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Genetic variants of *SMAD2/3/4/7* are associated with susceptibility to ulcerative colitis in a Japanese genetic background

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ABSTRACT

Purpose: Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is attributed to inappropriate inflammatory response in intestinal mucosa. Transforming growth factor β (TGF-β)/SMAD signaling plays key role in differentiation of naïve $CD4^+$ T cells to T helper 17 (Th17) cells or regulatory T (T_{reg}) cells. This study aimed to investigate associations between single nucleotide polymorphisms (SNPs) of *SMAD* family genes and susceptibility to IBD in a Japanese cohort to elucidate genetic determinants of IBD.

Methods: This study included 81 patients with CD, 108 patients with UC, and 199 healthy subjects as controls. A total of 21 SNPs in four genes (*SMAD2*, *SMAD3*, *SMAD4*, and *SMAD7*) involved in the TGF-β/SMAD signaling pathway were genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism, PCR-direct DNA sequencing, or PCR-high resolution melting curve analysis.

Results: Four SNPs (rs13381619, rs9955626, rs1792658, and rs1792671) within *SMAD2*, one SNP within *SMAD3* (rs41473580), two SNPs within *SMAD4* (rs7229678 and rs9304407), and one SNP within *SMAD7* (rs12956924) were significantly associated with susceptibility only to UC. rs13381619 within *SMAD2*, rs4147358 within *SMAD3*, rs9304407 within *SMAD4*, and rs12956924 within *SMAD7* exhibited the strongest association ($p < 0.001$, $p = 0.021$, $p = 0.005$, and $p = 0.001$, respectively). Furthermore, rs4147358 of *SMAD3* altered the expression of a luciferase reporter gene in Jurkat T cell line *in vitro*.

Conclusions: Genetic variants of several *SMAD* family of genes might alter the balance of differentiation between Th17 and T_{reg}, resulting in the development of IBD, especially UC.

1. Introduction

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is attributed to inappropriate inflammatory response in intestinal mucosa. Although the precise etiology of IBD remains unclear, its development was shown to involve environmental triggers such as food antigens and commensal microbiota as well as genetic predisposition [1]. Multiple genome-wide association (GWA) studies that have been conducted to date were successful in identifying IBD susceptibility loci. Two genome-wide meta-analyses identified 71 CD susceptibility loci in 2010 [2] and 47 UC susceptibility loci in 2011 [3]. With another genome-wide meta-analysis in 2012 that reported 71 new associations, a total of 163 IBD susceptibility loci have been reported to date, including 110 loci shared between CD and UC [4]. In addition to these GWA studies on European ancestry, GWA studies in the Japanese population identified 18 loci that were common with Europeans and 2 loci specific to Japanese [5].

These genetic studies reveal that multiple pathways are involved in the maintenance of intestinal homeostasis, including epithelial barrier function and restitution, microbial defense, innate and adaptive immune regulation, and autophagy [6]. Although some of these pathways are specific to either CD or UC, T cell differentiation, which are shared between CD and UC, appears to be a key pathway in which multiple components are implicated as genetic risk cofactors [6]. A balance between pro-inflammatory and anti-inflammatory responses is involved in gut homeostasis, and inflammatory diseases result from excessive activation of effector T helper (Th) cells together with an inadequate function of regulatory T (T_{reg}) cells [7,8]. Specifically, increased

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production of Th17-type cytokines was observed in the intestinal mucosa of both CD and UC patients [9,10]. Transforming growth factor-β (TGF-β) and the downstream effector SMAD proteins play a key role in differentiation of naïve T cells to either Th17 or T_{reg} cells [11–13]. Naïve T cells that respond to TGF-β alone lead to the upregulation of forkhead box P3 (FOXP3) and RORγt, which are master transcription factors in T_{reg} and Th17 cells, respectively [11]. TGF − β concentration and presence of other cytokines such as IL-6 decide the fate of T cells. TGF-β binding to its receptor leads to the phosphorylation of SMAD2 or SMAD3, which in turn interacts with SMAD4. The resultant SMAD4 translocation into the nucleus activates the transcription of target genes [12]. Importantly, dysregulated SMAD signaling was reported in the inflamed gut of patients with IBD [14]. Additionally, SMAD7, which blocks SMAD signaling by inhibiting the phosphorylation of SMAD3, was also shown to be upregulated in the gut of IBD patients [14]. European GWA studies identified an association between *SMAD3* and susceptibility to IBD [2,3]. However, previous Japanese GWA studies failed to find a similar association [5], and no candidate gene-based studies have thus far investigated the potential association between SMAD and IBD. Therefore, we conducted a candidate gene-based association using an original Japanese cohort of IBD to investigate whether genes in the SMAD pathway were associated with IBD susceptibility in Japanese.

2. Methods

2.1. Subjects

All study subjects were unrelated Japanese patients including 81 patients with CD and 108 patients with UC as well as 199 healthy volunteers as controls. Patients with IBD were enrolled from eight general health clinics in Nagasaki, Japan, from October 2003 to October 2008. General characteristics of the study subjects are shown in Table 1.

The diagnosis of IBD was based on endoscopic, radiological, histological, and clinical criteria of the World Health Organization Council for International Organizations of Medical Sciences and the International Organization for the Study of Inflammatory Bowel Disease [15–17]. Patients diagnosed with indeterminate colitis, multiple sclerosis, systemic lupus erythematosus, or other autoimmune diseases were excluded from this study. The study protocol was approved by the Committee for Ethical Issues dealing with the Human Genome and Gene Analysis at Nagasaki University. Written informed consent was obtained from all subjects.

2.2. Genotyping for single nucleotide polymorphisms

Genomic DNA was extracted from peripheral blood using the DNA Extractor WB-Rapid kit (WAKO Pure Chemical, Osaka, Japan). Single nucleotide polymorphisms (SNPs) in three candidate genes, *SMAD2* (NCBI gene ID: 4087), *SMAD4* (NCBI gene ID: 4089), and *SMAD7* (NCBI gene ID: 4092), all with minor allele frequencies (MAFs) of more than 0.05 in the Japanese population (JPT: Rel 24/phaseII Nov08, on NCBI B36 assembly, dbSNP b126) were selected for genotyping based on the data available on the International HapMap website. Among these SNPs, a total of 20 tag SNPs were selected by pairwise tagging using the Haploview 4.1 software ($r^2 > 0.8$) [18]. Regarding *SMAD3* (NCBI gene ID: 4088), rs4147358, an SNP that was reported to be associated with susceptibility to atopic dermatitis in Japanese and *SMAD3* mRNA expression in peripheral blood mononuclear cells [19], was genotyped. These SNPs were genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR-RFLP), PCR-high resolution melting curve analysis (PCR-HRM), or PCR-direct DNA sequencing (Table 2). Genotyping methods using PCR-RFLP, PCR-HRM, and PCR-direct sequencing were described previously [20]. The SNP rs4147358 was genotyped by PCR-unlabeled probe-HRM (PCR-pHRM), as described before [21]. Briefly, PCR-pHRM was performed in a 20-μL reaction mixture containing 10-ng genomic DNA, 60-nM forward primer, 300-nM reverse primer, with 300 nM of a 3′-amino modifier blocked oligonucleotide probe that targeted the SNP site and its flanking sequence and complemented the reverse primer strand, 2-μM fluorescent DNA intercalating dye SYTO® 9 (Life Technologies, Rockville, MD, USA), and 1× GoTaq® Colorless Master Mix (Promega, Madison, WI, USA). Fifty cycles of PCR amplification followed by a melting program was run using a LightCycler® 480 device (Roche Diagnostics, Basel, Switzerland). Raw melting curve data were analyzed using the melt curve genotyping program of the LightCycler® 480 software version 1.5 (Roche Diagnostics).

Table 1
The general characteristics of study subjects.

Characteristics	Patients with		Control subjects
	UC	CD	
Number	108	81	199
Age (mean ± SD, years)	(44.6 ± 16.9)*	(34.5 ± 13.1)	(32.5 ± 11.2)
Age range (years)	14 - 83	17 - 75	20 - 60
Male/female	56 / 52	49 / 32	126 / 73
(%)	(51.9 / 48.1)	(60.5 / 39.5)	(63.3 / 36.7)

Abbreviation: SD, standard deviation.
* $P < 0.01$ as compared to control subjects.

2.3. Cell culture

The T lymphocyte cell line Jurkat (clone E6-1, TIB-152™) was purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium (WAKO Pure Chemical) supplemented with 10% fetal bovine serum.

2.4. Reporter gene assay

The double-strand oligonucleotides, including A or C allele of *SMAD3* rs4147358, flanking sequences, and additional sticky ends (sense: 5′-CTACATACCAGAGCAGTACTTTGAA a/c TTGATTTTCTCCTC TGCCTCTGTGA-3′; antisense: 5′-CGCGTCACAGAGGCAGAGGAGAAAA TCAA t/g TTCAAAGTACTGCTCTGGTATGTAGGTAC-3′) were inserted into *Kpn*I and *Mlu*I sites of the pGL3-Promoter vector (Promega, Madison, WI). Direct DNA sequencing was performed to confirm successful insertion.

For reporter gene analysis, a day before the transfection, 1.5×10^5 cells were plated in each well of a 24-well plate in 500 μL of culture medium. The constructed plasmid (500 ng) was cotransfected with 25 ng of the *Renilla* luciferase plasmid pRL-CMV (Promega) using 2 μL of the ViaFect™ transfection reagent (Promega). Firefly and *Renilla* luciferase activities were measured 48 h after the transfection with the Dual Luciferase Assay system (Promega). Activity of the firefly luciferase was normalized to that of the *Renilla* luciferase. The value was further normalized to be presented as a value relative to the mean of control plasmid in each experiment.

2.5. Statistical analysis

Differences in age and sex of subjects were compared between healthy subjects and CD or UC patients using the Student's unpaired *t* test and the chi-square test. Relative luciferase activities using the reporter gene assay were compared among three groups (control, A allele, and C allele) by one-way analysis of variance with Tukey's multiple comparison test. Statistical analyses for these tests were performed with Prism statistical software version 5 (GraphPad, San Diego, CA, USA).

To examine whether each genotyped SNP was in Hardy-Weinberg equilibrium in our subjects, the chi-square test with Yate's correction was performed. Frequencies of alleles and genotypes among groups

Table 2
Information regarding SNP genotyping.

Gene	SNP	Sequence of oligonucleotides (5' to 3')		Genotyping method (Restriction enzyme)
SMAD2	rs4940086	F	GACTGATGGGGCCATAAAGA	PCR-RFLP (*Sfa*NI)
		R	GAGGGCCTTACTTGGAACCT	
	rs13381619	F	GGTCCCTATTCCCCATAACC	PCR-RFLP (*Cac*8I)
		R	ATGGTGGAGGGAACTCTGAA	
	rs9955626	F	GCAGGCTAAAGGCTTGGTCT	PCR-RFLP (*Hin*dIII)
		R	TGGTTTAGTCTGCCACTGGA	
	rs1792658	F	GGCATGGATGAATCTGGAAG	PCR-RFLP (*Bstz*17I)
		R	GAAGAAGCAACCCAGGATGA	
	rs1792684	F	TCTGGAGCTGAGGAAATAGACC	PCR-RFLP (*Nsi*I)
		R	TGTTCCCCTGGGTAGGATAA	
	rs1792689	F	AGTAGAGACGGGGTTTCACC	Sequencing
		R	CCGCAAAATGGTTGATCCTC	
	rs8086227	F	GCCATTTGCCAGTCACAAAG	PCR-RFLP (*Bsr*I)
		R	CCAGGTGGTACTTGCTGGTT	
	rs1792671	F	GATGGGGGTCAGAAGGTGTA	PCR-RFLP (*Tas*I)
		R	GCCACAGAGAAAGGAAAACA	
SMAD3	rs4147358	F	GGCCAAGAATTTCCTCAGTG	PCR-pHRM
		R	ACACATACCCCACCACCAAC	
		P	CCAGAGCAGTACTTTGAAATTTGATTTTCT	
SMAD4	rs12968012	F	CATGCCCCAGTCTAGGATCT	PCR-RFLP (*Bsm*AI)
		R	GTCAACAGTGCCAATGCAAG	
	rs10502913	F	GGGGTTGGTTGTCACTGCAG	PCR-HRM
		R	TGAGGGAGCATGGAAAGTTC	
	rs7229678	F	ATCCCCCTAGTGGTGGTTTT	PCR-RFLP (*Mva*I)
		R	AGCATCCCTGGAACCTAACA	
	rs17663887	F	GCCACCAAAACATTGTGTAA	PCR-HRM
		R	TTATGCTATGGACAAGAAGC	
	rs9304407	F	AGGCCTGATTTAGTGGCACA	PCR-RFLP (*Bsr*I)
		R	TTGGCCCACGAATACAAAAT	
SMAD7	rs3736242	F	CCCTGTGAACTGTGAAGTGC	Sequencing
		R	CCGGCTACACAGCACAAATA	
	rs1873190	F	TCAGCTTGGTGACTTTGGTG	PCR-RFLP (*Alu*I)
		R	ATACCTGTGAGCCCTGAGGA	
	rs2337107	F	AGGCAGGGGCAGCTTCTGTA	PCR-RFLP (*Hae*III)
		R	TCCCCCAGATGGTCTTCGTT	
	rs12956924	F	CTAACCCACCGCAGGGAGAC	PCR-RFLP (*Mva*I)
		R	GCAAAGCGTCCACACAGACC	
	rs7229639	F	GGGGACTGGAGCCTGAGATC	PCR-RFLP (*Tsp*RI)
		R	TAGTCCCGGCAGGAGGAGAC	
	rs6507874	F	CTGGCCAACAAAGCCTCTTC	PCR-RFLP (*Map*I)
		R	GCTGCTGCTGACACACTGAG	
	rs16950113	F	CTCCCTCCAAGCAGCAGAAG	PCR-RFLP (*Dra*I)
		R	TCCCCTTCCCTCCAAGAAGG	

Abbreviations: SNP, single nucleotide polymorphism; F, forward primer; R, reverse primer; P, probe; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; HRM, high-resolution melting curve analysis.

were compared by the chi-square test in three inheritance models: minor allele, minor allele dominant, and minor allele recessive. For multiple testing correction of SNP analysis, q values of false discovery rate (FDR) were calculated with the bootstrap method (λ range, 0 to 0.90; step interval, 0.05; bootstrap, 1000). SNP analyses were conducted using the SNPAlyze 7.1 standard software (Dynacom, Chiba, Japan). A p value of less than 0.05 was considered as statistically significant. For SNP association studies, significant p values with an FDR q value of less than 0.05 were considered to indicate significant associations.

3. Results

3.1. SNPs within SMAD genes are associated with UC development

The distribution of alleles and genotypes of 21 SNPs in four genes between UC and healthy subjects were compared. One SNP in *SMAD4*, rs12968012, was not in Hardy-Weinberg equilibrium and therefore excluded from the association study. Conversely, all four genes showed significant associations with UC development (Table 3).

Four tag SNPs (rs13381619, rs9955626, rs1792658, and rs1792671) within *SMAD2* showed significant differences in allele and/or genotype frequencies. The MAFs for the G allele of rs13381619, G allele of rs9955626, and A allele of rs1792671 were higher in the UC patients than the healthy subjects (p = 0.004, 0.027, and 0.015, respectively). In addition, the frequencies of C/G or G/G genotype of rs13381619 (p < 0.001, odds ratio [OR] = 2.400), A/G or G/G genotype of rs9955626 (p = 0.009, OR = 1.918), C/A or A/A genotype of rs1792658 (p = 0.003, OR = 2.482), and G/A or A/A of rs1792671 (p = 0.006, OR = 2.025) were higher in the UC patients than the healthy subjects. These results indicated that the possession of at least one minor allele at each SNP was associated with the risk of UC development.

With respect *SMAD3*, rs4147358 showed a significant difference in the minor allele dominant model. The frequency of A/C or C/C genotype was higher in the UC patients than in the healthy subjects (p = 0.021, OR = 1.974), indicating that the presence of at least one minor allele at the SNP was associated with the risk of UC development.

The two tag SNPs within *SMAD4*, rs7229678 and rs9304407, showed significant differences in the minor allele, minor allele dominant, and minor allele recessive models. The frequency of minor C allele of rs7229678 was lower in the UC patients than in the healthy subjects (p = 0.024). In contrast, the frequency of minor C allele of rs9304407 was higher in the UC patients than in the healthy subjects (p = 0.031). The frequency of C/C genotype of rs9304407 was higher in the UC patients than in the healthy subjects (p = 0.005, OR = 2.255).

Table 3
Allele and genotype comparisons in three inheritance models between control subjects and UC patients.

Gene	SNP		Control	UC	Model*	*P* value	Q value	OR (95% CI)
SMAD2	rs4940086	MAF	0.38	0.35	Allele	0.430	0.195	0.870 (0.616-1.229)
		T/T	74 (0.37)	47 (0.44)				
		T/C	99 (0.5)	47 (0.44)	Dominant	0.278	0.232	0.768 (0.477-1.238)
		C/C	26 (0.13)	14 (0.13)	Recessive	0.980	0.935	0.991 (0.494-1.989)
	rs13381619	MAF	0.12	0.2	Allele	0.004	0.016	1.91 (1.218-2.996)
		C/C	156 (0.78)	65 (0.6)				
		C/G	39 (0.2)	42 (0.39)	Dominant	0.001	0.007	2.4 (1.438-4.006)
		G/G	4 (0.02)	1 (0.01)	Recessive	0.474	0.913	0.456 (0.05-4.128)
	rs9955626	MAF	0.14	0.21	Allele	0.027	0.038	1.619 (1.053-2.488)
		A/A	145 (0.73)	63 (0.58)				
		A/G	51 (0.26)	44 (0.41)	Dominant	0.009	0.023	1.918 (1.17-3.143)
		G/G	3 (0.02)	1 (0.01)	Recessive	0.668	0.927	0.611 (0.063-5.942)
	rs1792658	MAF	0.48	0.53	Allele	0.214	0.142	1.235 (0.886-1.718)
		C/C	60 (0.3)	16 (0.15)				
		C/A	87 (0.44)	69 (0.64)	Dominant	0.003	0.015	2.482 (1.347-4.573)
		A/A	52 (0.26)	23 (0.21)	Recessive	0.347	0.913	0.765 (0.438-1.338)
	rs1792684	MAF	0.45	0.52	Allele	0.116	0.106	1.304 (0.936-1.818)
		C/C	64 (0.32)	23 (0.21)				
		C/T	90 (0.45)	58 (0.54)	Dominant	0.044	0.062	1.752 (1.013-3.032)
		T/T	45 (0.23)	27 (0.25)	Recessive	0.637	0.927	1.141 (0.66-1.973)
	rs1792689	MAF	0.18	0.14	Allele	0.302	0.164	0.785 (0.496-1.243)
		C/C	133 (0.67)	77 (0.71)				
		C/T	62 (0.31)	31 (0.29)	Dominant	0.422	0.301	0.811 (0.487-1.352)
		T/T	4 (0.02)	0 (0)	Recessive	0.138	0.523	-
	rs8086227	MAF	0.29	0.29	Allele	0.908	0.330	0.979 (0.679-1.411)
		G/G	99 (0.5)	55 (0.51)				
		G/A	84 (0.42)	44 (0.41)	Dominant	0.844	0.444	0.954 (0.597-1.524)
		A/A	16 (0.08)	9 (0.08)	Recessive	0.929	0.935	1.04 (0.443-2.439)
	rs1792671	MAF	0.13	0.21	Allele	0.015	0.037	1.713 (1.106-2.653)
		G/G	150 (0.75)	65 (0.6)				
		G/A	45 (0.23)	41 (0.38)	Dominant	0.006	0.018	2.025 (1.225-3.347)
		A/A	4 (0.02)	2 (0.02)	Recessive	0.924	0.935	0.92 (0.166-5.105)
SMAD3	rs4147358	MAF	0.45	0.53	Allele	0.066	0.069	1.365 (0.979-1.903)
		A/A	59 (0.3)	19 (0.18)				
		A/C	99 (0.5)	63 (0.58)	Dominant	0.021	0.041	1.974 (1.104-3.53)
		C/C	41 (0.21)	26 (0.24)	Recessive	0.482	0.913	1.222 (0.699-2.137)
SMAD4	rs10502913	MAF	0.39	0.33	Allele	0.136	0.110	0.767 (0.542-1.087)
		G/G	78 (0.39)	47 (0.44)				
		G/A	87 (0.44)	51 (0.47)	Dominant	0.462	0.308	0.837 (0.52-1.346)
		A/A	34 (0.17)	10 (0.09)	Recessive	0.062	0.389	0.495 (0.234-1.046)
	rs7229678	MAF	0.5	0.41	Allele	0.024	0.038	0.681 (0.487-0.951)
		G/G	51 (0.26)	40 (0.37)				
		G/C	96 (0.48)	48 (0.44)	Dominant	0.037	0.061	0.586 (0.354-0.97)
		C/C	52 (0.26)	20 (0.19)	Recessive	0.133	0.523	0.643 (0.36-1.147)
	rs17663887	MAF	0.04	0.04	Allele	0.847	0.324	0.918 (0.387-2.182)
		T/T	184 (0.92)	100 (0.93)				
		T/C	14 (0.07)	8 (0.07)	Dominant	0.967	0.484	0.981 (0.402-2.394)
		C/C	1 (0.01)	0 (0)	Recessive	0.461	0.913	-
	rs9304407	MAF	0.39	0.48	Allele	0.031	0.038	1.444 (1.034-2.018)
		G/G	74 (0.37)	35 (0.32)				
		G/C	96 (0.48)	43 (0.4)	Dominant	0.403	0.301	1.235 (0.753-2.026)
		C/C	29 (0.15)	30 (0.28)	Recessive	0.005	0.048	2.255 (1.267-4.013)

(*continued on next page*)

Table 3 (*continued*)

Gene	SNP		Control	UC	Model*	*P* value	Q value	OR (95% CI)
SMAD7	rs3736242	MAF	0.32	0.31	Allele	0.724	0.292	0.938 (0.656-1.34)
		G/G	91 (0.46)	51 (0.47)				
		G/A	87 (0.44)	47 (0.44)	Dominant	0.802	0.444	0.942 (0.589-1.506)
		A/A	21 (0.11)	10 (0.09)	Recessive	0.719	0.927	0.865 (0.392-1.91)
	rs1873190	MAF	0.4	0.39	Allele	0.706	0.292	0.937 (0.667-1.315)
		C/C	75 (0.38)	44 (0.41)				
		C/T	87 (0.44)	44 (0.41)	Dominant	0.600	0.375	0.88 (0.545-1.421)
		T/T	37 (0.19)	20 (0.19)	Recessive	0.987	0.935	0.995 (0.545-1.818)
	rs2337107	MAF	0.44	0.4	Allele	0.316	0.164	0.842 (0.601-1.178)
		G/G	58 (0.29)	34 (0.31)				
		G/A	105 (0.53)	61 (0.56)	Dominant	0.670	0.394	0.895 (0.539-1.488)
		A/A	36 (0.18)	13 (0.12)	Recessive	0.167	0.526	0.62 (0.313-1.226)
	rs12956924	MAF	0.29	0.4	Allele	0.003	0.016	1.68 (1.186-2.38)
		G/G	99 (0.5)	42 (0.39)				
		G/A	86 (0.43)	45 (0.42)	Dominant	0.068	0.085	1.556 (0.966-2.505)
		A/A	14 (0.07)	21 (0.19)	Recessive	0.001	0.021	3.19 (1.548-6.571)
	rs7229639	MAF	0.2	0.16	Allele	0.210	0.142	0.754 (0.485-1.173)
		G/G	129 (0.65)	78 (0.72)				
		G/A	61 (0.31)	26 (0.24)	Dominant	0.187	0.207	0.709 (0.425-1.183)
		A/A	9 (0.05)	4 (0.04)	Recessive	0.734	0.927	0.812 (0.244-2.701)
	rs6507874	MAF	0.21	0.18	Allele	0.298	0.164	0.798 (0.522-1.221)
		C/C	123 (0.62)	74 (0.69)				
		C/T	68 (0.34)	30 (0.28)	Dominant	0.242	0.232	0.744 (0.453-1.222)
		T/T	8 (0.04)	4 (0.04)	Recessive	0.891	0.935	0.918 (0.27-3.122)
	rs16950113	MAF	0.15	0.18	Allele	0.382	0.185	1.217 (0.783-1.892)
		T/T	143 (0.72)	71 (0.66)				
		T/C	51 (0.26)	35 (0.32)	Dominant	0.265	0.232	1.331 (0.804-2.202)
		C/C	5 (0.03)	2 (0.02)	Recessive	0.711	0.927	0.732 (0.14-3.838)

Abbreviations: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval *Allele, allele model; Dominant, the minor allele dominant model; Recessive, the minor allele recessive model.

Although an association of rs7229678 in the minor allele dominant model did not reach statistical significance after multiple test correction, the frequency of G/C or C/C genotype of the SNP was lower in the UC patients than in the healthy subjects ($p = 0.037$, OR = 0.586). Conversely, the G/G genotype of rs7229678 was more frequent in the UC patients than in the healthy subjects (OR = 1.706). These results indicated that the G/G genotype of rs7229678 and the C/C genotype of rs9304407 were associated with the risk of UC development.

Finally, rs12956924 within *SMAD7* showed significant differences in the minor allele and minor allele recessive models. The frequencies of the minor A allele ($p = 0.003$) and the homozygous A/A genotype ($p = 0.001$, OR = 3.190) were higher in the UC patients than in the healthy subjects. These results indicated that the G/G genotype of rs12956924 was associated with the risk of UC development.

3.2. SNPs within SMADs are not associated with CD development

The distribution of alleles and genotypes at 21 SNPs in four genes were also compared between the CD cases and healthy subjects, and the SNP rs12968012 within *SMAD4* was excluded from the association study. Only *SMAD2* showed a significant association with CD development, although the association did not meet the correction of multiple testing (Table 4).

Within *SMAD2*, the frequency of A/A genotype of rs1792658 was lower in the CD patients than in the healthy subjects ($p = 0.041$, OR = 0.492). However, the FDR q value of the association was above 0.05, indicating that the genes in the SMAD signaling pathway were not associated with CD development in the current study cohort.

3.3. The SMAD3 SNP associated with UC development alters transcription of a reporter gene

We next conducted a reporter gene assay to dissect the mechanism underlying the contribution of *SMAD* genes to UC development. Among the associated SNPs, *SMAD3* rs4147358 was previously shown to be associated with atopic dermatitis in Japanese and *SMAD3* mRNA expression in peripheral blood mononuclear cells [19]. In addition, *in silico* analysis using FATHMM [22] (http://fathmm.biocompute.org.uk) and RegulomDB [23] (http://regulomedb.org/index) indicated that this SNP had an effect on *SMAD3* expression. Therefore, rs4147358 in *SMAD3* was chosen for further analysis in the reporter gene assay.

The relative luciferase activity of the plasmid carrying the C allele of the SNP, *i.e.*, the risk allele for UC, was significantly higher than that of the A allele or the empty vector (Fig. 1), suggesting that *SMAD3* expression might be increased in subjects carrying the C allele of rs4147358.

4. Discussion

The current study demonstrated an association of *SMAD3*, which was reported as an IBD susceptibility gene in European ancestry [2,3], with UC development in a Japanese cohort. In addition, *SMAD2, SMAD4*, and *SMAD7* genes were also associated with the UC development. Results of the reporter gene assay revealed that the transcription of the reporter gene was higher with the risk allele of *SMAD3* rs4147358, one of the UC susceptibility SNPs. Overall, these results indicated that the genes involved in SMAD signaling were associated with UC development in a Japanese genetic background, at least partially through the modulation of SMAD protein expression.

The balance between pro-inflammatory effector Th cells and anti-

Table 4
Allele and genotype comparisons in three inheritance models between control subjects and CD patients.

Gene	SNP		Control	CD	Model*	*P* value	Q value	OR (95% CI)
SMAD2	rs4940086	MAF	0.38	0.34	Allele	0.375	0.150	1.189 (0.811-1.745)
		T/T	74 (0.37)	35 (0.43)				
		T/C	99 (0.5)	37 (0.46)	Dominant	0.349	0.229	0.778 (0.46-1.316)
		C/C	26 (0.13)	9 (0.11)	Recessive	0.654	0.923	0.832 (0.371-1.863)
	rs13381619	MAF	0.12	0.14	Allele	0.438	0.150	1.236 (0.723-2.112)
		C/C	156 (0.78)	59 (0.73)				
		C/G	39 (0.2)	21 (0.26)	Dominant	0.318	0.229	1.353 (0.746-2.452)
		G/G	4 (0.02)	1 (0.01)	Recessive	0.657	0.923	0.609 (0.067-5.537)
	rs9955626	MAF	0.14	0.15	Allele	0.736	0.155	1.092 (0.655-1.819)
		A/A	145 (0.73)	57 (0.7)				
		A/G	51 (0.26)	23 (0.28)	Dominant	0.673	0.283	1.131 (0.639-2)
		G/G	3 (0.02)	1 (0.01)	Recessive	0.861	0.977	0.817 (0.084-7.969)
	rs1792658	MAF	0.48	0.43	Allele	0.304	0.150	0.824 (0.571-1.190)
		C/C	60 (0.3)	23 (0.28)				
		C/A	87 (0.44)	46 (0.57)	Dominant	0.771	0.288	1.089 (0.616-1.925)
		A/A	52 (0.26)	12 (0.15)	Recessive	0.041	0.634	0.492 (0.247-0.98)
	rs1792684	MAF	0.45	0.47	Allele	0.716	0.155	1.07 (0.742-1.544)
		C/C	64 (0.32)	24 (0.3)				
		C/T	90 (0.45)	38 (0.47)	Dominant	0.679	0.283	1.126 (0.642-1.975)
		T/T	45 (0.23)	19 (0.23)	Recessive	0.879	0.977	1.049 (0.569-1.934)
	rs1792689	MAF	0.18	0.2	Allele	0.547	0.155	1.153 (0.725-1.836)
		C/C	133 (0.67)	53 (0.65)				
		C/T	62 (0.31)	24 (0.3)	Dominant	0.822	0.288	1.065 (0.617-1.836)
		T/T	4 (0.02)	4 (0.05)	Recessive	0.182	0.923	2.533 (0.618-10.381)
	rs8086227	MAF	0.29	0.31	Allele	0.686	0.155	1.085 (0.729-1.615)
		G/G	99 (0.5)	36 (0.44)				
		G/A	84 (0.42)	40 (0.49)	Dominant	0.421	0.229	1.238 (0.737-2.079)
		A/A	16 (0.08)	5 (0.06)	Recessive	0.591	0.923	0.753 (0.266-2.127)
	rs1792671	MAF	0.13	0.15	Allele	0.641	0.155	1.132 (0.672-1.906)
		G/G	150 (0.75)	58 (0.72)				
		G/A	45 (0.23)	22 (0.27)	Dominant	0.513	0.244	1.214 (0.679-2.17)
		A/A	4 (0.02)	1 (0.01)	Recessive	0.657	0.923	0.609 (0.067-5.537)
SMAD3	rs4147358	MAF	0.45	0.48	Allele	0.659	0.155	1.086 (0.753-1.566)
		A/A	59 (0.3)	19 (0.23)				
		A/C	99 (0.5)	47 (0.58)	Dominant	0.295	0.229	1.375 (0.757-2.499)
		C/C	41 (0.21)	15 (0.19)	Recessive	0.693	0.923	0.876 (0.454-1.69)
SMAD4	rs10502913	MAF	0.39	0.36	Allele	0.487	0.150	0.874 (0.598-1.277)
		G/G	78 (0.39)	37 (0.46)				
		G/A	87 (0.44)	30 (0.37)	Dominant	0.317	0.229	0.767 (0.455-1.292)
		A/A	34 (0.17)	14 (0.17)	Recessive	0.968	0.983	1.014 (0.512-2.01)
	rs7229678	MAF	0.5	0.54	Allele	0.459	0.150	1.148 (0.749-1.656)
		G/G	51 (0.26)	20 (0.25)				
		G/C	96 (0.48)	35 (0.43)	Dominant	0.870	0.290	1.051 (0.579-1.909)
		C/C	52 (0.26)	26 (0.32)	Recessive	0.312	0.923	1.336 (0.761-2.348)
	rs17663887	MAF	0.04	0.07	Allele	0.165	0.150	1.739 (0.789-3.834)
		T/T	184 (0.92)	71 (0.88)				
		T/C	14 (0.07)	9 (0.11)	Dominant	0.201	0.229	1.728 (0.742-4.025)
		C/C	1 (0.01)	1 (0.01)	Recessive	0.510	0.923	2.475 (0.153-40.054)
	rs9304407	MAF	0.39	0.34	Allele	0.293	0.150	0.814 (0.556-1.194)
		G/G	74 (0.37)	35 (0.43)				
		G/C	96 (0.48)	37 (0.46)	Dominant	0.349	0.229	0.778 (0.46-1.316)
		C/C	29 (0.15)	9 (0.11)	Recessive	0.443	0.923	0.733 (0.33-1.626)

(*continued on next page*)

Table 4 (*continued*)

Gene	SNP		Control	CD	Model*	*P* value	Q value	OR (95% CI)
SMAD7	rs3736242	MAF	0.32	0.31	Allele	0.831	0.166	0.958 (0.647-1.419)
		G/G	91 (0.46)	33 (0.41)				
		G/A	87 (0.44)	45 (0.56)	Dominant	0.446	0.229	1.226 (0.726-2.069)
		A/A	21 (0.11)	3 (0.04)	Recessive	0.063	0.634	0.326 (0.095-1.125)
	rs1873190	MAF	0.4	0.36	Allele	0.376	0.150	0.843 (0.578-1.23)
		C/C	75 (0.38)	32 (0.4)				
		C/T	87 (0.44)	39 (0.48)	Dominant	0.777	0.288	0.926 (0.545-1.573)
		T/T	37 (0.19)	10 (0.12)	Recessive	0.205	0.923	0.617 (0.291-1.308)
	rs2337107	MAF	0.44	0.39	Allele	0.226	0.150	0.794 (0.547-1.764)
		G/G	58 (0.29)	29 (0.36)				
		G/A	105 (0.53)	41 (0.51)	Dominant	0.275	0.229	0.738 (0.427-1.275)
		A/A	36 (0.18)	11 (0.14)	Recessive	0.360	0.923	0.712 (0.343-1.478)
	rs12956924	MAF	0.29	0.35	Allele	0.167	0.150	1.316 (0.891-1.944)
		G/G	99 (0.5)	32 (0.4)				
		G/A	86 (0.43)	42 (0.52)	Dominant	0.119	0.229	1.516 (0.897-2.563)
		A/A	14 (0.07)	7 (0.09)	Recessive	0.644	0.923	1.25 (0.485-3.221)
	rs7229639	MAF	0.2	0.14	Allele	0.116	0.150	0.668 (0.403-1.108)
		G/G	129 (0.65)	61 (0.75)				
		G/A	61 (0.31)	17 (0.21)	Dominant	0.089	0.229	0.604 (0.337-1.082)
		A/A	9 (0.05)	3 (0.04)	Recessive	0.759	0.949	0.812 (0.214-3.079)
	rs6507874	MAF	0.21	0.17	Allele	0.305	0.150	0.781 (0.487-1.254)
		C/C	123 (0.62)	54 (0.67)				
		C/T	68 (0.34)	26 (0.32)	Dominant	0.445	0.229	0.809 (0.47-1.393)
		T/T	8 (0.04)	1 (0.01)	Recessive	0.231	0.923	0.298 (0.037-2.426)
	rs16950113	MAF	0.15	0.2	Allele	0.148	0.150	1.413 (0.884-2.26)
		T/T	143 (0.72)	50 (0.62)				
		T/C	51 (0.26)	29 (0.36)	Dominant	0.097	0.229	1.583 (0.919-2.728)
		C/C	5 (0.03)	2 (0.02)	Recessive	0.983	0.983	0.982 (0.187-5.169)

Abbreviations: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval *Allele, allele model; Dominant, the minor allele dominant model; Recessive, the minor allele recessive model.

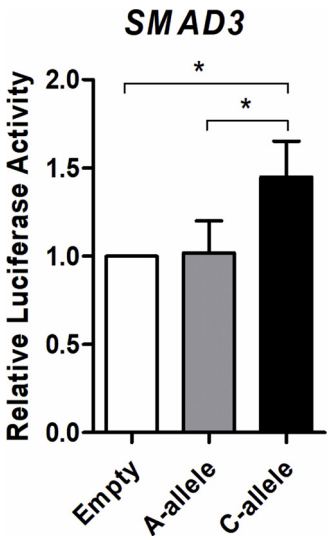

Fig. 1. Transcriptional activities of reporter genes affected by rs4147358. The DNA fragment including either the A or C allele of rs4147358 was inserted to the firefly gene plasmid pGL3-Promoter, and luciferase activity was measured 48 h after plasmid transfection of Jurkat cells. Firefly luciferase signal was normalized to that of *Renilla* luciferase. Data shown represent four independent experiments and presented as mean ± standard deviation. *$p < 0.05$, one-way analysis of variance with *post hoc* Tukey's test.

inflammatory T_{reg} cells is altered in the inflamed gut of IBD patients [7,8]. Targeted disruption of SMAD3 was shown to result in gut inflammation [24], and SMAD3 was demonstrated to be necessary for the expression of FOXP3, a master transcription factor of T_{reg} cell [25]. In addition, SMAD4 deficiency led to the increased activity of Th17 cells in the gut lamina propria and decreased *in vitro* polarization of $FOXP3^+$/$CD4^+$ T cells [26]. In the gut of IBD patients, increased SMAD7 inhibits the phosphorylation of SMAD3. In addition, blocking SMAD7 was shown to restore SMAD3 phosphorylation and ameliorate inflammation *in vitro* and *in vivo* [14,27]. These data imply key roles for the SMAD signaling in Th17 and T_{reg} cell differentiation in the context of gut inflammation, although the precise role of the TGF-β/SMAD pathway in IBD development remains unclear.

In the current study, we found an association between *SMAD3* and UC development. *SMAD3* was previously reported to be associated with both CD and UC in European ancestry, but such an association was not shown in a Japanese population. The *SMAD3* SNP rs17293632, which was associated with IBD in European ancestry, was distinct from and not associated with rs4147358, the novel SNP identified in the current study. We did not include rs17293632 in the current study, as the MAF of this SNP in the Japanese population was less than the MAF threshold of 0.05 used for the analysis. Specifically, the MAF of rs17293632 was much lower in Japanese (0.017 in HapMap JPT) than in Europeans (0.23 in HapMap CEU). Although it is not clear how rs17293632 and rs4147358 affect the gene function each other, the association of rs17293632 might mask the association of rs4147358 in previous European studies. In addition, there is a possibility that lack of association between *SMAD3* SNPs and IBD in previous Japanese GWA studies might result from the lower MAF of rs17293632, resulting in a relatively low statistical power compared with the European GWA studies.

In the cell-based assay, the reporter gene plasmid, which contained the SNP sequence and a firefly luciferase gene that was regulated by the SV40 promoter, exhibited a different luciferase activity than the control vector. The luciferase activity of the plasmid carrying the C allele

associated with the UC risk was higher than that carrying another allele in Jurkat cells, a T cell leukemia cell line. A previous study that showed increased *SMAD3* mRNA in peripheral blood mononuclear cells of subject with the C/C genotype of rs4147358 supports our *in vitro* data [19]. These results indicate that *SMAD3* expression might be higher in T cells of subjects with the C allele than those without the C allele of rs4147358. However, this proposed mechanism is in contrast to the previously speculated hypothesis, which states that lower expression of *SMAD3* due to the presence of the risk allele decreases the expression of FOXP3 and subsequently leads to altered T_{reg} function. Although it remains clear whether the inhibition of SMAD3 phosphorylation in the gut of IBD patients is a cause or a consequence of IBD, the results of the current study highlight a new aspect of SMAD3 function in disease development. Unphosphorylated SMAD3 was previously demonstrated to repress Th17 differentiation [28], whereas antigen-specific immunoglobulin A (IgA) was induced in the Peyer's patches not by $FOXP3^+$ regulatory T cells but by Th17 cells [29]. Gut mucosal IgA is involved in the maintenance of gut microbiota [30], as illustrated by the findings that disrupted IgA function exacerbated experimental colitis in mice [31,32] and that colitogenic bacteria were highly coated with IgA in the gut of IBD patients [33]. Higher basal expression of SMAD3 is suggested to repress Th17 cell differentiation, with a decrease in specific IgA in the intestines, resulting in increased susceptibility to IBD. However, further studies are necessary to reveal the precise role of SMAD3 in disease pathogenesis.

In addition to *SMAD3*, we demonstrated associations of *SMAD2*, *SMAD4,* and *SMAD7* with UC development. A locus flanking *SMAD7* showed an association with IBD in a genome-wide meta-analysis of European ancestry [4], supporting our finding. The associations of *SMAD2* and *SMAD4* with UC are novel findings of the current study, and these associations are likely specific to the Japanese population. The GWA studies in Japan reproduced 11% of the associations found in European GWA studies and identified East Asia-specific IBD susceptibility loci, implying the presence of similarities as well as differences in the genetic architecture of IBD susceptibility between the Europeans and the Japanese [5]. As the SNPs within *SMAD2/4/7* were selected as tag SNPs, dissecting the functions of associated SNPs or flanking causal SNPs is challenging. Although elevated *SMAD3* expression was suggested to associate with susceptibility to UC, it remains difficult to demonstrate that UC development is driven either by increased or decreased expression of SMAD2 and SMAD4 as SMAD2 and SMAD3 have different and/or opposing functions in certain signaling pathways. Indeed, a recent study demonstrated that the loss of SMAD2 and SMAD3 led to decreased and increased FOXP3 expression, respectively, implying opposing roles of SMAD2 and SMAD3 in T cell differentiation [28].

In the current study, we demonstrated associations between *SMAD* gene polymorphisms and susceptibility to UC. We previously demonstrated that tyrosine kinase 2 (TYK2) and signal transducer and activator of transcription 3 (STAT3), which are proposed to be involved in Th17 differentiation as well as the SMAD pathway, were associated with susceptibility to CD but not to UC [34]. Although our initial hypothesis was based on the anti-inflammatory roles of SMAD2/3/4 which are inhibited by SMAD7 in T cells, it might be important to focus also on their roles in different cell and tissue types such as the epithelial cells of the intestines.

Disclosure of conflicts of interest

There are no conflicts of interest to declare.

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