Courtney KD, Moore JA. 1971. Teratology studies with 2,4,5-trichlorophenoxyacetic acid and 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Appl Pharmacol 20:396–403.

Fujii K, Matsubara Y, Akanuma J, Takahashi K, Kure S, Suzuki Y, Imaizumi M, Iinuma K, Sakatsume O, Rinaldo P, Narisawa K. 2000. Mutation detection by TaqMan-allele specific amplification: Application to molecular diagnosis of glycogen storage disease type Ia and medium-chain acyl-CoA dehydrogenase deficiency. Hum Mutat 15:189–196.

Garcia AM, Fletcher T, Benavides FG, Orts E. 1999. Parental agricultural work and selected congenital malformations. Am J Epidemiol 149: 64–74.

Gullett BK, Ryan JV. 2002. On-road emissions of PCDDs and PCDFs from heavy duty diesel vehicles. Environ Sci Technol 36:3036–3040.

Hill WG, Robertson A. 1968. Linkage disequilibrium in finite populations. Theor Appl Genet 38:226–231.

Huwe JK. 2002. Dioxins in food: A modern agricultural perspective. J Agric Food Chem 50:1739–1750.

Kajihara S, Matsuhashi S, Yamamoto K, Kido K, Tsuji K, Tanae A, Fujiyama S, Itoh T, Tanigawa K, Uchida M. et al. 1995. Exon redefinition by a point mutation within exon 5 of the glucose-6-phosphatase gene is the major cause of glycogen storage disease type 1a in Japan. Am J Hum Genet 57:549–555.

Kozak KR, Abbott B, Hankinson O. 1997. ARNT-deficient mice and placental differentiation. Dev Biol 191:297–305.

Kruglyak L, Daly MJ, Reeve-Daly MP, Lander ES. 1996. Parametric and nonparametric linkage analysis: A unified multipoint approach. Am J Hum Genet 58:1347–1363.

Lewontin RC. 1964. The interaction of selection and linkage. I. General considerations; heterotic models. Genetics 49:49–67.

Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daack-Hirsch S, Semina EV, Johnson LR, Machida J, Burds A, Parnell TJ, Rubenstein JL, Murray JC. 1998. Association of MSX1 and TGFB3 with nonsyndromic clefting in humans. Am J Hum Genet 63:557–568.

Lorente C, Cordier S, Goujard J, Ayme S, Bianchi F, Calzolari E, De Walle HE, Knill-Jones R, Occupational Exposure and Congenital Malformation Working Group. 2000. Tobacco and alcohol use during pregnancy and risk of oral clefts. Am J Public Health 90:415–419.

Maestri NE, Beaty TH, Hetmanski J, Smith EA, McIntosh I, Wyszynski DF, Liang KY, Duffy DL, VanderKolk C. 1997. Application of transmission disequilibrium tests to nonsyndromic oral clefts: Including candidate genes and environmental exposures in the models. Am J Med Genet 73:337–344.

Mimura J, Yamashita K, Nakamura K, Morita M, Takagi TN, Nakao K, Ema M, Sogawa K, Yasuda M, Katsuki M, Fujii-Kuriyama Y. 1997. Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. Genes Cell 2:645–654.

Murray JC. 2002. Gene/environment causes of cleft lip and/or palate. Clin Genet 61:248–256.

Muto H, Takizawa Y. 1989. Dioxins in cigarette smoke. Arch Environ Health 44:171–174.

Scapoli L, Martinelli M, Pezzetti F, Carinci F, Bodo M, Tognon M, Carinci P. 2002. Linkage disequilibrium between *GABRB3* gene and non-syndromic familial cleft lip with or without cleft palate. Hum Genet 110:15–20.

Scheel J, Hussong R, Schrenk D, Schmitz HJ. 2002. Variability of the human aryl hydrocarbon receptor nuclear translocator (*ARNT*) gene. J Hum Genet 47:217–224.

Schutte BC, Murray JC. 1999. The many faces and factors of orofacial clefts. Hum Mol Genet 8:1853–1859.

Shaw GM, Wasserman CR, Lammer EJ, O'Malley CD, Murray JC, Basart AM, Tolarova MM. 1996. Orofacial clefts, parental cigarette smoking, and transforming growth factor-alpha gene variants. Am J Hum Genet 58:551–561.

Sogawa K, Fujii-Kuriyama Y. 1997. Ah receptor, a novel ligand-activated transcription factor. J Biochem (Tokyo) 122:1075–1079.

Spielman RS, McGinnis RE, Ewens WJ. 1993. Transmission test for linkage disequilibrium: The insulin gene region and insulin-dependent diabetes mellitus (IDDM). Am J Hum Genet 52:506–516.

van Rooij IA, Wegerif MJ, Roelofs HM, Peters WH, Kuijpers-Jagtman AM, Zielhuis GA, Merkus HM, Steegers-Theunissen RP. 2001. Smoking, genetic polymorphisms in biotransformation enzymes, and nonsyndromic oral clefting: A gene-environment interaction. Epidemiology 12: 502–507.

Wakamatsu N, Kobayashi H, Miyatake T, Tsuji S. 1992. A novel exon mutation in the human beta-hexosaminidase beta subunit gene affects 3' splice site selection. J Biol Chem 267:2406–2413.

Wyszynski DF, Beaty TH. 1996. Review of the role of potential teratogens in the origin of human nonsyndromic oral clefts. Teratology 53:309–317.

Wyszynski DF, Beaty TH, Maestri NE. 1996. Genetics of nonsyndromic oral clefts revisited. Cleft Palate Craniofac J 33:406–417.

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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.

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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REFERENCES

Asada H, Kawamura Y, Maruyama K, Kume H, Ding RG, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K. 1997. Cleft palate and decreased brain gamma-aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase. Proc Natl Acad Sci USA 94:6496–6499.

Condie BG, Bain G, Gottlieb DI, Capecchi MR. 1997. Cleft palate in mice with a targeted mutation in the gamma-aminobutyric acid-producing enzyme glutamic acid decarboxylase 67. Proc Natl Acad Sci USA 94:11451–11455.

Culiat CT, Stubbs L, Nicholls RD, Montgomery CS, Russell LB, Johnson DK, Rinchik EM. 1993. Concordance between isolated cleft palate in mice and alterations within a region including the gene encoding the beta 3 subunit of the type A gamma-aminobutyric acid receptor. Proc Natl Acad Sci USA 90:5105–5109.

Fujii K, Matsubara Y, Akanuma J, Takahashi K, Kure S, Suzuki Y, Imaizumi M, Iinuma K, Sakatsume O, Rinaldo P, Narisawa K. 2000. Mutation detection by TaqMan-allele specific amplification: Application to molecular diagnosis of glycogen storage disease type Ia and medium-chain acyl-CoA dehydrogenase deficiency. Hum Mutat 15: 189–196.

Hill WG, Robertoson A. 1968. Linkage disequilibrium in finite populations. Theor appl genet 38:175–195.

Homanics GE, DeLorey TM, Firestone LL, Quinlan JJ, Handforth A, Harrison NL, Krasowski MD, Rick CE, Korpi ER, Makela R, Brilliant MH, Hagiwara N, Ferguson C, Snyder K, Olsen RW. 1997. Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. Proc Natl Acad Sci USA 94:4143–4148.

Kajihara S, Matsuhashi S, Yamamoto K, Kido K, Tsuji K, Tanae A, Fujiyama S, Itoh T, Tanigawa K, Uchida M, et al. 1995. Exon redefinition by a point mutation within exon 5 of the glucose-6-phosphatase gene is the major cause of glycogen storage disease type 1a in Japan. Am J Hum Genet 57:549–555.

Lewontin RC. 1964. The interaction of selection and linkage. I. General considerations; heterotic models. Genetics 74:175–195.

Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daack-Hirsch S, Semina EV, Johnson LR, Machida J, Burds A, Parnell TJ, Rubenstein JL, Murray JC. 1998. Association of MSX1 and TGFB3 with nonsyndromic clefting in humans. Am J Hum Genet 63:557–568.

Maestri NE, Beaty TH, Hetmanski J, Smith EA, McIntosh I, Wyszynski DF, Liang KY, Duffy DL, VanderKolk C. 1997. Application of transmission disequilibrium tests to nonsyndromic oral clefts: Including candidate genes and environmental exposures in the models. Am J Med Genet 73:337–344.

Miller RP, Becker BA. 1975. Teratogenicity of oral diazepam and diphenylhydantoin in mice. Toxicol Appl Pharmacol 32:53–61.

Niu T, Qin ZS, Xu X, Liu JS. 2002. Bayesian haplotype inference for multiple linked single-nucleotide polymorphisms. Am J Hum Genet 70:157–169.

Safra MJ, Oakley GP Jr. 1975. Association between cleft lip with or without cleft palate and prenatal exposure to diazepam. Lancet 2:478–480.

Saxen I, Saxen L. 1975. Letter: Association between maternal intake of diazepam and oral clefts. Lancet 2:498.

Scapoli L, Martinelli M, Pezzetti F, Carinci F, Bodo M, Tognon M, Carinci P. 2002. Linkage disequilibrium between *GABRB3* gene and nonsyndromic familial cleft lip with or without cleft palate. Hum Genet 110:15–20.

Schutte BC, Murray JC. 1999. The many faces and factors of orofacial clefts. Hum Mol Genet 8:1853–1859.

Sobel E, Lange K. 1996. Descent graphs in pedigree analysis: Applications to haplotyping, location scores, and marker-sharing statistics. Am J Hum Genet 58:1323–1337.

Wagner T, Stoppa-Lyonnet D, Fleischmann E, Muhr D, Pages S, Sandberg T, Caux V, Moeslinger R, Langbauer G, Borg A, Oefner P. 1999. Denaturing high-performance liquid chromatography detects reliably BRCA1 and BRCA2 mutations. Genomics 62:369–376.

Wakamatsu N, Kobayashi H, Miyatake T, Tsuji S. 1992. A novel exon mutation in the human beta-hexosaminidase beta subunit gene affects 3′ splice site selection. J Biol Chem 267:2406–2413.

Wee EL, Zimmerman EF. 1983. Involvement of GABA in palate morphogenesis and its relation to diazepam teratogenesis in two mouse strains. Teratology 28:15–22.

Wyszynski DF, Beaty TH, Maestri NE. 1996. Genetics of nonsyndromic oral clefts revisited. Cleft Palate Craniofac J 33:406–417.

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ORIGINAL INVESTIGATION

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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

Association of haplotypes of the *C3* and *C5* genes with BA

We estimated the frequencies of the *C3* and *C5* gene haplotypes using an expectation-maximization algorithm (Excoffier and Slatkin 1995). Table 2 shows that the distribution of the *C3* haplotype frequency differed between the control and childhood BA (P=0.0078) and between the control and adult BA (P=0.00016). The most frequent haplotype in controls was 912G, 1692A, 1836G, and 4896C (*GAGC*), whereas the 912G, 1692A, 1836G, and 4896T (*GAGT*) haplotype was the most frequent in asthmatics. The frequency of the *GAGC* haplotype in the adult patients was significantly lower (χ^2=18.62, P=0.00011, odds ratio=0.58, CI=0.45–0.75) than that in the controls. The *GAGT* haplotype was significantly associated with both types of asthmatics (χ^2=15.47, P=0.0021, odds ratio=1.55, CI=1.22–1.97 for childhood BA; χ^2=11.18, P=0.0058, odds ratio=1.48, CI=1.18–1.86 for adult BA). There is a concern about the accuracy of the haplotype frequency results because we used inferred haplotypes when they could not be determined unambiguously. Thus we analyzed only samples whose certainty of haplotype was more than 0.99. The number of such alleles in controls was 510; the number in cases of childhood BA was 430; and in cases of adult BA was 538. All the differences mentioned above with all samples remained highly significant with the phase-certain samples (not shown).

Powerful evidence indicated that the *C5* haplotype is also associated with childhood and adult BA (Table 2). In comparison with the overall distribution, the P values of association for childhood BA was 2.0×10^{-6} and that for adult BA was 0.0021. The major difference resided in the frequency of the 433G, 1155G, 1632C, 2404G, and 4266A (*GGCGA*) haplotype. The frequency of this haplotype in the control population was 5.7%, whereas it was 0.55% in childhood BA (χ^2=27.16, $P=1.3\times10^{-6}$, odds ratio=0.10, CI=0.035–0.29) and 1.5% in adult BA (χ^2=15.38, P=0.00062, odds ratio=0.25, CI=0.12–0.53). The results suggested that the *GGCGA* haplotype of *C5* is an allele that protects against the disease. Again, the differences in the overall distribution and *GGCGA* haplotype of *C5* remained highly significant with the phase-certain samples (not shown).

Discussion

Because the genes in the complement system are promising candidates for the pathogenesis of BA, we systematically screened all exons of the *C3*, *C5*, *C3AR1*, and *C5R1* genes for common polymorphisms and performed case-control and case-only association studies of clinically characterized patients.

Among the SNPs in *C3*, only 4896C/T was weakly associated with BA. This association became evident when patient data were stratified by total IgE, atopy, or severity (Table 1). Given that high total IgE was related to higher severity, the primary target of the effect of *C3* variations is thus difficult to define. The *C3* gene may affect the total IgE level through Th2 effector functions or it may directly affect the late phase of airway inflammation. Evidence that *C3* is associated with BA is further supported by the results of the haplotype analysis. The frequency of the *GAGT* haplotype was significantly higher in childhood and in adult BA patients than in controls. The distribution of the haplotype frequency in the BA patients also differed from that of controls. The different haplotypes of the *C3* gene do not alter the amino acid sequence of C3 protein. It is therefore unlikely that this combination of nucleotide polymorphisms per se affects the function of *C3*. The nucleotide changes that are in linkage disequilibrium with this haplotype must exist in a part of the *C3* gene that we have not yet investigated. We are currently screening polymorphisms in the promoter region and introns of the *C3* gene to identify variations that might affect the gene expression.

The 1526G/A of the *C3AR1* was associated with the severe childhood BA in the case-control study. The results of the case-only association study supported the result of the case-control study. The group of patients with a milder condition can be regarded as controls in the case-only analysis. The *C3AR1* gene may be related with severity of childhood BA or with the severest childhood BA.

The case-control study showed that *C3* 4896C/T was associated with atopic childhood BA. The case-only study showed that *C3AR1* 1595A/G was associated with atopic dermatitis in patients with childhood BA. Because atopy and atopic dermatitis are strongly correlated, the C3 pathway might also be involved in the development of atopy or atopic dermatitis, at least in patients with childhood BA.

Two SNPs of the *C5* gene, 1632C/T and 2404A/G, were associated with onset of adult BA. We examined only whether childhood BA (onset <18 years old) was recorded among the patients with adult BA. Detailed data on the onset of adult BA should be collected to confirm the association of the age of onset and *C5* SNPs.

Among haplotypes in the *C5* gene, *GGCGA* was associated with both childhood BA and adult BA. The frequencies of this haplotype in the patient groups were significantly lower than that in the control group, suggesting that it protects against the development of BA. The *433G* and *2404G* haplotypes of the *C5* gene resulted in valine at 145 and 802 of the amino acid sequence of C5. This Val145-Val802 type of *C5* was not thought to directly reduce the risk of disease because the *GACGG* and *GGCGG* haplotypes that also produce the same C5 protein did not reduce the risk. As in *C3*, this haplotype of the *C5* gene is probably in linkage disequilibrium with unidentified functional nucleotide changes. Nevertheless, the *GGCGA* haplotype is a good marker of the allele that protects against BA. Compared with what is known about the C3 pathway, less is known about the mechanism of C5 in the development of BA. The production of IL-12 from macrophages is reduced in mice that are deficient in *C5* (Karp et al. 2000). Given that

IL-12 is a cytokine that drives the $CD4^+$ Th1 immune response, a deficiency of IL-12 would result in Th2 dominant responses. Exogenous IL-12 prevents airway hyperresponsiveness in ovalbumin-susceptible strains (Gavett et al. 1995). Thus, whether cells of the immune systems of individuals with the *GGCGA* haplotype produce more C5 and IL-12 should be investigated.

In conclusion, we have discovered evidence for the association of particular haplotypes of the *C3* and *C5* genes with the development of BA in the Japanese population. Our data also suggested that the *C3AR1* gene also affects disease severity. This is the first report to describe associations between SNPs of genes in the C3 and C5 pathways and BA in humans. Because the identified SNPs and haplotypes are probably markers for true susceptibility changes in the genes, we intend to extend our survey of polymorphisms in the promoter regions as well as introns of the genes. A significant association between promoters and intronic variants of genes and diseases has been reported (Niimi et al. 2002; Prokunina et al. 2002). Further investigations of other populations, and of alterations in gene function due to polymorphisms, are also required to establish the effect of the complement system on the pathogenesis of BA.

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References

Barnes KC, Marsh DG (1998) The genetics and complexity of allergy and asthma. Immunol Today 19:325–332

Barrington R, Zhang M, Fischer M, Carroll MC (2001) The role of complement in inflammation and adaptive immunity. Immunol Rev 180:5–15

Bautsch W, Hoymann HG, Zhang Q, Meier-Wiedenbach I, Raschke U, Ames RS, Sohns B, Flemme N, Meyer zu Vilsendorf A, Grove M, Klos A, Kohl J (2000) Cutting edge: guinea pigs with a natural C3a-receptor defect exhibit decreased bronchoconstriction in allergic airway disease: evidence for an involvement of the C3a anaphylatoxin in the pathogenesis of asthma. J Immunol 165:5401–5405

Brown CC (1981) The validity of approximation methods for interval estimation of the odds ratio. Am J Epidemiol 113:474–480

Burgi B, Brunner T, Dahinden CA (1994) The degradation product of the C5a anaphylatoxin C5adesarg retains basophil-activating properties. Eur J Immunol 24:1583–1589

CSGA (1997) A genome-wide search for asthma susceptibility loci in ethnically diverse populations. The collaborative study on the genetics of asthma (CSGA). Nat Genet 15:389–392

Daser A, Daheshia M, De Sanctis GT (2001) Genetics of allergen-induced asthma. J Allergy Clin Immunol 108:167–174

Drouin SM, Corry DB, Hollman TJ, Kildsgaard J, Wetsel RA (2002) Absence of the complement anaphylatoxin C3a receptor suppresses Th2 effector functions in a murine model of pulmonary allergy. J Immunol 169:5926–5933

Drouin SM, Corry DB, Kildsgaard J, Wetsel RA (2001a) Cutting edge: the absence of C3 demonstrates a role for complement in Th2 effector functions in a murine model of pulmonary allergy. J Immunol 167:4141–4145

Drouin SM, Kildsgaard J, Haviland J, Zabner J, Jia HP, McCray PB Jr, Tack BF, Wetsel RA (2001b) Expression of the complement anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. J Immunol 166:2025–2032

el-Lati SG, Dahinden CA, Church MK (1994) Complement peptides C3a- and C5a-induced mediator release from dissociated human skin mast cells. J Invest Dermatol 102:803–806

Excoffier L, Slatkin M (1995) Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. Mol Biol Evol 12:921–927

Fujii K, Matsubara Y, Akanuma J, Takahashi K, Kure S, Suzuki Y, Imaizumi M, Iinuma K, Sakatsume O, Rinaldo P, Narisawa K (2000) Mutation detection by TaqMan-allele specific amplification: application to molecular diagnosis of glycogen storage disease type Ia and medium- chain acyl-CoA dehydrogenase deficiency. Hum Mutat 15:189–196

Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M (1995) Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. J Exp Med 182:1527–1536

Gerard NP, Gerard C (2002) Complement in allergy and asthma. Curr Opin Immunol 14:705–708

Henson P (2000) Complementing asthma. Nat Immunol 1:190–192

Humbles AA, Lu B, Nilsson CA, Lilly C, Israel E, Fujiwara Y, Gerard NP, Gerard C (2000) A role for the C3a anaphylatoxin receptor in the effector phase of asthma. Nature 406:998–1001

Karp CL, Grupe A, Schadt E, Ewart SL, Keane-Moore M, Cuomo PJ, Kohl J, Wahl L, Kuperman D, Germer S, Aud D, Peltz G, Wills-Karp M (2000) Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. Nat Immunol 1:221–226

Khoury MJ (1998) Genetic epidemiology. In: Rothman KJ, Greenland S (eds) Modern epidemiology. Lippincott-Raven, Philadelphia, pp 609–622

Kohl J (2001) Anaphylatoxins and infectious and non-infectious inflammatory diseases. Mol Immunol 38:175–187

Krug N, Tschernig T, Erpenbeck VJ, Hohlfeld JM, Kohl J (2001) Complement factors C3a and C5a are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. Am J Respir Crit Care Med 164:1841–1843

Muller-Eberhard HJ (1988) Molecular organization and function of the complement system. Annu Rev Biochem 57:321–347

National Heart, Lung, and Blood Institute (1991) Guidelines for the diagnosis and management of asthma. National Heart, Lung, and Blood Institute. National Asthma Education Program. Expert panel report. J Allergy Clin Immunol 88:425–534

National Heart, Lung, and Blood Institute (1997) Guidelines for the diagnosis and management of ashtma. Second expert panel on the management of asthma. Publication 97-4051A

Niimi T, Munakata M, Keck-Waggoner CL, Popescu NC, Levitt RC, Hisada M, Kimura S (2002) A polymorphism in the human UGRP1 gene promoter that regulates transcription is associated with an increased risk of asthma. Am J Hum Genet 70:718–725

Niu T, Qin ZS, Xu X, Liu JS (2002) Bayesian haplotype inference for multiple linked single-nucleotide polymorphisms. Am J Hum Genet 70:157–169

Ober C, Cox NJ, Abney M, Di Rienzo A, Lander ES, Changyaleket B, Gidley H, Kurtz B, Lee J, Nance M, Pettersson A, Prescott J, Richardson A, Schlenker E, Summerhill E, Willadsen S, Parry R (1998) Genome-wide search for asthma susceptibility loci in a founder population. The collaborative study on the genetics of asthma. Hum Mol Genet 7:1393–1398

Drug Metab. Pharmacokin. 19 (4): 303–307 (2004).

Short Communication

Genotyping of Single Nucleotide Polymorphisms (SNPs) Influencing Drug Response by Competitive Allele-specific Short Oligonucleotide Hybridization (CASSOH) with Immunochromatographic Strip

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Full text of this paper is available at http://www.jssx.org

Summary: Using competitive allele-specific oligonucleotide hybridization with immunochromatographic strip (CASSOH), we have developed a simplified method for the detection of eight polymorphisms that are especially important in the identification of drug responders or non-responders and patients at increased risk of drug toxicity. The genotyping method is unambiguously determined by the presence or the absence of visible purple lines on the immunochromatographic strip, and results are obtained within 5 min after PCR. This method is rapid, highly sensitive, simplified, and should be suitable for point-of-care genotyping in clinical settings.

Key words: genotyping; single nucleotide; polymorphism; pharmacogenetics

Introduction

Clinical application of pharmacogenetic information is important in customizing the species, optimal dosage, and schedule of drug for individual patients. Pharmacogenetics involves determining the genetic polymorphisms influencing drug exposure levels. Specifically, increased toxicity or altered efficacy can result from variations in a gene coding for an important drug metabolizing enzyme or phase I or II enzyme. Known alterations in genes influencing the drug response include single nucleotide polymorphisms (SNPs) at loci for *CYP2C19*,[1,2] N-acetyltransferase 2 (*NAT2*),[3-5] thiopurine S-methyltransferase (*TPMT*),[6-9] uridine diphosphate glucuronosyltransferase 1A1 (*UGT1A1*),[10,11] and mitochondrial DNA (mtDNA).[12]

Prior to drug treatment, it is very desirable to use bedside genotyping methods to identify drug responders or non-responders as well as patients at increased risk of toxicity. Numerous SNP detection methods have been developed, including PCR-RFLP, allele-specific PCR,[13] PCR-SSCP,[14] oligonucleotide ligation assay,[15] TaqMan PCR,[16-18] invader assay,[19,20] pyrosequencing,[21] microarray,[22] and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry.[23] However these require expensive instrumentation and substantial technical expertise.

Matsubara and Kure[24] have recently developed the competitive allele-specific short oligonucleotide hybridization (CASSOH) with immunochromatographic strip for the detection of some prevalent disease-causing mutations and clinically important polymorphisms. This is a rapid and simplified method for SNP detection that demands neither technical expertise nor expensive instruments. In this report, we use this assay and either purified DNA or unprocessed whole blood as templates to successfully detect SNPs *CYP2C19* (*CYP2C19*3* [636G>A]), *NAT2* (*NAT2*5* [341T>C], *NAT2*6* [590G>A], and *NAT2*7* [857G>A]), *TPMT* (*TPMT*3C* [719A>G]), *UGT1A1* (*UGT1A1*6* [211G>A] and *UGT1A1*27* [686C>A]), and mitochondrial DNA (1555A>G).

Materials and Methods

Isolation of DNA from human blood: The local ethics committee of Tohoku Pharmaceutical University and Tohoku University Hospital approved the study, and all blood donors provided written, informed

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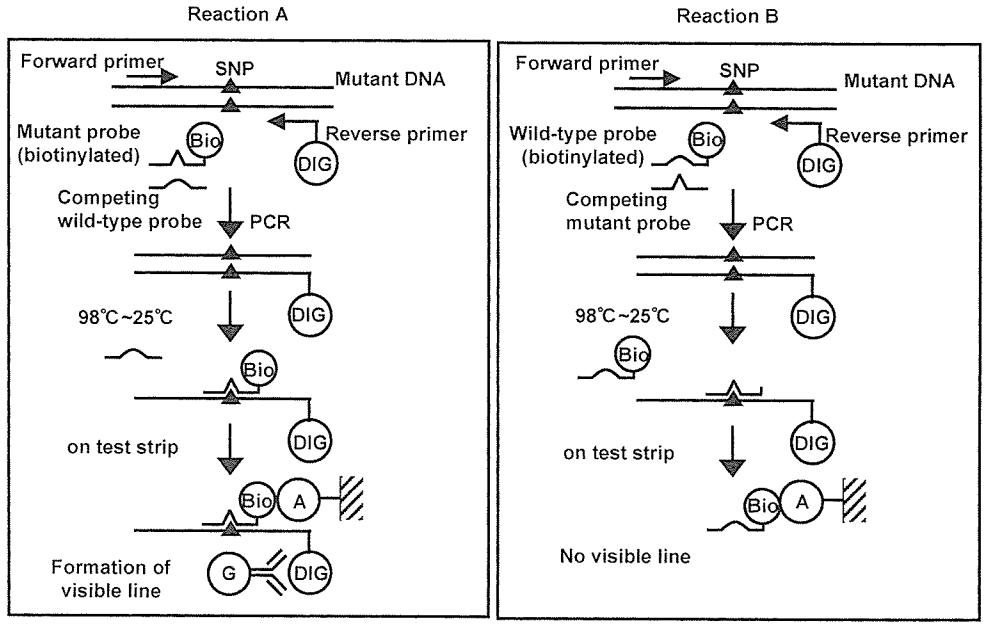

Fig. 1. CASSOH assay with immunochromatographic strip.
Analysis of mutant DNA using reaction A (left) for the detection of a mutant sequence or reaction B (right) for the detection of a wild-type sequence. Closed triangles indicate a SNP site. DIG, digoxigenin labeling; Bio, biotin labeling; A, streptavidin; G, gold particle conjugated to anti-digoxigenin antibody.

consent. DNA was isolated from K_2EDTA-treated anti-coagulated peripheral blood using a DNA Extractor WB-Rapid kit (Wako Pure Chemical Industries, Osaka, Japan) or a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

CASSOH assay: The CASSOH assay for the detection of polymorphisms of *CYP2C19* (*CYP2C19*3* [636G>A]), *NAT2* (*NAT2*5* [341T>C], *NAT2*6* [590G>A], and *NAT2*7* [857G>A]), *TPMT* (*TPMT*3C* [719A>G]), *UGT1A1* (*UGT1A1*6* [211G>A] and *UGT1A1*27* [686C>A]), and mitochondrial DNA (1555A>G) was carried out according to the method described by Matsubara and Kure[24] with minor modifications. The principle of the method is illustrated in **Fig. 1**. A target sequence containing a SNP site is amplified by PCR with a pair of PCR primers, one of which is labeled with digoxigenin (DIG) at its 5′-end. The PCR reaction mixture also contains two sets of hybridization probes. One set is used for the detection of the wild-type nucleotide sequence and consists of an oligonucleotide containing the wild-type sequence labeled with biotin (Bio) at its 3′-end and an unlabeled oligonucleotide containing the mutant sequence (reaction A). The second set consists of an oligonucleotide containing the mutant sequence labeled with Bio at its 3′-end and an unlabeled oligonucleotide containing the wild-type sequence (reaction B). All hybridization probes are designed on the strand opposite to a DIG-labeled PCR primer. A summary of the primers and probes used is presented in **Table 1**.

The standard PCR was carried out in a reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 250 μM each dNTP, 1 μM PCR forward primer, 1 μM PCR reverse primer, 600 nM biotinylated probe, 3 mM unlabeled competing probe, 1.25 U of Ex Taq DNA polymerase (TaKaRa, Otsu, Japan), and 0.04 to 100 ng of genomic DNA in a total volume of 20 μL. The PCR reactions were performed in a BIO-RAD iCycler (Hercules, CA, USA) with the following cycling conditions: denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, and, finally, successive incubations of 72°C for 3 min, 98°C for 3 min, 65°C for 1 min, 55°C for 1 min, 45°C for 1 min, 35°C for 1 min, and 25°C for 1 min.

The direct PCR using whole blood as template was carried out in a PCR reaction mixture containing 4 μL of 5X Ampdirect-A (Shimadzu, Kyoto Japan), 4 μL of 5X Amp Addition-1 (Shimadzu, Kyoto Japan), 250 μM each dNTP, 1 μM PCR forward primer, 1 μM PCR reverse primer, 600 nM biotinylated probe, 3 mM unlabeled competing probe, 1.25 U of Ex Taq DNA polymerase (TaKaRa, Otsu, Japan), and 0.2 to 1 μL of blood in a total volume of 20 μL. Whole blood was obtained by scratching of the fingertip with a needle. The PCR reactions were performed in the BIO-RAD iCycler with the following cycling conditions: denaturation at 94°C for 4.5 min, followed by 45 cycles of

Table 1. Sequences of PCR primers and hybridization probes

Gene (allele)	Forward primer	Reverse primer
PCR primers		
*CYP2C19 (*3)*	5′-tgatctgctccattattttccaga-3′	5′-DIG-cttggtcaatatagaattttggatttcc-3′
*NAT2 (*5)*	5′-ttggaaacattaactgacattcttgag-3′	5′-DIG-catctgggaggagcttccag-3′
*NAT2 (*6)*	5′-ttggaaacattaactgacattcttgag-3′	5′-DIG-tgtggttataaatgaagatgttggagac-3′
*NAT2 (*7)*	5′-agggtatttttacatccctccagtt-3′	5′-DIG-ggtagagaggatatctgatagcacataagt-3′
*TPMT (*3C)*	5′-cagtggtgtatttatttttactcttggga-3′	5′-DIG-attttcaattcctcaaaaacatgtcag-3′
*UGT1A1 (*6)*	5′-gaaagggtccgtcagcatga-3′	5′-DIG-agcagaggggacatgaaatag-3′
*UGT1A1 (*27)*	5′-acctgacgcctcgttgtacatc-3′	5′-DIG-tcacaaagtcacttctaaacagccag-3′
mtDNA	5′-cccaaactgggattagataccc-3′	5′-DIG-ttagctcagagcggtcaagttaag-3′

Gene (allele)	Substitution	Detection of normal sequence		Detection of mutant sequence	
		Biotinylated wild probe	Competing mutant probe	Biotinylated mutant probe	Competing wild probe
Hybridization probes					
*CYP2C19 (*3)*	636G>A	5′-ccctgGatcca-Bio-3′	5′-ccctgAatccag-3′	5′-ccctgAatccag-Bio-3′	5′-ccctgGatcca-3′
*NAT2 (*5)*	341T>C	5′-tgaccaTtgacg-Bio-3′	5′-tgaccaCtgacg-3′	5′-tgaccaCtgacg-Bio-3′	5′-tgaccaTtgacg-3′
*NAT2 (*6)*	590G>A	5′-aacctcGaacaa-Bio-3′	5′-gaacctcAaacaa-3′	5′-gaacctcAaacaa-Bio-3′	5′-aacctcGaacaa-3′
*NAT2 (*7)*	857G>A	5′-tgatgGatccct-Bio-3′	5′-gtgatgAatccct-3′	5′-gtgatgAatccct-Bio-3′	5′-tgatgGatccct-3′
*TPMT (*3C)*	719A>G	5′-aaagttatAtctacttac-Bio-3′	5′-aaagttatGtctactta-P-3′#	5′-aaagttatGtctactta-Bio-3′	5′-aaagttatAtctacttac-P-3′#
*UGT1A1 (*6)*	211G>A	5′-atgctcCgtctct-Bio-3′	5′-atgctcTgtctctg-3′	5′-atgctcTgtctctg-Bio-3′	5′-atgctcCgtctct-3′
*UGT1A1 (*27)*	686C>A	5′-ttcccCgtatgc-Bio-3′	5′-ttcccAgtatgc-3′	5′-ttcccAgtatgc-Bio-3′	5′-ttcccCgtatgc-3′
mtDNA	1555A>G	5′-aggagAcaagtcg-Bio-3′	5′-aggagGcaagtc-3′	5′-aggagGcaagtc-Bio-3′	5′-aggagAcaagtcg-3′

Underlined nucleotides indicate targeted substitutions.
DIG, digoxigenin; Bio, biotin; P, phosphorylation.
Competing probes for *TPMT (*3C)* detection were labeled with phosphoric acid on their 3′-end to prevent reaction as a primer during PCR amplification.

denaturation at 98°C for 20 sec, annealing at 55°C for 30 sec, and extension at 72°C for 60 sec, and, finally, successive incubations of 72°C for 3 min, 98°C for 3 min, 65°C for 1 min, 55°C for 1 min, 45°C for 1 min, 35°C for 1 min, and 25°C for 1 min.

A 5 μL portion of the PCR reaction mixture is pipetted onto the sample application pad of a DNA detection test strip (Roche Diagnostics, Mannheim, Germany). The DNA test strip uses a nitrocellulose membrane with a line of immobilized streptavidin together with a conjugate pad impregnated with gold particle-conjugated anti-DIG mouse monoclonal antibody. The bottom end of the strip is immersed in chromatographic buffer at room temperature for approximately 5 sec to initiate chromatography. As the buffer and gold-labeled anti-DIG antibody contained in the test strip pad migrate through the sample, the conjugated antibody binds to the DNA-oligonucleotide hybrid. This complex is further trapped by immobilized streptavidin on the test strip, forming a visible purple line after 5 min. The genotype of the specimen is determined by the presence or absence of purple lines in reactions A and B.

Results and Discussion

Figure 2 shows representative results for mtDNA (1555A>G) **(Fig. 2a)** and *NAT2*6* (590G>A) **(Fig. 2b)** when purified DNA was used as a template for the CASSOH assay. False positive or false negative signals were not observed. There was a 100% match in the genotyping results of the 1555A-homoplasmy and 1555G-homoplasmy of mtDNA and the 590G-homozygotes, heterozygotes, and 590A-homozygotes of *NAT2*. **Figure 2c** shows that, using the primers and probes listed in **Table 1**, similar results were obtained for the six other polymorphisms: *CYP2C19* (*CYP2C19*3*, [636G>A]), *NAT2* (*NAT2*5* [341T>C] and *NAT2*7* [857G>A]), *TPMT* (*TPMT*3C* [719A>G]), and *UGT1A1* (*UGT1A1*6* [211G>A] and *UGT1A1*27* [686C>A]). Although signal intensities between wild-type probes and mutant probes in each heterozygous samples of *CYP2C19*3*, *NAT2*5*, *NAT2*7*, *TPMT*3C* and *UGT1A1*27* were slightly different, no false-negative signals were observed under the conditions. To estimate the precision and reproducibility of the assay, selected samples (n=3 each) with a known genotype were analyzed in duplicate. All samples were tested for genotypes by sequencing and by CASSOH assay, and identical results were obtained by the two methods (data not shown). The detection limit of this assay was approximately 0.04 ng of template DNA. Test results on the dried DNA detection test strips were stable for at least 3 years at room temperature (data not shown).

SNP genotyping can generally be divided into two steps: purification of DNA from blood and allele

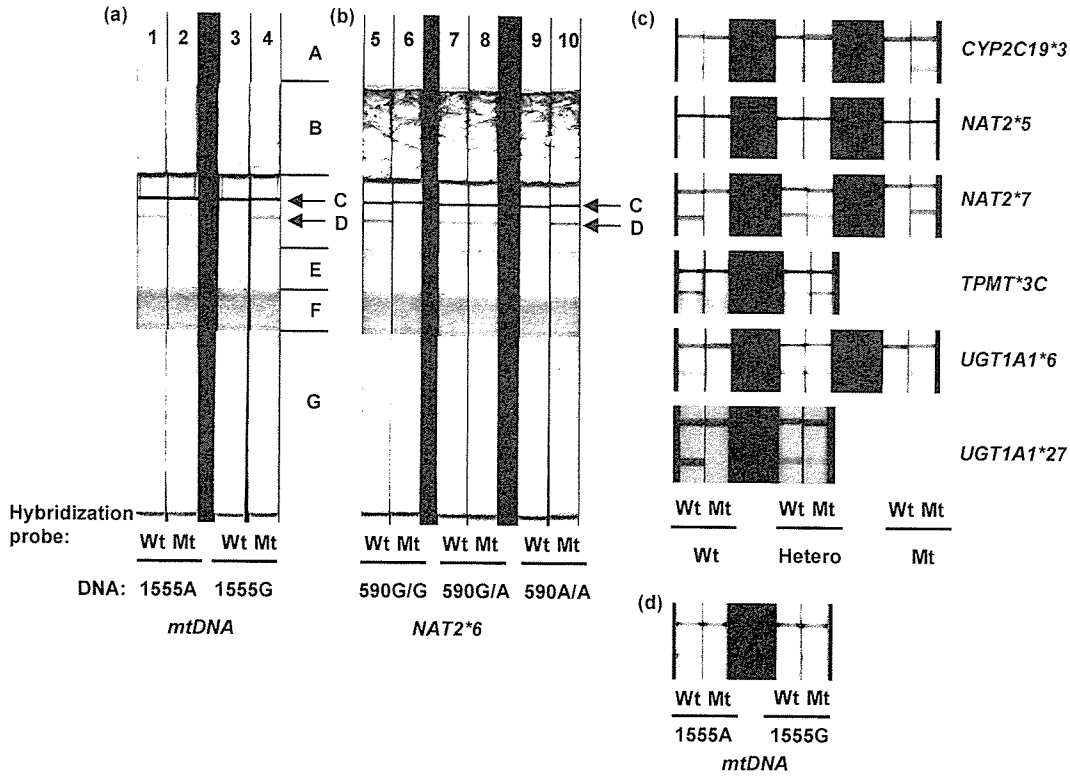

Fig. 2. Genotyping by CASSOH assay with immunochromatographic strip.
(a) Detection of the mtDNA 1555A > G polymorphisms. DNA samples included wild-type (lanes 1 and 2) and mutant homoplasmy (lanes 3 and 4). (b) Detection of the *NAT2* 590G > A polymorphisms. DNA samples included wild-type homozygous (lanes 5 and 6), heterozygous (lanes 7 and 8), and mutant homozygous (lanes 9 and 10). Immunochromatographic strips were as follows: A, handle; B, absorbent pad; C, control line (anti-mouse polyclonal antibody line; not visible before use); D, streptavidin line (diagnostic line, which binds the hybridized PCR product including the anti-DIG gold conjugate; not visible before use); E, sample application pad; F, gold conjugate pad; and G, immersion area. (c) Detection of the *CYP2C19*3* (636G > A), *NAT2*5* (341T > C), *NAT2*7* (857G > A), *TPMT*3C* (719A > G), *UGT1A1*6* (211G > A), and *UGT1A1*27* (686C > A) polymorphisms. (d) Detection of the mtDNA 1555A > G polymorphisms in whole blood using Ampdirect.

detection. The DNA purification step is required because DNA polymerases are susceptible to inhibition by endogenous substances, including haemoglobin and bile acids. Purification of DNA is often labor-intensive, time-consuming, and costly, and it enhances the risk of back- or cross-contamination of samples. In the present study, we tested whether Ampdirect-A and Amp Addition-1, a reagent cocktail that suppresses the inhibitors in blood, is useful on CASSOH assay. **Figure 2d** shows representative results for mtDNA (1555A > G) when whole blood was used as a template for the CASSOH assay. We found that all SNPs tested in this study could be detected by the CASSOH assay in the presence of this cocktail without prior extraction of the DNA (data not shown). In contrast, an amplified band was not produced by PCR when a blood sample was used in the absence of this reagent cocktail. Typically, a 1 μL sample of whole blood yielded 15 to 60 ng of DNA, and the detection limit for this assay in the presence of Ampdirect was approximately 0.2 μL whole blood (3 to 12 ng of DNA). The entire assay can be completed in less than 3 h, and costs approximately \$7 per sample. Thus, the CASSOH assay for SNP determination avoids the need for sample purification, leading to a considerable savings in time, cost, and effort.

In summary, we have developed a CASSOH-based genotyping method using whole blood as a template for eight polymorphisms that are especially important in the Japanese population for identification of drug responders or non-responders and patients at increased risk of drug toxicity. This method is rapid, highly sensitive, simple, and should be suitable for routine clinical genotyping.

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References

1) de Morais, S. M., Wilkinson, G. R., Blaisdell, J., Nakamura, K., Meyer, U. A. and Goldstein, J. A.: The major genetic defect responsible for the polymorphism of S-mephenytoin metabolism in humans. *J. Biol. Chem.*, **269**: 15419–15422 (1994).
2) de Morais, S. M., Wilkinson, G. R., Blaisdell, J., Meyer, U. A., Nakamura, K. and Goldstein, J. A.: Identification of a new genetic defect responsible for the polymorphism of (S)-mephenytoin metabolism in Japanese. *Mol. Pharmacol.*, **46**: 594–598 (1994).
3) Evans, D. A.: N-acetyltransferase. *Pharmacol. Ther.*, **42**: 157–234 (1989).
4) Okumura, K., Kita, T., Chikazawa, S., Komada, F., Iwakawa, S. and Tanigawara, Y.: Genotyping of N-acetylation polymorphism and correlation with procainamide metabolism. *Clin. Pharm. Ther.*, **61**: 509–517 (1997).
5) Hiratsuka, M., Kishikawa, Y., Takekuma, Y., Matsuura, M., Narahara, K., Inoue, T., Hamdy, S. I., Endo, N., Goto, J. and Mizugaki, M.: Genotyping of the N-acetyltransferase2 polymorphism in the prediction of adverse drug reactions to isoniazid in Japanese patients. *Drug Metab. Pharmacokin.*, **17**: 357–362 (2002).
6) McLeod, H. L., Lin, J. S., Scott, E. P., Pui, C. H. and Evans, W. E.: Thiopurine methyltransferase activity in American white subjects and black subjects. *Clin. Pharmacol. Ther.*, **55**: 15–20 (1994).
7) McLeod, H. L., Pritchard, S. C., Githang'a, J., Indalo, A., Ameyaw, M. M., Powrie, R. H., Booth, L. and Collie-Duguid, E. S. R.: Ethnic differences in thiopurine methyltransferase pharmacogenetics: evidence for allele specificity in Caucasian and Kenyan individuals. *Pharmacogenetics*, **9**: 773–776 (1999).
8) Hiratsuka, M., Inoue, T., Omori, F., Agatsuma, Y. and Mizugaki, M.: Genetic analysis of thiopurine methyltransferase polymorphism in a Japanese population. *Mutat. Res.*, **448**: 91–95 (2000).
9) Hiratsuka, M., Inoue, T., Omori, F., Agatsuma, Y., Kishikawa, Y. and Mizugaki, M.: Detection assay of rare variants of the thiopurine methyltransferase gene by PCR-RFLP using a mismatch primer in a Japanese population. *Biol. Pharm. Bull.*, **23**: 1090–1093 (2000).
10) Aono, S., Yamada, Y., Keino, H., Hanada, N., Nakagawa, T., Sasaoka, Y., Yazawa, T., Sato, H. and Koiwai, O.: Identification of defect in the genes for bilirubin UDP-glucuronosyl-transferase in a patient with Crigler-Najjar syndrome type II. *Biochem. Biophys. Res. Commun.*, **197**: 1239–1244 (1993).
11) Aono, S., Adachi, Y., Uyama, E., Yamada, Y., Keino, H., Nanno, T., Koiwai, O., Sato, H., Hanada, N., Nakagawa, T., Sasaoka, Y. and Yazawa, T.: Analysis of genes for bilirubin UDP-glucuronosyltransferase in Gilbert's syndrome. *Lancet*, **345**: 958–959 (1995).
12) Prezant, T. R., Agapian, J. V., Bohlman, M. C., Bu, X., Oztas, S., Qiu, W. Q., Arnos, K. S., Cortopassi, G. A., Jaber, L., Rotter, J. I., Shohat, M. and Fischel-Ghodsian, N.: Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. *Nat. Genet.*, **4**: 289–294 (1993).
13) Newton, C. R., Graham, A., Heptinstall, L. E., Powell, S. J., Summers, C., Kalsheker, N., Smith, J. C. and Markham, A. F.: Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res.*, **17**: 2503–2516 (1989).
14) Orita, M., Suzuki, Y., Sekiya, T. and Hayashi, K.: Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics*, **5**: 874–879 (1989).
15) Landegren, U., Kaiser, R., Sanders, J. and Hood, L.: A ligase-mediated gene detection technique. *Science*, **241**: 1077–1080 (1988).
16) Livak, K. J., Marmaro, J. and Todd, J. A.: Towards fully automated genome-wide polymorphism screening. *Nat. Genet.*, **9**: 341–342 (1995).
17) Fujii, K., Matsubara, Y., Akanuma, J., Takahashi, K., Kure, S., Suzuki, Y., Imaizumi, M., Iinuma, K., Sakatsume, O., Rinaldo, P. and Narisawa, K.: Mutation detection by TaqMan-allele specific amplification: application to molecular diagnosis of glycogen storage disease type Ia and medium-chain acyl-CoA dehydrogenase deficiency. *Hum. Mutat.*, **15**: 189–196 (2000).
18) Hiratsuka, M., Agatsuma, Y., Omori, F., Narahara, K., Inoue, T., Kishikawa, Y. and Mizugaki, M.: High throughput detection of drug-metabolizing enzyme polymorphisms by allele-specific fluorogenic 5′ nuclease chain reaction assay. *Biol. Pharm. Bull.*, **23**: 1131–1135 (2000).
19) Kwiatkowski, R. W., Lyamichev, V., de Arruda, M. and Neri, B.: Clinical, genetic, and pharmacogenetic applications of the Invader assay. *Mol. Diagn.*, **4**: 353–364 (1999).
20) Lyamichev, V., Mast, A. L., Hall, J. G., Prudent, J. R., Kaiser, M. W., Takova, T., Kwiatkowski, R. W., Sander, T. J., de Arruda, M., Arco, D. A., Neri, B. P. and Brow, M.: A. Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. *Nat. Biotechnol.*, **17**: 292–296 (1999).
21) Fakhrai-Rad, H., Pourmand, N. and Ronaghi, M.: Pyrosequencing: an accurate detection platform for single nucleotide polymorphisms. *Hum. Mutat.*, **19**: 479–485 (2002).
22) Pastinen, T., Raitio, M., Lindroos, K., Tainola, P., Peltonen, L. and Syvanen, A. C.: A system for specific, high-throughput genotyping by allele-specific primer extension on microarrays. *Genome Res.*, **10**: 1031–1042 (2000).
23) Pusch, W., Wurmbach, J. H., Thiele, H. and Kostrzewa, M.: MALDI-TOF mass spectrometry-based SNP genotyping. *Pharmacogenomics*, **3**: 537–548 (2002).
24) Matsubara, Y. and Kure, S.: Detection of single nucleotide substitution by competitive allele-specific short oligonucleotide hybridization (CASSOH) with immunochromatographic strip. *Hum. Mutat.*, **22**: 166–172 (2003).

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FULL PAPER

Association of the hCLCA1 gene with childhood and adult asthma

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Asthma is caused by bronchial inflammation. This inflammation involves mucus overproduction and hypersecretion. Recently, a mouse model of asthma showed that gob-5 is involved in the pathogenesis of asthma. The gob-5 gene is involved in mucus secretion and its expression is upregulated upon antigen attack in sensitized mice. The observation suggests that human homologue of gob-5, hCLCA1 (human calcium-dependent chloride channel-1), may be involved in human disease. We screened for single-nucleotide polymorphisms (SNPs) in hCLCA1 in the Japanese population. We identified eight SNPs, and performed association studies using 384 child patients with asthma, 480 adult patients with asthma, and 672 controls. In haplotype analysis, we found a different haplotype distribution pattern between controls and childhood asthma ($P < 0.0001$) and between controls and adult asthma ($P = 0.0031$). We identified a high-risk haplotype (CATCAAGT haplotype; $P = 0.0014$) and a low-risk haplotype (TGCCAAGT haplotype; $P = 0.00010$) in cases of childhood asthma. In diplotype analysis, patients who had the CATCAAGT haplotype showed a higher risk for childhood asthma than those who did not ($P = 0.0011$). Individuals who had the TGCCAAGT haplotype showed a lower risk for childhood asthma than those who did not ($P < 0.0001$). Our data suggested that variation of the hCLCA1 gene affects patients' susceptibility for asthma.
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Introduction

Asthma is a chronic illness characterized by reversible airway obstruction and airway hyper-responsiveness (AHR) that are caused by bronchial inflammation.[1,2] This inflammation involves epithelial damage, deposition of collagen beneath the basement membrane, eosinophilic and lymphocytic infiltration, and hypertrophy and hyperplasia of goblet cells, submucosal glands, and airway smooth muscle.[3,4] Ample evidence suggests that a combination of genetic and environmental factors causes asthma.[5–7] Candidate gene and genome-wide linkage studies have already identified several specific gene polymorphisms, regions of linkage to asthma, and asthma-related phenotypes.[8,9]

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Recently, a mouse model of asthma provided the evidence that the *gob-5* gene is involved in the pathogenesis of asthma.[10,11] Nakanishi *et al* reported that intratracheal administration of adenovirus-expressing antisense *gob-5* RNA into AHR-model mice efficiently suppressed the asthma phenotype, including AHR and mucus overproduction, and that an adenovirus-mediated overexpression of *gob-5* in airway epithelia exacerbated the asthma phenotype. Zhou *et al*[11] showed that *gob-5* is highly expressed in the lung epithelium of IL-9 transgenic mice, which exhibit many signs and symptoms characteristic of human asthma. Asthmatic patients showed upregulation of *hCLCA1* (human calcium-dependent chloride channel-1) mRNA in mucus-producing epithelium responsive to the interleukin IL-9.[12] Because IL-9 is one of the Th2 cytokines, these observations suggest the involvement of *hCLCA1* in the pathophysiology of Th2 cytokine-mediated asthma. In addition, *gob-5* was suggested to be involved in mucus production induced by respiratory syncytial virus infection that has strong impact on asthma pathogenesis.[13,14]