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Absence of mutation in the *NOD2/CARD15* gene among 483 Japanese patients with Crohn's disease

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Abstract Chronic inflammatory bowel diseases (IBDs), specifically Crohn's disease (CD) and ulcerative colitis (UC), have increased significantly in western countries and Japan over the last decade, but very little is known about their pathogenesis. A candidate-gene approach recently identified *NOD2/CARD15* as one susceptibility gene from the *IBD1* locus on chromosome 16. Alterations in this gene were found in many Caucasian patients with CD; in particular, two nonsynonymous substitutions (R702W and G908R) and a frameshift mutation (1007fs) were shown to be independent risk factors for CD. We investigated DNA from 483 Japanese CD patients to detect those three mutations in *NOD2/CARD15* by appropriate genotyping techniques, but found only an R702Q substitution in a single patient. Direct sequencing of DNA from 96 of our patients in the regions containing the three reported major mutations detected no sequence alterations of consequence. Our findings indicate that the *NOD2/CARD15* gene is not a major contributor to CD susceptibility in the Japanese population.

Key words Crohn's disease · Single-nucleotide polymorphism (SNP) · *NOD2/CARD15* · *IBD1* · Japanese population

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Introduction

The pathogenic processes leading to Crohn's disease (CD) and ulcerative colitis (UC), both classified as chronic inflammatory bowel diseases (IBDs), are little known. The combined prevalence of the two diseases in Western countries and Japan has increased significantly over the last decade. CD is characterized by recurrent inflammation extending through all layers of the affected areas in the small bowel and/or colon; UC entails diffuse and continuous inflammation involving only the innermost layers of the intestinal wall.

Attempts to localize genes conferring susceptibility to IBD through genome-wide linkage analyses have disclosed several possible candidate loci, on chromosomes 1, 3, 5, 6, 7, 12, 14, 16, and 19 (Hugot et al. 1996; Satsangi et al. 1996; Cho et al. 1998; Duerr et al. 2000; Rioux et al. 2000, 2001). Linkage of susceptibility to CD, but not UC, to a locus in the pericentromeric region on chromosome 16 (*IBD1*) was confirmed by repeated experiments using several independent populations (Cavanaugh and the IBD International Genetics Consortium 2001). Recently *NOD2/CARD15*, a member of the NOD1/Apaf1 family, was mapped to chromosome 16q12 and became a candidate for *IBD1* (Hugot et al. 2001; Ogura et al. 2001a). *NOD2/CARD15* includes two caspase-recruitment domains (CARDs) in its N-terminal region and multiple leucine-rich repeats (LRRs) in its C-terminal (Ogura et al. 2001b); the LRR region interacts with bacterial lipopolysaccharides (LPS). Three major genetic alterations, R702W, G908R, and 1007fs, identified within those leucine-rich regions have been shown to increase the risk for CD in Caucasian populations (Lesage et al. 2002).

To investigate whether *NOD2/CARD15* plays a role in the pathogenesis of CD in Japan, we examined this gene in a large clinical population. We report here that alterations in this gene are very rare among Japanese CD patients, and we therefore assume that clear differences pertain to the etiology of CD between Caucasian and Japanese populations.

Subjects and methods

Subjects and DNA

Blood samples were obtained with written informed consent from 483 Japanese CD patients at the Social Insurance Chuo General Hospital. DNA was prepared from each sample according to standard protocols.

Mutational analysis

DNA samples were examined for the R702W substitution (C→T) by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis. The PCR products (annealing temperature, 60°C) were digested with *Msp*I and loaded on a 15% polyacrylamide gel. We looked for the G908R substitution, on the other hand, by means of hybridization with allele-specific oligonuclotides (ASO): a 20-ng aliquot of each genomic DNA was amplified by polymerase chain reaction (PCR) and spotted onto Biodyne A nylon membranes (Pall, East Hills, NY, USA). Then, allele-specific probes labeled with ^{32}P were hybridized to the membrane at 39°C and washed in 6 × SSC at 42°C. The 1007fs mutation was sought by direct sequencing of the relevant region, carried out by using the BigDye Terminator RR Mix (Applied Biosystems, Foster City, CA, USA) with ABI 3700 sequencers (Applied Biosystems). Sequences of the DNA primers and probes used are listed in Table 1. Furthermore, to investigate LRRs for sequence variations, we carried out direct sequencing of those regions using the primers listed in Table 2; the regions we sequenced are indicated in Fig. 1. Nucleotide positions within *NOD2/CARD15* were derived from the GenBank database [accession numbers AF1738930 (mRNA) and NT_010419 (genomic DNA)].

Results and discussion

To examine a possible association between alterations in the *NOD2/CARD15* gene and susceptibility to Crohn's disease in the Japanese population, we genotyped 483 Japanese CD patients for the three major genetic substitutions (R702W, G908R, and 1007fs) that had been reported in Caucasians with CD. No G908R or 1007fs substitutions were found in any of the 483 patients examined. By PCR-RFLP analysis, we detected loss of the *Msp*I site at codon 702 in a single patient, but analysis of the DNA sequence disclosed that the substitution was not R702W (C2104T), but R702Q, resulting from a change in the second nucleotide (nt.) of this codon (nt. 2105 G→A). Hence, none of the 483 Japanese CD patients we examined possessed any of the three major genetic changes that reports from elsewhere had indicated as increasing the risk for CD.

To investigate further whether this gene might nevertheless have some role in the etiology of CD in the Japanese population, we examined longer DNA sequences from 96 of our CD patients, to cover coding exons that included the

Table 1. Genotyping procedures for the R702W, G908R, and 1007fs mutations

Mutations and genotyping methods	Sequences of specific primers and probes	Size of PCR products (bp)
R702W: PCR-RFLP	Forward primer: 5′-GCACAACCTTCAGATCACAGCAG-3′ Reverse primer: 5′-CTGGCGGGATGGAGTGGAAG-3′	Wild type: 47, 53, 68 Mutant type: 47, 121
G908R: ASO	Forward primer: 5′-GGAGGAGGACTGTTAGTTCATGTC-3′ Reverse primer: 5′-GTTCAAAGACCTTCAGAACTGGCC-3′ Probe for wild type: 5′-ATTCTGGGGCAACAG-3′ Probe for mutant type: 5′-ATTCTGGCGCAACAG-3′	251
1007fs direct sequencing	Forward primer: 5′-TCACTGATGGTACTGAGCCTTTG-3′ Reverse primer: 5′-CACATGCAGAATCATTCTCACTG-3′ Sequence primer: 5′-CTCAGACATGAGCAGGATGTGT-3′	405

PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ASO, allele-specific oligonucleotides

Table 2. Primers for direct sequencing of relevant LRRs in the *NOD2/CARD15* gene

Screened exon	Primers (forward primer: F; reverse primer: R)		Size of PCR products (bp)
	PCR primer	Sequencing primer	
4(1)	F: 5′-TTCTACCTGGCACTCAGTGCTG-3′ R: 5′-TCACCCACAGAGTTGTAGTCCAG-3′	F: 5′-TTGCTCAGACACCTCTTCAATTC-3′	541
4(2)	F: 5′-TCCGCAAGCACTTCCACTCCATC-3′ R: 5′-AAATTAAGGCCCAGAAGGGAAGGG-3′	R: 5′-AGTGTTGCAAAGCAGGGTGGCAGA-3′	478
8	F: 5′-CTCTTGGGTTAAGTTTGGCCAT-3′ R: 5′-CCATTGCCTAACATTGTGGGGTAG-3′	F: 5′-GGAGGAGGACTGTTAGTTCATGTC-3′ R: 5′-CCATTGCCTAACATTGTGGGGTAG-3′	407
11	F: 5′-TCACTGATGGTACTGAGCCTTTG-3′ R: 5′-CACATGCAGAATCATTCTCACTG-3′	F: 5′-CTCAGACATGAGCAGGATGTGT-3′	405

LRR, leucine-rich repeat

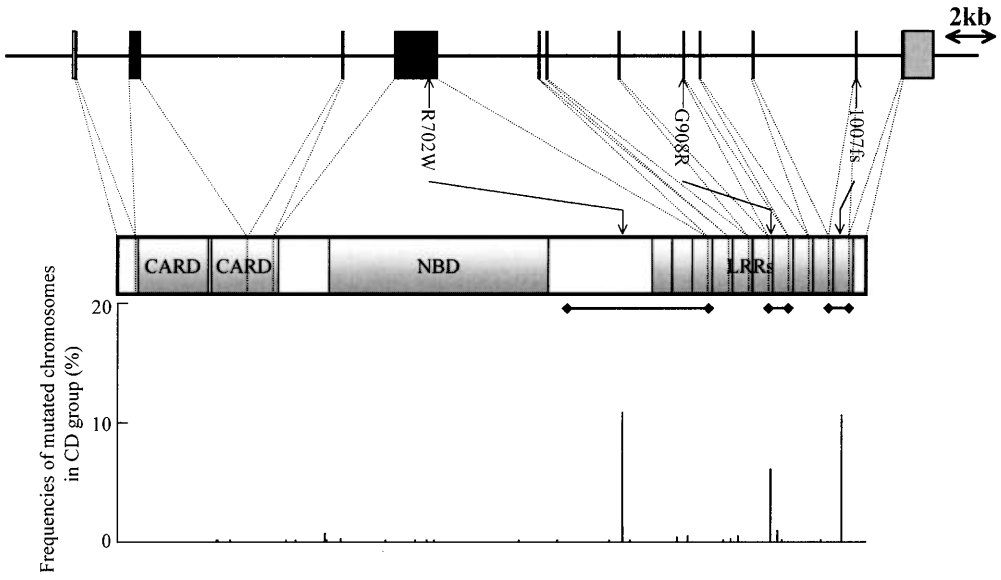

Fig. 1. Genomic structure, predicted functional domains, and frequencies of genetic alterations at each nucleotide position of the *NOD2/CARD15* gene reported among Caucasian Crohn's disease (CD) patients (Lesage et al. 2002). The regions we sequenced in DNAs from 96 Japanese individuals with CD are indicated by *horizontal lines* with *diamond ends*. *CARD*, caspase-recruitment domain; *NBD*, nucleotide-binding domain; *LRR*, leucinerich region

sites of the three reported genetic alterations and their flanking intronic sequences. Apart from the R702Q (G2105A) substitution described above, we found only one change, a G-to-C substitution at IVS8+6 in a single patient.

Multiple studies have yielded evidence for linkage of CD to the *IBD1* locus (Rioux et al. 2000; Cavanaugh and the IBD International Genetics Consortium 2001), and a positional-candidate approach has indicated that three major genetic substitutions in the *NOD2/CARD15* gene can contribute to susceptibility to CD (Hampe et al. 2001; Ahmad et al. 2002; Cuthbert et al. 2002). However, we clearly demonstrated that none of those substitutions was present in any of the 483 Japanese CD patients examined; we found only R702Q at codon 702 in a single case. In addition to the three major substitutions found among Caucasian CD patients, Lesage et al. (2002) identified SNPs in this gene, mainly in the LRRs. In contrast, apart from the R702Q substitution, we found no substitutions in the three relevant exons of *NOD2/CARD15* by directly sequencing DNA from 96 Japanese subjects. Since we investigated nearly 500 patients, the absence of genetic variations in this gene must reflect genetic heterogeneity in the cause of CD among different ethnic groups.

Genes other than the *NOD2/CARD15* gene have been suggested for association with susceptibility to CD. For example, *MUC3* (mucin 3), *TNF* (tumor necrosis factor), and *HLA class II* (DR, DQ, and DP) may be be candidates for CD genes in some populations (Kyo et al. 1999, 2001; Neguro et al. 1999; Yoshitake et al. 1999). Mucins are large and heterogeneous glycoproteins that are important for the protection of the epithelium. Buisine et al. (1999) reported that expression of mucins, especially *MUC3*, was significantly lower in IBD patients than in healthy individuals. The frequency of rare VNTR (variable number of tandem repeats) alleles of *MUC3* among Japanese and Caucasian patients with UC is higher than in controls (Kyo et al. 1999), and some variants of *MUC3A* are involved in susceptibility to both UC and CD (Kyo et al. 2001). *TNF* is a proinflammatory cytokine that plays important roles in the initiation and regulation of immune responses. The frequencies of the three polymorphisms found in the 5′-flanking region of the *TNF* gene are significantly higher in Japanese patients with CD than in controls (Neguro et al. 1999). Moreover, analysis of polymorphisms in *HLA class II* genes among Japanese patients with IBD has suggested that DQB1*0402 and DRB1*1502 are disease-resistance alleles of CD patents in this population, and that DRB1*1502 increases the risk for UC (Yoshitake et al. 1999). Variations in immune-related genes may indeed influence autoimmune responses and result in differences of susceptibilities to IBD.

The results presented here clearly indicate that ethnic differences exist as regards susceptibility to CD. Since CD and UC are complex diseases involving both environmental and genetic factors, we need to investigate the genetic factors in genome-wide studies and also to examine the interaction between potential genetic factors and environmental factors, particularly food intake. Novel technologies and construction of a high-density map of single-nucleotide polymorphisms (SNPs) should make it possible to perform epidemiological studies that would include both a large-scale genomic analysis and an environmental analysis involving a large number of cases and controls. Such studies would eventually shed more light on the etiology of IBD.

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