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• *CLINICAL RESEARCH* •

Crohn's disease in Japanese is associated with a SNP-haplotype of *N*-acetyltransferase 2 gene

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Abstract

AIM: To investigate the frequency and distribution of *N*-acetyltransferase 2 (*NAT2*) and uridine 5'-diphosphate (UDP)-glucuronosyltransferase 1A7 (*UGT1A7*) genes in patients with ulcerative colitis (UC) and Crohn's disease (CD).

METHODS: Frequencies and distributions of *NAT2* and *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-restriction fragment 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, *NAT2*7B*, 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 *NAT2* haplotypes and UC, or between any *UGT1A7* haplotypes and inflammatory bowel disease (IBD).

CONCLUSION: It is likely that the *NAT2* gene is one of the determinants for CD in Japanese. Alternatively, a new CD determinant may exist in the 8p22 region, where *NAT2* is located.

Key words: Crohn's disease; *N*-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[1,2]. 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 (*CARD15/NOD2*) located at 16q12. Although mutations in *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 *NOD2* is not a major determinant for CD in Japanese.

We have particularly focused on genes for *N*-acetyltransferase 2 (*NAT2*) and uridine 5'-diphosphate (UDP)-glucuronosyltransferase 1A7 (*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. *N*-acetyltransferases (NATs)

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-glucuronides, 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[13-15]. 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[16], drug toxicity[17,18], orolaryngeal cancer[19], esophageal cancer[20], colorectal cancer[21,22], pancreas cancer[23], hepatocellular 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[b]	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)

[b]$P<0.01$ *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 $MgCl_2$, 0.2 mmol/L each dNTP, 500 ng of forward primer: 5'-GGCTATAA-GAACTCTAGGAAC-3', 500 ng of reverse primer: 5'-AAGGGTTTATTTTGTTCCTTATTCTAAAT-3', and 2.0 U *Taq* DNA polymerase. The amplification protocol comprised initial denaturation at 94 ℃ for 5 min; 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 30 s; and a final extension at 72 ℃ for 5 min. PCR product of 896 bp was digested by restriction enzymes (TaKaRa Biomedical, Shiga, Japan). Three SNPs, C190T, G191A, and A434C, were detected by digestion with *Msp*I. Likewise, C282T, C481T, or G857A was detected by digestion with *Fok*I, *Kpn*I, or *Bam*HI, respectively. T111C, G590A, and C759T were detected by digestion with *Taq*I. 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 $MgCl_2$, 0.2 mmol/L each dNTP, 250 ng of forward primer: 5'-CACCTTCT-CCTGCAGGTGACCG-3' and reverse primer: 5'-TGTCAAGCAGAAAATGCAAGGC-3' for T341C and A803G, or 250 ng of forward primer: 5'-TGAGGAA-GAGGTTGAAGAAGTGCT-3' and reverse primer: 5'-AAGGGTTTATTTTGTTCCTTATTCTAAAT-3' for A845C, and 0.5 U *Taq* DNA polymerase. The amplification protocol comprised initial denaturation at 94 ℃ for 5 min; 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 62 ℃ for 30 s, and extension at 72 ℃ for 30 s; and a final extension at 72 ℃ for 5 min. The former nested PCR products were digested with *Aci*I and *Dde*I (New England BioLabs Inc., MA, USA) to detect T341C and A803G, respectively. The latter products were digested with *Dra*III (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[15]. 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

instrument (WAVE™, Transgenomic, CA, USA), and by direct DNA sequencing with ABI 310 (Applied Biosystems, Foster City, USA). A DNA fragment containing codons 129 and 131 was amplified by PCR using 125 ng of genomic DNA in a 25-μL reaction containing 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP, 500 ng of forward primer: 5'-CCGGGAGT-TCATGGTTTTT-3', 250 ng of reverse primer: 5'-CACAGAGGGGAGGGAGAAAT-3', and 1.0 U *Taq* DNA polymerase, generating a 260-bp fragment. Amplification protocol comprised initial denaturation at 94 ℃ for 5 min; 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 30 s; and a final extension at 72 ℃ for 5 min. PCR products were used for DHPLC analysis. The temperature required for successful resolution of heteroduplex molecules was determined to be 56.8 ℃ according to the manufacturer's protocol. Another SNP at codon 208 was detected by PCR-RFLP using primer pair of 5'-GCATGAGGTGGTCGTCGTCA-3'/5'-CATCACGGGTTTGGGATACT-3', as in the *NAT2* SNP-detection. After digestion of PCR products by *Rsa*I (Promega, WI, USA), the fragments were subjected to electrophoresis on 2% agarose gel, and visualized as described above.

Statistical analysis

Gender and age value among the subjects were evaluated by χ^2 test and unpaired Student's *t* test, respectively. Allele frequencies were estimated by the gene-counting method, and χ^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 χ^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[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*.[34], and Gross *et al*.[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).

Table 2 Distributions of six *NAT2*-haplotypes in patients with UC/CD and controls

Haplotype	SNP	Number (%) of subjects with haplotype		
		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)

Table 3 Comparisons of frequencies of *NAT2*-haplotypes among study subjects by multiple logistic regression analysis

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

Table 4 Number of subjects with or without haplotype *NAT2*7B*

*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)

CD patients *vs* controls: *P* = 0.032, OR = 2.076.

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, *UGT1A*4*, 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	SNP	Number (%) of subjects with haplotype		
		UC (allele = 190)	CD (allele = 120)	Control (allele = 400)
*UGT1A7*1*	None	120 (63.2)	69 (57.5)	242 (60.5)
*UGT1A7*2*	T387G, C391A, G392A	29 (15.3)	24 (20.0)	55 (13.8)
*UGT1A7*3*	T387G, C391A, G392A, T622C	41 (21.6)	27 (22.5)	103 (25.7)
*UGT1A7*4*	T622C	0 (0)	0 (0)	0 (0)

DISCUSSION

We have shown that a *NAT2* haplotype, *NAT2*7B,* 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[16,17]. Therefore, the present study is the first report documenting an association between *NAT2* genetic variation and CD.

Three *NAT2* haplotypes, *NAT2*5B*, *NAT2*6A*, and *NAT2*7B*, are estimated to show slow acetylator phenotypes[34,35]. The present study showed that slow acetylator carrying these haplotypes was not associated, with CD (data not shown). Although a role of the *NAT2*7B* haplotype in the susceptibility to CD is unknown, Fretland *et al*., demonstrated, that this haplotype is functionally related to low activity of *N*-acetylation[36]. It is likely, that low activity of *N*-acetylation due to *NAT2*7B* 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[10-12,15]. 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[37].

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)[5,38,39]. 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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