Table 2 Association study of the genes on chromosome 12

	Controls (frequency %)	Asthma (frequency %)	Significance level*
STAT6 exon 1 GT repeat			
Allelic distribution			
Allele 1 (13 repeat)	77 (22.4)	72 (31.6)	$Pc = 0.061$
Allele 2 (14 repeat)	1 (0.3)	4 (1.4)	$Pc = 0.34$
Allele 3 (15 repeat)	233 (67.7)	123 (53.8)	$Pc = 0.0044$
Allele 4 (16 repeat)	33 (9.6)	29 (12.7)	$Pc > 1.0$
Overall	344 (100.0)	228 (100.0)	$P = 0.0032$
Genotypic distribution			
Allele 1/allele 1	14 (8.1)	12 (10.5)	$Pc > 1.0$
Allele 1/allele 3	46 (26.7)	41 (36.5)	$Pc = 0.81$
Allele 1/allele 4	3 (1.7)	7 (6.1)	$Pc = 0.67$
Allele 2/allele 3	1 (0.6)	4 (3.5)	$Pc = 0.59$
Allele 3/allele 3	81 (47.1)	30 (26.3)	$Pc = 0.0035$
Allele 3/allele 4	24 (14.0)	18 (15.8)	$Pc > 1.0$
Allele 4/allele 4	3 (1.7)	2 (1.8)	$Pc > 1.0$
Overall	172 (100.0)	114 (100.0)	$P = 0.0054$
NOS1 intron 2 GT repeat			
Allelic distribution			
Allele 1 (14 repeat)	2 (0.5)	1 (0.5)	$Pc > 1.0$
Allele 2 (15 repeat)	11 (3.0)	2 (0.9)	$Pc = 0.88$
Allele 3 (16 repeat)	189 (51.4)	88 (40.0)	$Pc = 0.049$
Allele 4 (17 repeat)	3 (0.8)	7 (3.2)	$Pc = 0.27$
Allele 5 (18 repeat)	126 (34.2)	97 (44.1)	$Pc = 0.11$
Allele 6 (19 repeat)	37 (10.1)	25 (11.4)	$Pc > 1.0$
Overall	368 (100.0)	220 (100.0)	$P = 0.0082$
Genotypic distribution			
Allele 1/allele 3	2 (1.1)	1 (0.9)	$Pc > 1.0$
Allele 2/allele 3	10 (5.4)	2 (1.8)	$Pc > 1.0$
Allele 2/allele 5	1 (0.5)	0 (0.0)	$Pc > 1.0$
Allele 3/allele 3	53 (28.8)	15 (13.6)	$Pc = 0.030$
Allele 3/allele 4	3 (1.6)	1 (0.9)	$Pc > 1.0$
Allele 3/allele 5	50 (27.2)	43 (39.1)	$Pc = 0.42$
Allele 3/allele 6	18 (9.8)	11 (10.0)	$Pc > 1.0$
Allele 4/allele 5	0 (0.0)	6 (5.5)	$Pc = 0.028$
Allele 5/allele 5	28 (15.2)	18 (16.4)	$Pc > 1.0$
Allele 5/allele 6	19 (10.3)	12 (10.9)	$Pc > 1.0$
Allele 6/allele 6	0 (0.0)	1 (0.9)	$Pc > 1.0$
Overall	184 (100.0)	110 (100.0)	$P = 0.0019$
IFNG intron 1 CA repeat			
Allelic distribution			
Allele 1 (12 repeat)	39 (11.0)	24 (10.6)	$Pc > 1.0$
Allele 2 (13 repeat)	209 (58.7)	110 (48.7)	$P = 0.12$
Allele 3 (14 repeat)	6 (1.7)	7 (3.1)	$Pc > 1.0$
Allele 4 (15 repeat)	91 (25.6)	78 (34.5)	$P = 0.17$
Allele 5 (16 repeat)	7 (2.0)	3 (1.3)	$Pc > 1.0$
Allele 6 (17 repeat)	0 (0.0)	1 (0.4)	$Pc > 1.0$
Allele 7 (18 repeat)	4 (1.1)	3 (1.3)	$Pc > 1.0$
Overall	356 (100.0)	226 (100.0)	$P = 0.12$
Genotypic distribution			
Allele 1/allele 1	6 (3.4)	3 (2.7)	$Pc > 1.0$
Allele 1/allele 2	19 (10.7)	8 (7.1)	$Pc > 1.0$
Allele 1/allele 3	0 (0.0)	1 (0.9)	$Pc > 1.0$
Allele 1/allele 4	8 (4.5)	9 (8.0)	$Pc > 1.0$
Allele 2/allele 2	62 (34.8)	32 (28.3)	$Pc > 1.0$
Allele 2/allele 3	3 (1.7)	0 (0.0)	$Pc > 1.0$
Allele 2/allele 4	54 (30.3)	35 (31.0)	$Pc > 1.0$
Allele 2/allele 5	6 (3.4)	2 (1.8)	$Pc > 1.0$
Allele 2/allele 7	3 (1.7)	1 (0.9)	$Pc > 1.0$
Allele 3/allele 3	0 (0.0)	2 (1.8)	$Pc > 1.0$
Allele 3/allele 4	2 (1.1)	1 (0.9)	$Pc > 1.0$
Allele 3/allele 7	1 (0.6)	1 (0.9)	$Pc > 1.0$
Allele 4/allele 4	13 (7.3)	15 (13.3)	$Pc > 1.0$
Allele 4/allele 5	1 (0.6)	1 (0.9)	$Pc > 1.0$
Allele 4/allele 6	0 (0.0)	1 (0.9)	$Pc > 1.0$
Allele 4/allele 7	0 (0.0)	1 (0.9)	$Pc > 1.0$
Overall	178 (100.0)	113 (100.0)	$P = 0.289$
AICDA 465C/T			
Allelic distribution			
C	214 (58.8)	119 (57.8)	
T	150 (41.2)	87 (42.2)	
Overall	364 (100.0)	206 (100.0)	$P = 0.860$
Genotypic distribution			
C/C	63 (34.6)	41 (39.8)	
C/T	88 (48.4)	37 (35.9)	
T/T	31 (17.0)	25 (24.3)	
Overall	182 (100.0)	103 (100.0)	$P = 0.099$

* All significance levels were calculated by Fisher's exact methods. In allele-by-allele or genotype-by-genotype comparison, the *P* value was corrected by multiplying the number of alleles or genotypes and expressed as *Pc*

region is a little different from Wilkinson's and our peak regions. However, if we compare the region with MLS exceeding 1.0, the three regions overlap each other—our data 130–160 cM, Yokouchi et al. 110–150 cM, and Wilkinson et al. 130–160 cM. In an analysis of the Danish population, linkage with an MLS of 1.58 to atopic asthma at D12S392 (146 cM) was reported (Haagerup et al. 2002). More recently, linkage to airway responsiveness was also suggested at 147 cM by the Childhood Asthma Management Program (CAMP) study (Raby et al. 2003). Thus, all these studies shared a region from 140 to 150 cM. Several studies have reported linkage between asthma and the different regions of chromosome 12. The Collaborative Study on the Genetics of Asthma reported evidence of linkage to asthma of the 12q22 region. This study analyzed Caucasians, African Americans, and Hispanics and found a linkage peak at D12S2070 (125 cM) in Hispanics (CSGA 1997; Xu et al. 2001a). When the condition of loci of chromosome 14 was considered, this linkage peak became broader and greater and the region with an LOD score greater than 1.0 extended from 120 cM to the telomere (Xu et al. 2001a). The region detected in Barbados families was located at 12q21.1 (90 cM), which is clearly different from those found in the Japanese and English (Barnes et al. 1999; Barnes et al. 1996). Linkage to asthma of this region has also been suggested in German and Swedish populations (Wjst et al. 1999). A suggestive linkage (MLS = 2.81) for asthma was reported at a region around D12S390 (65 cM) in the Italian population (Malerba et al. 2000). The region was close to the region suggested by a CAMP study (Raby et al. 2003). In a founder population, Hutterites, Ober et al. (1998) suggested linkage of D12S375 (80 cM) to asthma in an earlier study, a finding not replicated in a more recent study (Ober et al. 2000). Chromosome 12 showed no evidence of linkage to asthma in Finnish families (Laitinen et al. 2001). A genome-wide study of the French population failed to detect a linkage to asthma but detected linkage to eosinophilia around the 130 cM region (Dizier et al. 2000). There was no evidence of

linkage to asthma-related phenotypes of chromosome 12 markers in the Chinese population, which is ethnically close to the Japanese population (Xu et al. 2001b).

In combination, the results of our and previous studies suggest that several genes located in 12q24–q33 are likely to be responsible for susceptibility to asthma. Some susceptibility genes of this region may be shared by several populations, and others may not. The MLS at the position of *NOS1* (128 cM), an association with which was detected in this study, was less than 1.0. The *NOS1* locus was 20 cM apart from the peak of the MLS. Thus, the region we detected in the present study most likely contains other asthma-susceptibility gene(s). In the 12q24 region, more than a hundred genes are listed in the Human Genome Map. Narrowing down the linked region by dense mapping and an intensive survey of SNPs in this region will be required to identify new susceptibility genes.

We investigated the existence of QTL for total IgE on chromosome 12 using asthma sib pairs. No region on chromosome 12 showed significant linkage to total IgE. Although type-2 error (false negative) could not be excluded due to the relatively small sample size, we speculate that loci in 12q22–q23 are asthma-susceptibility loci rather than QTLs that affect the total IgE level in the Japanese population. Using HE sib-pair QTL approaches, Barnes et al. (1996) reported that markers from 107 to 135 cM showed evidence of linkage to log (total IgE) in Barbados and Amish families. Xu et al. (2000) suggested linkage (LOD score = 2.73) to log(total IgE) of the region from *PAH* (108 cM) and D12S2070 (125 cM) in the Danish population using variance-component linkage analysis. Analysis of total IgE as a dichotomous trait (high versus normal IgE) was also performed by Nickel et al. (1997). They performed TDT in German children and presented evidence of association between markers located from 75 to 108 cM and high total IgE. On the other hand, many studies have shown negative linkage for total IgE. These include studies of the Australian population by Daniels et al. (1996), the Germans by Heinzmann et al. (2000a), the Germans and Swedish by Wjst et al. (1999), the Hutterites by Ober et al. (2000), the Chinese by Xu et al. (2001b), the French by Dizier et al. (2000), the Finnish by Leitinen et al. (2001), the Italians by Malerba et al. (2000), and the Danish by Haagerup et al. (2002). This inconstancy suggests that the relative importance of chromosome 12 loci in controlling total IgE varies from population to population.

We detected association of *STAT6* exon 1 GT repeat with Japanese childhood asthma. Our results clearly show association of the 15-repeat allele (allele 3) and asthma by allelic and genotypic distribution analyses. In Tamura's study using fewer samples than the present study, the number of heterozygote of allele 1 and allele 3 was significantly higher in patients with "allergic diseases" and that of homozygotes of allele 3 was lower in the patients with marginal statistical significance (Tamura et al. 2001). This observation was further confirmed by our present results: a change in the frequency of allele 3 homozygotes is more essential than that in the frequency of allele 1/allele 3 heterozygotes. Allele 3 appears to be a protective allele against the development of asthma in Japanese childhood asthma.

We also investigated the 2964G/A polymorphism in the 3'-UTR that was reported to be associated with adult asthma by Gao et al. (2000b) but failed to detect association in our samples (data not shown). Similar results were reported by Deutsch et al. (2002). These observations suggest that the *STAT-6* exon 1 GT repeat, but not 2964 G/A, is an asthma-susceptibility polymorphism. Further study is necessary to establish whether the polymorphism is functionally relevant to disease development or only a marker for the true functional polymorphism(s).

Association of the *NOS1* intron 2 GT repeat with asthma was also demonstrated in this study. Another study also describing the association of this polymorphism with asthma was carried out in the British population (Gao et al. 2000a). The 16-repeat allele of *NOS1* is a protective allele against asthma development in both British and Japanese populations, suggesting that this allele represents an old protective haplotype that evoked before the division of races. It is therefore possible that this polymorphism is associated with the disease in many ethnic groups. Moreover, Grasemann et al. (2000) have shown that *NOS1* exon 29 CA repeat in the 3'-UTR was associated with asthma in the US population. This is additional evidence that *NOS1* is a susceptibility gene for asthma in diverse populations. Again, further study is required to conclude whether these known polymorphisms are functionally relevant to the disease development or only markers for the true functional polymorphism(s).

We were unable to detect any association between *IFNG* and asthma in our series. Nakao et al. (2001) reported that allele frequency distribution differed between their 218 controls and 158 patients with atopic asthma in the Japanese population. Deviation of the distribution was primarily derived from the difference in frequency of allele 5 ($P = 0.0069$, $Pc = 0.048$). Frequencies in controls and patients of this allele were 4.8% and 1.3% respectively. In our study, the corresponding frequencies were 2.0% and 1.3% respectively. If we combine Nakao's and our data, P values become 0.012, which is not statistically significant after correction for the number of alleles ($Pc = 0.083$). More subjects are required to evaluate the significance of the association. Even if the association was confirmed, allele 5 would affect less than 5% of the population, i.e., the attributable fraction of this polymorphism would be low.

Although association of *AICDA* 465C/T (His155His) and asthma was suggested by TDT (Noguchi et al. 2001), our case-control study failed to prove the association of this polymorphism with asthma in the same population. In allelic distribution, allele frequency was essentially the same between the patient and control groups. Genotype distribution of the patients was not in

Hardy-Weinberg equilibrium. This did not result in significant difference in the genotype frequency between cases and controls. The data suggest that case samples were not well representative of the homogeneous population. We genotyped another set of 94 cases with this SNP. Genotypes CC, CT, and TT were 25, 50, and 19 cases respectively. Genotype frequency of the new set of patients was in Hardy-Weinberg equilibrium and not significantly different from the control value. Although we cannot exclude *AICDA* completely as a candidate gene for asthma, the effect of the 465C/T polymorphism on susceptibility to asthma was not as strong as the repeat polymorphisms of *STAT6* or *NOS1*.

In conclusion, we demonstrated that *STAT6* and *NOS1* loci are associated with childhood asthma in the Japanese population and showed evidence of "suggestive" linkage between region 12q24.23–q24.33 and asthma. This chromosome region most likely contains as yet unidentified asthma susceptibility gene(s).

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Significant Association Between Nonsyndromic Oral Clefts and Arylhydrocarbon Receptor Nuclear Translocator (ARNT)

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The etiology of nonsyndromic oral clefts (cleft lip, cleft palate, or cleft lip and palate) is still controversial, but is considered to involve both genetic and environmental factors. One of suspected environmental factors is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) found in tobacco, herbicides, contaminated soil, and food. TCDD administered during organogenesis in mice causes a high incidence of CP in fetuses. There is ample evidence that aryl hydrocarbon receptor (AHR), AHR nuclear translocator (ARNT), and cytochrome P450 1A1 (CYP1A1) are involved in TCDD metabolism. We assessed whether there is any association in the Japanese population of nonsyndromic oral clefts with single nucleotide polymorphisms (SNPs) in the *AHR*, *ARNT*, and *CYP1A1* genes using transmission disequilibrium test (TDT) and case-control study. We identified and investigated three SNPs in ARNT; 567G/C (V189V), IVS12-19T/G, and 2117C/T (P706L). Two amino acid substitutions, R554L in AHR and I462V in CYP1A1, were also investigated. In the TDT, the C allele of ARNT 567G/C was preferentially transmitted to patients ($P = 0.033$). When a haplotype consisting of 567G/C and IVS12-19T/G in ARNT was considered, the preferential transmission of the CT (567C-IVS12-19T) haplotype was observed ($P = 0.0012$). In a case-control study, a significant association of IVS12-19T/G in ARNT was observed ($P = 0.021$). The SNPs studied in AHR and CYP1A1 were not associated with the disease. Our results suggest that ARNT is involved in the development of nonsyndromic oral clefts in the Japanese population.

KEY WORDS: **nonsyndromic oral clefts; aryl hydrocarbon receptor (AHR); AHR nuclear translocator (ARNT); cytochrome P450 1A1 (CYP1A1); transmission disequilibrium test (TDT); 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)**

INTRODUCTION

Nonsyndromic oral clefts, which include cleft lip (CL), cleft lip and palate (CLP), and cleft palate (CP), are one of the most common congenital anomalies, with an incidence of 1/700 to 1/1,000 live births. Both environmental and genetic factors are thought to be involved in their pathogenesis [Murray, 2002]. Numerous studies have shown that genetic factors appear to play a significant role in the etiology of nonsyndromic oral clefts [Wyszynski et al., 1996]. However, the genetic characteristics of nonsyndromic oral clefts are complicated by an uncertain mode of inheritance, incomplete penetrance, and heterogeneity both within and among populations [Maestri et al., 1997]. Candidate genes for nonsyndromic oral clefts have been proposed based on the allelic association studies, linkage analysis and animal studies [Schutte and Murray, 1999]. Studies with knockout mice have identified putative genes that cause the cleft phenotype in the knockout mice without other physical or developmental anomalies and are expressed at critical times in tissues relevant to lip and palate development [Schutte and Murray, 1999]. Some knockout genes, such as the homeobox homologue 1 (*MSX1*), transforming growth factor-β_3 (*TGFB3*), the β_3 subunit of the gamma-aminobutyric acid receptor (*GABRB3*) genes, not only resulted in a cleft phenotype but also exhibited a significant association with human nonsyndromic oral clefts [Maestri et al., 1997; Lidral et al., 1998; Schutte and Murray, 1999; Scapoli et al., 2002], although some subsequent studies did not support these results [Murray, 2002].

One of the suspected environmental factors for the development of nonsyndromic oral clefts, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), causes a high percentage of CP in fetuses when administered during organogenesis in mice [Courtney and Moore, 1971]. TCDD is found in tobacco smoke, contaminated foods, contaminated soil, and gas exhaust from motor vehicles [Muto and Takizawa, 1989; Gullett and Ryan, 2002; Huwe, 2002]. TCDD in tobacco smoke is suspected to be involved in the development of nonsyndromic oral clefts, and maternal smoking during pregnancy has been identified as a risk factor for nonsyndromic oral clefts [Shaw et al., 1996; Lorente et al., 2000].

Aryl hydrocarbon receptor (AHR), AHR nuclear translocator (ARNT), and cytochrome P450 1A1 (CYP1A1) are involved in TCDD metabolism. TCDD binding with the AHR results in nuclear translocation and release of heat shock protein 90, followed by dimerization with ARNT, and DNA binding/

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transactivation. The *CYP1A1* gene is one of the targets of this complex [Sogawa and Fujii-Kuriyama, 1997]. Interestingly, the AHR, ARNT, and CYP1A1 mRNAs are expressed in developing craniofacial tissue of mice and the CYP1A1 mRNA was highly induced by TCDD exposure [Abbott et al., 1999]. When pregnant mice were administered with a dose of 40 μg TCDD/kg body weight by gavage at gestation day 12.5, almost all wild-type fetus suffered from CP, whereas none of AHR-null mutant fetuses were sensitive to TCDD [Mimura et al., 1997]. ARNT-null mice are not viable past gestation day 10.5, because most knockout embryos have retarded growth as indicated by significant decreases in crown rump and head length; abnormalities include neural tube closure defect, failure of the head folds to close completely, forebrain hypoplasia, delayed rotation of the embryos, placental hemorrhaging, and visceral arch abnormalities [Kozak et al., 1997].

These observations led us hypothesize that AHR, ARNT, and CYP1A1 may be involved in the pathogenesis of nonsyndromic oral clefts. Although there is only one association study between nonsyndromic oral clefts and polymorphisms of CYP1A1 [van Rooij et al., 2001], no association study on AHR or ARNT has been reported. In the present study, we assessed whether there is any association of nonsyndromic oral clefts and SNPs in AHR, ARNT, and CYP1A1.

MATERIALS AND METHODS

Families

A total of 148 Japanese nonsyndromic oral clefts probands and their parents were recruited for this study (36 CL cases, 8 CP cases, and 104 CLP cases). Each of ten probands had an affected parent. Each of six probands had an affected sib. One proband had both affected parent and a sib. The rest of the probands (131 cases) had no affected individual in the first-degree relatives. Written and oral informed consent was obtained from all subjects. A family history was obtained from each participant to determine the presence or absence of related individuals with nonsyndromic oral clefts and other congenital anomalies. This study was approved by the Ethics Committee of Tohoku University School of Medicine. Blood samples were collected from the probands, their parents, and 189 healthy Japanese volunteers as controls.

DNA analysis

DNA was prepared from whole blood samples using a GFX DNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, England). We amplified exons 1–22 and a 1 kb promoter region of ARNT using previously identified primers by standard PCR [Scheel et al., 2002]. The amplified products were purified (Qiagen K.K, Tokyo, Japan) and directly sequenced with ABI genetic analyzer 310 (Applied Biosystems, Foster City, CA). The sequences were analyzed by computer program ATGC (GENETYX, Tokyo, Japan).

Genotyping of SNPs in AHR, ARNT, and CYP1A1 was performed with a unique allele-specific TaqMan PCR method [Fujii et al., 2000] using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers used for TaqMan PCR are shown in Table I. PCR mixtures containing 7.5 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.4 μM each of PCR primer, 0.12 μM of TaqMan probe, and 10 ng of template DNA in a final volume of 15 μl were subjected to using the following thermal cycling program: 50°C for 2 min, 95°C for 3 min, and 45 cycles of 95°C for 15 sec and 60°C for 1 min.

The standard single-allele transmission disequilibrium test [Spielman et al., 1993] and haplotype TDT of ARNT were carried out by GENEHUNTER 2 [Kruglyak et al., 1996]. Pairwise linkage disequilibrium in each SNPs was estimated as D′ [Lewontin, 1964] and r^2 [Hill and Robertson, 1968]. Allele frequencies in patients and controls were compared by contingency Chi-square test. Fisher's exact P values were calculated using SPSS software version 11.0J (SPSS Japan, Inc., Tokyo, Japan).

RESULTS

Sequencing of ARNT in 17 cases, that had positive family history in the first-degree relatives, identified three SNPs

TABLE I. Primer and TaqMan Probe Sequences for the TaqMan Allele-Specific Amplification

(A) Allele-specific amplification of 1661A/G in the *AHR* gene	
Forward primer for A-allele	5′-GCATTGATTTTGAAGACATCCA-3′
Forward primer for G-allele	5′-GCATTGATTTTGAAGACATCCG-3′
Reverse primer	5′-CCATACAGCTTGAGTTCAGAGC-3′
TaqMan probe[a]	5′-CGGATGAAATCCTGACGTATGTCCAAGA-3′
(B) Allele-specific amplification of 567G/C in the *ARNT* gene	
Forward primer for G-allele	5′-GACAGGCAGGGTGGTGTATGCG-3′
Forward primer for C-allele	5′-GACAGGCAGGGTGGTGTATGCC-3′
Reverse primer	5′-GCTGCCAAACCATTCAGACTG-3′
TaqMan probe[a]	5′-CTGACTCCGTGACTCCTGTTTTGAACCAG-3′
(C) Allele-specific amplification of IVS12-19T/G in the *ARNT* gene	
Forward primer	5′-AACTCAGGAGGTGGAGGT-3′
Reverse primer for T-allele	5′-GAGTTCCTAGAATACAGAAAGAAA-3′
Reverse primer for G-allele	5′-GAGTTCCTAGAATACAGAAAGATC-3′
TaqMan probe[a]	5′-CAGTGAGCCAAGATCGCGC-3′
(D) Allele-specific amplification of 2117C/T in the *ARNT* gene	
Forward primer for C-allele	5′-CTTCCTTGCTGTATTTCTAGCTAC-3′
Forward primer for T-allele	5′-CTTCCTTGCTGTATTTCTAGCTTT-3′
Reverse primer	5′-ACACCCACACCCTCTGCTGT-3′
TaqMan probe[a]	5′-AGACTGGACAGACTGCAGGACAATTCC-3′
(E) Allele-specific amplification of 1384A/G in the *CYP1A1* gene	
Forward primer for A-allele	5′-CGGAAGTGTATCGGTGAGACTA-3′
Forward primer for G-allele	5′-CGGAAGTGTATCGGTGAGACTG-3′
Reverse primer	5′-AAGCACCTAAGAGCGCAGCTG-3′
TaqMan probe[a]	5′-CGTGAAGGTGGACATGACCCCCAT-3′

[a]Each TaqMan probe was labeled with 6-carboxyfluorescein (FAM, reporter dye) at the 5′-end and 6-carboxy-tetramethyl-rhodamine (TAMRA, quenching dye) at the 3′-end.

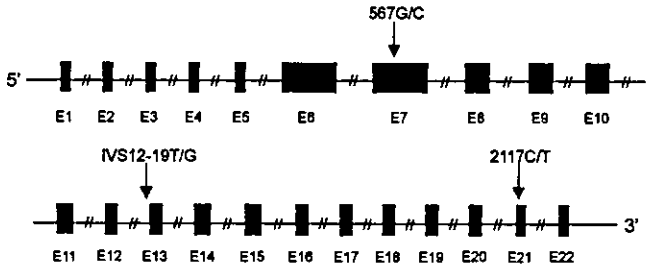

Fig. 1. The schematic structure of the human *ARNT* gene [Scheel et al., 2002]. Genetic variations are indicated by arrows. E, exon.

with the following allelic variations: 567G/C in exon 7 (GenBank accession number AJ404854), IVS12-19T/G in intron 12 (nt 16623, GeneBank accession number AJ404854), and 2117C/T in exon 21 (GeneBank accession number AJ251863) (Fig. 1). A substitution of G to C at nt 567 altered the third nucleotide of codon 189 (valine), but did not change the amino acid. A substitution of T to G at IVS12-19 did not affect consensus sequence for mRNA splicing. A substitution of C to T at nt 2117 altered the second nucleotide of codon 706 (proline), resulting in an amino acid change to leucine. All three SNPs are found in the dbSNP database (567G/C NCBI CLUSTER ID: rs no. 2228099, IVS12-19T/G rs no.3768017, and 2117C/T rs no. 2275237).

In 100 unaffected control subjects, allele frequencies of 567C, IVS12-19G, and 2117T were 0.4, 0.15, and 0.02, respectively. Two of three SNPs, 567G/C, IVS12-19T/G were subjected to TDT, whereas 2117C/T was not because of a low allelic frequency. The frequency of 2117C/T allele in 96 patients was 0.015 and not significantly different from the control value.

Pair-wise linkage disequilibrium coefficients between 567G/C and IVS12-19T/G were estimated. D′ and r^2 were 0.739 and 0.0718, respectively, both of which suggested that two SNPs were in linkage disequilibrium.

TDT was performed using 567G/C and IVS12-19T/G in ARNT, 1661A/G in AHR, and 1384A/G in CYP1A1 for 148 patient–parents trios (Table II). The 567C allele of ARNT was transmitted preferentially to children with nonsyndromic oral clefts ($P = 0.033$). When CP patients were excluded from the test, both 567C allele and IVS12-19T allele of ARNT were transmitted preferentially to the patient with the marginal significance (567C, $P = 0.054$; IVS12-19T, $P = 0.049$). Next, we tested transmission of haplotypes consisted with 567G/C and IVS12-19T/G. Among four haplotypes observed, the 567C and IVS12-19T haplotype (CT haplotype) was preferentially transmitted to the patients ($P = 0.0012$) (Table III). The 567G and IVS12-19G haplotype (GG haplotype) was transmitted to the patients less than 50% chance with the marginal significance ($P = 0.052$). Other haplotypes did not show any deviation of transmission. When CP patients were excluded, both overtransmission of CT haplotype and undertransmission of GG haplotype were significant ($P = 0.0023$ and $P = 0.023$, respectively). Other haplotypes did not show any deviation in their transmission from the parents to the patients. Association of 567G/C and IVS12-19T/G with the disease was also evaluated in the case-control samples (Table IV). None of the patients with oral clefts were homozygous for IVS12-19G, whereas 4.2% of the controls were homozygous for this allele. The exact P value for this change in total oral clefts was 0.010 and that for CL + CLP was 0.023. Decrease in frequency of homozygotes of the G allele corresponded to the undertransmission of IVS12-19G observed in the TDT (Table II). There was no difference in frequency of the heterozygote at this SNP between the cases and controls.

TABLE II. Results of TDT of Genes Involved in TCDD Metabolism

		CL + CLP + CP (148 families)				CL + CLP (140 families)			
Polymorphism	Allele	T	NT	χ^2	P	T	NT	χ^2	P
AHR (1661A/G)	A	65	59	0.29	0.59	60	57	0.08	0.78
ARNT (567G/C)	C	70	47	4.52	0.033*	64	44	3.70	0.054**
ARNT (IVS12-19T/G)	T	47	32	2.85	0.091	46	29	3.85	0.049*
CYP1A1 (1384A/G)	G	46	43	0.10	0.75	45	39	0.43	0.51

χ^2 and P values were calculated using the GENEHUNTER 2 program. CL, cleft lip; CLP, cleft lip and palate; CP, cleft palate; T, transmitted; NT, not transmitted.
*Significant.
**Marginally significant.

TABLE III. Results of ARNT Haplotype Transmission to Patients With Nonsyndromic Oral Clefts

	CL + CLP + CP (148 families)				CL + CLP (140 families)			
Haplotype	T	NT	χ^2	P	T	NT	χ^2	P
GG haplotype[a]	19	33	3.77	0.052**	17	33	5.12	0.023*
GT haplotype[b]	40	54	2.09	0.15	39	48	0.93	0.33
CG haplotype[c]	0	2	2.00	0.16	0	2	2.00	0.15
CT haplotype[d]	58	28	10.47	0.0012*	53	26	9.23	0.0023*

χ^2 and P values were calculated using the GENEHUNTER 2 program.
CL, cleft lip; CLP, cleft lip and palate; CP, cleft palate; T, transmitted; NT, not transmitted.
*Significant.
**Marginally significant.
[a]567G and IVS12-19G.
[b]567G and IVS12-19T.
[c]567C and IVS12-19G.
[d]567C and IVS12-19T.

TABLE IV. Association Between SNPs of ARNT and Patients With Nonsyndromic Oral Clefts

Name	Number	Genotype			OR (95% CI)	χ^2	*P*
ARNT (567G/C)		GG	GC	CC			
CL + CLP + CP	148	54	65	29	1.20 (0.69–2.09)[a]	0.401	0.821
CL + CLP	140	53	60	27	1.17 (0.67–2.07)[a]	0.332	0.853
Controls	189	72	85	32			
ARNT (IVS12-19T/G)		TT	TG	GG			
CL + CLP + CP	148	104	44	0	Could not be calculated	6.996	0.021*
CL + CLP	140	99	41	0	Could not be calculated	6.513	0.030*
Controls	189	134	47	8			

χ^2 and *P* values were calculated using the SPSS ver. 11.0J program.
OR, odds ratio; CI, confidence interval; CL, cleft lip; CLP, cleft lip and palate; CP, cleft palate.
[a]GG + GC versus CC.
*Significant.

The 1661A/G in AHR and 1384A/G in CYP1A1 showed no association with the disease (Table II).

DISCUSSION

Results of TDT of SNPs in the *ARNT* gene show evidence of association of this gene and nonsyndromic oral clefts in the Japanese population. The CT haplotype was transmitted to the patients more often than expected under the assumption of no association with the disease risk. This result suggests that the CT haplotype is associated with the disease-promoting nucleotide change(s). Although the significance was marginal, the GG haplotype was transmitted less often than expected. The GG haplotype may be associated with disease-protective change(s). The GT allele did not show any effect on the development of nonsyndromic oral clefts. Since the frequency of the CG haplotype was small, we could not evaluate the risk of this allele. In the case-control study, we observed significant association between nonsyndromic oral clefts and IVS12-19T/G of ARNT. None of the oral cleft patients was homozygous for IVS12-19G, whereas the frequency of the heterozygote among the patients was the same as that in the controls. This suggests that the IVS12-19G allele is a recessive protective allele against the disease. The observation is consistent with the result of the haplotype TDT that showed that the 567G and IVS12-19G haplotype was transmitted less often to the patients than 50% chance. Odds ratio and its 95% confidence interval of IVS12-19G/G could not be calculated because the number of homozygous patients was zero. To estimate these values, more patients should be analyzed.

Because neither the 567G/C nor the IVS12-19G/T haplotypes affects amino acid sequence or splicing consensus sequence, it is not likely that these SNPs are directly involved in the pathogenesis of nonsyndromic oral clefts. They are probably in linkage disequilibrium to unidentified genetic variation(s) that are responsible for the development of the disease. Alternatively, it is possible that these seemingly neutral nucleotide substitutions affect mRNA splicing and/or the gene expression as described in other genetic disorders [Wakamatsu et al., 1992; Kajihara et al., 1995]. Effects of these SNPs on splicing should be assessed to determine whether these SNPs are pathogenic of not. If these are not directly responsible for the pathogenesis of oral clefts, polymorphisms located in and near ARNT and in linkage disequilibrium to the GG haplotype or CT haplotype should be extensively examined for their effects on function and expression of ARNT.

Some of epidemiologic and embryologic studies suggest that CL with or without CP (CL + CLP) is a distinct entity from CP only [Murray, 2002]. Thus, we examined the association of SNPs in all oral clefts patients and CL + CLP patients. The results of the TDT and case-control study on ARNT were essentially the same as shown in Table II–IV. It was not possible to elucidate whether ARNT is associated with defective development of lips or palate or both.

A total of 17 patients had positive family history in the first-degree relatives. We have performed TDT analysis on the patients other than these 17 cases. The results of the TDTs were essentially the same (data not shown). There was no evidence for a different effect of ARNT between familial and sporadic cases.

Neither of two SNPs in AHR and CYP1A1 showed the association with oral clefts in this study. The observation did not preclude possible enrollment of these genes in the pathogenesis of oral clefts.

For mothers involved in agricultural activities during the month before conception and the first trimester of pregnancy, the adjusted odds ratio for carrying a fetus with malformations is 3.16 (95% confidence interval, 1.11–9.01) primarily due to an increased risk for nervous system defects, oral clefts, and multiple anomalies [Garcia et al., 1999]. Although controversial, an association between prenatal exposure to tobacco and oral clefts in humans has also been suggested in several studies [Lorente et al., 2000]. TCDD in herbicides and tobacco may increase the risk of nonsyndromic oral clefts [Wyszynski and Beaty, 1996; Garcia et al., 1999]. Interestingly, the teratogenic effect of TCDD has been reported to vary among mice of different genetic backgrounds [Courtney and Moore, 1971]. The importance of AHR–ARNT pathway in palate development has been further advocated by genetic approaches [Abbott et al., 1999].

In conclusion, our study employing TDT and case-control study on SNPs in the *ARNT* gene provides evidence, in addition to the previous AHR and ARNT knockout mice studies, for the possible involvement of the AHR–ARNT pathway in the pathogenesis of nonsyndromic oral clefts. To establish an association between the *ARNT* gene and nonsyndromic oral clefts, studies with a larger number of families from various ethnic groups may be necessary.

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Association Between Nonsyndromic Cleft Lip With or Without Cleft Palate and the Glutamic Acid Decarboxylase 67 Gene in the Japanese Population

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Nonsyndromic cleft lip with or without cleft palate (NSCLP) is one of the most common craniofacial malformations. Both genetic and environmental factors are involved in the pathogenesis. In addition to its role as an inhibitory neurotransmitter, γ-aminobutyric acid (GABA) synthesized by glutamic acid decarboxylase (GAD) is presumed to play a role in normal embryonic, especially facial, development. This notion has been substantiated by the fact that Gad67 knockout mice have been shown to have cleft palate. We hypothesized that GAD67 may be involved in the development of NSCLP and investigated the possible association between the GAD67 gene (*GAD67*) and NSCLP in Japanese patients. We screened 50 probands for single nucleotide polymorphisms (SNPs) in GAD67 using denaturing high performance liquid chromatography (DHPLC) and found seven SNPs. Since two SNPs showed complete linkage disequilibrium (LD) to the other SNPs, we constructed a 5-locus haplotype of GAD67. The frequency distribution of the haplotype differed between NSCLP patients and controls ($P = 0.0028$). The frequency of −445A, −292A, −147G, 111C, and IVS9−39T haplotype in the NSCLP patients was significantly lower than that in controls ($P = 0.00098$). In a transmission disequilibrium test (TDT) in 99 parent–offspring trios, we found −445C, −292C, −147G, 111C, and IVS9−39C haplotype was preferentially transmitted to the patients with cleft lip and palate ($P = 0.0077$). Our data suggest that GAD67 is involved in the pathogenesis of NSCLP in the Japanese population. © 2003 Wiley-Liss, Inc.

KEY WORDS: **nonsyndromic cleft lip and/or cleft palate; γ-aminobutyric acid; glutamic acid decarboxylase 67; single nucleotide polymorphism; transmission disequilibrium test**

INTRODUCTION

Nonsyndromic cleft lip with or without cleft palate (NSCLP, MIM 119530) is one of the most common congenital anomalies, with an incidence of 1/700 to 1/1,000 live births. Numerous studies have shown that genetic factors appear to play a significant role in the etiology of NSCLP [Wyszynski et al., 1996]. However, the genetic characteristics of NSCLP are complicated by an uncertain mode of inheritance, incomplete penetrance, and heterogeneity both within and among populations [Maestri et al., 1997]. Candidate genes for NSCLP have been proposed based on allelic association studies, linkage analysis, and animal studies [Schutte and Murray, 1999]. Previous knockout mouse studies produced many mutants that included cleft palate as a part of the phenotype. For a gene to be a strong candidate, a clefting phenotype must result from the knockout with no other physical or developmental anomalies, and be expressed at a critical time and in a tissue relevant to lip and palate development [Schutte and Murray, 1999]. Some knockout genes, such as

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homeobox homolog1 (*MSX1*), transforming growth factor β3 (*TGFB3*), and the β3 subunit of the γ-aminobutyric acid (GABA) receptor gene (*GABRB3*), not only resulted in a clefting phenotype but also exhibited a significant association with human NSCLP [Maestri et al., 1997; Lidral et al., 1998; Schutte and Murray, 1999; Scapoli et al., 2002].

The GAD67 gene (*GAD67*) is one of the strong candidate genes for NSCLP, because mice lacking in Gad67 developed an isolated cleft palate [Condie et al., 1997]. Glutamic acid decarboxylase (GAD) is a key enzyme that synthesizes GABA from glutamate. There are two GAD isozymes (GAD65 and GAD67) that are derived from two distinct genes. Selective elimination of each GAD isoform showed that Gad65 knockout mice were slightly more susceptible to seizures, whereas the Gad67 knockout mice died of severe cleft palate during the first morning after birth [Asada et al., 1997; Condie et al., 1997]. No other morphological abnormalities were noted in the Gad67 knockout mice. A Western blot analysis showed that GAD67 protein is expressed in the fetal maxillary tissue [Asada et al., 1997], suggesting that GAD67 plays a critical role in normal craniofacial development. These observations led us hypothesize that the *GAD67* gene may be involved in the pathogenesis of NSCLP in humans.

The aim of this study was to investigate whether genetic variations of GAD67 affect the susceptibility to NSCLP. We screened for single nucleotide polymorphisms (SNPs) in GAD67 among probands, using denaturing high performance liquid chromatography (DHPLC). A population-based association study and transmission disequilibrium test (TDT) were performed with the haplotype consistent with the identified SNPs.

MATERIALS AND METHODS

Patients

Ninety-nine Japanese NSCLP probands (78 probands were cleft lip with cleft palate) and their parents were recruited for this study. Written and oral informed consent was obtained from all subjects. This study was approved by the Ethics Committee of Tohoku University School of Medicine. A family history was obtained from each participant to determine the presence or absence of related individuals with NSCLP and other anomalies. Blood samples were collected from the probands and their parents.

Screening for Polymorphisms

DNA was prepared from whole blood samples using a GFR DNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, England). We screened for single SNPs in all 17 exons and the 5′-flanking regions of GAD67 with DHPLC. The DHPLC method is based on the aberrant electrophoretic mobility of DNA heteroduplex and is known to be highly sensitive for detecting nucleotide changes in PCR products [Wagner et al., 1999]. DNA was amplified by PCR using the primers that were designed based on the information obtained from a public genome database (accession number AC007405, NT005403, http://www.ncbi.nlm.nih.gov/). Thirty microliters of reaction mixture contained 10 ng of genomic DNA, 10 mM of Tris/HCl (pH 8.3), 50 mM of KCl, 1.5 mM of $MgCl_2$, 250 μM of each dNTP, 6 μM of each primer, and 1.5 U of Taq polymerase. Amplification conditions of the fragments were: 94°C for 1 min, followed by 40 cycles at 94°C for 30 sec, 55°C for 40 sec, and 72°C for 50 sec, ending with a single 10 min extension step at 75°C. Finally, each PCR product was denatured at 94°C for 5 min and gradually cooled to 4°C. Running temperatures of the chromatography were determined by Run, the DHPLC Melt Program (http://insertion.stanford.edu/index.html), using the sequences of the PCR products. Each PCR product (3 μl) was applied to a column (ZORBAX Eclipse dsDNA Analysis Column™, Agilent Technologies, CA) and the absorbance at 280 nm of the eluent was monitored using the DNA Screen™ System (Shimazu Corporation, Kyoto, Japan). When aberrant peak profiles were detected in DHPLC, the PCR products were used as templates for direct sequencing by a fluorescent dye-terminator cycle sequencing method, using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Forester, MA).

Genotyping

The −667C > G, −449C > T, −445C > A, −292A > C, −147G > A, 111T > C, and IVS9−39c > t polymorphisms (Table I) were genotyped in 99 parent–offspring trios and 99 healthy Japanese individuals by a unique TaqMan-allele specific amplification method using an ABI PRISM 7700 Sequence Detector System (Applied Biosystems) [Fujii et al., 2000]. Sequences of the PCR amplification primers and the TaqMan probes are shown in Table II.

Statistical Analysis

Pair-wise linkage disequilibrium (LD) in each SNP was estimated as D′ [Lewontin, 1964] and r^2 [Hill and Robertoson, 1968]. To infer the haplotype frequency in the population and most probable diplotype in individuals, the HAPLOTYPER program was used (http://www.people.fas.harvard.edu/~junliu/Haplo/docMain.htm)

TABLE I. Summary of Genetic Variations Detected in the *GAD67* Gene

Location	Position	Genetic variation	NCBI SNP ID
5′ Flanking[a]	−667	C/G (5′ UTR)	
5′ Flanking	−449	C/T	
5′ Flanking	−445	C/A	
5′ Flanking	−292	A/C	
Exon 1[b]	−147	G/A	rs3749034
Exon 3	111	T/C	rs769404
Intron 9	−39	C/T	rs701492

UTR, Untranslated region; NCBI, National Center for Biotechnology Information.
[a]Nt number of the 5′ flanking regions described relative to the transcription starting site.
[b]Nt number of the exon regions described relative to the adenine of the initiation codon.

TABLE II. Primer and TaqMan Probe Sequences for the TaqMan-Allele Specific Amplification

−677C > G	
Forward primer for C-allele	5′-CAGACACGCACAGACATCAAC-3′
Forward primer for G-allele	5′-GGGCGCCAAGAGCCCAGAGATG-3′
Reverse primer	5′-GGGCGCCAAGAGCCCAGAGATC-3′
TaqMan probe (FAM)	5′-AAGGCATGAAGAGGCAAGCCGGCGCGTAAC-3′
−449C > T	
Forward primer for C-allele	5′-ATGCGCGTGCAGCGGCGCAC-3′
Forward primer for T-allele	5′-ATGCGCGTGCAGCGGCGCCT-3′
Reverse primer	5′-CTCGCTTTGGCCCCCTTGGTGATG-3′
TaqMan probe (FAM)	5′-TGTCATCAACCTTCAAACGTGAT-3′
−445C > A	
Forward primer	5′-CAGCGGTTCTTTTAACTACGCC-3′
Reverse primer for C-allele	5′-ACGTTTGAAGGTTGATGACATG-3′
Reverse primer for A-allele	5′-ACGTTTGAAGGTTGATGACAGT-3′
TaqMan probe (FAM)	5′-AGGGAGAATCCTTAAAGCGCGTGAAATCGA-3′
−292A > C	
Forward primer for A-allele	5′-GTGGCCAGGTGTGGTACTTTAA-3′
Forward primer for C-allele	5′-GTGGCCAGGTGTGGTACTTTTC-3′
Reverse prime	5-GCAAAGGAGGCAGAAATGAGGG-3′
TaqMan probe (FAM)	5′-CAGGGCTGGATGAGGAAACTGTAATTCCTC-3′
−147G > A	
Forward primer for G-allele	5′-AGGTGACGCCGGGCAGATTAAG-3′
Forward primer for A-allele	5′-AGGTGACGCCGGGCAGATTAGA-3′
Reverse prime	5-ACTCATTTCGGGAGGTTGGGTGG-3′
TaqMan probe (FAM)	5′-CGGCTTCTCAACCAACCCCATCCCA-3′
111T > C	
Forward primer	5′-AGAGCTCTGGCAAAGTCCTCATCCT-3′
Reverse primer for T-allele	5′-CCCCAGTTTTCTGGTGCATCGA-3′
Reverse primer for C-allele	5′-CCCCAGTTTTCTGGTGCATCTG-3′
TaqMan probe (FAM)	5′-AAAACCATTGTCCTCCACCCATTTCCCCG-3′
IVS9-39c > t	
Forward primer for C-allele	5′-GTTTTTTTCATGTGCAATCTCATTGTC-3′
Forward primer for T-allele	5′-GTTTTTTTCATGTGCAATCTCATTGTT-3′
Reverse primer	5′-TCAGATGCACCACGGAGTACA-3′
TaqMan probe (FAM)	5′-AGGCAAAAATTCTTGAAGCCAAACAGAAGG-3′

[Niu et al., 2002]. Haplotype frequencies between patients and controls were evaluated both by the whole distribution with Fisher's exact test and by χ^2 tests of one haplotype against others (haplotype-wise test). In the haplotype-wise test, significance values were corrected for multiple comparisons by multiplying the *P*-value by the number of haplotypes compared (Bonferroni correction). The SPSS software version 11.0J (SPSS Japan, Inc., Tokyo, Japan) was used to perform the statistical analyses. Haplotypes for the TDT was determined by the SimWalk2 program (http://watson.hgen.pitt.edu/register) [Sobel and Lange, 1996], and the deviations of transmission from 50% chance were evaluated by chi-square test. We performed Bonferroni correction for the significance levels in TDT. *P*-value less than 0.05 was considered statistically significant.

RESULTS

Using DHPLC, we screened the nucleotide changes in all 17 exons that covered the entire coding sequence and their flanking introns and 5′-flanking region of GAD67 among 50 patients. Sequencing of the PCR products with aberrant chromatographic patterns identified seven SNPs: four in the 5′ flanking region, −677C > G, −449C > T, −445C > A, and −292A > C; one in exon 1, −147G > A; one in exon 3, 111T > C; and one in intron 9, IVS9−39c > t (nucleotide changes are according to the accession number AC007405, NT005403; Table I). The −147G > A was located in the 5′ untranslated region (UTR). The 111T > C substitution altered the third nucleotide of codon 37 (histidine), resulting in no amino acid change. The IVS9−39c > t substitution did not affect the consensus sequences for mRNA splicing. The −147G > A, 111T > C, and IVS9−39c > t were identical to the entry in the dbSNP (accession number [rs3749034], [rs769404], [rs701492], respectively). The −677C > G, −449C > T, −445C > A, and −292A > C were not found in the current SNP databases. All genotypes of the SNPs in the parents were in Hardy–Weinberg equilibrium. To examine LD between identified SNPs, pairwise LD coefficients, D′ and r^2, were calculated (Table III). The −677C > G and the −449C > T polymorphism were in complete LD with the −147G > A and the −445C > A, respectively. Therefore, the −667C > G and −449C > T were excluded from further analyses.

We estimated the frequency of 5-locus haplotype (−445C > A, −292A > C, −147G > A, 111T > C, and IVS9−39c > t) of the *GAD67* gene with the aid of the HAPLOTYPER program. Table IV shows the different haplotype distribution pattern between NSCLP and control ($P = 0.0028$). The frequency of −445A, −292A, −147G, 111C, IVS9−39T (AAGCT) haplotype in NSCLP was significantly lower than that in controls ($\chi^2 = 14.533$, $P = 0.00098$).

TABLE III. Analysis of LD for All Possible Two-Way Comparisons Among Seven SNPs

Polymorphism	−677C>G	−449C>T	−445C>A	−292A>C	−147G>A	T111C	IVS9-39c>t
Physical distance		228 bp	4 bp	153 bp	695 bp	5.15 kbp	23.9 kbp
−677C>C							
D′		−0.999	−0.999	−0.999	1.000	−1.000	−0.726
r^2		0.124	0.124	0.199	1.000	0.584	0.062
−449C>T							
D′			1.000	−0.916	−0.999	1.000	0.154
r^2			1.000	0.162	0.124	0.212	0.023
−445C>A							
D′				−0.916	−0.999	1.000	0.154
r^2				0.163	0.124	0.212	0.023
−292A>C							
D′					−0.999	1.000	−0.440
r^2					0.199	0.341	0.036
−147G>A							
D′						−1.000	−0.726
r^2						0.584	0.062
T111C							
D′							−0.103
r^2							0.006

TDT was performed in 99 parent and patient trios using haplotypes with the aid of SimWalk2 program. Table V shows that the −445C, −292C, −147G, 111C, and IVS9−39C (CCGCC) haplotype transmitted preferentially to NSCLP children (transmitted = 43, not-transmitted = 24, $\chi^2 = 5.388$). After Bonferroni correction, the *P*-value for this transmission deviation did not reach a significant level ($P = 0.122$). However, when the patients were limited to cleft lip with cleft palate, the corrected *P*-value maintained a significant level ($\chi^2 = 10.37$, $P = 0.0077$). The AAGCT haplotype transmitted only twice from 11 heterozygous parents to NSCLP children. Although the corrected *P*-value of this deviation did not reach to a significant level even when limited to cleft lip with cleft palate patients, the trend was consistent with the result of the case-control study in which the frequency of the AAGCT haplotype was lower in the patients.

DISCUSSION

We identified seven SNPs, −667C>G, −449C>T, −445C>A, −292A>C, −147G>A, T111C, and IVS9−39 c>t, in the *GAD67* gene. Five of the seven SNPs facilitated the association between NSCLP and GAD67. In the case-control study, we observed a significant difference in the distribution pattern of the haplotype frequency between cases and controls. The difference in the frequency of the AAGCT haplotype contributed greatly to the difference in the whole frequency distribution. The lower frequency of the AAGCT haplotype in the patients suggested that this is a disease-protective allele. This notion was supported by the result of TDT. Although the number of heterozygous parents was small and the significance of the transmission deviation remained marginal, the AAGCT haplotype transmitted to the patients less frequently than the 50% expectation. The consistent results of the case-control study and TDT suggested an association of the AAGCT haplotype and NSCLP in the Japanese population.

The frequency of the CCGCC haplotype in NSCLP children was not significantly different from that of the controls, but the CCGCC haplotype was significantly over-transmitted to the patients from their parents who were heterozygous for this haplotype. The reason for this inconsistency is considered to be as follows. The over-transmission of the CCGCC haplotype (43 alleles transmitted from 67 heterozygous parents) reflected exactly the frequency change of this haplotype from 0.276 in parents to 0.308 in NSCLP patients. However, because the frequency in the parents was lower than that in the controls (0.323) in this study, this over-transmission rather diminished the difference of the frequency between patients and control. The frequency difference of the three groups was only a few points and not statistically significant with the number of samples used in this study. To establish the association of the CCGCC haplotype with the disease, confirmation with a new set of samples both for TDT and case-control studies is necessary.

The SNPs found in this study do not affect the amino acid sequence or splicing consensus sequence. The SNPs in the 5′-flanking region may affect the expression of GAD67. It is also possible that seemingly innocent SNPs affect mRNA splicing and/or the gene expression level as described in other genetic disorders [Wakamatsu et al., 1992; Kajihara et al., 1995]. Alternatively, the SNPs found in this study may not be functionally meaningful, but are in LD to an unidentified genetic polymorphism(s) responsible for the development of the disease.

Recently, a linkage study between GAD67 and NSCLP among Italian patients was reported [Scapoli et al., 2002]. The study involved 38 Italian pedigrees and showed no evidence of LD. Discrepancy between this study and ours might be due to the difference in the genetic markers studied. The genetic marker employed

TABLE IV. Frequencies of 5-locus Haplotypes in *GAD67*

					Freq.			Control vs. cases		Parent vs. cases	
−445C>A	−292A>C	−147G>A	T111C	IVS9−39c>t	Controls (198)[b]	Parents (370)[b]	Cases (198)[b]	χ^2 (df=1)	P*	χ^2 (df=1)	P*
Haplotypes[a]											
C	C	G	C	C	0.323	0.276	0.308	0.105	>1	0.662	>1
C	A	A	T	C	0.253	0.278	0.258	0.013	>1	0.282	>1
A	A	G	C	C	0.157	0.135	0.177	0.291	>1	1.757	>1
C	A	G	T	T	0.106	0.154	0.141	1.141	>1	0.162	>1
A	A	G	C	T	0.096	0.024	0.010	14.533	0.00098	1.374	>1
C	C	G	C	T	0.030	0.081	0.081	4.813	0.196	0.000129	>1
Others					0.035	0.051	0.025				
Whole distribution					1.000	1.000	1.000		0.0028**		0.453**

[a]Six predominant haplotypes are listed. the "Others" category includes minor haplotypes with <1% frequency in controls.
[b]Analyzed allele number.
*Bonferroni correction with 7× of raw *P*-values.
**P-value for the whole distribution comparison calculated with Fisher's exact test.

TABLE V. Results of TDT

					Cleft lip with or without cleft palate				Cleft lip with cleft palate			
−445C>A	−292A>C	−147G>A	T111C	IVS9-39c>t	Transmitted	Not transmitted	χ^2	P[a]	Transmitted	Not transmitted	χ^2	P[a]
Haplotypes												
C	C	G	C	C	43	24	5.388	0.122	37	14	10.37	0.0077
C	A	A	T	C	26	28	3.272	0.422	18	24	0.857	>1
A	A	G	C	C	27	16	2.814	0.561	23	13	2.778	>1
C	A	G	T	T	16	18	0.118	>1	12	12	0.000	>1
A	A	G	C	T	2	9	4.455	0.209	1	9	6.400	0.068
C	C	G	C	T	15	16	0.032	>1	14	13	0.037	>1

[a]Bonferroni correction with 6× of raw *P*-values.

in the Italian study was a microsatellite marker D2S335 that lies in close proximity (2 cM) to GAD67, but the precise distance between this marker and GAD67 has not yet been determined. In contrast, our study used five SNPs within the gene, reducing the chance of a recombination between the marker and the disease locus. Alternatively, the discrepancy between the two studies might also be due to differences in the populations investigated.

Neuropharmacological studies have suggested that GABA may be involved in the development of palate [Miller and Becker, 1975; Wee and Zimmerman, 1983]. GABA functions as an inhibitory neurotransmitter in the palate of mice. When diazepam, a neuropharmacologic agent which mimics GABA, was administered to pregnant dams, it inhibited palate reorientation and caused clefting in their offspring [Wee and Zimmerman, 1983]. Interestingly, the teratogenic effect of diazepam as well as the GABAergic system varied among mice of different genetic background. Although controversial, an association between prenatal exposure to diazepam and oral clefts in humans has also been suggested in several studies [Safra and Oakley, 1975; Saxen and Saxen, 1975]. The importance of the GABAergic system in palate development has been further advocated by genetic approaches. Mice with mutations in Gabrb3 showed defects in forming the second palate [Culiat et al., 1993; Homanics et al., 1997]. In addition, LD between GABRB3 and NSCLP was observed in the above mentioned 38 Italian pedigrees ($P = 0.008$ in the allele-wise analysis for multiallelic markers) [Scapoli et al., 2002]. Our study with SNPs in GAD67 and the previous Gad67 knockout mice study provide additional evidence for the possible involvement of the GABAergic system in the pathogenesis of NSCLP.

In conclusion, we have presented evidence of an association between GAD67 and NSCLP in the Japanese. Studies with a larger number of families from various ethnic groups are necessary to confirm our results. Novel intragenic SNPs identified in this study would facilitate future investigations.

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Variations in the C3, C3a receptor, and C5 genes affect susceptibility to bronchial asthma

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Abstract Bronchial asthma (BA) is a common chronic inflammatory disease characterized by hyperresponsive airways, excess mucus production, eosinophil activation, and the production of IgE. The complement system plays an immunoregulatory role at the interface of innate and acquired immunities. Recent studies have provided evidence that C3, C3a receptor, and C5 are linked to airway hyperresponsiveness. To determine whether genetic variations in the genes of the complement system affect susceptibility to BA, we screened single nucleotide polymorphisms (SNPs) in *C3*, *C5*, the C3a receptor gene (*C3AR1*), and the C5a receptor gene (*C5R1*) and performed association studies in the Japanese population. The results of this SNP case-control study suggested an association between 4896C/T in the *C3* gene and atopic childhood BA (P=0.0078) as well as adult BA (P=0.010). When patient data were stratified according to elevated total IgE levels, 4896C/T was more closely associated with adult BA (P=0.0016). A patient-only association study suggested that severity of childhood BA was associated with 1526G/A of the *C3AR1* gene (P=0.0057). We identified a high-risk haplotype of the *C3* gene for childhood (P=0.0021) and adult BA (P=0.0058) and a low-risk haplotype for adult BA (P=0.00011). We also identified a haplotype of the *C5* gene that was protective against childhood BA ($P=1.4\times10^{-6}$) and adult BA (P=0.00063). These results suggest that the C3 and C5 pathways of the complement system play important roles in the pathogenesis of BA and that polymorphisms of these genes affect susceptibility to BA.

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Introduction

The incidence of allergic diseases has significantly increased over the past few decades. These allergies are complex conditions caused by a combination of genetic and environmental factors (Barnes and Marsh 1998). Bronchial asthma (BA) is an allergic condition characterized by three key phenotypes: intermittent airway obstruction, inflammatory cell infiltrates, and airway hyperresponsiveness (AHR) (Daser et al. 2001), which are all closely linked to Th2 lymphocyte functions.

The complement system defends the host from invading microorganisms by initiating inflammatory and immunological responses and by promoting cell lysis (Barrington et al. 2001; Kohl 2001; Muller-Eberhard 1988). Complement activation leads to the production of several fragments; among these, anaphylatoxins (including C3a and C5a) can stimulate respiratory burst in macrophages, neutrophils, and eosinophils (Drouin et al. 2001b; Gerard

and Gerard 2002). The anaphylatoxins C3a and C5a causes histamine release from basophils and mast cells (Burgi et al. 1994; el-Lati et al. 1994), exhibit properties resembling IgE-mediated anaphylaxis, contract smooth muscle, activate mast cells, and increase vascular permeability (Henson 2000).

Recent studies indicate that the complement system plays an important role in the pathogenesis of BA. Mice and guinea pigs that are deficient for the C3a receptor (C3aR) are protected against bronchoconstriction and AHR that develops after allergen challenges (Bautsch et al. 2000; Drouin et al. 2002; Humbles et al. 2000). Mice deficient in C3 or C3aR have decreased AHR and lung eosinophilia after allergen challenges (Drouin et al. 2001a). On the other hand, strains of mice that are naturally deficient in C5 have increased susceptibility to allergen-induced AHR via decreased IL-12 production (Karp et al. 2000). These animal studies suggest that C3a and C5a have opposite effects. Both C3a and C5a levels are increased in the bronchoalveolar lavage fluid of patients with BA after allergen provocation compared with healthy control individuals (Humbles et al. 2000; Krug et al. 2001; van de Graaf et al. 1992), suggesting that the C3 and C5 pathways are involved in human BA.

Several genome-wide screens for susceptibility to BA and atopy have identified candidate loci, including chromosomes 9q32-34 and 19q13.3-q13.4, where *C5* and the gene encoding the C5a receptor (*C5R1*) are respectively localized (CSGA 1997; Ober et al. 1998; Wjst et al. 1999).

Clinical, experimental, and genetic evidence suggests that genes involved in the C3 and C5 pathways are strong candidates for BA susceptibility genes. We therefore screened SNPs in *C3*, *C5*, *C3AR1* (encoding C3aR) and *C5R1*, and performed association studies of clinically characterized Japanese patients with childhood or adult BA.

Materials and methods

Subjects

In this study, we classified patients who were under 17 years of age at entry as having childhood BA and those who were 18 years of age or older as having adult BA regardless of the age of onset. We recruited 384 patients with childhood BA and 96 patients with adult BA from Osaka Prefectural Habikino Hospital and 384 patients with adult BA from the Miyatake Asthma Clinic. All participants with BA were selected according to the American Thoracic Society criteria (National Heart, Lung, and Blood Institute 1991). The patients with childhood BA had a mean age of 9.8 years (range, 1–17 years). The patients with adult BA had a mean age of 45.9 years (range, 18–83 years). The mean onset of childhood BA was 3.3 years. Of the patients with adult BA, 22% showed onset under 18 years old. Total serum IgE level and eosinophil count were measured from blood samples for genetic analyses. The mean of $\log_{10}$ [total IgE (tIgE) (IU/ml)] of patients with childhood BA was 2.59; that of patients with adult BA was 2.32. The mean of $\log_{10}$[-eosinophil count (percentage of white blood cells)] in patients with childhood BA was 0.77; that of patients with adult BA was 0.66. In this study, "high IgE" levels were defined as those values in the 75th percentile or higher of total IgE. The 75th percentile value of $\log_{10}$(tIgE) in patients with childhood BA was 3.04 [=$\log_{10}$(1,092 IU/ml)]. That in patients with adult BA was 2.68 [=$\log_{10}$(480 IU/ml)]. In adult patients, we also examined the presence of first- to third-degree relatives with BA (positive: 54%), aspirin-induced BA (history of at least two attacks due to aspirin or related drugs; positive: 5.6%), and nasal polyps (positive: 13%). The severity of adult BA was classified according to the criteria of the National Heart, Lung, and Blood Institute (1997) by physicians who are experts in allergic diseases. The severity of childhood BA was defined according to the degree of therapy required to control symptoms at the time of entry into this study. The grades were as follows: grade 1, β stimulants only; grade 2, sodium cromoglycate and/or theophylline; grade 3, inhaled beclomethasone, 400 μg/day or less; grade 4, inhaled beclomethasone of more than 400 μg/day. Patients with grades 3 and 4 were treated with inhaled steroids and they accounted for 46% of all patients. We also examined the number of parents of the proband who also had BA, the number of siblings with BA, and the level of IgE specific for house dust mites in the patients with childhood BA. Specific IgE was considered positive when values exceeded 0.35 U_A/ml (RAST score ≥1) according to an enzyme immunoassay. Atopy was defined as positive mite-specific IgE. Of the patients with childhood BA, 80% had atopic BA. Atopic dermatitis was observed in 45% of the patients with childhood BA. Of the patients with childhood BA, 26% had a parent with BA. As controls, we analyzed 384 randomly selected population-based individuals (mean age 41.5; range, 18–81 years; male/female ratio=2.05:1.00) who had no history of BA.

Peripheral blood was sampled for genetic analyses. We screened SNPs using cDNA synthesized from the mRNAs of 12 volunteers. We used standard procedures to obtain mRNA and synthesize cDNA, as well as to prepare genomic DNAs for SNP genotyping. 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).

SNP screening and genotyping

We designed specific primer sets based on the *C3*, *C5*, *C3AR1*, and *C5R1* cDNA sequences in the GenBank DNA database (accession numbers K02765, M57729, U62027 and M62505, respectively) to screen their entire coding regions. We first amplified 40 ng of cDNA from 12 individuals by the polymerase chain reaction (PCR) method. Information on the primers and reaction condi-

tions of PCR will be provided on request. Each amplification product was reacted with BigDye Terminator RR mix (Applied Biosystems, Foster City, Calif., USA) using the same primers as for the PCR amplification, and then sequences were determined using an ABI Prism 3700 DNA autosequencer (Applied Biosystems). Sequences were analyzed with SEQUENCHER 3.1 software (Gene Codes, Ann Arbor, Mich., USA).

We genotyped single-nucleotide polymorphisms (SNPs) by PCR-directed sequencing or by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. Specific primers were designed based on genomic sequences containing these genes obtained from the GenBank DNA database (accession numbers AC008760 in *C3*, AC006430 in *C5*, AC006511 in *C3AR1*, and AC099491 in *C5R1*). Information on the primer sequences and thermal cycling conditions will be provided on request. We genotyped all SNPs by direct sequencing, except *C5* 1632T/C, which was genotyped by the PCR-RFLP method. The PCR product for *C5* 1632T/C was digested with *Rsa*I at 37°C. Digested PCR fragments were resolved by electrophoresis on 4% agarose gels.

Statistical analysis

Serum IgE levels and eosinophil counts were $\log_{10}$-transformed before analysis. The contingency chi-square test compared allele frequencies in patients with BA and controls. In the association study between individual SNPs and asthma or a BA-related phenotype, we performed many statistical tests; therefore, inflation of false positive results (type 1 error) was a concern. Because all SNPs in each gene were significantly in linkage disequilibrium and because asthma-related phenotypes (seven variables for children and seven variables for adults, see Results) are significantly related, multiplication of *P* values by the number of SNPs or phenotypes tested is too conservative and the appropriate value for the correction is not evident. Thus, to deal with the multiple comparisons, we did not apply Bonferroni corrections but set the significant *P* value at 0.01 rather than 0.05. Odds ratios were estimated according to Brown (1981). Haplotypes were analyzed with a maximum-likelihood method using ARLEQUIN software Ver. 2.0 (Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva, Switzerland) (Excoffier and Slatkin 1995). The program HAPLOTYPER (http://www.people.fas.harvard.edu/~junliu/Haplo/docMain.htm) determined the most probable haplotype and diplotype in individuals with uncertain genotype phases (Niu et al. 2002). Haplotype frequencies between patients and controls were evaluated both by the entire distribution with Fisher's exact test and by chi-square tests of one haplotype against others (haplotype-wise test). In this test, probability values were corrected for multiple comparisons by multiplying the *P* value by the number of haplotypes compared. Effects of genotypes or haplotypes on $\log_{10}$-transformed IgE levels and eosinophil counts were evaluated using the analysis of variance (ANOVA) or *t*-test. Statistical analyses were performed with SPSS software, version 11.0J (SPSS Japan, Tokyo, Japan), unless otherwise noted.

Results

Polymorphisms in the complement system genes

Complements and their receptor genes were screened for variations in 12 Japanese individuals. From the screening of cDNA sequences, we identified nine SNPs in the *C3* gene, five SNPs in the *C5* gene, three SNPs in the *C3AR1* gene, and two SNPs in the *C5R1* gene. Seven SNPs of *C3* (912G/A: rs3815752, 1692G/A: rs3745559, 1836G/A: rs3745560, 2421C/G: rs428453, 2745C/T: rs423490, 4311C/T: rs11569554, 4896C/T: rs4807893) were already registered in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). Two (1872CT and 2811A/T) were not confirmed at the genomic DNA level and not investigated further. In the *C5* gene, four SNPs (1155A/G: rs10985126, 1632C/T: rs25681, 2404A/G: rs17611, 4266G/A: rs12237774) were found in the dbSNP and one SNP (433G/A: ss23140500) was not. All three SNPs (210C/T: rs11567805, 1526G/A: rs2230318, 1595A/G: rs7842) in *C3AR1* were found in the dbSNP. The two SNPs we identified in the *C5R1* were new (1289C/A ss23141118, 1337C/T: ss23141119). Among 17 SNPs identified in the four genes, two were non-synonymous substitutions [*C5* 433G/A (Val145Ile), *C5* 2404A/G (Ile802Val)], four were located in the 3′-untranslated regions (*C3AR1* 1526G/A and 1595A/G, *C5R1* 1289C/A and 1337C/T), and the rest were synonymous substitutions. Thirteen SNPs (*C3*: 912G/A, 1692G/A, 1836G/A, 4896C/T; *C5*: 433G/A, 1155A/G, 1632C/T, 2404A/G, 4266G/A; *C3AR1*: 1526G/A, 1595A/G; *C5R1*: 1289C/A, 1337C/T) were used for the association studies.

Association of SNPs with bronchial asthma

We genotyped 384 patients with childhood BA, 480 patients with adult BA, and 384 controls. The cohort represents the largest number of samples among published genetic studies of BA in Japanese patients. All genotype results of the SNPs in the control samples were in Hardy-Weinberg equilibrium, except *C5* 4266G/A. All control samples were retyped by the TaqMan PCR method (Fujii et al. 2000). The result was consistent with that by the sequencing method, suggesting no indication of typing errors with this SNP. In the association study of childhood BA, we stratified patient data by age at onset, high total IgE level, mite-specific IgE (atopy), high eosinophil count, severity, existence of atopic dermatitis, and family history. In the study of adult BA, we stratified patient data by age at onset, high total IgE level, eosinophil count, severity, aspirin-induced asthma, nasal polyps, and family history.

Table 1 shows the results of the case-control study, where *P* values of association of the SNPs with patients

with BA with and without data stratification were no more than 0.010 in genotypic association tests. When all patients were examined, only the association of *C3* 4896C/T with adult BA was marginally significant (odds ratio=1.53, CI=1.11–3.59, P=0.010). We next performed association studies of patients stratified by the BA-related phenotypes. Association between *C3* 4896C/T and BA was evident in childhood BA with atopy (P=0.0078), or with severity ≥3 (P=0.0041). This SNP and adult BA with high total IgE (≥480 IU/ml) were also associated (P=0.0016). An association between *C3AR1* 1526G/A and the patients with the most severe cases of childhood BA was suggested (P=0.0050).

We next investigated associations between BA-related phenotypes and SNPs within patients with BA (case-only association study). With this method, spurious associations can be avoided due to population stratification (Khoury 1998; Reich and Goldstein 2001). Table 1 also includes results of the case-only study. The 1526G/A of *C3AR1* was associated with the severity of childhood BA (P=0.0057). The 1595G/A of *C3AR1* was associated with the existence of atopic dermatitis in childhood BA (P=0.0092). The 1632C/T and 2404G/A of *C5* were associated with onset of adult BA to a similar degree because these SNPs are in strong linkage disequilibrium (P=0.0053 and P=0.0054, respectively).

There was no significant association between SNPs in the *C5R1* gene and BA. An evaluation of total IgE level and eosinophil count by ANOVA did not reveal any significant differences among the studied SNP genotypes.

Table 1 Association between SNPs of complement system genes and asthma (*95% CI* 95% confidence interval, *df* degrees of freedom)

Gene	SNP	No. of cases	Genotype			Odds ratio (95% CI)	χ^2 (*df*=1)	*P*
			CC	CT	TT			
C3	4896C/T							
	Controls	364	103	184	77	Reference		
	Childhood BA							
	Positive mite-specific IgE)	299	58	167	74	1.64 (1.14–2.37)[b]	7.07	0.0078
	Severity≥3	172	29	100	43	1.95 (1.23–3.08)[b]	8.23	0.0041
	Adult BA							
	All patients	447	107	210	130	1.53 (1.11–2.11)[c]	6.64	0.010
	High total IgE[a]	105	24	43	38	2.11 (1.32–3.39)[c]	11.88	0.0016
			GG	GA	AA			
C3AR1	1526G/A							
	Controls	264	240	24	0	Reference		
	Childhood BA							
	Severity=4	83	66	16	1	2.58 (1.31–5.08)[d]	7.86	0.0050
	Severity<4	252	229	23	0	Reference		
	Severity=4	83	66	16	1	2.57 (1.29–5.08)[d]	7.66	0.0057
			AA	AG	GG			
	1595A/G							
	Childhood BA							
	Without atopic dermatitis	199	114	77	8	Reference		
	With atopic dermatitis	166	117	43	6	0.562 (0.363–0.869)[e]	6.78	0.0092
			CC	CT	TT			
C5	1632C/T							
	Adult BA							
	Onset≥18 years old	362	89	187	86	Reference		
	Onset<18 years old	104	17	48	39	1.93 (1.21–3.07)[b]	7.77	0.0053
			AA	AG	GG			
	2404A/G							
	Adult BA							
	Onset≥18 years old	350	83	182	85	Reference		
	Onset<18 years old	101	38	46	17	0.515 (0.321–0.826)[e]	7.73	0.0054

[a]Total IgE ≥480 IU/m (=75 percentile of adult BA patients' value)
[b]CC vs CT+TT
[c]CC+CT vs TT
[d]GG vs GA+AA
[e]AA vs AG+GG

Table 2 Association of the haplotype of the *C3* and *C5* genes with BA (*95% CI* 95% confidence interval, *df* degree of freedom)

Gene	Haplotype[a]					Frequency[a]			Control vs childhood asthma			Control vs adult asthma		
						Control	Childhood BA	Adult BA	χ^2 (*df*=1)	P^b	Odds ratio (95% CI)	χ^2 (*df*=1)	P^b	Odds ratio (95% CI)
C3	912	1692	1836	4896		(708)[c]	(704)[c]	(852)[c]						
	G	A	G	C		0.253	0.216	0.164	2.68	0.714	0.81 (0.64–1.04)	18.62	0.00011	0.58 (0.45–0.75)
	G	A	G	T		0.223	0.309	0.298	13.1	0.0021	1.55 (1.22–1.97)	11.18	0.0058	1.48 (1.18–1.86)
	A	G	A	C		0.210	0.201	0.209	0.17	>1	0.95 (0.73–1.37)	0.005	>1	0.99 (0.78–1.27)
	A	G	A	T		0.183	0.164	0.178	1.01	>1	0.87 (0.66–1.14)	0.07	>1	0.97 (0.75–1.25)
	G	G	G	C		0.059	0.063	0.081	0.06	>1	1.06 (0.68–1.64)	2.75	0.68	1.40 (0.94–2.08)
	G	G	G	T		0.043	0.031	0.035	1.23	>1	0.73 (0.42–1.28)	0.54	>1	0.83 (0.49—1.38)
	Others					0.029	0.016	0.035						
	Entire distribution									0.0078[d]			0.00016[d]	
C5	433	1155	1632	2404	4266	(476)[c]	(666)[c]	(664)[c]						
	G	A	T	A	G	0.487	0.479	0.492	0.078	>1	0.96 (0.76–1.22)	0.029	>1	1.02 (0.81–1.29)
	G	A	C	G	G	0.211	0.265	0.248	4.45	0.245	1.35 (1.02–1.79)	2.29	0.91	1.24 (0.94–1.65)
	A	G	C	G	A	0.158	0.175	0.174	0.651	>1	1.14 (0.83–1.56)	0.58	>1	1.13 (0.82–1.56)
	G	G	C	G	A	0.057	0.0055	0.015	27.04	1.40×10^{-6}	0.10 (0.035–0.29)	15.32	0.00063	0.25 (0.12–0.56)
	G	G	C	G	G	0.030	0.037	0.034	0.556	>1	1.29 (0.66–2.50)	0.24	>1	1.18 (0.60–2.33)
	G	A	T	A	A	0.025	0.023	0.022	0.087	>1	0.89 (0.41–1.92)	0.082	>1	0.89 (0.41–1.93)
	Others					0.032	0.016	0.016						
	Entire distribution									0.0000020[d]			0.0021[d]	

[a]Six predominant haplotypes are listed; the "others" category includes minor haplotypes with <1% frequency in controls
[b]Bonferroni-type adjustment with ×7 of raw *P* values
[c]Number of alleles analyzed
[d]*P* value for the entite distribution calculated with exact test