Crohn’s disease in Japanese is associated with a SNP-haplotype of \( \gamma \)-acetyltransferase 2 gene

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Abstract

AIM: To investigate the frequency and distribution of \( \gamma \)-acetyltransferase 2 (\( \gamma \text{AT2} \)) and uridine 5’-diphosphate (UDP)-glucuronosyltransferase 1A7 (\( \text{UGT1A7} \)) genes in patients with ulcerative colitis (UC) and Crohn’s disease (CD).

METHODS: Frequencies and distributions of \( \gamma \text{AT2} \) and \( \text{UGT1A7} \)SNPs as well as their haplotypes were investigated in 95 patients with UC, 60 patients with CD, and 200 gender-matched, unrelated, healthy, control volunteers by PCR-fragment restriction length polymorphism (RFLP), PCR-denaturing high-performance liquid chromatography (DHPLC), and direct DNA sequencing.

RESULTS: Multiple logistic regression analysis revealed that the frequency of haplotype, \( \gamma \text{AT2}^\#B \), significantly increased in CD patients, compared to that in controls (\( P = 0.0130, OR = 2.802, 95\% CI = 1.243-6.316 \)). However, there was no association between \( \gamma \text{AT2} \) haplotypes and UC, or between any \( \text{UGT1A7} \)haplotypes and inflammatory bowel disease (IBD).

CONCLUSION: It is likely that the \( \gamma \text{AT2} \) gene is one of the determinants for CD in Japanese. Alternatively, a new CD determinant may exist in the 8p22 region, where \( \gamma \text{AT2} \) is located.

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Key words: Crohn’s disease; \( \gamma \)acetyltransferase 2 gene; Polymorphism; Disease-susceptible gene; Association study; Japanese population

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INTRODUCTION

Chronic inflammatory bowel disease (IBD) is a multi-factorial disorder characterized by non-specific inflammation of the gastrointestinal tract with an increase in the permeability to xenobiotics in the intestinal mucosa, finally resulting in intestinal malabsorption and immune defense abnormalities\[1-3\]. Ulcerative colitis (UC) and Crohn’s disease (CD) are the major forms of IBD. Although the precise etiology of IBD remains unknown, not only several environmental factors, such as dietary components and microorganisms, but also genetic factors may contribute to the occurrence of this disorder\[3-4\]. Recently, extensive molecular genetic studies have been launched to identify genes underlying the etiology\[5\]. One of them is the caspase activating recruitment domain 15/nucleotide oligomerization domain 2 gene (\( \text{CARD15/NOD2} \)) located at 16q12. Although mutations in \( \text{NOD2} \) are observed frequently in Caucasian patients with CD, but not with UC\[6,7\], they have rarely been found in Japanese CD patients\[8,9\], suggesting that \( \text{NOD2} \) is not a major determinant for CD in Japanese.

We have particularly focused on genes for \( \gamma \)-acetyltransferase 2 (\( \gamma \text{AT2} \)) and uridine 5’-diphosphate (UDP)-glucuronosyltransferase 1A7 (\( \text{UGT1A7} \)) as candidates susceptible to IBD, because they are expressed in the gastrointestinal tract and play a role in biochemical barriers against internal and external xenobiotics\[10-12\]. Diminution or disturbance of these barriers might result in increased permeability to xenobiotics in the gastrointestinal tract, and subsequently their accumulation in the body, probably leading to the development of IBD. \( \gamma \)-acetyltransferases (\( \gamma \text{ATs} \))
are the enzymes catalyzing N-acetylation (deactivation) of a variety of carbocyclic and heterocyclic arylamines by means of transferring acetyl-CoA to the amino or hydroxyl side chain of arylamines in metabolism of the phase II reaction[10]. NATs are encoded by two genes, NAT1 and NAT2, both are located at 8p22. NAT1 is ubiquitously expressed, while the expression of NAT2 is confined to the gastrointestinal tract and liver[10]. The UDP-glucuronosyltransferase 1 family genes located at 2q37 consist of nine functional genes, UGT1A1, UGT1A3-10, which catalyze the glucuronidation of small lipophilic agents by means of conversion of hydrophobic substrates to inactive hydrophilic UDP-glucurononides, and are expressed in a tissue-specific fashion in the gastrointestinal tract and liver. In particular, UGT1A7 is expressed exclusively in the gastrointestinal tract and lung, but not in the liver[11-13]. The degree of metabolism with regard to both NAT2 and UGT1A7 varies among individuals, suggesting the presence of genetic variations contributing to the metabolic activation capacity. Current studies have shown an association between NAT2 or UGT1A7 polymorphisms and various diseases, i.e., systemic sclerosis and systemic lupus erythematosus[14], drug toxicity[15,18], orolaryngeal cancer[19], esophageal cancer[20], colorectal cancer[21,22], pancreas cancer[23], hepato cellular carcinoma[15,24], or bladder cancer[25].

Here we report the results of studies on association between NAT2 or UGT1A7 and IBD in Japanese using six and three polymorphic haplotypes in the two genes, respectively.

**MATERIALS AND METHODS**

**Subjects**

The subjects studied comprised 95 patients with UC, 60 patients with CD, and 200 gender-matched, unrelated, healthy volunteers, and were further characterized as listed in Table 1. All participants were Japanese, who were randomly recruited from eight general health clinics in the Nagasaki area in Japan. The study protocol was approved by the Committee for the Ethical Issue on Human Genome and Gene Analysis in Nagasaki University, and written informed consent was obtained from each participant. Diagnosis of IBD was made according to endoscopic, radiological, histological, and clinical criteria provided by both the Council for International Organizations of Medical Sciences in WHO and the International Organization for the Study of Inflammatory Bowel Disease[26-28]. Patients with indeterminate colitis, multiple sclerosis, systemic lupus erythematosus, or other recognized autoimmune diseases were excluded from the subjects studied.

**Table 1 Clinical characteristics of study subjects**

| Characteristic | Disease | Control |
|---------------|---------|---------|
|               | UC      | CD      |          |
| Number of subjects | 95      | 60      | 200      |
| Age range (yr)  | 14-83   | 17-75   | 20-60    |
| Age (mean±SD)   | 44.4±16.4       | 35.0±12.6   | 32.5±11.1 |
| Male/female (%) | 53 (55.8)/42 (44.2) | 35 (58.3)/25 (41.7) | 125 (62.5)/75 (37.5) |

*P<0.01 vs control.

**Determination of NAT2 polymorphisms**

Genomic DNA was extracted from peripheral whole blood of each individual using the DNA Extractor WB-rapid Kit (Wako, Osaka, Japan) according to the manufacturer’s protocol. Single nucleotide polymorphisms (SNPs) of NAT2 deposited in SNP-database[29] were determined with the PCR-restriction fragment length polymorphism (RFLP) method using primer pairs and protocol described by Leff et al.[30]. The PCR-RFLP method was modified in order to distinguish among all known NAT2 SNPs[29]. In brief, polymorphic region in NAT2 was amplified by PCR with a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using 250 ng of genomic DNA in a 50-µL reaction containing 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.2 mmol/L of each dNTP, 500 ng of forward primer: 5'-GGCTATAAGAACCTCTAGGAAC-3', 500 ng of reverse primer: 5'-AAGGTTATTATTTTGTCCCTATTCTAAAT-3', and 2.0 U Taq DNA polymerase. The amplification protocol comprised initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min. PCR product of 896 bp was digested for 30 s, and extension at 72 °C for 5 min. The former nested PCR product of 115 bp was digested for 30 s, and extension at 72 °C for 5 min. The latter products were digested with restriction enzymes (TaKaRa Biomedical, Shiga, Japan). Three SNPs, C190T, G191A, and A434C, were detected by digestion with MspI. Likewise, C282T, C481T, or G857A were detected by digestion with FokI, KpnI, or BamHI, respectively. T111C, G590A, and C759T were detected by digestion with TaqI. These fragments were subjected to electrophoresis on 2% agarose or 5% polyacrylamide gel, and visualized with UV transilluminator (Alpha Innotech, CA, USA) after ethidium bromide staining. Moreover, T341C, A803G, and A845C were detected by further nested PCR. Amplified NAT2 product (1 µL) was used as a template in a 25-µL reaction containing 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.2 mmol/L of each dNTP, 250 ng of forward primer: 5'-CACCTTCTTGAGCTACAGG-3', and reverse primer: 5'-TGATCAAGCAGAATGCAAGGC-3' and reverse primer: 5'-TGATCAAGCAGAATGCAAGGC-3' and reverse primer: 5'-TGATCAAGCAGAATGCAAGGC-3' and reverse primer: 5'-TGATCAAGCAGAATGCAAGGC-3' and reverse primer: 5'-TGATCAAGCAGAATGCAAGGC-3' and reverse primer: 5'-TGATCAAGCAGAATGCAAGGC-3' and reverse primer: 5'-TGATCAAGCAGAATGCAAGGC-3' and reverse primer: 5'-TGATCAAGCAGAATGCAAGGC-3'. The amplification protocol comprised initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min. The former nested PCR products were digested with AsI and DdeI (New England BioLabs Inc., MA, USA) to detect T341C and A803G, or 250 ng of forward primer: 5'-TGAGGAA-GAGGTTAAGAATGCT-3' and reverse primer: 5'-AAGGTTATATTTTGTCCCTATTCTAAAT-3' for A845C, and 0.5 U Taq DNA polymerase. The amplification protocol comprised initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min. The former nested PCR products were digested with AsI and DdeI (New England BioLabs Inc., MA, USA) to detect T341C and A803G, respectively. The latter products were digested with DraIII (New England BioLabs Inc.) to detect A845C. All these products were subjected to electrophoresis on 6% polyacrylamide gel, and visualized as described above.

**Determination of UGT1A7 polymorphisms**

Four SNPs have been known within UGT1A7-exon 1[31]. A SNP at codon 11 is a silent mutation. SNPs at codons 129 and 131 lying in a linkage disequilibrium (LD) block were detected by PCR-denaturing high-performance liquid chromatography (DHPLC) with an automated HPLC
Statistical analysis

Gender and age value among the subjects were evaluated by $\chi^2$ test and unpaired Student’s $t$ test, respectively. Allele frequencies were estimated by the gene-counting method, and $\chi^2$ test was used to identify significant departures from the Hardy-Weinberg equilibrium. Subsequently, the odds ratio (OR) with 95% confidence interval (95%CI) was calculated by multiple logistic regression analysis using the JMP program package (version 5, SAS Institute, Cary, NC, USA) and the StatView program package (version 5, SAS Institute). Haplotype and genotype frequencies were compared between individuals with and without haplotype or genotype, using $\chi^2$ test. A $P$ value of 0.05 or less was considered statistically significant.

RESULTS

Haplotype frequencies of NAT2

We identified six haplotypes composed of six SNPs among the subjects examined (Table 2). The haplotype “NAT2*4” comprising 69.5% of controls was wild-type, while five other haplotypes were variants. Distributions of the haplotypes in our study population were well corresponded to the Hardy-Weinberg equilibrium (Table 2). The results implied that the population we studied had a homogeneous genetic background, being consistent with the previous observations\cite{31-33}. However, since the frequencies of three haplotypes, NAT2*5B, NAT2*11, and NAT2*13, were very low, they were not considered for subsequent multiple logistic regression analysis.

The frequency of haplotype “NAT2*7B” composed of two SNPs (C282T and G857A) significantly increased in patients with CD, compared to that in controls ($P = 0.0130$, OR = 2.802, 95%CI: 1.243-6.316, Table 3). In contrast, there was no difference in frequency of NAT2*7B between patients with UC and controls ($P = 0.3338$, OR = 1.436, 95%CI: 0.689-2.992). Of the 60 CD patients, 17 (28.3%) had NAT2*7B, the incidence being significantly higher than that (32/200, 16.0%) in controls ($P = 0.032$, OR = 2.076, Table 4). These results indicated that the haplotype NAT2*7B was associated with the susceptibility to CD, but not to UC.

Cascorbi et al\cite{34}, and Gross et al\cite{35}, have shown a relationship between genotypes of NAT2 polymorphism and phenotypes. The haplotypes NAT2*4, NAT2*11, and NAT2*13, code for the rapid acetylator phenotype, while NAT2*5B, NAT2*6A, and NAT2*7B, code for the slow acetylators. According to their reports, we divided the subjects in to two groups: the rapid acetylators comprised homozygous and heterozygous carriers of the haplotypes NAT2*4, NAT2*11, or NAT2*13 and the slow acetylators comprised all homozygous carriers of the other haplotypes. The frequency and distribution were compared between these groups, but there were no significant differences in frequencies of these estimated phenotypes among patients with UC, CD, and controls (data not shown).

| Haplotype | SNP | UC (allele = 190) | CD (allele = 120) | Control (allele = 400) |
|-----------|-----|------------------|------------------|------------------------|
| NAT2*4    | None | 122 (64.2)       | 77 (64.2)        | 278 (69.5)             |
| NAT2*5B   | T341C, C481T, A803G | 3 (1.6)          | 1 (0.8)          | 2 (0.5)                |
| NAT2*6A   | C282T, G590A | 43 (22.6)        | 21 (17.5)        | 79 (19.75)             |
| NAT2*7B   | C282T, G857A | 20 (10.5)        | 18 (15.0)        | 35 (8.75)              |
| NAT2*11   | C481T  | 0 (0)            | 1 (0.8)          | 1 (0.25)               |
| NAT2*13   | C282T  | 2 (1.1)          | 2 (1.7)          | 5 (1.25)               |

| Haplotype | $P$ | Odds ratio | 95% confidence interval |
|-----------|-----|------------|-------------------------|
| UC patients vs controls | | | |
| NAT2*4 | 0.6823 | 0.809 | 0.293–2.232 |
| NAT2*6A | 0.5621 | 1.183 | 0.671–2.084 |
| NAT2*7B | 0.3338 | 1.436 | 0.689–2.992 |
| CD patients vs controls | | | |
| NAT2*4 | 0.2616 | 2.162 | 0.563–8.304 |
| NAT2*6A | 0.3898 | 1.349 | 0.682–2.670 |
| NAT2*7B | 0.0130 | 2.802 | 1.243–6.316 |

| NAT2*7B | UC (n = 95, %) | CD (n = 60, %) | Control (n = 200, %) |
|---------|---------------|---------------|----------------------|
| Presence | 19 (20.0)     | 17 (28.3)     | 32 (16.0)            |
| Absence | 76 (80.0)     | 43 (71.7)     | 168 (84.0)           |

Haplotype frequencies of UGT1A7

We detected two SNPs at codons 129 and 131 of UGT1A7 by DHPLC with 100% accuracy, as confirmed by direct
DNA sequencing. Subsequently, on the basis of the results by PCR-DHPLC and PCR-RFLP, three haplotypes, UGT1A7*1, UGT1A7*2, and UGT1A7*3, were determined in the Japanese population studied (Table 5). The UGT1A7*1 haplotype was wild-type, UGT1A7*2 and UGT1A7*3 were identified as variants, while another haplotype, UGT1A4*, was not observed, indicating that it was very rare in Japanese. There were no significant differences in frequencies of haplotypes and genotypes among patients UC, CD, and controls (data not shown).

Table 5 Distributions of three UGT1A7 haplotypes among study subjects

| Haplotype | Number (%) of subjects with haplotype |
|-----------|--------------------------------------|
|           | UC (allele = 190) | CD (allele = 120) | Control (allele = 400) |
| UGT1A7*1  | 120 (63.2)        | 69 (57.5)         | 242 (60.5)            |
| UGT1A7*2  | 29 (15.3)         | 24 (20.0)         | 103 (25.7)            |
| UGT1A7*3  | 41 (21.6)         | 27 (22.5)         | 0 (0)                 |
| UGT1A7*4  | 0 (0)             | 0 (0)             | 0 (0)                 |

DISCUSSION

We have shown that a NAT2 haplotype, NAT2*B7, is associated with CD, and thus, NAT2 could be one of the genetic factors for the predisposition to the onset and/or development of CD, although its contribution to this disease appears relatively small. In contrast, we could not find any association between UGT1A7 polymorphism and IBD, suggesting that UGT1A7 never confers to these diseases. Although there are previous reports demonstrating an association between certain NAT2 variants and diseases, they deal with phenotypical variations, such as rapid, intermediate, and slow acetylators in different conditions such as systemic sclerosis, systemic lupus erythematosus, and drug-induced agranulocytosis. Therefore, the present study is the first report documenting an association between NAT2 genetic variation and CD.

Three NAT2 haplotypes, NAT2*B5, NAT2*B6A, and NAT2*B7B, are estimated to show slow acetylator phenotypes. The present study showed that slow acetylator carrying these haplotypes was not associated with CD (data not shown). Although a role of the NAT2*B7B haplotype in the susceptibility to CD is unknown, Freeland et al., demonstrated, that this haplotype is functionally related to low activity of N-acetylation. It is likely, that low activity of N-acetylation due to NAT2*B7B might fail to metabolite xenobiotics in the state of increased permeability in the gastrointestinal tract and subsequently accumulates them in the body since NAT2 functions as a biochemical barrier against xenobiotics including dietary intake, intestinal bacteria, and toxins. Our hypothesis may be partly supported by clinical evidence that total parenteral nutrition and elemental diet placing the gastrointestinal tract “at rest” can successfully improve CD, and refeeding by oral conventional diet aggravates the activity of CD.

Recent genome-wide linkage analyses and candidate gene-based association studies have shown possible IBD susceptibility regions at 16q12 (IBD1), 12p13 (IBD2), 6p21 (IBD3), 14q11 (IBD4), 19p13 (IBD5), 5q31-q33 (IBD6), 1p36 (IBD7), and at 16p (IBD8). Our results indicate the existence of a new CD determinant at an LD region of 8p22, even if it is not NAT2 it-self. It remains to be confirmed whether the association is reproducible in larger Japanese samples as well as in other populations.

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