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• *CLINICAL RESEARCH* •

Association of polymorphic alleles of *CTLA4* with inflammatory bowel disease in the Japanese

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Abstract

AIM: To examine an association between the cytotoxic T-lymphocyte antigen 4 (*CTLA4*) gene that plays a role in downregulation of T-cell activation and inflammatory bowel disease consisting of ulcerative colitis (UC) and Crohn's disease (CD) in the Japanese.

METHODS: We studied 108 patients with UC, 79 patients with CD, and 200 sex-matched healthy controls, with respect to three single nucleotide polymorphisms (SNPs) in *CTLA4*, such as C-318T in the promoter region, A+49G in exon 1 and G+6230A in the 3′ untranslated region (3′-UTR) by a PCR-restriction fragment length polymorphism method, and to an $(AT)_n$ repeat polymorphism in 3′-UTR by fragment analysis with fluorescence-labeling on denaturing sequence gels. Frequency of alleles and genotypes and their distribution were compared statistically between patients and controls and among subgroups of patients, using χ^2 and Fisher exact tests.

RESULTS: The frequency of "A/A" genotype at the G+6230A SNP site was statistically lower in UC patients than in controls (3.7% *vs* 11.0%, $P = 0.047$, odds ratio (OR) = 0.311). Moreover, the frequency of "G/G" genotype at the A+49G SNP site was significantly higher in CD patients with fistula (48.6%) than those without it (26.2%) ($P = 0.0388$, OR=2.67).

CONCLUSION: The results suggest that *CTLA4* located at 2q33 is a determinant of UC and responsible for fistula formation in CD in the Japanese.

Key words: Ulcerative colitis; *CTLA4* gene; Disease-susceptible gene; Crohn's disease; Fistula formation

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INTRODUCTION

Chronic inflammatory bowel disease (IBD) is a multifactorial disorder characterized by non-specific inflammation of the gastrointestinal tract, resulting in intestinal malabsorption and immune defense abnormalities, especially an exaggerated T-cell response[1,2]. Ulcerative colitis (UC) and Crohn's disease (CD) are common major forms of IBD. Although the etiology of IBD remains unknown, both environmental and genetic factors may contribute to the occurrence of this disorder[3,4]. Genome-wide linkage analyses and candidate gene-based association studies have shown possible IBD-susceptibility loci at 16q12 (IBD1), 12p13 (IBD2), 6p21 (IBD3), 14q11 (IBD4), 5q31-q33 (IBD5), 19p13 (IBD6), 1p36 (IBD7), and 16p (IBD8)[5-7]. The caspase activating recruitment domain 15/nucleotide oligomerization domain 2 gene (*CARD15/NOD2*) located at 16q12 is one of them, and its mutations were associated with CD in the Caucasians, but not in the Japanese[8-11]. This may be due to different genetic background between the races.

As a candidate gene susceptible to IBD, we focused on the cytotoxic T-lymphocyte antigen 4 (*CTLA4*) gene located at 2q33, because CTLA4 is a T-cell receptor that binds to B7-1 (CD80) and B7-2 (CD86) during antigenic stimulation of T cells, and plays a role in downregulation of T-cell activation against another competitive receptor, CD28, which operates on upregulation of T-cell activation[12-14]. Since *CTLA4*-deficient mice developed a lethal lymphoproliferative

disease characterized by massive T-lymphocytic infiltration in all tissues[15,16], diminution of downregulation of T-cell activation through CTLA4 may result in an exaggerated T-cell response and subsequent continuous inflammation in the gastrointestinal mucosae, probably leading to the development of IBD. Three single nucleotide polymorphisms (SNPs) in the human *CTLA4*, i.e., a C-318T SNP in the promoter region[17], an A+49G SNP in exon 1[12], and a G+6230A SNP in the 3' untranslated region (3'-UTR)[18], and an $(AT)_n$ repeat polymorphism in 3'-UTR[19] have been reported. Current studies showed an association of *CTLA4* polymorphic alleles with inhibitory function of CTLA4 at the mRNA and protein levels in peripheral blood mononuclear cells[20,21], and also with various autoimmune diseases, such as Graves' disease[18,22], rheumatoid arthritis[23], multiple sclerosis[24], type I diabetes mellitus[18,25], Hashimoto's disease[18,26], and others[27-29], of which pathoetiology is probably similar to IBD. However, there was no association of two *CTLA4* SNPs, C-318T and A+49G, with IBD in both the Dutch and Chinese populations[30].

In this study, we examined on whether three *CTLA4* SNPs, C-318T, A+49G, and G+6230A, and an $(AT)_n$ repeat polymorphism in 3'-UTR are associated with IBD in the Japanese.

MATERIALS AND METHODS

Subjects

The subjects studied comprised 108 patients with UC, 79 patients with CD, and 200 gender-matched unrelated healthy volunteers as controls (Table 1). All participants were Japanese who were randomly recruited from eight general health clinics in the Nagasaki district, 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[31-33]. 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

Characteristics	Patients with UC	Patients with CD	Controls
Number of subjects	108	79	200
Age range (yr)	14-83	17-75	20-60
Age (mean±SD)	44.0±16.9[b]	34.5±12.7	32.5±11.1
Male / female (%)	57 (52.8) / 51 (47.2)	47 (59.5) / 32 (40.5)	125 (62.5) / 75 (37.5)

[b]$P<0.01$ *vs* controls.

Patients with UC were classified into three subgroups according to age at onset (<40 or ⩾40 years), localization and extension of disease (pancolitis, left-sided colitis, or proctitis), and presence or absence of colectomy as an indicator of severity. Likewise, patients with CD were divided into subgroups according to age at onset (<40 or ⩾40 years), localization and extension of lesions (ileum, ileocolon, or colon), presence or absence of fistula, and performance of operation such as partial resection of intestine, and stricture plasty.

Determination of three SNPs and $(AT)_n$ repeat polymorphism

Genomic DNA was extracted from whole blood of each subject using the DNA Extractor WB-rapid Kit (Wako, Osaka, Japan) according to the manufacturer's protocol. Presence or absence of polymorphic alleles at three SNP sites in the human *CTLA4*, a C/T SNP at nt -318 (C-318T) in the promoter region[17], an A/G SNP at nt +49 in exon 1 (A-49G)[12], and a G/A SNP at nt +6 230 (G+6230A) in 3'-UTR[18], were determined with the PCR-restriction fragment length polymorphism methods. Polymorphic region was amplified by PCR with a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using 150 μg of genomic DNA in a 25-μL reaction solution containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each dNTPs, 15 pmol of forward primer: 5'-AATGAATTGGACTGGATGG-3' and reverse primer: 5'-TTACGAGAAAGGAAGCCGTG-3' for C-318T SNP[17]; forward primer: 5'-CTGAACACCGCTC-CCATAAA-3' and reverse primer: 5'-CCTCCTCCATCTT-CATGCTC-3' for A+49G SNP; or forward primer: 5'-TGATTCATTCAGTATCTGGTGGAG-3' and reverse primer: 5'-AGGGGAGGTGAAGAACCTGT-3' for G+6230A SNP, and 1 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 60 ℃ (C-318T), at 65 ℃ (A+49G), and at 62 ℃ (G+6230A) for 30 s, and extension at 72 ℃ for 30 s, and final extension at 72 ℃ for 5 min. The PCR products were digested with *Mse*I (New England BioLabs Inc., Beverly, MA, USA), *Bbv*I (New England BioLabs Inc.), and *Tai*I (MBI Fermentas Inc., Hanover, MD, USA), to detect C-318T, A+49G, and G+6230A, respectively. All these products were subjected to electrophoresis on a 6% polyacrylamide gels and visualized with UV transilluminator (Alpha Innotech Co., San Leandro, CA, USA).

A $(AT)_n$ repeat polymorphism in 3'-UTR of *CTLA4*[19] was investigated by fragment analysis with fluorescence-labeling on denaturing sequence gels. Polymorphic region was amplified by PCR using 150 μg of genomic DNA in a 25-μL reaction solution containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each dNTPs, 15 pmoL of forward primer labeled with 6-carboxyfluorescein dye (Applied Biosystems): 5'-GCCAG-TGATGCTAAAGGTTG-3' and reverse primer: 5'-AACATACGTGGCTCTATGCA-3', and 1 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 64 ℃ for 30 s, and extension at 72 ℃ for 30 s, and final extension at 72 ℃ for 5 min. The PCR products were analyzed on a 6% denaturing sequence gel with an internal size marker, GeneScan 500XL ROX (Applied Biosystems), by ABI Prism 377 genetic analyzer and ABI Prism 3100 genetic analyzer (Applied Biosystems).

Statistical analysis

Gender and age values between UC or CD patients and controls 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. SNP and genotype frequencies and their distributions were compared between UC or CD patients and controls, between individuals with and without a genotype, and among subgroups of UC or CD patients, using χ^2 and Fisher exact tests. Odds ratio (OR) with 95% confidence interval was calculated by multiple logistic regression analysis using the JMP program package (version 5, SAS Institute Inc., Cary, NC, USA) and the StatView program package (version 5, SAS Institute Inc.). A *P* value of 0.05 or less was considered statistically significant.

RESULTS

Frequencies and distributions of CTLA4 polymorphic alleles

We identified frequencies and distributions of alleles at the three SNP sites and seven alleles of the $(AT)_n$ repeat polymorphism of *CTLA4* among the subjects examined (Table 2). Distributions of *CTLA4* polymorphic alleles in our study population well corresponded to the Hardy–Weinberg equilibrium (Table 2). The results imply that the population we studied has a homogeneous genetic background. The alleles, "C" at nt -384, "A" at nt +49, "G" at nt +6 230, and "$(AT)_7$" in 3'-UTR, are wild types, while other alleles are variants. Since the frequencies of two alleles, $(AT)_{20}$ and $(AT)_{22}$, were very low (<2%), they were not considered for subsequent multiple logistic regression analysis. There were no significant differences in frequency of any alleles between IBD and controls.

Frequencies and distributions of CTLA4 genotypes

Of a total of 108 UC patients, 4 (3.7%) had "A/A" genotype at the G+6230A SNP site, the incidence being significantly lower than that (22/200, 11.0%) in the controls ($P = 0.047$, OR = 0.311) (Tables 3 and 4). There were no significant

Table 2 Distribution of *CTLA4* polymorphic alleles among study subjects

Polymorphic site	Allele	Number (%) of alleles in		Controls
		UC	CD	
nt –318	C	192 (88.9)	140 (88.6)	362 (90.5)
	T	24 (11.1)	18 (11.4)	38 (9.5)
nt +49	A	84 (38.9)	59 (37.3)	159 (39.8)
	G	132 (61.1)	99 (62.7)	241 (60.2)
nt +6 230	G	158 (73.1)	115 (72.8)	278 (69.5)
	A	58 (26.9)	43 (27.2)	122 (30.5)
$(AT)_n$ in 3'-UTR	$(AT)_7$	107 (49.5)	83 (52.5)	205 (51.3)
	$(AT)_{15}$	45 (20.8)	22 (13.9)	82 (20.5)
	$(AT)_{16}$	50 (23.1)	35 (22.2)	87 (21.8)
	$(AT)_{17}$	3 (1.4)	15 (9.5)	16 (4.0)
	$(AT)_{18}$	11 (5.1)	1 (0.6)	8 (2.0)
	$(AT)_{20}$	0	2 (1.3)	0
	$(AT)_{22}$	0	0	2 (0.5)
Total number of alleles		216	158	400

Table 3 Distribution of *CTLA4* genotypes among study subjects

Polymorphic site	Genotype	Number (%) of subjects with genotype		Controls (*n* = 200)
		UC (*n* = 108)	CD (*n* = 79)	
nt –318	C/C	84 (77.8)	63 (79.8)	163 (81.5)
	C/T	24 (22.2)	14 (17.7)	36 (18.0)
	T/T	0	2 (2.5)	1 (0.5)
nt +49	A/A	14 (13.0)	9 (11.4)	33 (16.5)
	A/G	56 (51.8)	41 (51.9)	93 (46.5)
	G/G	38 (35.2)	29 (36.7)	74 (37.0)
nt +6 230	G/G	54 (50.0)	39 (49.4)	100 (50.0)
	G/A	50 (46.3)	37 (46.8)	78 (39.0)
	A/A	4 (3.7)	3 (3.8)	22 (11.0)
$(AT)_n$ in 3'-UTR	$(AT)_7/(AT)_7$	52 (48.1)	41 (51.9)	101 (50.5)
	$(AT)_7/(AT)_{15}$	2 (1.9)	1 (1.3)	1 (0.5)
	$(AT)_7/(AT)_{16}$	1 (0.9)	0	2 (1.0)
	$(AT)_{15}/(AT)_{15}$	16 (14.8)	5 (6.3)	31 (15.5)
	$(AT)_{15}/(AT)_{16}$	11 (10.2)	9 (11.4)	18 (9.0)
	$(AT)_{15}/(AT)_{17}$	0	2 (2.5)	1 (0.5)
	$(AT)_{16}/(AT)_{16}$	18 (16.7)	13 (16.4)	30 (15.0)
	$(AT)_{16}/(AT)_{17}$	2 (1.9)	0	6 (3.0)
	$(AT)_{16}/(AT)_{18}$	0	0	1 (0.5)
	$(AT)_{17}/(AT)_{17}$	0	6 (7.6)	3 (1.5)
	$(AT)_{17}/(AT)_{18}$	1 (0.9)	1 (1.3)	3 (1.5)
	$(AT)_{18}/(AT)_{18}$	5 (4.6)	0	2 (1.0)
	$(AT)_{20}/(AT)_{20}$	0	1 (1.3)	0
	$(AT)_{22}/(AT)_{22}$	0	0	1 (0.5)

Table 4 Number of subjects with or without "G" allele at the G+6230A SNP site of *CTLA4*

Genotype	Number (%) of subjects with genotype		
	UC (n = 108)	CD (n = 79)	Control (n = 200)
G/G+G/A	104 (96.3)	76 (96.2)	178 (89.0)
A/A	4 (3.7)[a]	3 (3.8)	22 (11.0)

[a]P<0.05 *vs* controls (P = 0.047, OR = 0.311).

differences in frequency of genotypes at three other polymorphic sites between patients with IBD and the controls.

Frequencies and distributions of genotypes among UC and CD subgroups classified according to clinical features were shown in Tables 5 and 6, respectively. With respect to A+49G SNP, the frequency of "G/G" genotype was significantly higher in CD patients with fistula (48.6%) than

Table 5 Number of UC patients classified by clinical features

Polymorphic site	Genotype	Number of patients (n = 108, %)	Age at onset (yr)	
			<40	⩾40
C–318T	C/C	84 (77.8)	55	29
	C/T	24 (22.2)	18	6
	T/T	0	0	0
A+49G	A/A	14 (13.0)	8	6
	A/G	56 (51.8)	40	16
	G/G	38 (35.2)	25	13
G+6230A	G/G	54 (50.0)	39	15
	G/A	50 (46.3)	31	19
	A/A	4 (3.7)	3	1
$(AT)_n$ in 3'-UTR	$(AT)_7/(AT)_7$	52 (48.1)	33	19
	$(AT)_7/(AT)_x$ or others	56 (51.9)	40	16

(Continued)

Location			Colectomy	
Pancolitis	Left-sided colitis	Proctitis	Yes	No
42	29	13	7	77
10	13	1	1	23
0	0	0	0	0
9	4	1	0	14`
28	20	8	3	53
15	18	5	5	33
20	27	7	6	48
28	15	7	2	48
4	0	0	0	4
31	14	7	1	51
23	26	7	7	49

Table 6 Number of CD patients classified by clinical features

Polymorphic site	Genotype	Number of patients (n = 79, %)	Age at onset (yr)	
			<40	⩾40
C–318T	C/C	63 (79.8)	54	9
	C/T	14 (17.7)	12	2
	T/T	2 (2.5)	2	0
A+49G	A/A	9 (11.4)	8	1
	A/G	41 (51.9)	35	6
	G/G	29 (36.7)	25	4
G+6230A	G/G	39 (49.4)	35	4
	G/A	37 (46.8)	30	7
	A/A	3 (3.8)	3	0
$(AT)_n$ in 3'-UTR	$(AT)_7/(AT)_7$	41 (51.9)	34	7
	$(AT)_7/(AT)_x$ or others	38 (48.1)	34	4

(Continued)

Location of lesion			Operation		Fistula	
Ileocolon	Ileum	Colon	Yes	No	Presence	Absence
40	14	9	33	30	27	36
11	1	2	9	5	8	6
2	0	0	2	0	2	0
7	2	0	5	4	5	4
25	11	5	20	21	14	27
21	2	6	19	10	18	11
25	5	7	21	18	19	20
25	8	4	22	15	18	19
1	2	0	1	2	0	3
27	10	4	24	17	19	22
26	9	3	20	18	17	21

those without it (26.2%) (P = 0.0388, OR = 2.67; Table 7). There were no significant differences in frequency of other genotypes among any other subgroups of IBD patients.

Table 7 Relationship between genotype at the A+49G SNP site and presence/absence of fistula in CD patients

Genotype	No. (%) of patients	
	With fistula (n = 37)	Without fistula (n = 42)
A/A+A/G	19 (51.4)	31 (73.8)
G/G	18 (48.6)[a]	11 (26.2)

[a]P<0.05 *vs* without fistula (P = 0.0388, OR = 2.67).

DISCUSSION

We have shown that "A/A" genotype at the G+6230A SNP site of *CTLA4* is associated with insusceptibility to UC. This suggests that individuals with "A/A" genotype at nt +6 230 may have some resistance to UC, or reversely, those with "G/G" or "G/A" genotypes are susceptible to UC. Moreover, "G/G" genotype at the A+49G SNP site was more frequently observed in CD patients with fistula than those without it. These findings suggest that *CTLA4* is one of genetic factors for the predisposition to the onset and/or development of UC and CD. However, since the number of UC patients with "A/A" genotype at the G+6230A SNP site in our study population is small (Table 5), it remains to be confirmed whether the association is reproducible in larger Japanese samples as well as in other populations. Although a previous study in the Dutch and Chinese populations did not find an association between *CTLA4* and IBD, it never dealt with the G+6230A SNP site[30]. Therefore, the present study is the first report on an association of *CTLA4* polymorphisms with IBD.

CTLA4 consists of four exons that encode leader peptide, ligand-binding domain, transmembrane domain, and cytoplasmic tail, respectively. In humans, there are two isoforms of *CTLA4*, which are a full-length isoform (*flCTLA4* transcript) and a soluble isoform (*sCTLA4* transcript) which lacks exon 3 by alternative splicing. Especially, sCTLA4 is secreted and circulating in human sera[34,35]. It binds CD80/86 molecules and subsequently inhibits T-cell proliferation *in vitro*[35]. Expression of the human *CTLA4* mRNA isoforms by alternative splicing correlates genotype, G+6230A SNP[18]. The ratio of *sCTLA4* to *flCTLA4* at mRNA level in unstimulated T cells was 50% lower in individuals with "G/G" genotype at nt +6 230 than in those with "A/A" genotype[18]. Although expression of *CTLA4* isoforms at protein level and activities of T-cell signal pathway were not examined, individuals with "G/G" genotype at nt +6 230 may reduce the production of *sCTLA4* transcript, and subsequently diminish the inhibition of T-cell activation, probably leading to an increase in T-cell proliferation and chronic inflammation in epithelial cells of the colon. Moreover, the A+6230G SNP was associated with the susceptibility to autoimmune diseases, i.e., Grave's disease, autoimmune hypothyroidism, and type 1 diabetes mellitus[18]. As well as these autoimmune diseases, autoantibodies against colonic epithelial cells, such as anticolon antibodies, antitropomyosin antibodies, and antineutrophil cytoplasmic antibodies, are frequently found in sera of patients with UC[36-38]. Thus, it is plausible that UC is also an autoimmune disease and some genetic factors are common between UC and autoimmune diseases.

Fistula formation in CD patients is one of the indicators of severity. Our results indicated that CD patients with "G/G" genotype at nt +49 more frequently had fistula. Since intracellular distribution of CTLA4 in individuals with "G/G" genotype at nt +49 was qualitatively different from that with "A/A", and downregulation of T-cell activation in individuals with "G/G" genotype was reduced[39], CD patients with "G/G" genotype may show progressive and severe clinical course. It remains to be investigated why the A+49G SNP is associated with fistula formation in Japanese CD patients.

In conclusion, our study showed that *CTLA4* is one of the determinants of UC and responsible for fistula formation in CD in the Japanese.

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