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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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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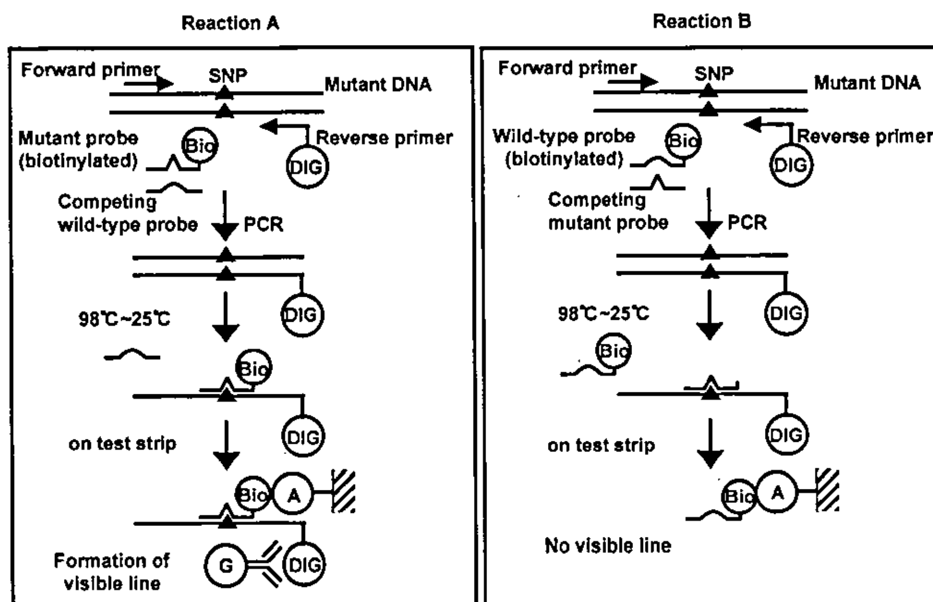

Fig. 1. CASSOH assay with immunochromatographic strip.
Analysis of mutant DNA using reaction A (left) for the detection of a mutant sequence or reaction B (right) for the detection of a wild-type sequence. Closed triangles indicate a SNP site. DIG, digoxigenin labeling; Bio, biotin labeling; A, streptavidin;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

consent. DNA was isolated from K_2EDTA-treated anticoagulated 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

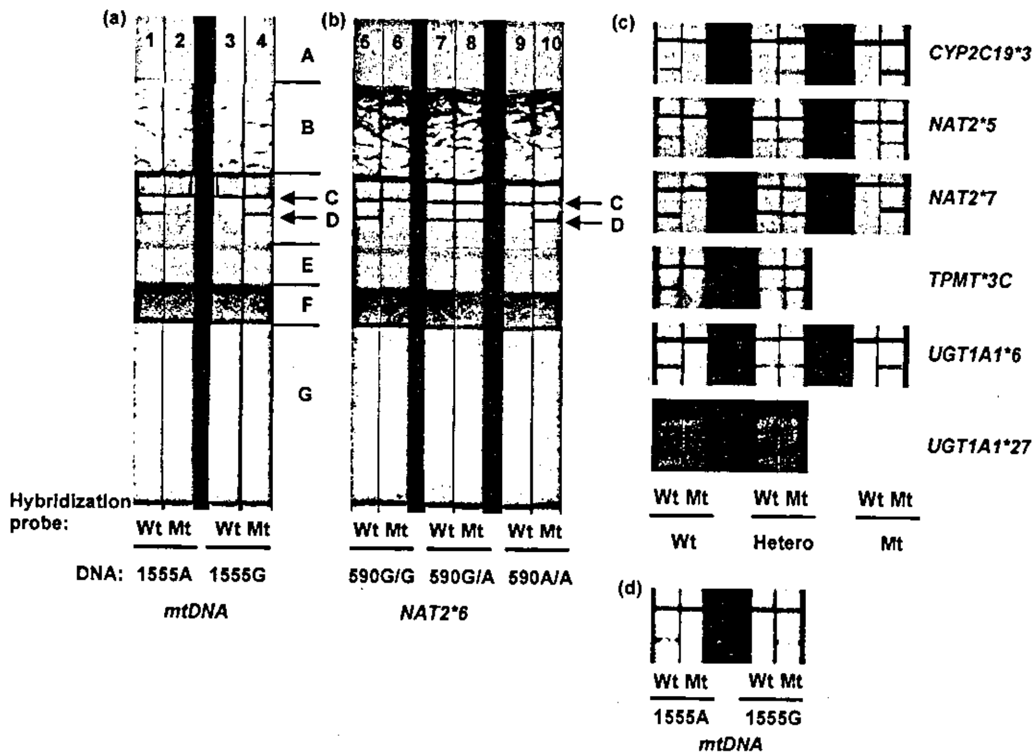

Fig. 2. Genotyping by CASSOH assay with immunochromatographic strip.
(a) Detection of the mtDNA 1555A>G polymorphisms. DNA samples included wild-type (lanes 1 and 2) and mutant homoplasmy (lanes 3 and 4). (b) Detection of the *NAT2* 590G>A polymorphisms. DNA samples included wild-type homozygous (lanes 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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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]

The human homologue of *gob-5* is *hCLCA1*, which is expressed in intestinal epithelia, uterus, testes, and kidneys where *gob-5* is also expressed.[15,16] The human *hCLCA1* gene is one of the members of the Ca^{2+}-activated Cl^- channel family. The *hCLCA1* enhances mucus secretion by mediating the active transport of chloride ions.[10] Currently, the family includes two bovine homologues (*bCLCA1* and *bCLCA2*),[17–19] four murine homologues (*mCLCA1*, *mCLCA2*, *mCLCA3* (*gob-5*), *mCLCA4*),[20–24] and four human homologues (*hCLCA1*, *hCLCA2*, *hCLCA3*, *hCLCA4* (*hCaCC2*)).[15,16,25,26] All human *CLCA* genes identified are clustered on the short arm of chromosome 1 (1p22–31).[16,27] The *hCLCA1* gene is 31 902 bp in length, contains 15 exons, and encodes a protein of 914 amino acids.[16]

Mouse experiments on *gob-5*[10,11] and observations on bronchial epithelia from asthma patients[12] suggest that overexpression of *hCLCA1* in the bronchial epithelia exacerbates AHR and mucus hypersecretion in asthma patients. We therefore hypothesized that polymorphisms that affect expression or function of the *hCLCA1* gene would be associated with asthma phenotypes. In this study, we screened single-nucleotide polymorphisms (SNPs) in the *hCLCA1* gene and performed case-control and case-only association studies using clinically characterized Japanese patients with childhood or adult asthma.

Results

Characteristics of asthma patients

Clinical characteristics of the patients are described in detail elsewhere.[28] Briefly, 80% of the children with asthma were found to be positive for mite-specific IgE (atopic), 54% were found to have high serum IgE concentrations (>400 U/ml), and 46% were found to have atopic dermatitis. Of the adults with asthma, 28% were found to have high serum IgE concentrations and 22% experienced the onset of asthma before the age of 18 years.

Polymorphisms in the *hCLCA1* gene

During the course of the Japanese Single Nucleotide Polymorphisms project in Japan (http://snp.ims.u-tokyo.ac.jp/),[29,30] SNPs were identified in the *hCLCA1* gene. Two SNPs were located in exons. One (JST083354) was located in exon 6 and the other (JST046987 (SNP8 in this study)) in exon 15 (Table 1). Both SNPs were synonymous substitutions (Val215Val and Thr812Thr, respectively). Because the SNP of exon 6 showed complete linkage disequilibrium to SNP2 (JST120332) (Table 1) in 94 normal samples (data not shown), it was excluded from any further investigation. In addition to the exonic SNPs, seven SNPs found in the introns with a minor allele frequency of more than 20% were investigated in this study (Table 1). In the haplotype and diplotype analyses, PHASE and HAPLOTYPER programs showed essentially the same results. Results obtained with PHASE software are shown in Tables 2, 4, and 5.

To examine the linkage disequilibrium between identified SNPs, pairwise linkage disequilibrium coefficients D'[30] and r^2[31] and P-value were calculated using 592 controls (Table 2). Strong but not complete linkage disequilibrium was found between the following pairs: SNP1 and SNP3, SNP2 and SNP3, SNP4 and SNP5, SNP4 and SNP6, and SNP6 and SNP7. Weak linkage disequilibrium was found between the following pairs: SNP1 and SNP7, SNP2 and SNP7, SNP2 and SNP8, SNP4 and SNP8, and SNP5 and SNP7.

Association of each SNP with asthma and asthma related-phenotypes

Eight SNPs were genotyped in 384 patients with childhood asthma, 480 patients with adult asthma, and 672 controls. All genotype results of the SNPs in the control samples were in the Hardy–Weinberg equilibrium. The results of case–control and case-only association studies with significant ($P<0.01$) P-values are shown in Table 3.

In the case–control study, an association of SNP4 with the most severe cases of adult asthma was observed (odds ratio (OR) = 0.26, 95% confidence interval (CI) = 0.12–0.60, $\chi^2 = 11.43$, $P = 0.0032$, corrected OR (cOR) = 0.15). Both SNP2 and SNP8 showed significant association with aspirin-induced asthma (AIA) ($P = 0.0057$ and 0.0050, respectively). SNP8 showed some association with adult asthma cases that had a high (>10%) eosinophil count ($P = 0.0079$).

In the case-only study, associations between asthma-related phenotypes and the SNPs of *hCLCA1* were investigated within the group of asthma patients. There were significant differences in the genotype frequency of SNP2 and SNP8 between AIA and non-AIA asthma ($P = 0.0074$ and 0.0084, respectively). We found an association between SNP6 and the complication of atopic dermatitis in childhood asthma ($P = 0.0046$). There was no significant association

Table 1 SNPs in the *hCLCA1* gene

Name	*JSNP ID*	*SNP*[a]	*Position*[b]	*Amino acid*	*Minor allele frequency (%)*[c]
SNP1	IMS-JST049517	I5:908+246C>T	10047		42
SNP2	IMS-JST120332	I5:908+2199G>A	12000		43
SNP3	IMS-JST120333	I6:1086+589T>C	16220		45
SNP4	IMS-JST120334	I6:1087−754C>A	17837		20
SNP5	IMS-JST120335	I6:1087−705A>G	17886		33
SNP6	IMS-JST120341	I8:1533+997A>C	21007		42
SNP7	IMS-JST120342	I8:1534−808G>C	21445		33
SNP8	IMS-JST046987	E15:2787T>C	33001	T812T	49

[a]Numbering according to the cDNA sequence of *hCLCA1*.
[b]Numbering according to the genomic sequence of *hCLCA1*.
[c]SNPs were genotyped in this study.

Table 2 Pairwise linkage disequilibrium for all possible two-way comparisons among eight SNPs

	SNP1	*SNP2*	*SNP3*	*SNP4*	*SNP5*	*SNP6*	*SNP7*	*SNP8*
SNP1	D'	0.745*	0.749*	0.544*	0.480*	0.377*	0.337*	0.322*
	r^2	0.290*	0.489*	0.053*	0.079*	0.070*	0.040*	0.070*
SNP2		D'	0.987*	0.577*	0.409*	0.406*	0.271*	0.290*
		r^2	0.583*	0.060*	0.058*	0.081*	0.050*	0.064*
SNP3			D'	0.397*	0.386*	0.301*	0.322*	0.342*
			r^2	0.048*	0.059*	0.075*	0.063*	0.091*
SNP4				D'	1.000*	0.964*	0.474*	0.206*
				r^2	0.523*	0.340*	0.112*	0.010*
SNP5					D'	0.463*	0.212*	0.520*
					r^2	0.150*	0.043*	0.122*
SNP6						D'	0.914*	0.472*
						r^2	0.609*	0.144*
SNP7							D'	0.785*
							r^2	0.289*

*$P<0.05$.

Table 3 Association between SNPs of the *hCLCA1* gene and asthma or asthma-related phenotypes

Name	*Number*	*Genotype frequencies*			*OR (95% CI)*	χ^2 *(df=1)*	*P*
SNP2 (908+2199G>A)		AA	AG	GG			
Controls	640	0.166	0.512	0.322	Reference		
Adult asthma							
Aspirin-induced asthma	25	0.400	0.280	0.320	0.30 (0.13–0.68)[a] 0.29 (0.12–0.71)[b]	9.18[a]	0.0057[a]
Adult asthma							
With aspirin-induced asthma	25	0.400	0.280	0.320	Reference		
Without aspirin-induced asthma	441	0.170	0.485	0.345	0.31 (0.13–0.71)[a] 0.32 (0.14–0.73)[b]	8.39[a]	0.0074[a]
SNP4 (1087−754C>A)		CC	CA	AA			
Controls	642	0.629	0.340	0.031	Reference		
Adult asthma							
Severity=4	83	0.651	0.241	0.108	0.26 (0.12–0.60)[c] 0.15 (0.05–0.43)[b]	11.43[c]	0.0032[c]
SNP6 (1533+997A>C)		AA	AC	CC			
Childhood asthma							
With atopic dermatitis	169	0.278	0.556	0.166	Reference		
Without atopic dermatitis	203	0.424	0.419	0.157	1.91 (1.23–2.95)[d] 1.92 (1.24–2.97)[e]	8.50[d]	0.0046[d]
SNP8 (2787T>C)		TT	TC	CC			
Controls	636	0.285	0.465	0.250	Reference		
Adult asthma							
Eosinophil⩾10%	71	0.296	0.591	0.113	2.63 (1.23–5.60)[f] 2.93 (1.34–6.43)[b]	6.68[f]	0.0079[f]
Aspirin-induced asthma	25	0.040	0.720	0.240	9.55 (1.28–71.10)[g] 6.69 (0.88–50.97)[b]	7.21[g]	0.0050[g]
Adult asthma							
With aspirin-induced asthma	25	0.040	0.720	0.240	Reference		
Without aspirin-induced asthma	441	0.277	0.521	0.202	9.18 (1.23–68.59)[g] 8.94 (1.19–66.88)[b]	6.82[g]	0.0084[g]

[a]GG+AG *vs* AA.
[b]OR adjusted for age and sex.
[c]CC+CA *vs* AA.
[d]CC+AC *vs* AA.
[e]OR adjusted for sex.
[f]TT+TC *vs* CC.
[g]CC+TC *vs* TT.

between atopy (positive mite-specific IgE) and any SNPs of *hCLCA1* among childhood asthma patients, suggesting that *hCLCA1* is primarily associated with asthma and/or dermatitis but not atopy.

Association between haplotypes of the *hCLCA1* gene and asthma

We used eight SNPs to construct the haplotype of the *hCLCA1* gene and estimated the frequency of each

haplotype in the control, childhood asthma, and adult asthma groups (Table 4). The frequency pattern of the haplotype differed between the control and childhood asthma groups ($P<0.0001$) and between the control and adult asthma groups ($P=0.0031$). Association with childhood asthma was observed with the TGCCAAGT haplotype (haplotype 6) (OR = 0.27, CI = 0.14–0.50, $\chi^2=19.742$, P_c (corrected P-value) = 0.00010) and the CATCAAGT haplotype (haplotype 18) (OR = 3.44, CI = 1.87–6.29, $\chi^2=17.800$, $P_c=0.0014$). Association between the CGCCACCT haplotype (haplotype 22) and adult asthma showed marginal significance ($P_c=0.014$).

Table 4 Frequencies of haplotypes constructed from eight SNPs and ORs in the control group and in both asthma groups

Name	*SNP1 C/T*	*SNP2 G/A*	*SNP3 T/C*	*SNP4 C/A*	*SNP5 A/G*	*SNP6 A/C*	*SNP7 G/C*	*SNP8 T/C*	*Controls (1184)*[a] *Frequency*	*Childhood asthma (712)*[a] *Frequency*	*Adult asthma (878)*[a] *Frequency*	*OR (95% CI) Controls vs childhood asthma Controls vs adult asthma*	χ^2 *(df = 1)*	*P*
Haplotype 1	C	A	T	C	A	A	G	C	0.151	0.180	0.139	1.23 (0.96–1.58) 0.91 (0.71–1.16)	2.679 0.605	>1* >1*
Haplotype 2	T	G	C	C	A	A	G	C	0.123	0.112	0.091	0.90 (0.67–1.20) 0.71 (0.53–0.95)	0.508 5.355	>1* 0.583*
Haplotype 3	T	G	C	C	A	C	C	T	0.084	0.118	0.109	1.47 (1.08–1.99) 1.35 (1.00–1.81)	6.021 3.896	0.414* >1*
Haplotype 4	C	A	T	C	A	C	C	T	0.065	0.052	0.071	0.79 (0.53–1.18) 1.09 (0.77–1.55)	1.344 0.250	>1* >1*
Haplotype 5	C	A	T	C	G	A	G	T	0.062	0.044	0.056	0.69 (0.45–1.07) 0.90 (0.62–1.31)	2.815 0.310	>1* >1*
Haplotype 6	T	G	C	C	A	A	G	T	0.060	0.017	0.058	0.27 (0.14–0.50) 0.97 (0.67–1.40)	19.742 0.032	0.00010* >1*
Haplotype 7	C	G	T	C	A	A	G	C	0.053	0.051	0.032	0.95 (0.62–1.44) 0.59 (0.37–0.92)	0.063 5.432	>1* 0.583*
Haplotype 8	C	G	C	A	G	C	C	T	0.041	0.024	0.028	0.57 (0.32–0.99) 0.68 (0.42–1.11)	4.057 2.429	>1* >1*
Haplotype 9	C	G	T	A	G	C	G	C	0.032	0.032	0.035	1.01 (0.59–1.70) 1.10 (0.68–1.79)	0.001 0.161	>1* >1*
Haplotype 10	T	G	C	A	G	C	G	C	0.026	0.035	0.039	1.35 (0.79–2.31) 1.50 (0.91–2.46)	1.237 2.598	>1* >1*
Haplotype 11	T	G	C	C	G	A	G	T	0.025	0.044	0.022	1.75 (1.05–2.92) 0.85 (0.48–1.52)	4.731 0.297	0.833* >1*
Haplotype 12	C	A	T	A	G	C	C	T	0.024	0.031	0.025	1.27 (0.72–2.23) 1.02 (0.58–1.80)	0.697 0.007	>1* >1*
Haplotype 13	C	A	T	C	A	C	C	C	0.024	0.022	0.035	0.92 (0.49–1.70) 1.46 (0.87–2.44)	0.078 2.087	>1* >1*
Haplotype 14	T	A	T	C	A	A	G	T	0.019	0.024	0.016	1.23 (0.65–2.33) 0.82 (0.42–1.60)	0.426 0.347	>1* >1*
Haplotype 15	C	G	T	C	G	A	G	T	0.016	0.027	0.028	1.68 (0.88–3.19) 1.80 (0.98–3.29)	2.562 3.728	>1* >1*
Haplotype 16	T	G	C	C	A	A	C	T	0.015	0.014	0.015	0.92 (0.42–2.01) 0.97 (0.47–2.00)	0.041 0.005	>1* >1*
Haplotype 17	C	G	C	A	G	C	G	C	0.014	0.007	0.026	0.49 (0.18–1.32) 1.85 (0.98–3.47)	2.086 3.714	>1* >1*
Haplotype 18	C	A	T	C	A	A	G	T	0.014	0.045	0.017	3.44 (1.87–6.29) 1.27 (0.62–2.58)	17.800 0.434	0.0014* >1*
Haplotype 19	T	A	T	A	G	C	C	T	0.013	0.001	0.009	0.11 (0.01–0.83) 0.72 (0.30–1.70)	6.742 0.578	0.210* >1*
Haplotype 20	T	A	T	C	A	A	G	C	0.013	0.010	0.018	0.77 (0.31–1.91) 1.45 (0.71–2.94)	0.312 1.050	>1* >1*
Haplotype 21	T	G	C	A	G	C	C	T	0.013	0.004	0.022	0.33 (0.10–1.14) 1.72 (0.87–3.41)	3.380 2.502	>1* >1*
Haplotype 22	C	G	C	C	A	C	C	T	0.012	0.013	0	1.07 (0.46–2.49)	0.025 10.453	>1* 0.014*
Haplotype 23	C	A	T	A	G	C	G	C	0.011	0.014	0.009	1.28 (0.56–2.94) 0.83 (0.34–2.01)	0.349 0.175	>1* >1*
Haplotype 24	C	G	C	C	A	A	G	T	0.011	0.008	0.015	0.77 (0.29–2.02) 1.35 (0.62–2.93)	0.292 0.593	>1* >1*
Haplotype 25	C	G	T	A	G	C	C	T	0.010	0.008	0.010	0.83 (0.31–2.22) 1.01 (0.42–2.41)	0.138 0.001	>1* >1*
Others									0.069	0.063	0.075			
Overall									1.000	1.000	1.000		75.088 45.465	<0.0001 0.0031

Bonferroni-type adjustment is corrected with ×26. A total of 25 predominant haplotypes are listed. The 'Others' category includes 36 minor haplotypes (<1% frequency in controls).
*P-value corrected with Bonferroni correction (raw P-values were multiplied by 26).
[a]Analyzed allele number.

Table 5 Frequencies of diplotypes and ORs in the control group and in both asthma groups

Name	*Number*	*Diplotype frequencies*			*OR (95% CI)*	χ^2 *(df=1)*	P_c[a]
Haplotype 6		Homozygote	Heterozygote	Others[b]			
Controls	592	0.002	0.116	0.882			
Childhood asthma	356	0	0.034	0.966	0.26 (0.14–0.49)[c] 0.26 (0.14–0.50)[d]	20.11[c]	<0.0001[c]
Serum IgE≥400 IU/ml	193	0	0.016	0.984	0.12 (0.04–0.38)[c] 0.12 (0.04–0.39)[d]	18.20[c]	<0.0001[c]
Positive mite-specific IgE	289	0	0.024	0.976	0.19 (0.08–0.41)[c] 0.19 (0.09–0.42)[d]	21.52[c]	<0.0001[c]
Onset <3 years old	180	0	0.022	0.978	0.17 (0.06–0.47)[c] 0.17 (0.06–0.47)[d]	14.68[c]	0.00089[c]
Haplotype 18		Homozygote	Heterozygote	Others[e]			
Controls	592	0	0.027	0.973			
Childhood asthma	356	0	0.090	0.910	3.56 (1.92–6.58)[f] 3.61 (1.92–6.72)[d]	18.28[f]	0.0011[f]
Serum IgE≥400 IU/ml	193	0	0.093	0.907	3.70 (1.85–7.41)[f] 3.61 (1.79–7.23)[d]	15.41[f]	0.0081[f]
Severity≥2	162	0	0.099	0.901	3.95 (1.93–8.06)[f] 3.92 (1.90–8.06)[d]	16.11[f]	0.0064[f]
Haplotype 22		Homozygote	Heterozygote	Others[g]			
Controls	592	0	0.024	0.976			
Adult asthma	439	0	0	1.000		10.53[h]	0.013[h]

[a] *P*-value corrected with Bonferroni correction (raw *P*-values were multiplied by 26).
[b] Diplotype consists of haplotype other than haplotype 6.
[c] Haplotype 6/haplotype 6+haplotype 6/others *vs* others/others.
[d] OR adjusted for sex.
[e] Diplotype consists of haplotype other than haplotype 18.
[f] Haplotype 18/haplotype 18+haplotype 18/others *vs* others/others.
[g] Diplotype consists of haplotype other than haplotype 22.
[h] Haplotype 22/haplotype 22+haplotype 22/others *vs* others/others.

As shown in Table 5, we examined the association of the diplotypes of *hCLCA1* with asthma. The results suggested that a heterozygote of haplotype 6 showed a lower risk for childhood asthma (OR=0.26, CI=0.14–0.49, $\chi^2=20.11$, $P_c<0.0001$, cOR=0.26) and a heterozygote of haplotype 18 showed a higher risk for childhood asthma (OR=3.56, CI=1.92–6.58, $\chi^2=18.28$, $P_c=0.0011$, cOR=3.61) when compared to other diplotypes. We next studied the association of diplotypes with patients stratified by the asthma-related phenotypes. Significant associations were found between haplotype 6 and children with high IgE levels ($P_c<0.0001$), positive mite-specific IgE results ($P_c<0.0001$), and early age of asthma onset (<3 years old) ($P_c=0.00089$). The association between haplotype 18 and childhood asthma was also significant when patients were limited to those with higher IgE levels ($P_c=0.0081$) or those with severe symptoms ($P_c=0.0064$). The significance of association between haplotype 22 and adult asthma was marginal ($P_c=0.013$). In childhood asthma patients, there was no significant association between haplotype 6 or haplotype 18 and atopy, suggesting that these haplotypes are primarily associated with asthma development.

Discussion

We performed case–control and case-only association studies of SNPs in the *hCLCA1* gene using clinically characterized asthma patients. When each SNP in *hCLCA1* was studied for its association in all asthma samples, no association with childhood asthma or with adult asthma was suggested. However, when patients were stratified by asthma-related phenotypes, subgroups of patients showed associations with SNPs. The genotype frequencies of SNP2 and SNP8 in patients with AIA were different from those of the controls and those of patients without AIA. These data suggest an association of the *hCLCA1* gene with AIA and different pathophysiologies between patients with AIA and those without AIA. Because the number of patients with AIA is relatively small, an association study of *hCLCA1* with a larger number of patients with AIA may be required to confirm our observation. Adult patients with asthma and high eosinophil counts showed a significant association with SNP8. Because the SNP8 genotype frequency in adults with asthma and a low eosinophil count is similar to that of the controls (data not shown), only adult patients with high eosinophil counts showed different genotype frequencies from controls or other patient groups. The data may reflect different pathophysiologies between patients with eosinophilia and those without eosinophilia. An association was found between SNP6 and the complication of atopic dermatitis among childhood asthma patients. Because atopic dermatitis was closely related with total IgE levels and positive mite-specific IgE status, it is possible that *hCLCA1* is involved in atopy or the development of atopic dermatitis. Since

we could not detect significant association between *hCLCA1* and atopy in the case-only studies, it is likely that *hCLCA1* is primarily associated with dermatitis itself.

The evidence of association of the *hCLCA1* gene with asthma was shown more clearly by the haplotype analyses. The entire distributions of the haplotype frequency in childhood and adult asthma patients were different from that of the controls. The frequency of haplotype 6 was significantly lower in childhood asthma patients than in the controls. From the diplotype construction analysis, individuals who were heterozygous for haplotype 6 showed a lower risk for childhood asthma (OR = 0.26). These data suggest that it is a protective haplotype against asthma development. About 10% of Japanese individuals have this haplotype and they are expected to show a lower susceptibility to childhood asthma than those who do not. On the other hand, the frequency of haplotype 18 was significantly higher in childhood asthma patients than in the controls. From the diplotype analysis, individuals who harbor haplotype 18 showed a higher risk for childhood asthma (OR = 3.56). These data suggest that it is a high-risk haplotype for asthma development. About 3% of Japanese individuals have this haplotype and they are expected to show a higher susceptibility to childhood asthma than those who do not.

Our observations support the results from an earlier AHR-model mice study. The *gob-5* mRNA is strongly expressed in the airway epithelium, especially in the goblet cells, the function of which is to secrete mucins.[10] Mucus overproduction is thought to be responsible for the small-airway obstruction and lung dysfunction that are closely linked to morbidity and mortality in asthma cases.

This study is the first to investigate the association between SNPs of the *hCLCA1* gene and asthma in humans. It showed evidence of associations between particular haplotypes of the *hCLCA1* gene and asthma development in the Japanese population. None of the SNPs investigated in this study change the amino-acid sequence of the *hCLCA1* protein. Therefore, it is unlikely that the nucleotide changes *per se* affect the function of *hCLCA1*. Any nucleotide changes that are in linkage disequilibrium to this haplotype may exist in or near the *hCLCA1* gene. It will be necessary to extend the survey of polymorphisms in the promoter regions as well as introns and exons of the *hCLCA1* gene to identify any functional genetic changes. Several studies have reported that there was a significant association between promoter and intronic variants of a gene and a disease.[32,33] Demonstrating the alteration of gene functions as the result of polymorphisms is necessary to further validate the involvement of the *hCLCA1* gene in the pathogenesis of asthma.

While this manuscript was being prepared, the association of *hCLCA1* and chronic obstructive lung disease (COLD) in the Japanese population was published.[34] Among the eight SNPs in our study, only one SNP (SNP8) was also investigated in Hegab's study; therefore, the comparison of results between our studies is difficult. Association of *hCLCA1* with both asthma and COLD may imply a common pathogenic background between these two diseases.

Materials and methods

Subjects

We recruited 384 children with asthma and 96 adults with asthma from Osaka Prefectural Habikino Hospital and another 384 adults with asthma from the Miyatake Asthma Clinic. Details of these patients are described elsewhere.[28] All participants with asthma were selected according to the American Thoracic Society criteria.[35] Regarding the children with asthma, we recorded their age, sex, age at asthma onset, serum total IgE level, mite-specific IgE level, eosinophil count, clinical severity, incidence of atopic dermatitis, and the number of parents and siblings affected with asthma. Specific IgE was considered positive when values exceeded $0.35\,U_A/ml$ (RAST score ≥ 1) according to an enzyme immunoassay. The severity of childhood asthma was defined according to the degree of therapy required to control symptoms at the time of entry into this study. The grades were defined as follows: Grade 1, β stimulants only; Grade 2, sodium chromoglycate and/or theophylline; Grade 3, inhaled beclometasone, 400 μg/day or less; and Grade 4, inhaled beclometasone of more than 400 μg/day. Regarding the adults with asthma, we recorded their age, sex, age at asthma onset, serum total IgE level, eosinophil count, clinical severity, incidence of AIA, presence or absence of nasal polyps, and the number of relatives second-degree or closer with asthma. The severity of adult asthma was classified according to the system from the National Heart, Lung, and Blood Institute.[36] In this study, patients who were under 18 years of age at the time of entry were classified as having childhood asthma and those who were 18 years of age or older were classified as having adult asthma regardless of their age at the time of onset. We selected 672 control subjects who did not have atopy-related diseases from the general population in the Tokyo and Osaka areas. Genomic DNAs were prepared in accordance with standard protocols. 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) and the Tohoku University School of Medicine.

SNP genotyping

Genotyping of SNPs was performed by using the Invader assay[37,38] or the TaqMan™ allele-specific amplification (TaqMan-ASA) method.[39] Specific primers were designed on the basis of genomic sequences obtained from the GenBank DNA database (accession number AF039401). The primer sequences and genotyping methods are shown in Table 6. The mixture for the Invader assay contained 5 ng of genomic DNA, 1.25 mM dNTPs, 5.9 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 10 mM β-mercaptoethanol, a pair of specific primers (12.5 pmol each), and *Ex-Taq* DNA polymerase (0.5 U; TaKaRa, Japan) in a final volume of 10 μl. Samples were amplified in the GeneAmp™ PCR system 9700 (Applied Biosystems, USA). Thermoprofiles were initial denaturation at 95°C for 2 min, followed by 37 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 3 min, with a final extension at 72°C for 10 min. The Invader assay was performed as previously described.[40]

The polymerase chain reaction (PCR) mixture for the TaqMan-ASA method contained 7.5 μl of 2 × TaqMan™

Table 6 Primers for genotyping

Primers for Invader assay			
Name	*Forward primer (5′–3′)*	*Reverse primer (5′–3′)*	
SNP1	GGGACCTTGTACACCATGTTGAG	CCCTGAAGGCACCGAAGAG	
SNP2	ATCAAGTGCACTGCTGCCTC	TGGCAGTGTCTCTAGTGTTTCCC	
SNP3	TGCTCCAAGGGAGAATCAAAG	CTGGAAGACAAGAGAGACTTCCC	
SNP4	TGAAGCTATAAAGGGTGGAATGG	CCCATCTGCTCCTCCTCCTC	
SNP5	TGAAGCTATAAAGGGTGGAATGG	CCCATCTGCTCCTCCTCCTC	
SNP6	AACCCTTGCTGTGCAAGACC	CCATAACTGCTGTGTGTCATGG	
SNP7	TTCTCTCACCTGCCAACACC	CAGGATACTCCTTCTGCCAATG	
Primers for TaqMan-ASA method			
Name	*Common forward primer (5′–3′)*	*Reverse primer (5′–3′)*	*TaqMan probe (5′–3′)*
SNP8	CAACAGCCTGAATAGCAATG	CAATGAATCTCTTCAAGTGAATACTAGT CAATGAATCTCTTCAAGTGAATACTAAC	ACTTCCTCAGAGTTGGCTTCCTTTGG

Universal PCR Master Mix (Applied Biosystems), 0.4 μM of each PCR primer, 0.12 μM of TaqMan probe, and 5 ng of template DNA in a final volume of 15 μl. The samples were analyzed with the GeneAmp™ PCR System 7700 (Applied Biosystems). The thermoprofiles were 50°C for 2 min, 95°C for 10 min, then 45 cycles of 95°C for 15 s, and finally 60°C for 1 min.

Statistical analysis

In all genotypic, haplotypic, and diplotypic distribution analyses, *P*-values were calculated using Fisher's exact test. Allele frequencies in asthma patients and controls were compared by the contingency χ^2 test. ORs were estimated according to Brown.[41] Corrections of ORs for age and sex were performed with logistic regression formula in the adult case analysis. Because the range of age of the controls (18–81 years) was not comparable with child cases (1–17 years), CI of OR becomes very broad in the childhood asthma analysis. Thus, ORs corrected for only sex were presented in childhood asthma analysis. As shown in Tables 3 and 5, the correction had little effects on the estimated OR values. Pairwise linkage disequilibrium between SNPs was estimated as D'[30] and r^2.[31] To infer the frequencies of haplotype in control and patient groups and diplotype in individuals with uncertain phases of genotypes, software programs PHASE™ version 2.0.2 (http://www.stat.washington.edu/stephens/software.html)[42] and HAPLO-TYPER™ (http://www.people.fas.harvard.edu/~junliu/Haplo/docMain.htm)[43] were used. Haplotype frequencies between cases and controls were evaluated both by a whole distribution with Fisher's exact test and by χ^2 tests of one haplotype against others (haplotype-wise test). In the association study between a single SNP and asthma or an asthma-related phenotype, we performed many statistical tests; therefore, inflation of the false-positive results (type 1 error) is a concern. Because all eight SNPs were significantly in linkage disequilibrium (Table 2) and asthma-related phenotypes (seven variables for children and seven variables for adults) are significantly related,[28] the simple 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 rather set the significant *P*-value at 0.01 rather than 0.05. In the haplotype-wise test, comparisons were repeated as many as 26 times (25 haplotypes and 'another minor haplotype'; Tables 4 and 5). In this case, we assumed each comparison was independent and performed the Bonferroni correction (raw *P*-values were multiplied by 26). The corrected *P*-values were designated as P_c. The P_c-values less than 0.01 were judged to be significant. The software SPSS™ version 11.0J (SPSS Japan Inc., Tokyo, Japan) was used for all statistical analyses.

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Mutation analysis of the *MMAA* and *MMAB* genes in Japanese patients with vitamin B_{12}-responsive methylmalonic acidemia: identification of a prevalent *MMAA* mutation

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Abstract

Methylmalonic acidemia (MMA) is caused by the deficient activity of L-methylmalonyl-CoA mutase, which is a vitamin B_{12} (or cobalamin, Cbl)-dependent enzyme. MMA due to the effect of insufficient Cbl metabolism is classified into three forms (*cblA*, *cblB*, and *cblH*). Recently, the genes responsible for *cblA* and *cblB* were identified as *MMAA* and *MMAB*, respectively. The *MMAA* protein likely transports Cbl into the mitochondria for adenosylcobalamin synthesis, while the MMAB protein appears to be an adenosyltransferase. We performed a mutation analysis of 10 unrelated Japanese patients with vitamin B_{12}-responsive MMA. Seven patients had mutations in *MMAA*, whereas the other three patients showed no disease-causing substitutions in either *MMAA* or *MMAB*. Five novel mutations were identified in *MMAA* (R22X, R145X, L217X, R359G, and 503delC). The 503delC mutation was observed in five of the seven *MMAA* patients, suggesting that the mutation is prevalent in Japanese patients. This finding may facilitate the DNA diagnosis of vitamin B_{12}-responsive MMA within the Japanese population.
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Keywords: Vitamin B_{12}-responsive methylmalonic acidemia; L-methylmalonyl-CoA mutase; Adenosylcobalamin; *MMAA*; *MMAB*

Introduction

Methylmalonic acidemia (MMA) is an autosomal recessive disorder of organic acid metabolism caused by a defect in the isomerization of L-methylmalonyl-CoA to succinyl-CoA [1]. The reaction is catalyzed by L-methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) which requires adenosylcobalamin (AdoCbl) as a cofactor. MMA is subdivided into two forms. One is a defect in the MCM apoenzyne (*mut* MMA or vitamin B_{12}-unresponsive MMA, MIM 251000), and the other is a defect in the steps of AdoCbl synthesis (*cbl* MMA or vitamin B_{12}-responsive MMA) [2]. The patients with *cbl* MMA usually respond to pharmacological doses of hydroxycobalamin.

The intracellular synthesis of AdoCbl has been extensively investigated [2]. The cobalt atom of cob(III)alamin is reduced in steps; the first step is the reduction of cob(III)alamin to form cob(II)alamin, and the second step is the reduction of cob(II)alamin to cob(I)alamin. Within these reactions, a transport from the cytosol to the mitochondrial matrix occurs. The final step of AdoCbl synthesis is adenosylation by

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cob(I)alamin adenosyltransferase. Intact fibroblasts from patients with *cbl* MMA accumulated negligible amounts of AdoCbl [3].

Mahoney et al. [4] found that patients with *cbl* MMA were further subdivided into two classes by an AdoCbl synthesis assay using cell extracts. One type, *cblA* (MIM 251100), showed normal AdoCbl synthesis by cell extracts, in spite of a decreased accumulation of AdoCbl in intact fibroblasts. While the other type, *cblB* (MIM 251110), showed severe deficiency of AdoCbl synthesis in cell extracts. These two classes may also be distinguished by somatic cell complementation analyses [5]. It has been speculated that *cblB* is caused by a deficiency of adenosyltransferase [6], and that *cblA* mutants affect the activity of cob(III)alamin reductase or the mitochondrial transport of Cbl [4]. Watanabe et al. [7] reported that mitochondrial NADPH-linked aquacobalamin reductase was decreased in a cell line of *cblA*.

Laboratory diagnoses of *cbl* MMA have been hampered by complicated requirements for the biochemical assays, which are able to be offered by few laboratories worldwide. Recently, the genes responsible for *cblA* and *cblB* were identified as *MMAA* and *MMAB*, respectively, and the disease-causing mutations were reported [8,9]. The MMAA protein is speculated to be involved in the translocation of Cbl into mitochondria and the MMAB protein shows a similarity to bacterial adenosyltransferase. The identification of the two genes opened the way for the DNA diagnosis of *cbl* MMA. In this study, we performed a mutation analysis of ten Japanese patients with vitamin B_{12}-responsive MMA to examine the mutation spectrum within the population and to explore the possibility of a molecular diagnosis.

Methods

Patients

Ten apparently unrelated Japanese MMA patients were studied. There were no consanguineous marriages among the parents of these patients except for patient 2. All patients were symptomatic during their neonatal or infantile period. Diagnoses of MMA were confirmed by urinary organic acid analysis using gas chromatography/mass spectrometry. MCM activity was measured by isomerization of L-methylmalonyl-CoA to succinyl-CoA [10] and/or the incorporation of ^{14}C-propionate [11]. Since MCM activities in the cell cultures from the 10 patients recovered to normal ranges in the presence of AdoCbl or hydroxycobalamin, we assigned a diagnosis of vitamin B_{12}-responsive MMA to these patients. A complementation study was not performed.

The Ethics Committee of the Tohoku University School of Medicine approved this study.

Direct sequencing of the MMAA and MMAB genes

Genomic DNA was extracted from cultured fibroblasts, EBV-transformed lymphoblasts, or leukocytes with the aid of a Sepa Gene Kit (Sanko Junyaku, Tokyo, Japan). All coding exons including flanking introns in *MMAA* and *MMAB* were amplified by PCR (Table 1). To facilitate the cycle sequencing analysis, the M13 forward primer sequence or the M13 reverse primer sequence was attached to the 5′ end of the sense primers or the antisense primers, respectively. Direct sequencing of the PCR products was performed using a Big Dye Primer Cycle Sequencing kit and an ABI 310 Genetic Analyzer (PE Applied Biosystems, Foster city, CA, USA).

Table 1
Primers for the amplification of the *MMAA* and *MMAB* genes

Sense primers	Antisense primers
MMAA	
F-MMAA-Ex2: 5′-F-AATCACATTGAGCCAAAACG-3′	R-MMAA-Ex2: 5′-R-ACAGAATACAGAGAATTTGT-3′
F-MMAA-Ex3: 5′-F-CTCAGTAAAACTGATCGTAG-3′	R-MMAA-Ex3: 5′-R-TAGAGGTCACCCAACTGTGC-3′
F-MMAA-Ex4: 5′-F-GGAACTGGCTGATAATTGAC-3′	R-MMAA-Ex4: 5′-R-GTCACTCATCTTTATATAGC-3′
F-MMAA-Ex5: 5′-F-GTGACCATGAGTATGAGTAA-3′	R-MMAA-Ex5: 5′-R-GCCAACATGAATGATATTTC-3′
F-MMAA-Ex6: 5′-F-GATTCTTGGCATCCAGGGCT-3′	R-MMAA-Ex6: 5′-R-CTATCATCTTCACATAGAAG-3′
F-MMAA-Ex7: 5′-F-TAACTGGCAGGTATCAGCGT-3′	R-MMAA-Ex7: 5′-R-AGAAGACAAGAGCACCATAC-3′
MMAB	
F-MMAB-Ex1: 5′-F-GCCAGCTGTGGGTGGAGTCA-3′	R-MMAB-Ex1: 5′-R-CGACGACACCACGATTCACG-3′
F-MMAB-Ex2: 5′-F-AGGTTACAAGCAGCAAGCTG-3′	R-MMAB-Ex2: 5′-R-AAATGGTGTATGCCATGAGT-3′
F-MMAB-Ex3: 5′-F-CAGCATATCAGGAAAACAGA-3′	R-MMAB-Ex3: 5′-R-CATACTCGACTCAAACGCAA-3′
F-MMAB-Ex4: 5′-F-GCCTGCCACCTGAGAATCTA-3′	R-MMAB-Ex4: 5′-R-TGGATGCTGAGTCCCGTGAT-3′
F-MMAB-Ex5: 5′-F-TATTAGGTGGCGCCTCTGCA-3′	R-MMAB-Ex5: 5′-R-AGATGGTGACCCTAGGAGAG-3′
F-MMAB-Ex6: 5′-F-GTGATGGCCTCATGGCAGTT-3′	R-MMAB-Ex6: 5′-R-CATGTGTGTCTGTCACTGAA-3′
F-MMAB-Ex7: 5′-F-GGCTGGACTTCAGAGGAGCT-3′	R-MMAB-Ex7: 5′-R-TCAGAGATGGCCCTGCTGTA-3′
F-MMAB-Ex8: 5′-F-TGCTGCTCAAGGTTTAGGCC-3′	R-MMAB-Ex8: 5′-R-AATGCTGCCCCACACTGCTT-3′
F-MMAB-Ex9: 5′-F-GAAGACCCAGTTAGCGTTGA-3′	R-MMAB-Ex9: 5′-R-CTTTGAGCCTCTCTGGGTGA-3′

F, M13 Forward sequence (5′-TGTAAAACGACGGCCAGT-3′).
R, M13 Reverse sequence (5′-CAGGAAACAGCTATGACC-3′).

PCR with restriction fragment analyses for the identified mutations

PCR amplification of exons 2, 3, 4, and 7 in *MMAA* was performed as described above. PCR products were digested with *Taq*I (for R145X), *Dde*I (for 503delC), *Mse*I (for L217X), or *Hyp*99I (for R359G) (all from New England Biolabs, Beverly, MA, USA). Fragments were separated on agarose gels and visualized by ethidium bromide staining. The R22X mutation was detected by a PCR

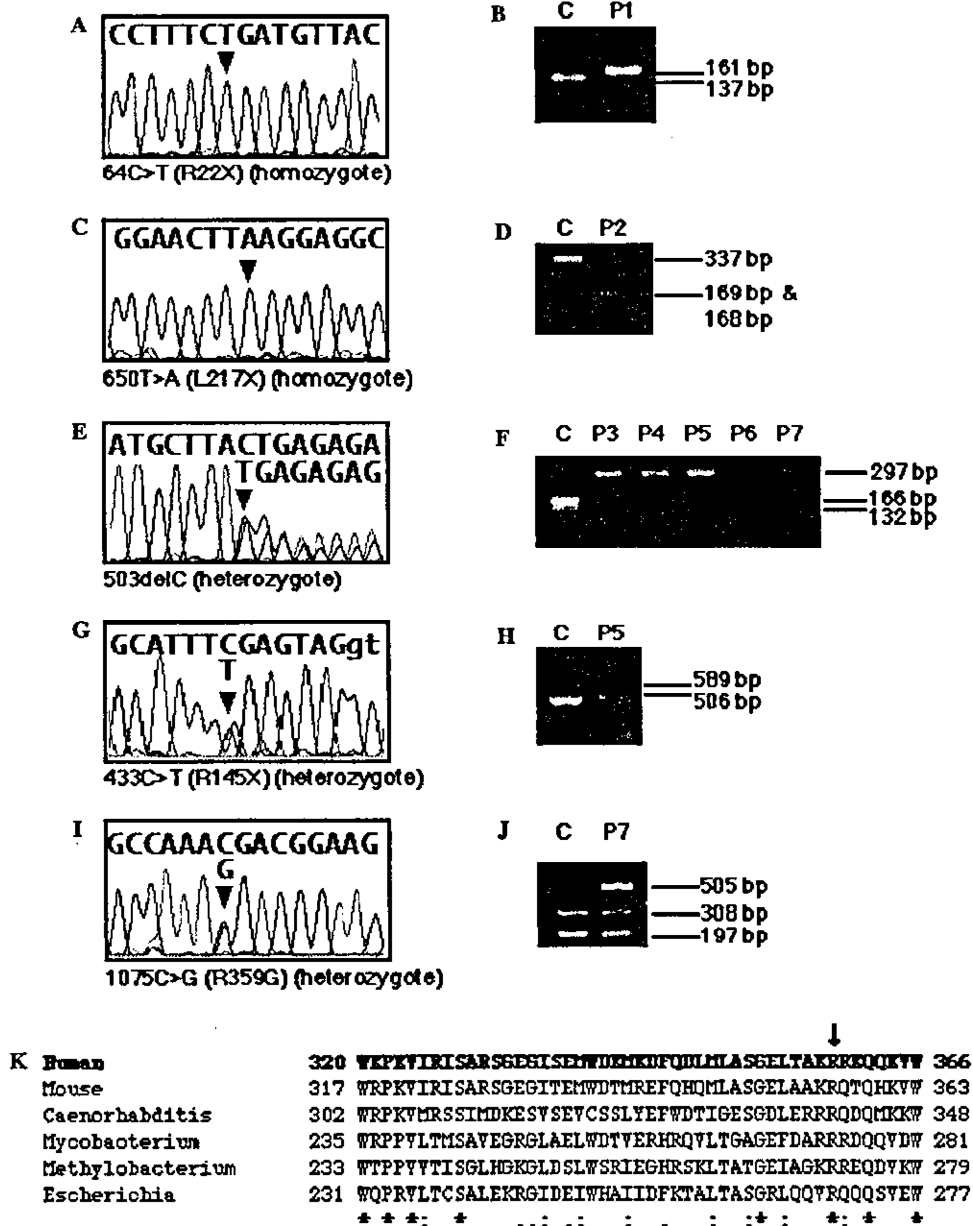

Fig. 1. Mutation analysis by sequencing (A, C, E, G, and I) and PCR-restriction fragment analysis (B, D, F, H, and J). (A) Sequencing analysis in patient 1 (P1). A C-to-T substitution at nucleotide (nt) 64 (R22X) was found in a homozygous pattern. (B) The AS-MMAA-R22X primer, which includes one mismatch, creates an *Hpa*II site in the wild-type sequence. The left lane (C, control) showed a digested fragment. R22X abolishes this site. Patient 1 (P1) was homozygous for R22X. (C) In patient 2 (P2), a T-to-A substitution at nt 650 (L217X) was found in a homozygous pattern. (D) L217X creates an *Mse*I site. Patient 2 was homozygous for L217X. (E) Sequence of 503delC in a heterozygous pattern (patient 5, P5). (F) 503delC abolishes the *Dde*I site. Patients 3, 4, and 6 were homozygous, and patients 5 and 7 were heterozygous for 503delC. (G) Sequencing analysis in patient 5 (P5). A C-to-T substitution at nt 433 (R145X) was found in a heterozygous pattern. (H) R145X abolishes the *Taq*I site. Patient 5 was a heterozygote of R145X. (I) In patient 7 (P7), a C-to-G substitution at nt 1075 (R359G) was found in a heterozygous pattern. (J) R359G abolishes the *Hpy*99I site. Patient 7 was a heterozygote of R359G. (K) Comparisons between human MMAA protein and MMAA homologues of other species were analyzed by the Clustal W program. Arginine at 359 (arrow) is conserved in the MMAA homologues of other species (mouse, *C. elegans*, *M. tuberculosis*, *M. extorquens*, and *E. coli*).

method incorporating a restriction fragment length polymorphism using a mismatched primer. The PCR was conducted by a sense primer (F-MMAA-Ex2) and an antisense primer (AS-MMAA-R22X: 5′-GTGAAAGATGAAGT GGTAACAC-3′) that contained one mismatch (bold type) to create an *Hpa*II site with a wild sequence. After digestion of the PCR products with *Hpa*II (New England Biolabs), electrophoresis was performed using agarose gel.

Results

Seven of the 10 patients with vitamin B_{12}-responsive MMA (patients 1–7) showed mutations in *MMAA*. No disease-causing substitutions were found in either *MMAA* or *MMAB* among the three remaining patients (patients 8, 9, and 10).

In patient 1, we found a C-to-T substitution at nucleotide (nt) 64 in a homozygous pattern (Figs. 1A and B). This substitution created a stop codon (R22X) within exon 2. Patient 2 showed a 650T > A substitution (L217X) in exon 4 in a homozygous pattern (Figs. 1C and D). One base (C) deletion at nt 503 in exon 3 was detected in five patients; patients 3, 4, and 6 were homozygotes and patients 5 and 7 were heterozygotes (Figs. 1E and F). Another mutation found in patient 5 was a C-to-T substitution at nt 433 in exon 2, which created a stop codon (R145X) (Figs. 1G and H). In patient 7, we found a C-to-G substitution at nt 1075 in a heterozygous pattern (Figs. 1I and J). The 1075C > G resulted in the replacement of arginine with glycine (R359G) in exon 7. The missense R359G mutation was not identified in DNA samples from 50 healthy volunteers (data not shown).

Patients 1, 2, 3, 4, and 6 showed a homozygous pattern for the mutations. A consanguineous marriage was found among the parents of patient 2 only. These patients were considered "presumptive" homozygotes because the possibility of being hemizygotes with a deleted second allele could not be ruled out.

We also found 1089G > C in *MMAA* (patient 9), and 56–57GC > AA (patients 2, 5, and 9) and 716T > A (patients 2, 5, 6, 7, 8, and 9) in *MMAB* (data not shown). In the database of Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/) [12], the 1089G > C (Q363H) in *MMAA* has been reported as a polymorphism (IMS-JST065247) (G; 0.9167, C; 0.0833). The 56–57GC > AA (R19Q) and 716T > A (M239K) substitutions have been reported in the dbSNP (http://www.ncbi.nlm.nih.gov/SNP). According to the dbSNP, the heterozygosity of 716T > A (rs9593) is 0.492 and that of 56–57GC > AA (rs10774774 and 10774775) is not reported.

Discussion

Three nonsense mutations (R22X, R145X, and L217X), one deletion (503delC), and one missense mutation (R359G) were identified in Japanese patients with *cbl* MMA. Dobson et al. [8] found three premature-termination mutations (260insATAAACTT, 592delACTG, and Q95X), and one missense mutation (Y207C). There was no overlap of the mutation spectrum between Caucasian and Japanese patients.

Among seven patients diagnosed as *cblA* in this study, three were homozygotes and two were heterozygotes of 503delC; thus the allelic frequency was 57%. It suggests that the 503delC mutation is the predominant *MMAA* gene mutation among Japanese vitamin B_{12}-responsive MMA patients (see Table 2). A similarity in the mutation spectrum among Japanese patients and other Asian patients has been reported in other single-gene disorders, such as propionic acidemia [13–15], phenylketonuria [16] and glycogen storage disease type Ia [17,18]. It would be interesting to study whether the 503delC mutation is also prevalent among vitamin B_{12}-responsive MMA patients in other Far East Asian countries.

The missense mutation R359G observed in patient 7 was not found in 100 alleles in healthy volunteers. Arginine at 359 is conserved in MMAA homologues of other species (mouse, *Caenorhabditis elegans*, *Mycobacterium tuberculosis*, *Methylobacterium extorquens*, and *Escherichia coli*) (Fig. 1K). These data suggest that R359G is likely to be a disease-causing mutation.

Table 2
Mutations in the *MMAA* gene identified in this study

Patient	Mutation 1		Mutation 2	
	Nucleotide change	Effect on coding sequence	Nucleotide change	Effect on coding sequence
1	64C > T	R22X	64C > T	R22X
2	650T > A	L217X	650T > A	L217X
3	503delC	Frame shift	503delC	Frame shift
4	503delC	Frame shift	503delC	Frame shift
5	433C > T	R145X	503delC	Frame shift
6	503delC	Frame shift	503delC	Frame shift
7	503delC	Frame shift	1075C > G	R359G
8	Not detected		Not detected	
9	Not detected		Not detected	
10	Not detected		Not detected	

In the other three patients (patients 8, 9, and 10), no mutations except polymorphisms were detected in either *MMAA* or *MMAB*. Because we had not done complementation studies, it was not possible to know whether these three patients were *cblA*, *cblB*, or belong to another complementation group (*cblH*) [19,20].

In conclusion, we have identified five novel *MMAA* mutations in seven of ten Japanese patients with vitamin B_{12}-responsive MMA. The 503delC mutation in *MMAA* was observed in five patients. The identification of the prevalent mutation would facilitate DNA diagnoses of vitamin B_{12}-responsive MMA within the population.

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