

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Introduction

Bronchial asthma (BA) is an inflammatory airway disease, the development of which is affected by both environmental and genetic factors (Barnes and Marsh 1998). The complement system belongs to the groups of ancient pattern-recognition systems and sensing exogenous (microorganisms) and endogenous (altered-self) danger-associated molecular patterns (Kohl and Wills-Karp 2007). The three pathways of complement activation are the classical, alternative, and lectin pathways, and all complement activation pathways converge at C3. On activation, C3 breaks into a large C3b fragment and a small C3a fragment that is called anaphylatoxin. C3b can

form C5 convertase and activate another component of the complement, C5. C5 is cleaved into C5a anaphylatoxin and C5b, and both fragments exert physiological roles. Both anaphylatoxins also show proinflammatory and immunoregulatory actions. It has been shown that complement-C3-deficient mice have reduced inflammation of asthmatic airways (Barrington et al. 2001; Kohl 2001; Muller-Eberhard 1988). Mice and guinea pigs lacking the C3a receptor (C3aR) essential for C3a action were protected against bronchoconstriction, airway hyperresponsiveness (AHR), and airway inflammation developing after allergen challenge (Bautsch et al. 2000; Drouin et al. 2002; Humbles et al. 2000). Compared with wild-type mice, C3-deficient mice also exhibit diminished AHR and lung eosinophilia when challenged with an allergen (Drouin et al. 2001). They also showed decreased numbers of interleukin (IL)-4-producing lung cells and decreased serum-antigen-specific immunoglobulin E (IgE) levels. Dendritic cells (Zhou et al. 2007) and activated T cells express C3a receptor (Werfel et al. 2000), and C3 and C3a have been shown to regulate interactions between dendritic and T cells (Kawamoto et al. 2004; Zhou et al. 2007). These results suggested that the C3 pathway is involved in allergen sensitization. In BA patients, increased levels of C3a (Humbles et al. 2000; Krug et al. 2001) and C5a (Krug et al. 2001) were observed in bronchoalveolar lavage fluid after segmental allergen provocation, suggesting involvement of the complement system in allergen-induced airway inflammation in humans. Elevation of plasma C3a level was observed in BA patients on acute exacerbation (Nakano et al. 2003). These data are consistent with the notion that the C3 gene plays an important role in allergic sensitization and allergic inflammation. Furthermore, the C3 gene is located at chromosome 19p, where several studies suggested linkage to BA and related phenotypes (Blumenthal et al. 2004; Lee et al. 2000; Venanzi et al. 2001). Therefore, the C3 gene is a strong candidate gene for allergic BA.

Our previous analysis of four single nucleotide polymorphisms (SNPs) in exons of the C3 gene suggested that variations of this gene conferred susceptibility to both childhood and adult BA (Hasegawa et al. 2004). Barnes et al. reported that a haplotype spanning introns 19–23 showed significant association with BA, log (total IgE) and log [interleukin (IL)-13]/log [interferon (IFN)- γ] (Barnes et al. 2006). The C3 gene, however, is a large gene consisting of 41 exons and containing hundreds of SNPs; thereby, further investigation is necessary to identify functional SNPs conferring susceptibility to BA. In this study, we analyzed 26 SNPs of the C3 gene to narrow down the regions showing association with childhood and adult BA.

Methods

Subjects

All participants were Japanese. Three hundred and forty-six childhood BA patients, 518 adult BA patients, and 550 healthy adult controls were recruited in Osaka City area, Japan. Details of these patients are described in a previous report (Nakashima et al. 2006). All participants with BA were diagnosed and selected by physicians according to the American Thoracic Society (ATS) criteria and using questionnaires based on the recommendation of the ATS, Division of Lung Disease (ATS-DLD) (Ferris 1978). In brief, patients showed repeated episodes of at least one of the following symptoms: cough, wheezing, shortness of breath, chest tightness, and sputum production. Spirometry was performed in all patients to confirm the obstructive pattern of the lung function and response to a bronchodilator. Improvement of their forced expiratory volume in 1 s (FEV₁) measurement was at least 12% after β_2 agonist inhalation. The diagnosis of atopic BA was based on one or more positive skin-scratch-test responses to a range of seven common allergens in the presence of a positive histamine control and a negative vehicle control. The seven allergens were house dust, *Felis domesticus* dander (Feld), *Canis familiaris* dander, *Dactylis glomerata*, Ambrosia, *Cryptomeria japonica*, and *Alternaria alternata*. The numbers of atopic childhood and adult BA were 304 and 371, respectively (Table 1). In this study, we only analyzed atopic BA patients. As child control subjects, 411 child volunteers with ages between 6 and 12 years (male:female = 1.0:1.04) were recruited in Chiba City, Japan. Total and eight specific IgE levels in serum were measured in this group (*Dermatophagoides pteronyssinus*, *C. familiaris*, *F. domesticus*, *A. alternata*, *C. japonica*, *D. glomerata*, egg white, golden/black bellied/Hungarian hamster). Questionnaires based on the International Study of Asthma and Allergies in Childhood (Asher et al. 1995) were used to exclude children with BA and/or atopic dermatitis. Three

Table 1 Clinical characteristics of patients and controls

	Childhood asthma	Adult asthma	Child control	Adult control
Number	304	371	333	550
Age mean (years)	9.69	45.13	9.22	44.57
Age range (years)	4–15	20–75	6–12	20–75
Gender (male:female)	1.54:1.0	1.11:1.0	1.0:1.04	2.81:1.0
Atopic asthma (%)	100	100		
Mean total IgE [log (IU/ml)]	2.66	2.54	1.90	ND

IgE immunoglobulin E, ND not determined

hundred and thirty-three children were used as a child control group in association studies. If atopy was defined as those who showed positive specific IgE (>0.35 IU/ml) to one or more allergens, 71% of the child controls were atopic. Details of the adult controls were described previously (Nakashima et al. 2006). Adult volunteers were interviewed by physicians, and those who were diagnosed as having BA, atopic dermatitis, and/or allergic rhinitis were excluded from the adult control group. Measurement of serum IgE levels and skin-prick tests were not performed in the adult controls. All patients and volunteers provided written informed consent to participate in the study in accordance with the rules of the process committee at the SNP Research Center (RIKEN). This study was approved by the ethics committee of Chiba University Graduate School of Medicine.

Screening and selection of SNPs

We previously investigated four SNPs in the exons of the *C3* gene (Hasegawa et al. 2004): 912G/A, 1692AG, 1936GA, and 4896 CT in our previous paper, which correspond to SNP6, SNP7, SNP13, and SNP25, respectively, in this paper (Table 2). SNP25 showed association with mite-positive childhood BA and adult BA. Because SNP25 is located in exon 41, the last exon of the *C3* gene, SNPs located around this exon were intensively investigated (SNP17–SNP26). We searched the dbSNP database to list up SNPs with the following criteria: minor allele frequency was more than 0.3, and distance of the nearest two SNPs did not exceed 2 kbp. Considering the potential importance of the gene's promoter region for gene expression, where several regulatory elements have been reported, we searched SNPs up to 5 kbp upstream of exon 1. Four SNPs (SNP1–SNP4) were identified. In addition, we included SNP14, SNP15, and SNP16 in this study to confirm the results of Barnes et al. (2006). To estimate the linkage disequilibrium (LD) status of these SNPs, we genotyped them in 96 adult controls. Haploview 3.32 program (Barrett et al. 2005) was used to show an LD map. LD block was defined with the solid spine of the LD method implemented in the Haploview program. Of the 26 SNPs investigated, 24 composed four LD blocks (Fig. 1). We selected tag SNPs from each LD block with the aid of the Tagger routine incorporated in the Haploview program.

Genotyping

Genomic DNA was prepared from whole blood samples using a standard protocol. Whole genome amplification was performed using the illustra GenomiPhi V2

Table 2 Locations and allele frequencies of single nucleotide polymorphisms (SNPs) of the *C3* gene

SNP	Location	Allele (1/2) ^a	Position ^b	Minor allele frequency (%) ^c	dbSNP number
SNP1	Promoter	T/C	6678365	0.330	rs171094
SNP2	Promoter	C/T	6674037	0.157	–
SNP3	Promoter	A/G	6673635	0.298	rs163913
SNP4	Promoter	A/C	6673022	0.306	rs339392
SNP5	Intron 2	A/G	6669534	0.270	rs2250656
SNP6	Exon 9	G/A	6663291	0.426	rs2230201
SNP7	Exon 14	G/A	6669848	0.468	rs2230204
SNP8	Exon 14	G/A	6663704	0.414	rs2230205
SNP9	Intron 14	G/A	6660074	0.148	rs11569429
SNP10	Intron 14	G/A	6660050	0.016	rs4807984
SNP11	Intron 17	A/G	6656246	0.403	rs11672613
SNP12	Intron 19	A/C	6648829	0.134	rs366510
SNP13	Exon 21	C/T	6648406	0.064	rs423490
SNP14	Intron 23	C/T	6647342	0.128	rs2287848
SNP15	Intron 23	C/T	6647178	0.371	rs10410674
SNP16	Intron 23	C/G	6646001	0.435	rs10402876
SNP17	Intron 33	G/A	6634846	0.436	rs344549
SNP18	Intron 33	C/G	6633953	0.441	rs344550
SNP19	Intron 33	T/–	6633534	0.446	rs11569553
SNP20	Intron 35	A/G	6631937	0.468	rs344552
SNP21	Intron 35	C/A	6631928	0.394	rs344553
SNP22	Intron 36	A/G	6630563	0.457	rs2277983
SNP23	Intron 36	G/A	6630511	0.447	rs2277984
SNP24	Intron 38	T/C	6629753	0.456	rs11569562
SNP25	Exon 41	C/T	6628989	0.447	rs4807893
SNP26	3' Downstream	T/G	6627442	0.414	rs379527

^a Base expressed in the direction the gene

^b Based on National Center for Biotechnology Information (NCBI) Build 35.1 reference group label

^c In 96 adult control subjects

amplification kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's standard protocol. Amplified deoxyribonucleic acid (DNA) was typed by allele-specific polymerase chain reaction (AS-PCR) using either the modified TaqMan AS amplification (TaqMan-ASA) method (Fujii et al. 2000) or SYBR Green detection. The primer and TaqMan probe sequences are shown in Table 3. For the TaqMan-ASA method, 2× Platinum qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA) was used as master mix, whereas 2× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) or 2× SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) (for only SNP24) was used for AS-PCR together with SYBR Green detection. For the ASA method, the PCR mixture contained 5 µl of 2× PCR master mix, 0.4 µM of each PCR

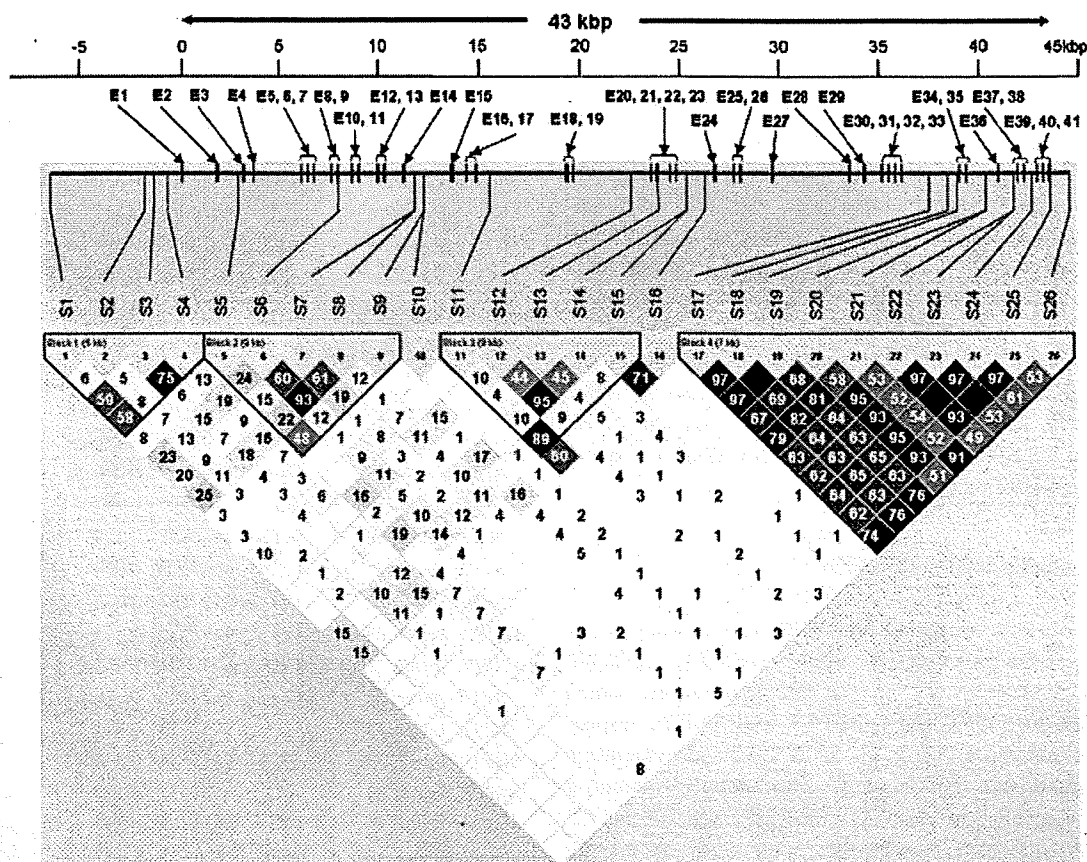


Fig. 1 Structure of the *C3* gene and location of single nucleotide polymorphisms (SNPs) and their linkage equilibrium (LD) map. Exons are indicated by black boxes. Numbers starting with *E* stand for exons and those starting with *S* for SNPs. Pair-wise LD (r^2) was

estimated from 96 control subjects. LD blocks were defined by the solid spine of LD using the Haploview program. Number in each cell represents r^2 ($\times 100$); black cells $r^2 = 1$; white cells $r^2 = 0$. Each cell is colored in a graduated manner according to the strength of LD

primer, 0.12 μ M of the TaqMan probe (when needed), and 5 ng of amplified template DNA in a final volume of 10 μ l. The samples were analyzed using an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) and Chromo4 Real-Time System (Bio-Rad). The thermoprofile was 50°C for 2 min, 95°C for 2 min, then 45 cycles of 95°C for 15 s and 60°C for 30 s (for SNP4: 45 cycles of 95°C for 15 s, 55°C for 30 s, and 60°C for 30 s). For SNP2, PCR was initially performed using 0.2 μ M of each first PCR primers and *Taq* DNA polymerase (Promega, Madison, WI, USA) according to the manufacturer's standard protocol. The thermoprofile of the first PCR was 95°C for 3 min, followed by 20 cycles at 95°C for 20 s, 60°C for 30 s, and 72°C for 7 min. In the second PCR (TaqMan-ASA method), a reaction mixture of the first PCR diluted tenfold with water was used as a template. Because an SNP was located within the sequence where the AS primer for SNP9 was designed, two primers were mixed and used in the reaction mixture for SNP9 (Table 3).

Statistical analysis

Pairwise LD in SNPs was estimated as r^2 (Hill and Robertson 1968). A 2×2 contingency χ^2 test of independence was performed to evaluate the significance of an association between allele frequency and disease status. Haplotype inference and a case-control association study were performed using SNPalyze ver. 4.1 program (DYNACOM, Mobarra, Japan). The effects of genotypes on \log_{10} -transformed total serum IgE levels were evaluated using analysis of variance (ANOVA). Statistical analysis was performed with SPSS software (ver. 15.0 J; SPSS Japan, Tokyo, Japan). In SNP association studies, corrected *P* (P_{cor}) values were calculated by multiplying the number of SNPs tested in this study (15). In haplotype association studies, P_{cor} values were calculated by multiplying the total number of haplotypes tested in the entire gene (27). Association of an SNP or haplotype with the disease was judged as significant if P_{cor} for the test of allele/haplotype frequency was <0.05 .

Table 3 Primers for genotyping single nucleotide polymorphisms (SNPs) in the C3 gene

SNP genotyped	Sequence
SNP1	
F: T allele	5'-GGCATAAACAAGGGTTAAATGT
F: C allele	5'-GGCATAAACAAGGGTTAAATGC
R: common	5'-GCTCACAAACGCCTATGA
TaqMan probe	5'-TGAATAGATAAGTTGCTGCCACCCG
SNP2	
F: C allele	5'-GCCTGGCCAACATGGCGAACC
F: T allele	5'-GCCTGGCCAACATGGCGAAGT
R: common	5'-TGCCTCCTGGGTCAAGTGATTCTC
TaqMan probe	5'-TAGTGGCGCATGCCTGTGATCCCAGCTCT
First PCR F:	5'-TTCCAAGAGTGTGTGCGAA
First PCR R:	5'-CCTGCTTCATAGAGTTGTCGT
SNP3	
F: A allele	5'-TTTGGCAATATCTAGCAAGATTACCTA
F: G allele	5'-TTTGGCAATATCTAGCAAGATTACCTG
R: common	5'-CCTTACCACCTGCTTCATAGAGTTG
TaqMan probe	5'-TGACCCAACATATTTCCCTTCATGCAACG
SNP4	
F: C allele	5'-CCTGTAACTGTAAGAATGAGAC
F: A allele	5'-CCTGTAACTGTAAGAATGAGTA
R: common	5'-CAAAGTGTGTTGTAAGTACTG
TaqMan probe	5'-TAGTATGTGCTATGTGCTGTCC
SNP5	
R: A allele	5'-GCCTGCCATTATTCTTGGTCCT
R: G allele	5'-GCCTGCCATTATTCTTGGTCAC
F: common	5'-CCTGTGAGCTCTTCTTTTGAGTTC
SNP6	
R: G allele	5'-CACCCCGTCCAGCAGTACCTAC
R: A allele	5'-CACCCCGTCCAGCAGTACCTAT
F: common	5'-AACAGAGGATTTCCCTGCCTGAA
TaqMan probe	5'-CCCTCAAGCGCATTCGG
SNP7	
F: A allele	5'-AACAGAGGATTTCCCTGCCTGTA
F: G allele	5'-AACAGAGGATTTCCCTGCCTGGG
R: common	5'-CACCCCGTCCAGCAGTACCTTC
TaqMan probe	5'-CCCTCAAGCGCATTCGG
SNP8	
F: G allele	5'-TGCTGAATAAGAAGAACAACACTGAGG
F: A allele	5'-TGCTGAATAAGAAGAACAACACTGAGA
R: common	5'-TGCTCCGCCTCTCTCA
SNP9	
F: G allele	5'-TAGGGACGAAGATGGAGATGTG
G allele	5'-TAGGGACGAAGATGGAGATGAG
F: A allele	5'-TAGGGACGAAGATGGAGATGTA
A allele	5'-TAGGGACGAAGATGGAGATGAA
R: common	5'-CTTATCTCCATTTCCCTCTGATTC
SNP10	
R: G allele	5'-TGATTCCATCTGCATTCCCAAC
R: A allele	5'-TGATTCCATCTGCATTCCCAAT
F: common	5'-TTCCGGAGTAGGGACGAAGA

Table 3 continued

SNP genotyped	Sequence
SNP11	
F: A allele	5'-AGGGTCACTGGGAAAATTAGACATA
F: G allele	5'-AGGGTCACTGGGAAAATTAGACAGG
R: common	5'-ATGGGCCAAAGGAATTACACAAT
SNP12	
F: A allele	5'-ACTCCCCGACCTTGACACTAA
F: C allele	5'-ACTCCCCGACCTTGACACTAC
R: common	5'-CCTGCATTACTGTGACCTCGAA
TaqMan probe	5'-CCCGAGCAGGGATCTGTGTGGCA
SNP13	
F: C allele	5'-GGAAGTGGAAAGTCAAGGTGGC
F: T allele	5'-GGAAGTGGAAAGTCAAGGTGGT
R: common	5'-GGGTGCCCAAGCACTCA
TaqMan probe	5'-CCATCATTTCATCAGTGACGGTGTGAGGAA
SNP14	
R: T allele	5'-GAATGAGATGGAATTTGGCTCGA
R: C allele	5'-GAATGAGATGGAATTTGGCTCTG
F: common	5'-CAAGTCCCGACACCGAGTCT
SNP15	
R: C allele	5'-CAGCGAGCTGAGGTCGGG
R: T allele	5'-CAGCGAGCTGAGGTCGTA
F: common	5'-CATCTGTGATCTGTTTTCCCTCTTTAC
SNP16	
R: C allele	5'-GAGTGTCTCACTTAATAGTCAACGATG
R: G allele	5'-GAGTGTCTCACTTAATAGTCAACGATC
F: common	5'-TGGTGAGGCTGGTCTTGAACCT
SNP17	
F: G allele	5'-CTGCCAAAGTTTTGGGATCACTG
F: A allele	5'-CTGCCAAAGTTTTGGGATCACTA
R: common	5'-CCACACCCGGCCATTTCC
SNP18	
R: C allele	5'-AATGCCAGAAGTGAACCTCAAAGTG
R: G allele	5'-AATGCCAGAAGTGAACCTCAAAGTTC
F: common	5'-CAGCAGGGTCAACATCACCATA
SNP19	
F: T allele	5'-GGCTGCCTGTATTCTTGCCTAT
F: delT allele	5'-GGCTGCCTGTATTCTTGCCTCG
R: common	5'-TGGATTCAAATTCAGCTCTAAATAAC
SNP20	
F: A allele	5'-ATTCCAAGCATGAGCCACGA
F: G allele	5'-ATTCCAAGCATGAGCCACGG
R: common	5'-GGAGAGGAGAAAGCCCAAATCA
SNP21	
R: A allele	5'-GATGGAGAGAAAATAACAGAAGAGTT
R: C allele	5'-GATGGAGAGAAAATAACAGAAGAGCG
F: common	5'-ATGTTGCTCAAGTTGGTCTCAAAT
SNP22	
R: A allele	5'-GGCCTCCCTCCAAAGACCTT
R: G allele	5'-GGCCTCCCTCCAAAGACCTC
F: common	5'-CGTGTCCAGGAATCTATGAATTT

Table 3 continued

SNP genotyped	Sequence
SNP23	
R: G allele	5'-ACCGGGTACAGCTTTCCTCTAC
R: A allele	5'-ACCGGGTACAGCTTTCCTCTTT
F: common	5'-GGCTTCTGTGAGTTGAGAGTCTAAGAGA
SNP24	
F: T allele	5'-CATGGCCATGAGGCTACAGTATAT
F: C allele	5'-CATGGCCATGAGGCTACAGTATAC
R: common	5'-CCCATGTCACCATCCACACA
SNP25	
F: T allele	5'-ACACTTGGGTGGAGCACTGGCAT
F: C allele	5'-ACACTTGGGTGGAGCACTGGCTC
R: common	5'-GGTCCTGGCATTGTTTCTGGTTCTC
TaqMan probe	5'-AGGAGGACGAATGCCAAGACG
SNP26	
R: T allele	5'-GGTGAGAATGTGGGCAAGAAGA
R: G allele	5'-GGTGAGAATGTGGGCAAGAAGC
F: common	5'-ACCTACATCCTCTCCGGTGAGTGT

PCR polymerase chain reaction, F forward primer, R reverse primer. All TaqMan probes were labeled with 6-carboxyfluorescein (FAM, reporter dye) at the 5' end and 6-carboxy-tetramethyl-rhodamine (TAMRA, quenching dye) at 3' end

Results

Polymorphisms in the C3 gene

We selected and characterized 26 SNPs from the C3 gene to investigate mainly genetic variations of the promoter region, SNPs forming a haplotype that showed significant association with BA in African Caribbean families, and the 3' end region where a SNP showed significant association with BA in our previous study (Table 2). The location of the SNPs and LD map is shown in Fig. 1. If an LD block was defined by the solid spine of LD, out of the 26 SNPs investigated, 24 composed four LD blocks. SNP10 showed r^2 values <0.033 to any other SNPs investigated in the current study and excluded from any LD block under this definition. Although SNP16 showed moderate LD to SNP15 ($r^2 = 0.71$) and SNP11 ($r^2 = 0.60$), it did not belong to LD block 3, where SNP15 and SNP11 are located. This is because of the characteristic of the "solid spin of LD" definition. When SNP12 and SNP14, both of which showed very low r^2 (<0.01) to SNP16, were omitted from LD block 3, SNP16 was found to be included in LD block 3. As tag SNPs, four SNPs each were selected in LD blocks from 1 to 3 and three SNPs in LD block 4 (Fig. 1).

Association study of childhood and adult BA

We genotyped 15 tag SNPs in 304 childhood BA, 371 adult BA, 333 child controls, and 550 adult controls (Table 1).

All loci were in Hardy–Weinberg equilibrium in the control groups. The genotype and allele frequencies of each SNP in the patient and control groups are shown in Table 4. Results of association tests for allele frequency between the patient and control groups are also shown. Allelic frequency of SNP24 was significantly different between adult BA and adult controls ($P = 0.002$). The P value for the difference remained significant ($P_{\text{cor}} = 0.030$) after correction for the number of SNPs tested (15). In a recessive model, the odds ratio (OR) and its 95% confidence interval (95% CI) range for this SNP were 1.55 and 1.15–2.09, respectively. In a dominant model, the OR and 95% CI range were 1.37 and 1.01–1.88, respectively. The difference in the allele frequency of SNP24 between all BA and all control showed a similar tendency. However, the P value was not significant ($P = 0.004$, $P_{\text{cor}} = 0.060$). None of the other SNPs showed a significant association with either childhood or adult BA.

The frequencies of haplotypes consisting of tag SNPs in four LD blocks are shown in Table 5. A significant difference was observed in LD block 4 (Table 5). In LD block 4 containing exons 24–41, the frequency of the major haplotype CCC in adult BA (54.1%) was significantly higher than that in controls (46.4%) ($P = 0.0014$, $P_{\text{cor}} = 0.038$). The frequencies of C alleles of SNP18, SNP19, and SNP24 were higher in adult BA than in adult controls. These increased frequencies were straightforwardly associated with the increased frequency of the CCC haplotype. The frequency of this haplotype in childhood BA (51.0%) was not significantly higher than that in adult controls (48.6%) ($P_{\text{cor}} > 1.0$). The OR of the CCC haplotype of LD block 4 was about 1.4 for both childhood and adult BA (Table 6). In LD block 2 containing at least exons 3–14, the frequency of the GGAG haplotype in childhood BA (5.6%) was higher than that in child controls (2.5%) ($P = 0.0044$). This difference, however, did not reach a genome-wide significance ($P_{\text{cor}} = 0.12$). In LD blocks 1 and 3, there was no haplotype showing any difference in frequency between patients and controls. LD block 1 contains the promoter region of the C3 gene; therefore, it is not likely that genetic variations of the promoter region have significant effect on susceptibility to BA in the Japanese population.

Barnes et al. reported that a 3-SNP haplotype consisting of the SNPs identical to SNP14, SNP15, and SNP16 showed significant association with BA, log (total IgE) and log (IL-13)/log (IFN- γ) in the Afro-Caribbean families (Barnes et al. 2006). To assess the reproducibility of their results, we investigated whether this 3-SNP haplotype shows association with either childhood BA, adult BA, or log (total IgE) in our samples. We found that this haplotype showed no significant association with any of these phenotypes.

Table 4 Single nucleotide polymorphism (SNP) association study of the C3 gene

	Childhood asthma						Adult asthma						Child control						Adult control						Childhood asthma versus all control		Adult asthma versus all control	
	Childhood asthma			Adult asthma			Child control			Adult control			Childhood asthma versus child control			Adult asthma versus control			Childhood asthma versus all control		Adult asthma versus all control							
	11 ^a	12 ^b	22 ^c	1 ^d	2 ^d	11 ^a	12 ^b	22 ^c	1 ^d	2 ^d	11 ^a	12 ^b	22 ^c	1 ^d	2 ^d	11 ^a	12 ^b	22 ^c	1 ^d	2 ^d	11 ^a	12 ^b	22 ^c	1 ^d	2 ^d	P ^e	P ^e	
SNP1	0.382	0.473	0.144	0.619	0.381	0.438	0.432	0.130	0.654	0.346	0.351	0.508	0.141	0.605	0.395	0.395	0.457	0.149	0.623	0.377	>1	>1	>1	>1	>1	>1	>1	>1
SNP2	0.755	0.222	0.024	0.865	0.135	0.785	0.171	0.044	0.871	0.130	0.754	0.192	0.055	0.849	0.151	0.769	0.205	0.027	0.871	0.129	>1	>1	>1	>1	>1	>1	>1	>1
SNP3	0.440	0.443	0.117	0.662	0.339	0.431	0.456	0.113	0.659	0.341	0.375	0.502	0.123	0.626	0.374	0.424	0.467	0.109	0.658	0.343	>1	>1	>1	>1	>1	>1	>1	>1
SNP4	0.433	0.406	0.161	0.636	0.364	0.437	0.439	0.124	0.657	0.344	0.402	0.475	0.123	0.640	0.361	0.426	0.430	0.143	0.642	0.358	>1	>1	>1	>1	>1	>1	>1	>1
SNP5	0.542	0.405	0.054	0.744	0.256	0.616	0.323	0.061	0.778	0.223	0.562	0.369	0.069	0.747	0.254	0.628	0.310	0.062	0.783	0.217	>1	>1	>1	>1	>1	>1	>1	>1
SNP6	0.411	0.461	0.128	0.642	0.359	0.371	0.454	0.175	0.598	0.402	0.381	0.469	0.150	0.616	0.385	0.327	0.485	0.188	0.570	0.431	>1	>1	>1	>1	>1	>1	>1	>1
SNP7	0.201	0.497	0.302	0.450	0.551	0.256	0.497	0.247	0.505	0.496	0.255	0.482	0.264	0.496	0.504	0.285	0.485	0.230	0.528	0.473	>1	>1	>1	>1	>1	>1	>1	>1
SNP9	0.737	0.232	0.030	0.854	0.146	0.750	0.219	0.031	0.860	0.141	0.709	0.273	0.018	0.846	0.155	0.753	0.219	0.028	0.863	0.138	>1	>1	>1	>1	>1	>1	>1	>1
SNP11	0.340	0.505	0.155	0.593	0.408	0.331	0.489	0.180	0.576	0.425	0.324	0.502	0.174	0.575	0.425	0.312	0.478	0.210	0.551	0.449	>1	>1	>1	>1	>1	>1	>1	>1
SNP13	0.862	0.131	0.007	0.928	0.073	0.889	0.106	0.006	0.941	0.059	0.880	0.120	0.000	0.940	0.060	0.890	0.110	0.000	0.945	0.055	>1	>1	>1	>1	>1	>1	>1	>1
SNP14	0.769	0.228	0.003	0.883	0.117	0.779	0.202	0.019	0.880	0.120	0.778	0.213	0.009	0.885	0.116	0.786	0.206	0.008	0.889	0.111	>1	>1	>1	>1	>1	>1	>1	>1
SNP15	0.379	0.480	0.141	0.619	0.381	0.348	0.514	0.138	0.605	0.395	0.363	0.469	0.168	0.598	0.403	0.316	0.503	0.181	0.568	0.433	>1	>1	>1	>1	>1	>1	>1	>1
SNP18	0.305	0.557	0.138	0.584	0.417	0.403	0.442	0.156	0.623	0.377	0.348	0.454	0.198	0.575	0.425	0.328	0.506	0.167	0.580	0.420	>1	>1	>1	>1	>1	>1	>1	>1
SNP21	0.367	0.522	0.111	0.628	0.372	0.433	0.444	0.122	0.656	0.344	0.369	0.471	0.159	0.605	0.395	0.358	0.498	0.144	0.607	0.393	>1	>1	>1	>1	>1	>1	>1	>1
SNP24	0.248	0.537	0.215	0.517	0.484	0.225	0.456	0.319	0.453	0.547	0.282	0.450	0.267	0.508	0.492	0.285	0.482	0.233	0.526	0.474	>1	>1	>1	>1	>1	>1	>1	>1

^a Frequency of homozygote for major allele

^b Frequency of heterozygote

^c Frequency of homozygote for minor allele

^d Allele frequency

^e Corrected P value (raw P values were multiplied by number of SNPs, 15) for allele frequency difference

Table 5 Haplotype association study of the C3 gene

Haplotype	Frequency				P_{cor}^a		
	Childhood asthma	Adult asthma	Child control	Adult control	Childhood asthma vs. control	Adult asthma vs. control	All-asthma vs. control
Block 1^b							
TCAA	0.441	0.483	0.436	0.467	>1	>1	>1
CCGC	0.295	0.263	0.279	0.280	>1	>1	>1
TTAA	0.129	0.108	0.121	0.103	>1	>1	>1
CCAA	0.051	0.043	0.041	0.042	>1	>1	>1
TCGC	0.032	0.051	0.040	0.021	>1	0.222	>1
CCGA	0.009	0.014	0.032	0.020	0.192	>1	0.297
CCAC	0.019	0.010	0.019	0.016	>1	>1	>1
TCAC	0.018	0.009	0.004	0.017	0.324	>1	>1
Others	0.006	0.022	0.029	0.027			
Block 2^c							
AAGG	0.322	0.361	0.333	0.403	>1	>1	>1
AGAG	0.337	0.301	0.310	0.291	>1	>1	>1
GGAA	0.124	0.110	0.141	0.115	>1	>1	>1
AGGG	0.058	0.067	0.072	0.063	>1	>1	>1
GGGG	0.049	0.055	0.053	0.051	>1	>1	>1
GGAG	0.056	0.042	0.025	0.037	0.121	>1	0.729
AAAG	0.016	0.018	0.015	0.015	>1	>1	>1
AGAA	0.014	0.024	0.013	0.010	>1	0.567	>1
GAGG	0.019	0.015	0.036	0.009	>1	>1	>1
Others	0.006	0.006	0.002	0.005			
Block 3^d							
ACCC	0.460	0.446	0.445	0.424	>1	>1	>1
GCCT	0.365	0.382	0.384	0.418	>1	>1	>1
ATTC	0.066	0.058	0.055	0.054	>1	>1	>1
ACTC	0.049	0.059	0.057	0.055	>1	>1	>1
GCCC	0.041	0.041	0.038	0.034	>1	>1	>1
ACCT	0.013	0.012	0.014	0.013	>1	>1	>1
Others	0.006	0.002	0.006	0.003			
Block 4^e							
CCC	0.510	0.541	0.486	0.464	>1	0.038	0.079
GAT	0.365	0.340	0.387	0.375	>1	>1	>1
CCT	0.066	0.078	0.081	0.098	>1	>1	>19
GCT	0.053	0.033	0.038	0.044	>1	>1	>1
Others	0.007	0.011	0.008	0.019			

^a Corrected *P* value (raw *P* values were multiplied by the number of haplotypes tested, 27) for haplotype frequency difference

^b This haplotype is consisted of SNPs 1, 2, 3, and 4

^c This haplotype is consisted of SNPs 5, 6, 7, and 9

^d This haplotype is consisted of SNPs 11, 13, 14, and 15

^e This haplotype is consisted of SNPs 18, 21, and 24

Table 6 Odds ratio (OR) and its 95% confidence interval (CI) of the CCC haplotype for bronchial asthma (BA)

	Diplotype frequency (%)				
	Case		Control		Odds ratio (95% CI)
	CCC/CCC and CCC/other	Other/other	CCC/CCC and CCC/other	Other/other	
Childhood BA	77.8	22.2	71.2	28.8	1.42 (0.99–2.04)
Adult BA	76.9	23.1	71.0	29.0	1.36 (1.00–1.86)
All BA	77.3	22.7	71.1	28.9	1.39 (1.10–1.75)

Diplotype of each individual was inferred with SNPalyze ver. 4.1. OR and its (95% CI) were determined with SPSS ver. 15.0

Effect of genetic variations of the C3 gene on serum total IgE level

To examine the effect of genetic variations of the C3 gene on serum IgE level, 15 tag SNPs and the CCC haplotype were analyzed by analysis of variance (ANOVA) for log-transformed serum total IgE values (Table 7). *P* values <0.05 were observed in SNP14 in adult BA patients (*P* = 0.042) and in SNP13 (*P* = 0.018) and SNP18 (0.041) in child controls. If we want to maintain type 1 error at 0.05 in each patient/control group, a *P* value <0.0031 (0.05/16) should be considered as significant. Thus, we deduced that these SNPs did not significantly affect serum total IgE level. The genotype of SNP24 and the CCC haplotype, both of which showed an association with adult BA, were not significantly associated with the IgE level.

Table 7 Effect of single nucleotide polymorphisms (SNPs) and haplotypes of the C3 gene on log₁₀-transformed total immunoglobulin E (IgE)

Locus	<i>P</i> value in ANOVA test ^a		
	Childhood asthma	Adult asthma	Child control
Block 1			
SNP1	0.347	0.242	0.250
SNP2	0.657	0.960	0.320
SNP3	0.276	0.051	0.720
SNP4	0.329	0.058	0.182
Block 2			
SNP5	0.537	0.794	0.119
SNP6	0.860	0.533	0.805
SNP7	0.952	0.556	0.497
SNP9	0.368	0.795	0.270
Block 3			
SNP11	0.463	0.678	0.937
SNP13	0.917	0.100	0.018
SNP14	0.485	0.042	0.261
SNP15	0.486	0.816	0.688
Block 4			
SNP18	0.197	0.890	0.041
SNP21	0.065	0.280	0.097
SNP24	0.356	0.661	0.526
CCC haplotype	0.175	0.586	0.505

^a Significance of difference in the mean of log₁₀ (total IgE) among individuals with different genotypes (major allele homozygote, heterozygote, minor allele homozygote) was tested with analysis of variance (ANOVA). Three groups (childhood asthma, adult asthma, and child control) were evaluated separately. Serum IgE level was not determined in the adult control group

Discussion

In this study, we investigated the association of SNPs in the C3 gene with childhood and adult BA. We observed a significant association of SNP24 and the CCC haplotype in LD block 4 with adult BA. As the CCC haplotype was discriminated from other major haplotypes with a frequency of >1% by the SNP24 allele (C/T), this haplotype association may just be a reflection of the association of SNP24. There was no significant difference in genotype frequency of SNP24 between the adult control group and the child control group, suggesting that the significant association observed in the adult patients–control comparison was not due to skewed genotype frequency of adult controls but to changes in adult BA. The frequency of the T/T genotype in childhood BA was decreased (from 0.282 to 0.248), as in adult BA. However, the change in allele frequency in childhood BA compared with that in child controls was small (T allele: 0.517 vs. 0.508). This SNP (or that with strong LD to this SNP) may not be a risk-modifying variation for childhood BA. Although it was not significant at the gene-wide level, we observed a tendency of the frequency of the GGAG haplotype of LD block 2 to show a difference between childhood BA and child controls (*P* = 0.0045, *P*_{cor} = 0.121). To determine and definitively conclude whether this LD block confers risk for childhood BA, an association study with more childhood samples and functional analyses of this region will be necessary.

LD block 4 is about 20 kb long and contains exon 41 coding the C-terminal end of the C3 protein and 3' untranslated region as well as upstream 17 exons and introns. Because we could not find SNPs that change the amino acid sequences in exons in this LD block, functional variation(s) in this region should be those affecting either expression of the gene, ribonucleic acid (RNA) splicing, or RNA stability. To the best of our knowledge, there is as yet no study investigating elements affecting gene expression in this region of the C3 gene. Currently, we do not have sufficient data to discuss the underlying mechanisms of the association between the LD block and adult BA. Further genetic and functional analyses of the LD blocks are necessary to pinpoint genetic variation(s) responsible for differences in susceptibility to BA.

It is possible that some of the control subjects involved in this study will develop asthma. Judging from the prevalence of the asthma in Japan, the chance of developing the disease is expected to be several percent. If we exclude atopic subjects from the control group, the chance may be reduced but not completely eliminated. This issue cannot be thoroughly controlled in a case–control study. The fact that our control group contained presymptomatic BA patients reduces the statistical power to detect an association between genotypes and the disease. We must be aware of this issue

when obtaining negative results. However, the main result (i.e., the significant association of the haplotype with the disease) cannot be changed by this issue. If we could eliminate presymptomatic BA patients, estimated ORs would be higher than the values we presented in this study.

We also investigated whether genetic variations of the *C3* gene affect total serum IgE level using childhood and adult BA patients and child controls. The IgE level was not significantly affected by any SNPs or haplotypes, including those showing a significant association with adult BA. This was true with mite-specific IgE level (data not shown). These results suggest that genetic variations of LD block 4 showing susceptibility to BA have a slight effect on sensitization to allergens but are more relevant to the effector phase of allergic inflammation. The roles of anaphylatoxins in the pathogenesis of BA can be divided into two phases: sensitization to allergens and effector phase of allergic BA (Kohl and Wills-Karp 2007). Several roles of C5a signaling at the interface between dendritic cells and T cells are evident, but those of C3a remain unclear. In a C3aR knockout experiment, different Th2 cytokine production responses were reported in various strains of mice (C57BL/6 and BALB/c) (Drouin et al. 2002; Humbles et al. 2000), suggesting that the effect of C3a is influenced by genetic background. As observed in mice, a difference in genetic background may explain the fact that association of the haplotype consisting of SNP14, SNP15, and SNP16 with total IgE level observed in Afro-Caribbean families was not confirmed in the Japanese population. Discrepancy between our results and those of Barnes et al. may also be due to a difference in the environmental factors of the two study populations. There are a number of studies showing that the effects of genetic variation (–159C/T) of the CD14 gene on allergic sensitization and BA risk differ greatly due to environmental factors such as mite or lipopolysaccharide concentration in dust (Ober et al. 2000; Simpson et al. 2006; Vercelli 2003; Zambelli-Weiner et al. 2005). It may be possible that *C3* gene variations also show this type of gene–environmental interaction and cause discrepant results in studies with different populations.

In summary, our results suggest that the LD block containing exons 24–41 of the *C3* gene confer susceptibility to adult BA in the Japanese population. Because this region showed a slight effect on serum IgE level in both BA patients and normal individuals, this region may be involved in the effector phase of allergic inflammation. The effect of variations of the *C3* gene on allergic sensitization and BA susceptibility may differ according to genetic background and environmental factors.

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2007, at the patient's request. After approximately 1½ years of normal eosinophil levels, the patient's eosinophilia worsened during late 2008 (Fig 1). A single osseous plasmacytoma was found and irradiated but progressed to multiple lytic lesions and multiple myelomas confirmed by means of bone marrow biopsy. The patient died in December 2008 as a result of this disease.

HES can occur as a myeloproliferative, lymphoproliferative, or, most frequently, idiopathic variant. Some myeloproliferative patients respond to imatinib mesylate³ and possess a mutant gene on chromosome 4.⁴ Deletion of approximately 800 kB on chromosome 4 results in a *FIP1L1/PDGFR*A fusion gene and formation of a kinase potentially inhibited by imatinib mesylate. In our patient the *FIP1L1/PDGFR*A fusion gene was not detected, and imatinib mesylate failed to control eosinophilia. In the lymphoproliferative HES variant, T-lymphocyte clones produce cytokines, especially IL-5, that stimulate eosinophil production in the bone marrow. Mepolizumab, an anti-IL-5 drug, is used to inhibit eosinophil proliferation stimulated by IL-5; however, for unknown reasons, 16% of patients do not respond to mepolizumab.⁵ In this case mepolizumab had no effect, suggesting that eosinophilia was not IL-5 dependent or that other cytokines, such as IL-3 or GM-CSF, were supporting eosinophil growth. Alternatively, the patient might have been producing so much IL-5 that the levels might have outpaced mepolizumab injections. IFN- α treatment is often effective because of a shift in the cytokine milieu from T_H2, which is supportive of eosinophil growth, to a T_H1-type response. In this case IFN- α caused a decrease in eosinophil counts, although not to normal levels, and the patient experienced the side effects of IFN- α .

Alemtuzumab is an anti-CD52 antibody that can bind to both eosinophils and T cells, potentially inhibiting either the myeloproliferative, lymphoproliferative, or idiopathic variant.⁶ CD52 is a glycosylphosphatidylinositol-anchored molecule expressed on human eosinophils, lymphocytes, macrophages, and monocytes but not on neutrophils.⁷ Alemtuzumab is approved for the treatment of B-cell chronic lymphocytic leukemia and is also used to treat small lymphocytic lymphoma and mantle cell lymphoma in conjunction with other treatments. Side effects include infusion reactions (often severe), lymphopenia, anemia, thrombocytopenia, and infections. Alemtuzumab was used successfully as a treatment for HES in 2 prior cases, 1 lymphoproliferative and 1 myeloproliferative, both of which did not respond to imatinib mesylate or IFN- α and that were not tested for the *FIP1L1/PDGFR*A fusion.^{8,9} Alemtuzumab controlled our patient's eosinophilia for 1½ years, and the patient's quality of life appeared improved. Our patient most likely had the idiopathic HES variant. However, the occurrence of thromboembolism and an increased B12 level point to a possible myeloproliferative HES variant. The patient had a plasmacytoma and then multiple lytic lesions and multiple myelomas, suggesting involvement of 2 cell lineages by a single mutation or possibly independent mutations. Overall, the results in our patient and the previously reported cases suggest that alemtuzumab might be an effective treatment for the myeloproliferative, idiopathic, and lymphoproliferative HES variants.

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CD14 and IL4R gene polymorphisms modify the effect of day care attendance on serum IgE levels

To the Editor:

The cause of atopy is generally traced to the interplay of genetic and environmental factors.¹ Day care appears to be one of the most frequently investigated environmental factors. Although the results of studies investigating the association between day care attendance and atopy, as assessed by skin prick test responses, were inconsistent, all studies²⁻⁴ measuring serum IgE levels have thus far shown a constant decreasing effect on serum IgE levels.

Among the genes that show a gene-environment interaction for the development of atopy or allergic diseases, the most frequently investigated is the *CD14* gene.¹ However, there is no report that investigates interaction of this gene and day care attendance. *CD14* is a pattern-recognition receptor involved in the clearance of bacterial endotoxin and is also known as a receptor of respiratory syncytial virus. We investigated *CD14*-159C/T (rs2569190) and *CD14*-550C/T (rs744455) polymorphisms in Japanese patients with severe respiratory syncytial virus-induced bronchiolitis and found that *CD14*-550C/T but not *CD14*-159C/T was significantly associated with the condition.⁵

The IL-4 receptor α gene (*IL4R*) is also one of the most frequently investigated genes and has been shown to be associated with atopy and atopic diseases.⁶ The Ile50Val polymorphism (rs1805010) of the *IL4R* gene is a functional polymorphism and has been reported to be strongly associated with atopy and atopic asthma in the Japanese population. To date, only one study has reported the interaction of the *IL4R* Ile50Val polymorphism and day care attendance in the first year of life.⁷ The result showed a

TABLE I. Characteristics of the subjects

Total no. of participants	473
Age (mo)	
Mean \pm SD	111.1 \pm 19.9
Range	76-147
Sex ratio (male:female)	1.00:1.01
Day care attendance before age 2 y (%)	14.5
Total IgE (IU/mL), mean \pm SD	
Male	254 \pm 340
Female	241 \pm 469
Prevalence of atopy (%)	
Male	76.9
Female	68.0
Prevalence of allergic disorders (%)	
Asthma	
Male	14.1
Female	6.6
Atopic dermatitis	
Male	11.5
Female	9.7
Allergic rhinitis	
Male	42.1
Female	31.2
Food allergy	
Male	3.0
Female	3.4

significant gene-environment interaction for IFN- γ production at 1 year of age. However, it is not known whether this modified cytokine response affects the chance of having atopy or allergic diseases in the later period of life.

Here we report a relationship between serum total and specific IgE levels in Japanese elementary school children and day care attendance during earlier life. Our results suggest that day care attendance is associated with serum IgE levels, and this effect is modified by *CD14-550C/T* and *IL4R* Ile50Val polymorphisms. This is the first report that suggests an interaction between early-life day care attendance and genetic variations on IgE levels in later life.

Children attending an elementary school located in the central area of Chiba city (population of approximately 930,000) were recruited for this study. We first asked all ($n = 843$) children for participate in the survey. We then sent a detailed questionnaire to those who had a positive response ($n = 582$). Children with congenital heart diseases and lung diseases caused by immature birth were excluded. A total of 473 school children aged 6 to 12 years were enrolled. Blood samples were collected from 411 children on 2 separate days (July 3 and 12, 2006) for serum and DNA preparation. A complete set of information on total and 8 specific IgE levels, genotypes, and environmental factors was obtained from 375 children. All parents provided written informed consent. The study protocol was approved by the Ethics Committee of Chiba University Graduate School of Medicine.

The status of allergic diseases was evaluated by using questions based on the International Study of Asthma and Allergies in Childhood. We asked whether the child regularly attends a day care center where time is spent with other children at or before 2 years of age. For parents who responded yes to this question, the age of entry of their child to the day care center was obtained. The questionnaire also included the following items to assess possible confounding factors: number of siblings; number of older

siblings; allergic diseases of parents and siblings (family history: scored as positive if parents, siblings, or both had any of 4 allergic diseases [asthma, allergic rhinitis, atopic eczema, and food allergy]); residential area (6 categories), type of house structure (5 categories), and floor type of bedroom (5 categories); yogurt/fermented food consumption; pet ownership; and smoking among family members.

Genotyping of the *CD14-550C/T* polymorphism was performed as described previously,⁵ whereas that of the *IL4R* Ile50Val (rs1805010) polymorphism was carried out with the TaqMan allele-specific PCR method.⁸ Primer sequences were as shown in this article's Online Repository at www.jacionline.org.

Table I shows the characteristics of the investigated population. The percentage of children who had regularly attended day care before 2 years of age was 14.5%. Atopy was defined as the presence of positive (≥ 0.35 IU/mL) specific IgE level against at least 1 of the 8 allergens. Although the prevalences of asthma, atopic dermatitis, and food allergy were compatible with those in a recent large study,⁹ prevalences of allergic rhinitis and atopy were about 10 to 20 points higher, suggesting that children who had allergic rhinitis were more likely to attend this study.

Table II shows the association between day care attendance and serum IgE levels or atopy after being stratified with the *CD14-550C/T* genotype. Day care significantly decreased total IgE levels ($P = 9.7 \times 10^{-5}$), mite-specific IgE levels ($P = .0016$), and rate of atopy ($P = .00041$) in individuals with the C/T or T/T genotype, whereas the effect of day care was not observed in those with the C/C genotype. Numbers of children with the C/T+T/T genotype and those with the C/C genotype were similar, suggesting that the difference is not likely due to the statistical power for detecting association. Multivariate analyses with confounding factors were performed to evaluate the significance of this gene-environment interaction. The interaction between the *CD14-550C/T* polymorphism and day care was significant for \log_{10} (total IgE) ($P = .0046$), mite-specific IgE classes ($P = .00047$), and atopy ($P = .0097$) after adjusting for age, sex, family history, and number of siblings.

Table III shows the association between day care attendance and serum IgE levels or atopy after being stratified with the *IL4R* Val50Ile genotype. The effects of day care on total and some specific IgE levels were significant in Val/Ile heterozygotes but not in Val/Val or Ile/Ile homozygotes. In Val/Ile individuals day care significantly decreased total IgE levels ($P = .0012$), mite-specific ($P = .011$) and cedar pollen-specific ($P = .034$) IgE levels, and rate of atopy ($P = .018$). No such trend was observed in Val/Val or Ile/Ile individuals. The numbers of Val/Val and Val/Ile individuals were similar. It is therefore unlikely that the lack of significant association in Val/Val individuals was due to smaller statistical power for detecting association. When the significance of gene-environment interaction was assessed with the confounding factors, the interaction term between *IL4R* and day care attendance was significant for \log_{10} (total IgE) ($P = .019$) and mite-specific ($P = .0025$) and cedar pollen-specific ($P = .040$) IgE classes but not for atopy.

Total IgE levels in 4 genotype groups (group 1: *CD14* C/C, *IL4R* Ile/Ile+Val/Val; group 2: *CD14* C/C, *IL4R* Val/Ile; group 3: *CD14* C/T+T/T, *IL4R* Ile/Ile+Val/Val; and group 4: *CD14* C/T+T/T, *IL4R* Val/Ile) were compared to evaluate the combined effect of 2 polymorphisms on total IgE levels. Fig 1 shows the box

TABLE II. Effects of day care attendance on IgE levels when stratified by *CD14*-550C/T genotype

	C/C				C/T + T/T				Gene-environment interaction <i>P</i> value*
	Day care attendance		Effect size or odds ratio (95% CI)	<i>P</i> value	Day care attendance		Effect size or odds ratio (95% CI)	<i>P</i> value	
	No	Yes			No	Yes			
No. of subjects	169	22			157	28			
Log ₁₀ (total IgE)									
Mean	1.88	1.98	0.094 (-0.21 to 0.39)¶	0.54†	2.09	1.58	-0.50 (-0.26 to -0.76)¶	9.7 × 10 ⁻⁵ †	.0046**
SD	0.77	0.76			0.63	0.51			
Specific IgE (positive‡ rate)									
Mite	0.49	0.59	1.50 (0.61 to 3.69)#	.51§	0.61	0.32	0.30 (0.13 to 0.71)#	.0016§	.00047††
Cedar pollen	0.45	0.46	1.02 (0.42 to 2.45)#	.92§	0.57	0.32	0.35 (0.15 to 0.83)#	.032§	.116††
Atopy (rate)	0.77	0.68	1.60 (0.56 to 4.55)#	.38	0.81	0.50	0.24 (0.10 to 0.55)#	.00041	.0097††

Boldface indicates statistically significant values.

*Adjusted for age, sex, number of siblings, and family history.

†Analysis of variance for log₁₀(total IgE [in international units per milliliter]).

‡Class ≥1 (≥0.35 IU/mL).

§Kruskal-Wallis test for IgE value (in international units per milliliter).

||χ² Test of independence.

¶Effect size.

#Odds ratio.

**General liner model.

††Generalized linear model (Poisson distribution, log link function).

‡‡Logistic regression.

TABLE III. Effects of day care attendance on IgE levels when stratified by *IL4R* Val50Ile genotype

	Val/Val				Val/Ile				Ile/Ile				Gene-environment interaction <i>P</i> value*
	Day care attendance		Effect size or odds ratio (95% CI)	<i>P</i> value	Day care attendance		Effect size or odds ratio (95% CI)	<i>P</i> value	Day care attendance		Effect size of odds ratio (95% CI)	<i>P</i> value	
	No	Yes			No	Yes			No	Yes			
No. of subjects	125	18			152	27			49	5			
Log ₁₀ (total IgE)													
Mean	1.94	1.91	-0.058 (-0.38 to 0.27)¶	.72†	1.88	1.55	-0.44 (-0.71 to -0.18)¶	.0012†	1.99	2.32	0.33 (-0.31 to 0.97)¶	.12†	.019**
SD	0.64	0.72			0.57	0.56			0.69	0.52			
Specific IgE (positive‡ rate)													
Mite	0.57	0.56	0.95 (0.35 to 2.57)#	.51§	0.52	0.30	0.39 (0.16 to 0.94)#	.011§	0.59	0.80	2.76 (0.29 to 26.5)#	.36§	.0025††
Cedar pollen	0.50	0.50	1.01 (0.38 to 2.73)#	.93§	0.51	0.30	0.41 (0.17 to 0.99)#	.034§	0.55	0.40	0.54 (0.083 to 3.54)#	.91§	.040††
Atopy (rate)	0.74	0.72	0.93 (0.31 to 2.82)#	.90	0.74	0.52	0.37 (0.16 to 0.86)#	.018	0.76	0.80	1.30 (0.13 to 12.8)#	.82	.118††

Boldface indicates statistically significant values.

*Adjusted for age, sex, number of siblings, and family history.

†Analysis of variance for log₁₀(total IgE [in international units per milliliter]).

‡Class ≥1 (≥0.35 IU/mL).

§Kruskal-Wallis test for IgE value (in international units per milliliter).

||χ² Test of independence.

¶Effect size.

#Odds ratio.

**General liner model.

††Generalized linear model (Poisson distribution, log link function).

‡‡Logistic regression.

plot of log₁₀(total IgE) in 4 genotype groups. Among children who attended day care compared with group 1, the mean log₁₀(total IgE) values of groups 2, 3, and 4 decreased by 0.41, 0.35, and 0.69, respectively. This magnitude of change suggests that the effects of *CD14* and *IL4R* were additive. The children in group 4 showed significantly (*P* = .0046) lower total IgE levels than

those in group 1. On the other hand, among children who did not attend day care, the log₁₀(total IgE) levels of children in groups 3 (*P* = .031) and 4 (*P* = .036) were significantly higher than those of children in group 1. The *CD14* C/T and T/T genotypes appeared to show the opposite effect on the serum total IgE level in children who did not attend day care compared

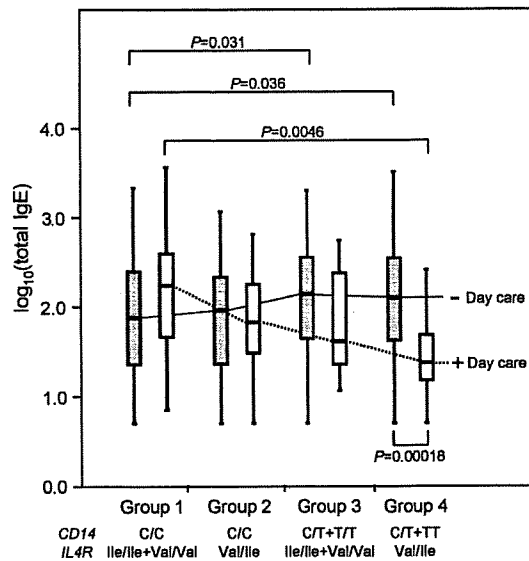


FIG 1. Total IgE levels in 4 groups of children classified based on a combination of *IL4R* and *CD14* genotypes. Box plot of \log_{10} (total IgE) values is shown for children who attended day care (+ Day care) and for those who did not (- Day care). Results are presented as medians and interquartile ranges. Only significant *P* values (<.05) are shown.

with those who did attend day care. When we examined the effect of day care in each genotype group, the effect was not sufficiently large to show a significant change in IgE level in groups 2 and 3, in which individuals had only 1 IgE level-decreasing genotype. However, in group 4, in which individuals had 2 IgE level-decreasing genotypes, the effect was sufficiently large to show a significant difference ($P = .00018$). Significance of interaction between the *CD14* and *IL4R* genotypes was also evaluated by using general linear models in which age, sex, family history, number of siblings, and day care were included as variables. The interaction term of the 2 genes was not significant, suggesting an independent effect of the *CD14* and *IL4R* genes.

The interaction of the *CD14* gene with day care attendance suggests that the mechanism of the effect of day care involves at least in part a response to infection, environmental endotoxin exposure, or both. The interaction of the *IL4R* gene with day care attendance suggests that the mechanism also involves those related to T_H2 cell proliferation and IgE production. These results suggest that the complex nature of mechanisms underlies the effect of day care attendance on serum IgE levels.

Environmental factors investigated in the present study were determined based on a questionnaire on past day care attendance, and therefore recall bias can be a potential problem. The number of subjects investigated in this study was not so large and might be the acceptable minimum for investigating gene-environment interactions. The subjects evaluated were children who attended a single school and lived in a medium-populated city, thus representing those living in rather small regional environments in Japan. Nevertheless, these characteristics of the present sample might have contributed to minimizing the variances of background and outcome parameters and might have resulted in the positive findings obtained from a relatively small number of subjects. It is necessary to perform a cohort study to follow children with or without day care attendance until they reach school age to validate the current observations.

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Mucormycosis in chronic granulomatous disease: Association with iatrogenic immunosuppression

To the Editor:

Chronic granulomatous disease (CGD) results from mutations in either X-linked (*gp91^{phox}*) or autosomal (*p47^{phox}*, *p67^{phox}*, and *p22^{phox}*) genes encoding the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Impaired generation of reactive oxygen species predisposes to recurrent life-threatening bacterial and fungal infections. Septated hyaline molds (particularly *Aspergillus* species) are the primary fungal pathogens in CGD. Fungi of the order Mucorales (pauciseptated molds) are environmentally ubiquitous and cause mucormycosis in select immunocompromised patient populations, such as those with diabetic ketoacidosis and hematologic malignancy and recipients of transplants or deferoxamine. We investigated the prevalence of mucormycosis in patients with CGD.

PRIMERS FOR *IL4R* GENOTYPING (5' TO 3')

TaqMan probe (FAM-TACAGGTGACCAGCCTAACCCAGC
CCCTGT-TAMRA); common primer (TGGAGGCATGTCCCG
GACAC); Ile (A) allele primer (CGCCTCCGTTGTTCTCAG
GGGT); and Val (G) allele primer (CGCCTCCGTTGTTCTC
AGGGGC).