Table 9   Four SNP haplotype (P1, P2, P8, and P12) frequency among normotensive (NT) and hypertensive (HT) subjects in the combined population (population 1 and 2)

	Haplotype[a]	Male subjects			Female subjects		
		NT ($n = 602$)	HT ($n = 615$)	P^b	NT ($n = 845$)	HT ($n = 658$)	P^b
H1	C-C-A-A	806 (0.671)	816 (0.663)	0.682	1215 (0.719)	858 (0.652)	$8.07 \times 10^{-5*}$
H2	C-T-G-A	167 (0.139)	171 (0.139)	0.989	175 (0.104)	191 (0.145)	$6.07 \times 10^{-4**}$
H3	T-T-G-G	129 (0.107)	144 (0.117)	0.446	177 (0.104)	146 (0.111)	0.578
H4	C-C-G-G	45 (0.038)	45 (0.036)	0.846	64 (0.038)	60 (0.045)	0.306
H5	C-C-A-G	29 (0.024)	38 (0.031)	0.263	22 (0.013)	37 (0.028)	0.003*
H6	C-C-G-A	21 (0.017)	9 (0.008)	0.033	24 (0.014)	20 (0.015)	0.906
	Others	5 (0.004)	7 (0.006)		13 (0.008)	6 (0.004)	

[a] Four loci are P1, P2, P8, and P12, and six predominant haplotypes are listed; 'others' category includes minor haplotypes with less than 1% frequency. [b] Significant P value after Bonferroni's correction for major six haplotypes is 0.0083 (0.05/6). * Difference was statistically significant.

for Pr-H2/Pr-H1, $P = 0.001$ and 0.88 for Pr-H2/Pr-H5, $P = 0.001$; Fig. 2c). There was no significant difference in promoter activity between the Pr-H1 and Pr-H5 constructs in both cells. These results suggest that expression of TNFRSF4 mRNA in cells is lower in individuals who have the H2 haplotype than in cells from individuals who have other types of haplotypes. To clarify the responsible SNP(s) for the lower promoter activity of Pr-H2, we performed an additional assay using a series of promoter constructs that contained only one polymorphic change (Pr-P2-T, Pr-P3-T, Pr-P4-del, Pr-P6-G, Pr-P8-G, Pr-P9-G,

Pr-P10-T, and Pr-P11-G). In COS-7 cells, promoter activities of Pr-P2-T, Pr-P6-G, and Pr-P11-G were significantly lower than that of Pr-H1 (0.69 for Pr-P2-T/Pr-H1, $P < 0.0001$, 0.90 for Pr-P6-G/Pr-H1, $P = 0.016$, and 0.88 for Pr-P11-G/Pr-H1, $P = 0.015$; Fig. 2b). In HEK293 cells, as in COS-7 cells, Pr-P2-T showed significantly lower promoter activity when compared with Pr-H1 (0.71 for Pr-P2-T/Pr-H1, $P = 0.0001$; Fig. 2d). The results of other constructs, however, were different: promoter activities of Pr-P8-G and Pr-P11-G were significantly higher than that of Pr-H1 (1.04 for Pr-P8-G/Pr-H1, $P = 0.002$ and 1.10 for

Fig. 2
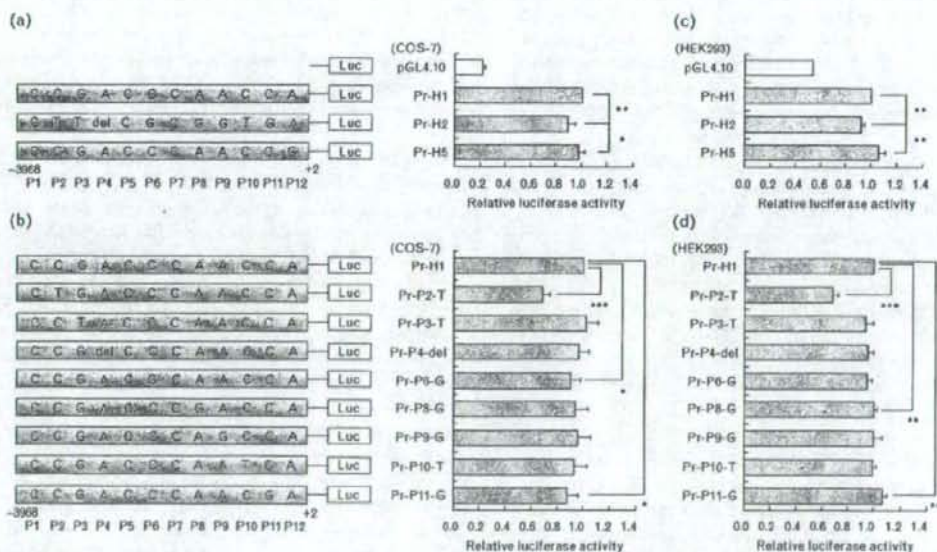
Effect of haplotypes and each polymorphism on the transcriptional activity of the TNFRSF4 promoter. (a and c) Effect of haplotypes on the transcriptional activity of the TNFRSF4 promoter. (b and d) Effect of each polymorphism on the transcriptional activity of the TNFRSF4 promoter. Relative luciferase activities after transient transfection in COS-7 (a and b) and HEK293 (c and d) cell lines are shown. Activities of the Pr-H1 constructs were considered as 100%. Each experiment was conducted in triplicate for each sample, and the results are expressed as mean ± SD for six (COS-7) or five (HEK293) independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Pr-P11-G/Pr-H1, $P = 0.003$; Fig. 2d). Only Pr-P2-T showed consistent change in promoter activity in the two different cell lines. These results suggest that P2 had the largest impact on the decreased promoter activity of the H2 haplotype.

Discussion

The significance of *TNFRSF4* in the pathogenesis of female subjects with EH was indicated in two independent sets of population. Haplotype analysis using four SNPs (P1: −3948C>T, P2: −3606C>T, P8: −1725A> G and P12: −530A>G) in the 5′ upstream region showed that the frequency of H1 (C-C-A-A) was significantly low among female HT patients when compared with female NT controls in both population 1 ($P = 0.031$) and population 2 ($P = 8.48 \times 10^{-4}$). The frequency of H2 (C-G-T-A) of female HT patients was significantly higher than that of female NT controls in population 2 ($P = 6.46 \times 10^{-4}$), but not in population 1. In the combined population, both significantly lower frequency of H1 ($P = 8.07 \times 10^{-5}$) and significantly higher frequency of H2 ($P = 6.07 \times 10^{-4}$) were observed in female HT patients compared with female NT controls. No difference in haplotype frequencies between the HT and NT groups was observed in the male subjects of either the combined or separate population. These results of association of the *TNFRSF4* haplotype with hypertension suggested that the H1 haplotype is a protective allele and that the H2 haplotype is a high-risk allele for EH in women. The promoter activity of the H2 haplotype was significantly lower than that of the H1 and H5 (C-C-A-G) haplotypes. Furthermore, the Pr-P2-T construct showed lower promoter activity than other constructs. Allelic association of P2 (−3606C>T, rs12036216) with female HT patients was significant in population 2 and the combined population (data not shown), but not in population 1. These data suggested that P2 is the responsive SNP that modifies the risk for hypertension in females, although it is possible that unidentified variant(s) in LD with this haplotype have function(s) that influence disease susceptibility. We also observed a significant difference in frequency of the H5 haplotype in the combined population ($P = 0.003$) and in population 1 ($P = 6.78 \times 10^{-5}$), but not in population 2. We, however, could not find any transcriptional effect of H5 haplotype.

The TNFRSF4–TNFSF4 interactions on T lymphocytes enhance proliferation and differentiation of the cells as well as generation and survival of memory CD4$^+$ T cells in the process of inflammation and immune response [15–18]. Several inflammatory markers, such as soluble leukocyte adhesion molecules, cytokines, specific growth factors, heat shock proteins, CD40L, and C-reactive protein (CRP), were reported to increase in patients with EH [32–41]. Although the relationship between inflammation and hypertension has not been well established, a growing body of evidence indicates that vascular inflam-

mation may be involved in both the initiation and development of hypertension [42–46]. Sesso *et al.* [46] showed that elevated plasma CRP, a well-known marker of inflammation, was associated with the future development of hypertension in a dose-dependent manner. Furthermore, hypertension has been suggested to trigger inflammation through the increased expression of several mediators, including leukocyte adhesion molecules, chemokines, specific growth factors, heat shock proteins, endothelin-1, and angiotensin [47–54]. Given our findings that variants of the *TNFRSF4* gene, which might affect the inflammatory cascade, were associated with EH among women, it is likely that inflammation may play a role in initiation and/or development of hypertension.

Inflammatory process [21] and T-lymphocyte activation [12,19,20] are implicated to be involved in the pathogenesis of atherosclerosis. Thus, alteration(s) in the TNFRSF4–TNFSF4 pathway could influence atherosclerosis formation. Indeed, Wang *et al.* [22] found that polymorphisms of *TNFSF4* are associated with MI in women. Furthermore, a polymorphism in *TNFRSF4* was also reported to be associated with MI [23]. These studies strongly suggested that genes involved in the TNFRSF4–TNFSF4 pathway play a role in the pathogenesis of atherosclerosis and MI, particularly in women.

Our findings combined with those of the studies mentioned above suggested that genetic variations in the TNFRSF4–TNFSF4 pathway may be involved in the pathogenesis of both atherosclerosis and hypertension. So, which comes first, atherosclerosis or hypertension? Hypertension is one of the principal risk factors for atherosclerosis and MI [24], but the exact mechanism underlying the association is not fully understood. Although arterial stiffness, which is a predictor of atherosclerosis [55,56], has been thought to be the result of hypertension rather than its cause, recent studies suggested that arterial stiffness is related to the development of hypertension [57,58]. These data indicated that the relationship between hypertension and arterial stiffness may be bidirectional [59]. Therefore, three different scenarios are possible to explain the results that genetic variations in the TNFRSF4–TNFSF4 pathway are associated with both hypertension and MI. First, inflammation may directly increase arterial stiffness and induce the development of an atherosclerotic lesion, which may lead to the development of hypertension. Second, inflammation may induce hypertension, which may result in increase in arterial stiffness and atherosclerosis. Third, inflammation may promote the development of hypertension and atherosclerosis by different pathways. Although it is not clear whether atherosclerosis is a cause of hypertension, our findings and that of previous studies indicate that the inflammation may be an important part of the link between hypertension and atherosclerosis and cardiovascular events, such as MI.

TNFSF4 is also a potential candidate for a susceptibility gene involved in the pathogenesis of EH in women. We, therefore, examined the putative association between polymorphisms in the *TNFSF4* gene and hypertension in population 1. The allele frequencies of four SNPs (rs1234315, rs3850641, rs1234313, and rs3861950) and its haplotype did not significantly differ between the HT group and the NT group for women (data not shown). In contrast to the case of MI in which susceptibility was affected by variations of both *TNFRSF4* and *TNFSF4*, susceptibility for hypertension may be affected only by *TNFRSF4*, though more extensive studies are required before we conclude an association of *TNFSF4* with hypertension.

In the present study, we found that variations of *TNFRSF4* affected hypertension susceptibility only in women. This is an interesting similarity to women-specific MI susceptibility exerted by *TNFSF4* and *TNFRSF4*. Some case–control studies [5,60,61] have identified gene variants associated with sex-specific susceptibility to EH. Recently, Nakayama *et al.* [5] reported that an SNP in the 5′-untranslated region of the follicle-stimulating hormone receptor (*FSHR*) gene, in which mutations were reported to cause hereditary hypergonadotropic ovarian failure [62], was associated with EH in women and affected the levels of transcriptional activity. In this study, the functional mutation of the gene was clearly identified in patients with EH in a sex-specific manner. Currently, the reason for women-specific association of *TNFRSF4* with EH is an open question. One possibility is the involvement of the female sex hormone, estrogen. After menopause, women are at increased risk of inflammatory cardiovascular diseases such as atherosclerosis and coronary heart disease, suggesting that estrogens modulate the initiation and progress of inflammation [63–65]. Recently, Xing *et al.* [66] suggested that estrogen may exert anti-inflammatory effects by inhibiting tumor necrosis factor-α-mediated chemokine production in vascular smooth muscle cells. Estrogen, however, is also known to increase CRP, which is an inflammatory marker [63]. These findings indicate that estrogen may modulate the production of several proinflammatory molecules in distinct pathways. It is possible that TNFRSF4 and estrogen cross talk in inflammation networks.

In conclusion, the present study revealed that haplotypes of the *TNFRSF4* gene were associated with EH among women in two Japanese population, suggesting an involvement of the *TNFRSF4* gene in the pathogenesis of female EH.

Acknowledgements

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ORIGINAL ARTICLE

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

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Abstract Bronchial asthma (BA) is a multifactorial dis-
order, 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 comple-
ment-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 sus-
ceptibility 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 asso-
ciation 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 rele-
vant to the effector phase of allergic inflammation.

Keywords Complement · *C3* gene · Asthma · SNP ·
Haplotype · Association · Total IgE

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Introduction

Bronchial asthma (BA) is an inflammatory airway dis-
ease, 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 exoge-
nous (microorganisms) and endogenous (altered-self)
danger-associated molecular patterns (Kohl and Wills-
Karp 2007). The three pathways of complement activa-
tion 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 (FEV_1) 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 (*Dermatophagouides 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
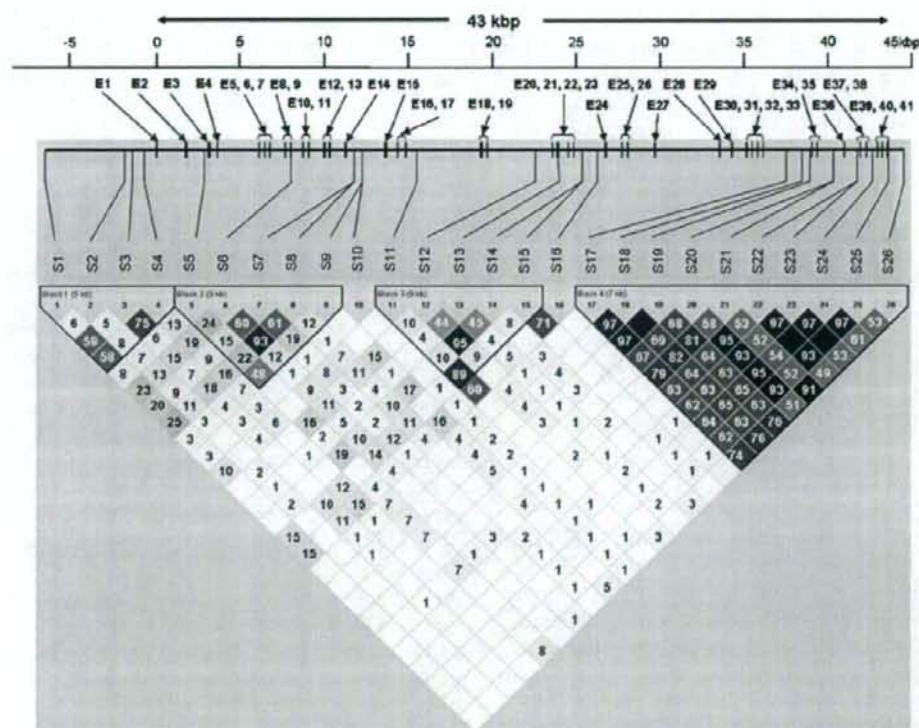
Fig. 1 Structure of the *C3* gene and location of single nucleotide polymorphisms (SNPs) and their linkage equilibrium (LD) map. Exons are indicated by *black boxes*. *Numbers* starting with *E* stand for exons and those starting with *S* for SNPs. Pair-wise LD (r^2) was estimated from 96 control subjects. 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 μM of the TaqMan probe (when needed), and 5 ng of amplified template DNA in a final volume of 10 μl. 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 μM 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, Mobara, 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'-GGCATAAACAAGGGTTAAAATGT
F: C allele	5'-GGCATAAACAAGGGTTAAAATGC
R: common	5'-GCTCACAAACGCCTATGA
TaqMan probe	5'-TGAAATAGATAAGTTGCTGCCACCCG
SNP2	
F: C allele	5'-GCCTGGCCAACATGGCGAACC
F: T allele	5'-GCCTGGCCAACATGGCGAAGT
R: common	5'-TGCCTCCTGGGTTCAAGTGATTCTC
TaqMan probe	5'-TAGTGGCGCATGCCTGTGATCCCAGCTCT
First PCR F:	5'-TTCCAAAGAGTGTGTCGCAA
First PCR R:	5'-CCTGCTTCATAGAGTTGTCGT
SNP3	
F: A allele	5'-TTTGGCAATATCTAGCAAGATTACCTA
F: G allele	5'-TTTGGCAATATCTAGCAAGATTACCTG
R: common	5'-CCTTACCACCTGCTTCATAGAGTTG
TaqMan probe	5'-TGACCCAACATATTTCCTTTCATTGCAACG
SNP4	
F: C allele	5'-CCTGTAACCTGTAAGAATGAGAC
F: A allele	5'-CCTGTAACCTGTAAGAATGAGTA
R: common	5'-CAAAGTGCTGGTGTGAACTACTG
TaqMan probe	5'-TAGTATGTGCTATGTGCTGTCC
SNP5	
R: A allele	5'-GCCTGCCCATTATTCTTGGTCCT
R: G allele	5'-GCCTGCCCATTATTCTTGGTCAC
F: common	5'-CCTTGTGAGCTCTTCTTTTTGAGTTC
SNP6	
R: G allele	5'-CACCCCGTCCAGCAGTACCTAC
R: A allele	5'-CACCCCGTCCAGCAGTACCTAT
F: common	5'-AACAGAGGATTTCCCTGCCTGAA
TaqMan probe	5'-CCCTCAAGCGCATTCCG
SNP7	
F: A allele	5'-AACAGAGGATTTCCCTGCCTGTA
F: G allele	5'-AACAGAGGATTTCCCTGCCTGGG
R: common	5'-CACCCCGTCCAGCAGTACCTTC
TaqMan probe	5'-CCCTCAAGCGCATTCCG
SNP8	
F: G allele	5'-TGCTGAATAAGAAGAACAAACTGAGG
F: A allele	5'-TGCTGAATAAGAAGAACAAACTGAGA
R: common	5'-TGCCTCCGCCTCTTCTCA
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'-TTTCCGGAGTAGGGACGAAGA

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'-GGAAGTGGAAGTCAAGGCTGGC
F: T allele	5'-GGAAGTGGAAGTCAAGGCTGGT
R: common	5'-GGGGTGCCCCAAGCACTCA
TaqMan probe	5'-CCATCATTTCATCAGTGACGGTGTCAGGAA
SNP14	
R: T allele	5'-GAATGAGATGGAATTTGGCTCGA
R: C allele	5'-GAATGAGATGGAATTTGGCTCTG
F: common	5'-CAAGTCCCGGACACCGAGTCT
SNP15	
R: C allele	5'-CAGCGAGCTGAGGTCGGG
R: T allele	5'-CAGCGAGCTGAGGTCGTA
F: common	5'-CATCTGTGATCTGTTTTCCCTCTTTTAC
SNP16	
R: C allele	5'-GAGTGTCTCACTTAATAGTCAACGATG
R: G allele	5'-GAGTGTCTCACTTAATAGTCAACGATC
F: common	5'-TGGTCAGGCTGGTCTTGAACTC
SNP17	
F: G allele	5'-CTGCCAAAGTTTTGGGATCACTG
F: A allele	5'-CTGCCAAAGTTTTGGGATCACTA
R: common	5'-CCACACCCGGCCATTTCC
SNP18	
R: C allele	5'-AATGCCAGAAGTGAACTTCAAAGTG
R: G allele	5'-AATGCCAGAAGTGAACTTCAAAGTC
F: common	5'-CAGCAGGGTCAACATCACCATA
SNP19	
F: T allele	5'-GGCTGCCTGTATTCTTGCCTAT
F: delT allele	5'-GGCTGCCTGTATTCTTGCCTCG
R: common	5'-TGGATTCAAATTCCAGCTCTAAATAAC
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'-ATGTTGCTCAAGTTGGTCTCAAACT
SNP22	
R: A allele	5'-GGCCTCCCTCCAAAGACCCT
R: G allele	5'-GGCCTCCCTCCAAAGACCTC
F: common	5'-CGTGTCCCAGGAATCTATGAATTT

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 ($P_{cor} = 0.030$) 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 ($P = 0.004$, $P_{cor} = 0.060$). 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%) ($P = 0.0014$, $P_{cor} = 0.038$). 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%) ($P_{cor} > 1.0$). 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 gene-wide significance ($P_{cor} = 0.12$). 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 child control $P_{cor}{}^e$	Adult asthma versus adult control $P_{cor}{}^e$	All asthma versus all control $P_{cor}{}^e$
	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			
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
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
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
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
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
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
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
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
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
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
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
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
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
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	0.540	0.555
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	0.030	0.060

[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 (%)				Odds ratio (95% CI)
	Case		Control		
	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 $\log_{10}$-transformed total immunoglobulin E (IgE)

Locus	P 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 $\log_{10}$ (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 lipopolysacharide 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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Association of the *GABRB3* Gene With Nonsyndromic Oral Clefts

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Objective: Nonsyndromic oral clefts are common craniofacial anomalies classified into two subgroups: cleft lip with or without cleft palate and isolated cleft palate. Nonsyndromic oral clefts are multifactorial diseases, with both genetic and environmental factors involved in their pathogenesis. The inhibitory neurotransmitter, γ-aminobutyric acid plays a role in normal embryonic, and particularly facial, development and γ-aminobutyric acid receptor type A β-3 subunit (GABRB3) knockout mice have been shown to have cleft palate. The *GABRB3* gene is therefore a strong candidate gene for nonsyndromic oral clefts. We investigated here whether genetic variations of the *GABRB3* gene affect the risk for nonsyndromic oral clefts.

Method: In this case-control study, a total of 178 Japanese patients with cleft lip with or without cleft palate and 374 unrelated controls were recruited and were genotyped for six single nucleotide polymorphisms and a dinucleotide repeat marker of the *GABRB3* gene.

Results: None of the single nucleotide polymorphisms showed complete linkage disequilibrium with other single nucleotide polymorphisms. In a case-control association study with the six-locus haplotype of the gene, TGTGCT haplotype frequency in patients with cleft lip with or without cleft palate was significantly higher than in the controls (corrected *p* value = .029). None of the alleles of the dinucleotide repeat marker showed significant association with cleft lip with or without cleft palate.

Conclusions: Our data suggest that the *GABRB3* gene is involved in the pathogenesis of cleft lip with or without cleft palate in the Japanese population.

KEY WORDS: *cleft palate, GABRB3 gene, γ-aminobutyric acid (GABA), non-syndromic cleft lip with or without cleft palate, oral clefts, single nucleotide polymorphism*

Craniofacial abnormalities are among the most common of all birth defects, and the most frequent of these are oral clefts (OC) (Wyszynski et al., 1996). Oral clefts can occur in isolation (nonsyndromic) or as a feature of certain syndromes (syndromic). On the basis of anatomical, embryological, and genetic observations, nonsyndromic OC (NSOC) typically are classified into two subgroups: cleft lip with or without cleft palate (CL $\pm$ P; MIM 119530) and isolated cleft palate (CP; MIM 119540) (Murray, 2002). The prevalence of CL $\pm$ P at

birth ranges from 1 in 300 to 1 in 2500 births and that of CP is about 1 in 1500 births (Wyszynski et al., 1996). Numerous studies have shown that genetic factors appear to play a significant role in the etiology of NSOC (Marazita et al., 1986; Farrall and Holder, 1992; Mitchell and Risch, 1992; Maestri et al., 1997; Schliekelman and Slatkin, 2002). Craniofacial development is a highly complicated process that involves multiple genes and signaling molecules (Cox, 2004; Helms et al., 2005). Among these, the importance of the metabolism and function of γ-amino butyric acid (GABA) has been demonstrated by multiple studies on mice, both teratological (Wee and Zimmerman, 1983; Wee et al., 1986; Ding et al., 2004) and genetic (Culiat et al., 1993, 1994, 1995; Asada et al., 1997; Condie et al., 1997; Homanics et al., 1997; Hagiwara et al., 2003). Furthermore, recent microarray and immunohistologic analyses have shown that levels of GABA, glutamic acid decarboxylase (GAD), and GABA receptor type A β-3 subunit (GABRB3) in developing palatal tissues change dramatically during palatogenesis (Brown et al., 2003). GAD is a key enzyme that synthesizes GABA from glutamic acid, and the two GAD isozymes are derived from two distinct genes, *GAD65*

and *GAD67*. The *GAD67* knockout mice die of severe cleft palate soon after birth (Asada et al., 1997; Condie et al., 1997). GABA receptors are ligand-gated Cl⁻ channels consisting of five subunits that are highly expressed in brain and certain peripheral tissues. Several mice studies have demonstrated the relevance of the *GABRB3* gene. Radiation-induced pink-eyed dilution (*p*) locus mutants in mice often have nonpigmentation phenotypes caused by disrupting nearby genes (Brilliant, 1992; Johnson et al., 1995). Of these, p^{cp} (*p* with cleft palate) shows cleft palate phenotype (Phillips, 1977; Culiat et al., 1993; Nakatsu et al., 1993). The p^{cp} mutation is a large deletion that includes the *p* gene and a cluster of three $GABA_A$ receptor subunit genes. The critical region of the cleft palate phenotype was mapped to *Gabrb3* (Culiat et al., 1993; Nakatsu et al., 1993) and was confirmed by transgenic rescue of the mutant phenotype by expression of the β_3 subunit minigene (Culiat et al., 1995). The suggestion that a deficiency of the *Gabrb3* gene causes cleft palate was confirmed further by two independent knockout mice experiments (Homanics et al., 1997; Hagiwara et al., 2003).

We have previously reported an association between the single nucleotide polymorphisms (SNPs) of the *GAD67* gene and CL ± P in the Japanese population (Kanno et al., 2004). A significant association between five-locus haplotype and CL ± P was detected in both case-control studies and transmission disequilibrium tests (TDT). The results suggested that GABA is necessary not only for palate formation but also for lip formation in humans. Scapoli et al. (2002) performed a TDT with a multi-allelic CA repeat of the *GABRB3* gene in Italian families and found significant transmission disequilibrium. However, a case-control study in the Japanese population failed to show association between the same marker and CL ± P (Tanabe et al., 2000).

The aim of this study was to investigate whether genetic variations of the *GABRB3* gene are associated with NSOC in the Japanese population. Population-based association studies were performed with six SNPs and a CA repeat of the *GABRB3* gene.

MATERIALS AND METHODS

Subjects

A total of 189 Japanese families with at least one NSOC patient were recruited. A family history was obtained from the patients or their parents to determine the presence or absence of related individuals with NSOC and other congenital anomalies. Of the probands, 181 were CL ± P and 11 were CP. Affected sib-pairs were observed in three families. Affected parents were found in seven families. From families with a sib-pair, one patient was selected for the case-control study. Thus, 178 CL ± P probands from 178 families were used in the case-control study. As control subjects, 374 healthy Japanese adult volunteers were recruited from among students and staff at Tohoku University Hospital. Because no phenotyping information was obtained from the controls, we estimated,

TABLE 1   Locations and Allele Frequencies of Single Nucleotide Polymorphisms (SNPs) in the *GABRB3* Gene

SNP	Location	Allele*	Position†	Minor Allele Frequency	dbSNP Number
SNP1	promoter	T/C	22261025	0.369	rs4906902
SNP2	intron3	G/A	22254361	0.357	rs12437535
SNP3	intron3	A/T	22238758	0.362	rs2059574
SNP4	intron3	A/G	22198692	0.231	rs981778
SNP5	intron3	A/C	22163638	0.481	rs890317
SNP6	intron8	C/T	22039220	0.286	rs2081648

* Base expressed in the direction of the gene.
† Based on NCBI Build 35.1 reference group label.

based on a birth prevalence of 1 in 700, that less than one CLP case could occur in this group. This would have a negligible effect on the study outcome. After written and oral informed consents were obtained, peripheral blood samples were collected from patients and controls. This study was approved by the Ethics Committees of Tohoku University School of Medicine.

Genotyping

DNA was prepared from whole blood samples using a GFR DNA purification kit (GE Healthcare Bio-Sciences Corp., Tokyo, Japan).

To test association of the *GABRB3* gene and NSOC, six SNPs were genotyped in probands and controls (Table 1). SNP1 was located in the promoter region of the *GABRB3* gene, which extends about 4 kb upstream of exon 1 (Kirkness and Fraser, 1993). Other SNPs that showed a minor allele frequency of more than 20% and had a variety of minor allele frequencies were selected from the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). We thought that by selecting SNPs with different minor frequencies, we could avoid selecting SNPs with complete linkage disequilibrium (LD).

A modified TaqMan–allele specific amplification method using an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA) was used for SNP typing (Fujii et al., 2000). For typing SNP1 and SNP2, the TaqMan probe was not used and allele specific amplification was detected with SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Sequences of polymerase chain reaction primers and TaqMan probes are shown in Table 2.

Typing of a CA repeat marker (Mutirangura et al., 1992) of the *GABRB3* gene was carried out as described previously (Scapoli et al., 2002).

Statistical Analysis

Pair-wise LD in each SNPs was estimated as D′ (Lewontin, 1964) and r^2 (Hill and Robertson, 1968). The SNPAlyze V4.1 Pro program (DYNACOM Co., Ltd., Chiba, Japan) was used for calculation of LD parameters and inference of haplotype frequency. Differences in haplotype frequencies between patients and controls were evaluated by chi-square tests of one haplotype against others (haplotype-wise test), and significance

TABLE 2   Primers for Genotyping SNPs in the *GABRB3* Gene

SNP Name	Primer Orientation	Sequence
SNP1	F: common*	5'-GGATGCTATATTTATCTCTGAATCTTTCAGGTA
	R: T-allele	5'-ACGTTGGCATGTTTCTGTGCATCA
	R: C-allele	5'-ACGTTGGCATGTTTCTGTGCATCG
SNP2	F: common	5'-AGCTATGGGTTCTGTTTCCAAGTC
	R: G-allele	5'-AATGGGCATCTGGAAACCTTC
	R: A-allele	5'-AATGGGCATCTGGAAACCTTT
SNP3	F: A-allele	5'-TGATTTTCAGGCAAACTATGCA
	F: T-allele	5'-TGATTTTCAGGCAAACTATGCT
	R: common	5'-AGACATGCCACCTCAGATAC
	TaqMan Probe†	5'-AATGGGTGCTTGGCAAGGAGGAAA
SNP4	F: A-allele	5'-AGGTTGGAGCACAGGGCCTATA
	F: G-allele	5'-AGGTTGGAGCACAGGGCCTATG
	R: common	5'-CCTTCACATCCTCCCCAGTCTTC
	TaqMan Probe	5'-AGGCCAGGGAGGTGGGCAGAG
SNP5	F: A-allele	5'-CTCTTCCATGATTGAAATGGCA
	F: C-allele	5'-CTCTTCCATGATTGAAATGGCC
	R: common	5'-GTCCTGCCCTTTTGATCTAA
	TaqMan Probe	5'-CGATAAGTTGGATCCCAGTTGCCTCTTT
SNP6	F: A-allele	5'-TCGATTGTATTAGAATGTCCAGCATCAC
	F: T-allele	5'-TCGATTGTATTAGAATGTCCAGCATCAT
	R: common	5'-GTGAACCTGGGAGGCAAAGC
	TaqMan Probe	5'-TCACCCAGGCTGGAGTGCAGTGG
	1st PCR F:	5'-CGGGAGAATGGTGTGAACCT
	1st PCR R:	5'-ATGCTTGGTACCCCACCAAATA

* 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 the 3' end.

values were corrected for multiple comparisons by multiplying the *p* value by the number of haplotypes tested in the association study (Bonferroni correction). Raw *p* values and corrected *p* values are abbreviated as *praw* and *pcor*, respectively. SPSS software version 11.0J (SPSS Japan Inc., Tokyo, Japan) was used to perform all statistical analyses unless otherwise stated. A *pcor* value less than .05 was considered statistically significant. The attributable fraction (AF) of a risk haplotype was calculated according to the definition by Miettinen (1974).

RESULTS

Genotypes of all SNPs in patients and controls were in Hardy-Weinberg equilibrium. Using control samples, pair-wise

linkage disequilibrium coefficients (D' and r^2) were determined (Table 3). SNP1, SNP2, and SNP3 were in strong LD with each other (D' > 0.8); SNP3, SNP4, and SNP5 were in weaker LD; SNP6 showed little LD with other SNPs. Because none of the SNP pairs showed complete LD, we used all SNPs for further analyses. Linkage disequilibriums between the CA repeat and each SNP were not strong (D' < 0.25).

None of the SNPs showed a significant association with the disease in single-SNP association studies (data not shown). Using the SNPAlyze program (DYNACOM), we estimated the frequency of the six-locus haplotype of the *GABRB3* gene in controls and NSOC patients (SNP1 to SNP6; Table 4). Haplotypes with an estimated frequency of less than 1% in the entire sample were combined and were denoted as "Others."

TABLE 3   Pair-wise Linkage Disequilibrium for All Possible Two-Way Comparisons Among SNPs and a CA Repeat Marker in the *GABRB3* Gene

First SNP*		Second SNP/Repeat Polymorphism					
		SNP2	SNP3	SNP4	SNP5	SNP6	CA repeat†
SNP1	D'‡	0.980	0.818	-5.39×10^{-3}	-0.122	-7.57×10^{-4}	0.079
	r^2§	0.902	0.214	5.35×10^{-6}	8.66×10^{-3}	3.78×10^{-7}	
SNP2	D'		0.894	5.75×10^{-3}	-0.083	0.013	0.105
	r^2		0.240	2.04×10^{-5}	3.66×10^{-3}	3.28×10^{-5}	
SNP3	D'			0.421	0.173	0.09	0.228
	r^2			0.032	0.017	5.20×10^{-3}	
SNP4	D'				0.241	-0.0799	0.089
	r^2				0.0188	5.74×10^{-3}	
SNP5	D'					0.105	0.062
	r^2					4.08×10^{-3}	
SNP6	D'						0.246

* SNP = single nucleotide polymorphism.
† CA repeat polymorphic marker (Mutirangura et al., 1992).
‡ Pair-wise LD parameter by Lewontin (1964).
§ Pair-wise LD parameter by Hill and Robertson (1968).

TABLE 4   Six-locus Haplotype Association Study of the *GABRB3* Gene

Haplotype	Frequency			praw*	pcor†	OR‡	95% CI§	
	Overall	Control	Patient				Low	High
CATGAC	0.129	0.119	0.177	0.010	0.124	1.59	1.12	2.26
TGAGCC	0.118	0.114	0.125	0.57	>1.0	1.13	0.77	1.66
TGAGAC	0.09	0.0970	0.0657	0.084	>1.0	0.64	0.39	1.04
TGTGCC	0.0859	0.0979	0.0641	0.063	>1.0	0.64	0.39	1.04
CATGCC	0.0658	0.0645	0.0611	0.83	>1.0	0.96	0.57	1.62
TGAGAT	0.0606	0.0677	0.0527	0.34	>1.0	0.77	0.45	1.33
TGTGAC	0.0593	0.0578	0.0561	0.91	>1.0	0.98	0.57	1.69
CATGAT	0.0416	0.0349	0.0388	0.75	>1.0	1.14	0.59	2.21
CATACC	0.0375	0.0409	0.0325	0.50	>1.0	0.81	0.41	1.59
TGTACC	0.0354	0.0485	0.0194	0.020	0.39	0.40	0.18	0.90
TGTAAC	0.0291	0.0245	0.0215	0.76	>1.0	0.93	0.40	2.17
TGTGCT	**0.0287**	**0.0168**	**0.0504**	**0.0015**	**0.029**	**3.01**	**1.46**	**6.22**
TGTACT	0.026	0.0286	0.0127	0.10	>1.0	0.49	0.18	1.32
CATGCT	0.0249	0.0301	0.0208	0.37	>1.0	0.63	0.270	1.49
TGAAAC	0.0226	0.0173	0.0453	0.0066	0.13	2.66	1.27	5.59
TGTGAT	0.0206	0.0146	0.0348	0.029	0.58	2.34	1.02	5.35
TGAGCT	0.0199	0.0128	0.0348	0.014	0.29	2.57	1.10	6.02
CATAAC	0.0194	0.0248	9.89×10^{-9}	0.0027	0.055	0.109‖	0.014‖	0.815‖
TGAAAT	0.0176	0.0138	0.0214	0.35	>1.0	1.70	0.66	4.34
CATACT	0.0154	0.0158	0.0189	0.71	>1.0	1.23	0.48	3.15
Others	0.0521	0.0587	0.0470					

* Raw *p* values.
† Corrected *p* values using Bonferroni correction (raw *p* values were multiplied by 20).
‡ Odds ratio of each haplotype against the remaining haplotypes.
§ 95% confidence interval of odds ratio.
‖ Approximate calculation assuming that this haplotype has a frequency of 1/356.

Association of a haplotype with the disease was evaluated by means of chi-square tests of 2×2 contingency tables using the SPSS program (SPSS Japan). The TGTGCT haplotype frequency (bolded in Table 4) was significantly higher in patients than in controls (0.0504 versus 0.0168, $\chi^2 = 10.13$ [$df = 1$], $praw = .0015$, $pcor = .029$). The odds ratio (OR) and its 95% confidence interval (CI) were 3.01 and 1.46 to 6.22, respectively. The attributable fraction of this haplotype was 0.034. The CATAAC haplotype frequency was lower in patients than in controls (Table 4). This trend did not reach significance after a Bonferroni correction ($pcor = .055$). Because the estimated CATAAC haplotype frequency in patients was nearly zero, the OR was approximated (Table 4). On the assumption that this haplotype appeared once in 356 alleles, the OR and CI were 0.11 and 0.014 to 0.82, respectively.

The CA repeat marker of the *GABRB3* gene, which showed significant LD with CL $\pm$ P in Italian families and no association with CL $\pm$ P in a Japanese case-control study, was investigated for association with CL $\pm$ P (Table 5). The alleles with more than 1% of frequency in overall samples were sub-

TABLE 5   CA Repeat Association Study of the *GABRB3* Gene

Allele*	Number of alleles† (%)			praw‡	pcor§	OR‖	95% CI¶	
	Overall	Control	Patient				Low	High
172	2 (0.2)	1 (0.1)	1 (0.3)					
174	2 (0.2)	0 (0.0)	2 (0.6)					
176	6 (0.6)	1 (0.1)	5 (1.5)					
178	65 (6.0)	39 (5.3)	26 (7.6)	0.143	>1	1.47	0.88	2.45
180	39 (3.6)	25 (3.4)	14 (4.1)	0.575	>1	1.21	0.62	2.36
182	755 (69.8)	516 (69.9)	239 (69.5)	0.883	>1	0.98	0.74	1.29
184	137 (12.7)	101 (13.7)	36 (10.5)	0.138	>1	0.74	0.49	1.10
186	17 (1.6)	15 (2.0)	2 (0.6)	0.074	0.592	0.28	0.06	1.24
188	1 (0.1)	1 (0.1)	0 (0.0)					
190	14 (1.3)	11 (1.5)	3 (0.9)	0.402	>1	0.58	0.16	2.10
192	26 (2.4)	17 (2.3)	9 (2.6)	0.754	>1*	1.14	0.50	2.58
194	15 (1.4)	9 (1.2)	6 (1.7)	0.492	>1	1.44	0.51	4.07
196	1 (0.1)	1 (0.1)	0 (0.0)					
198	2 (0.2)	1 (0.1)	1 (0.3)					

* Allele was scored as polymerase chain reaction product size estimated by our experiments. All size values were estimated to be one base larger than those of previous reports (Mutirangura et al., 1992; Scapoli et al., 2002).
† Five controls and six patients could not be genotyped. Total numbers of alleles in controls and patients were 738 and 344, respectively.
‡ Raw *p* values; chi-square tests were performed for eight alleles that showed more than 1% frequency in overall samples.
§ Correct *p* using Bonferroni correction (raw *p* values were multiplied by 8).
‖ Odds ratio of each haplotype against the remaining alleles.
¶ 95% confidence interval of odds ratio.

jected to chi-square tests. None of the alleles tested showed significant association with CL ± P.

DISCUSSION

Because the genes involved in the GABA system are promising candidates for the pathogenesis of NSOC, we investigated the association between the *GABRB3* gene and the disease. In the six-SNP haplotype case-control study, we observed one significant risk-changing haplotype in the *GABRB3* gene. The higher frequency of the TGTGCT haplotype in patients suggests that this is a disease-promoting allele. Because estimation of lower haplotype frequency by an expectation-maximization algorithm method (SNPAlyze program; DYNACOM) tends to be inaccurate, the significance observed in this study may be overestimated. To establish more firmly the association of this haplotype with the disease, confirmation with a larger number of samples is necessary. Although the estimated frequency of the most frequent haplotype, CATGAC, may be more reliable than that for the TGTGCT haplotype, the difference in frequency of the CATGAC haplotype between patients and controls did not reach significance after Bonferroni correction (*praw* = .010, *pcor* = .124). Again, a larger number of samples is required; if the current haplotype frequencies were observed in 356 patients (twice as many as in this study) and 374 controls, the *praw* value for the association would be .00178, and the *pcor* (.036), therefore, would remain significant.

Two previous studies have investigated the association of the *GABRB3* gene and NSOC. The earlier study investigating an association between a CA repeat marker (Mutirangura et al., 1992) and CL ± P in 43 patients and 73 controls in the Japanese population (Tanabe et al., 2000) failed to show a significant association. However, the later study by Scapoli et al. (2002) investigated the same marker in 38 nuclear and extended Italian families and showed significant evidence of LD. Because the sample sizes of the two previous studies were small, the results may be due to chance. If we assume the two previous results are true, the following interpretation would be possible. Our result showed no evidence of association of this marker with CL ± P and was consistent with the result of the previous Japanese study. The reason for discrepancy between the six-SNP haplotype result and the CA repeat result in the Japanese study may be explained by the lack of strong LD between the CA repeat and the SNPs (Table 3). Scapoli and colleagues' (2002) positive results may suggest that the LD between the CA repeat and SNPs that affect disease-susceptibility is greater in Italians than in Japanese.

We previously reported an association study of the *GAD67* gene (Kanno et al., 2004). Although the sample size was smaller than that of the current study, we observed an association of one haplotype with a *pcor* value of .00098 with CL ± P. The strength of the association with the *GAD67* gene seems to parallel or even to exceed that found in the current study. Evidence of an association between NSOC and the genetic variations of the two critical genes for GABA synthesis and function further stresses the importance of the GABAergic sys-

tem in the pathogenesis of NSOC. We investigated whether the risk haplotypes of GAD67 and GABRB3 showed significant interaction using logistic regression formulas or chi-square contingency tables (data not shown). We found no evidence of interaction between these two genes. However, the possibility of a small degree of gene-gene interaction cannot be excluded due to the limited number of samples in the current study.

There is ample evidence from animal studies to suggest that GABA is involved in the development of palate (Miller and Becker, 1975; Wee and Zimmerman, 1983; Wee et al., 1986; Ding et al., 2004). Administration of diazepam, a neuropharmacologic agent that mimics GABA, to pregnant female mice inhibited palate reorientation and caused clefting in offspring (Wee and Zimmerman, 1983). An association between prenatal exposure to diazepam and oral clefts in humans also has been suggested by several studies (Safra and Oakley, 1975; Saxen, 1975). A study using picrotoxin, an antagonist of GABA receptor, and 3-mercaptopropionic acid, a GAD inhibitor, provides further evidence for the requirement of GABA for normal palate development (Ding et al., 2004). The importance of the GABAergic system in palate development also has been supported by experiments with genetically modified mice; mice lacking in the *Gabrb3* gene showed defects in forming the second palate (Culiat et al., 1993; Homanics et al., 1997), and recently Hagiwara et al. (2003) showed the importance of nonneuronal expression of GABRB3 for normal palate development in mice. These observations suggest that GABA is not only a neurotransmitter but also a growth factor for organogenesis.

In this study, we demonstrated the association of a haplotype of the *GABRB3* gene with CL ± P in the Japanese population. Currently, more than 800 SNPs are listed in the *GABRB3* gene region in the dbSNP database. Thus, further efforts are needed to clarify the relationship between genetic variations of the *GABRB3* gene and the development of NSOC.

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