ORIGINAL ARTICLE

Association of *CARD8* with inflammatory bowel disease in Koreans

Suk-Kyun Yang¹, Hyeri Kim², Myunghee Hong², Jiyoung Lim², Eunkyong Choi², Byong Duk Ye¹, Soo-Kyung Park¹ and Kyuyoung Song²

Caspase recruitment domain (CARD)-containing protein 8 (*CARD8*) is a potential candidate risk gene for inflammatory bowel disease (IBD) because of its role as a component of the NALP3 inflammasome and as an inhibitor of nuclear factor-kappa B. Previous studies examining the association of a *CARD8* single-nucleotide polymorphism (SNP) (rs2043211, p.Cys10X) with IBD yielded mixed results in Caucasians that may result from interaction with *NALP3* or *NOD2* (nucleotide-binding oligomerization domain 2) variants. To understand the genetic association between *CARD8/NALP3* and IBD in Koreans, we investigated seven *CARD8*, four *NALP3* and four *NOD2* SNPs in 650 Crohn's disease (CD), 660 ulcerative colitis (UC) patients and 688 controls from the Korean population. rs2043211 of *CARD8* showed significant association with UC ($P=0.011$; odds ratio=1.50, 95% confidence intervals=1.12–2.00, $P=0.006$ under recessive model). In contrast, an SNP in intron 1, rs1972619, was associated with CD only ($P=0.033$). None of the *NALP3* or *NOD2* SNPs was significantly associated with CD or UC in the Korean populations. The stop allele of rs2043211 was associated with higher serum interleukin-1 β levels only in female patients with UC ($P=0.027$). Our data suggest that *CARD8* variants might have roles in the pathogenesis of CD and UC in Koreans. *Journal of Human Genetics* (2011) 56, 217–223; doi:10.1038/jhg.2010.170; published online 20 January 2011

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic recurrent disorders, clinically composed of Crohn's disease (CD) and ulcerative colitis (UC). Among the two IBD subtypes, a genetic contribution to CD risk is better documented than that of UC.^{1–10} To date, linkage and genome-wide association studies in Caucasian populations have identified more than 30 CD susceptibility loci.^{1–6} Some CD candidate genes were also associated with UC in Caucasians.^{11,12}

Although recent genome-wide association studies have successfully mapped susceptibility loci for IBD, novel potential candidate genes remain to be identified. Caspase recruitment domain (CARD)-containing protein 8 (*CARD8*) is a potential candidate risk gene for IBD, based on gene location (in the IBD 6 locus) and biological function.^{13–16} *CARD8* acts as a negative regulator of nuclear factor-kappa B^{13,14} and is a suppressor of apoptosis.^{15,16} Recent studies found that *CARD8* interacted with NALP3 (NACHT; *NAIP*, *CIIA*, *HET-E*, and *TP1*; Neuronal apoptosis inhibitor protein (NAIP), MHC Class II transcription activator (*CIIA*), Incompatibility locus protein from *Podospira anserina* (*HET-E*), and Telomerase-associated protein (*TP1*) domain, leucine-rich repeat and pyrin domain-containing protein 3) and NOD2 (nucleotide-binding oligomerization

domain 2), members of the NOD-like receptor family, implying a role for *CARD8* in the regulation of multiple inflammatory pathways.^{17,18}

Tumor necrosis factor- α , interleukin-1 β (IL-1 β), and IL-6 are characteristically overproduced in CD patients.¹⁹ Production of mature IL-1 β requires two distinct events: enhanced precursor synthesis stimulated by nuclear factor-kappa B and precursor processing through caspase-1 activation accomplished by a caspase-1-activating protein complex known as the inflammasome.²⁰ *CARD8* has an important role in cytokine regulation by interacting with the NALP3 inflammasome.^{21,22} This inflammasome, consisting of *CARD8*, ASC (apoptosis-associated speck-like protein), and NALP3, regulates the activity of caspase-1 and the maturation and release of IL-1 β . NOD2 is additionally implicated in caspase-1 activation through the NALP1 inflammasome, in the release of active IL-1 β , and in pro-IL-1 β transcription.^{23,24} Moreover, *CARD8* was shown to interact with NOD2 and negatively regulate NOD2-mediated signaling by interfering with NOD2 oligomerization.¹⁸ It remains unclear whether NOD2 requires NALP3 and/or NALP1 for activation of caspase-1. Pan *et al.*¹⁷ showed that both NOD2 and NALP3 were necessary for IL-1 β secretion after stimulation of murine macrophages

¹Department of Gastroenterology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea and ²Department of Biochemistry and Molecular Biology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea

Correspondence: Dr K Song, Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, 388-1 Pungnap-Dong, Songpa-Gu, Seoul 138-736, Korea.

E-mail: kysong@amc.seoul.kr

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by muramyl dipeptides. In contrast, Hsu *et al.*²³ reported that NOD2 interacted with NALP1, but not with NALP3.

Earlier studies examining the association of a nonsynonymous single-nucleotide polymorphism (SNP) (C10X) in *CARD8* with IBD reported conflicting results in Caucasians.^{25–28} Some reported a positive association of C10X with CD; but, the associated alleles were inconsistent.^{25,26} Other studies, including a genome-wide association studies, found no evidence of CD association with any allele.^{5,27,28} Subsequent reports found that patients homozygous for the C10X polymorphism still expressed an immunoreactive isoform of *CARD8*, although at a reduced level.²⁹ Recently, variants in another component of the NALP3 inflammasome, *NALP3* and/or *NOD2*, and a combination of *NALP3* (Q705K) and *CARD8* (C10X) genotypes, were found to be associated with disease severity of rheumatoid arthritis^{30,31} and CD in the absence of *NOD2* mutations.^{32,33}

Analysis of *CARD8*, *NALP3* and *NOD2* in the Korean UC and CD patients of this study found no evidence of *NALP3* or *NOD2* association with either disease. However, *CARD8* was positively associated with CD and/or UC in our study population. Interestingly, the SNPs associated with the two diseases were distinct, suggesting that the pathogenic mechanisms of CD and UC overlap but remain discrete. We additionally observed a correlation between presence of the stop allele of rs2043211 and higher serum IL-1 β levels, but only in female patients with UC.

MATERIALS AND METHODS

Study population

The study population consisted of 650 CD patients (441 men and 209 women), 660 UC patients (344 men and 316 women) and 688 unrelated healthy controls (351 men and 337 women). Part of this cohort was studied previously.^{34–36} All patients with CD or UC were diagnosed at the IBD Clinic of Asan Medical Center, Seoul, Korea on the basis of clinical, radiologic, endoscopic and histopathologic criteria.^{37–39} Patients with indeterminate colitis were excluded from the study. Disease-free individuals recruited from the Health Promotion Center of the Asan Medical Center (based on routine health checkups) were employed as controls. The phenotypic subgroups of CD and UC were determined by two independent investigators (SKY and SKP in CD; SKY and BDY in UC), blinded to the results of the genotype analysis, using the Montreal classification with minor modifications.^{35,40} Briefly, CD patients were subgrouped according to age at diagnosis (A1: ≤ 16 years; A2: 17–40 years; A3: ≥ 40 years), disease location (L1, ileum; L2, colon; L3, ileocolon) and disease behavior (B1, inflammatory; B2, stricturing; B3, penetrating). Upper gastrointestinal (GI) and perianal disease modifiers of the Montreal system were not included in our classification scheme. UC patients were grouped on the basis of maximal endoscopic extent as E1 (ulcerative proctitis), E2 (left-sided UC), or E3 (extensive UC). The demographic and clinical characteristics of the study population are summarized in Table 1. Gender distributions were statistically similar in the UC and control groups, but not age distributions. Neither gender nor age distributions were similar between the CD and control groups. This study was approved by the Institutional Review Board of Asan Medical Center, and written informed consent was obtained from all subjects.

SNP genotyping

Genomic DNA was isolated from 5 ml samples of ethylenediaminetetraacetic acid (EDTA) anticoagulated venous blood by standard methods using proteinase K and phenol/chloroform extraction. In total, 16 SNPs were selected and genotyped, including seven *CARD8* (rs1965759, rs1062808, rs4389238, rs2288877, rs2043211, rs2288876 and rs1972619), five *NALP3* (rs10802501, rs1539019, rs2027432, rs76291085 and rs35829419) and four *NOD2* (rs8057341, rs1861759, rs751271 and rs1077861). Samples were genotyped using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based system (Sequenom, San Diego, CA, USA) at the Analytical Genetics Technology Centre, Princess Margaret Hospital/University Health Network in Toronto, Canada. Genotype call rates were $>97.5\%$: markers

Table 1 Clinical characteristics of the study subjects

	CD (n=650)	UC (n=660)	Controls (n=688)
	No. (%)	No. (%)	No. (%)
Sex			
Male	441 (67.8)	344 (52.1)	351 (51.0)
Female	209 (32.2)	316 (47.9)	337 (49.0)
Mean age at diagnosis (year)	24.3 $\pm$ 7.7	36.6 $\pm$ 12.9	
Mean age at sampling (year)	27.4 $\pm$ 8.1	40.8 $\pm$ 13.3	31.5 $\pm$ 11.9
Age group at diagnosis			
≤ 16	63 (9.7)	21 (3.2)	
17–40	560 (86.2)	374 (56.7)	
≥ 40	27 (4.1)	265 (40.2)	
Location			
Ileum	123 (18.9)		
Colon	31 (4.8)		
Ileocolon	496 (76.3)		
Extent			
Proctitis		200 (30.3)	
Left-sided colitis		208 (31.5)	
Extensive colitis		252 (38.2)	
Behavior			
Inflammatory	296 (45.5)		
Stricturing	135 (20.8)		
Penetrating	219 (33.7)		
Perianal fistula			
No	278 (42.8)		
Yes	372 (57.2)		

Abbreviations: CD, Crohn's disease; UC, ulcerative colitis.

rs1965759, rs1062808, rs4389238 and rs1972619 were typed in 601 controls, and markers rs2288877, rs2043211 and rs2288876 were typed in 688 controls.

Measurement of the IL-1 β concentration in serum

Serum samples were stored at -20°C . Quantitative detection of IL-1 β in serum was performed using human IL-1 β enzyme-linked immunosorbent assay kit II (BD Biosciences, San Diego, CA, USA). In brief, microtiter plates (BD Biosciences) were coated with 100 μl per well of a mouse monoclonal antibody against IL-1 β , according to the manufacturers' instructions. Standards and serially diluted human serum samples were added to the wells and incubated for 2 h at room temperature. After washing five times with washing buffer, 100 μl of biotinylated antihuman IL-1 β antibody was added and incubated for 1 h at room temperature. Following a second wash, 100 μl of streptavidin-horseradish peroxidase was added to the wells and incubated for 30 min. Color was developed by adding 100 μl of tetramethyl-benzidine substrate solution, and the color reaction stopped by the addition of 50 μl of STOP solution. Optical density was measured at 450 nm using an enzyme-linked immunosorbent assay plate reader. A standard curve was prepared on the basis of seven IL-1 β dilutions, and the IL-1 β sample concentrations determined. The assay had a detection range of 1.95–125 pg ml $^{-1}$.

Statistical analysis

Genotype frequencies between cases and controls were assessed separately for consistency with those expected from Hardy–Weinberg equilibrium using SNP Alyze software, Version 5.0 (Dynacom, Yokohama, Japan). The associations were examined by comparing allele and genotype frequencies from different groups of subjects using the χ^2 -test with a 1000 permutation correction in

PLINK 2.050 (<http://pngu.mgh.harvard.edu/purcell/plink/>)⁴¹ and Version 4.2 of the Haploview program (<http://www.broadinstitute.org/haploview/haploview>). Allelic frequencies were compared between cases and controls using logistic regression to calculate age- and gender-adjusted odds ratios (OR) and 95% confidence intervals (CI). Genotype–phenotype analyses were performed for SNPs in situations where the *P*-value was less than 0.2 in case-control analysis. Additionally, logistic regression analyses were conducted to examine any significant associations between polymorphisms and disease phenotypes.

The pair-wise linkage disequilibrium values, *D'* and *r*², and *P*-values obtained from χ^2 -tests were calculated using the SNP Alyze software package and Version 4.2 of the Haploview program. Pair-wise linkage disequilibrium among the seven polymorphisms in *CARD8* spanning 43 349 base pairs on chromosome 19q13.3 was rather low ($0.08 < r^2 < 0.51$) (data not shown). The results of haplotype analyses were similar to those of single marker analyses. Differences in the IL-1 β levels among various genotype groups were evaluated using analysis of variance and the *t*-test. Statistical significance was assumed at *P* < 0.05. Data were analyzed using SPSS version 18.0.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Association analysis of *CARD8* polymorphisms with IBD in Koreans

Seven SNPs of *CARD8* were analyzed in 1 310 IBD patients (650 with CD and 660 with UC) and 688 healthy controls. The SNPs examined did not significantly deviate from Hardy–Weinberg equilibrium. Moreover, no SNP showed a significant association with IBD in Koreans. The differences in the allele frequencies of the seven SNPs between controls and IBD patients ranged from 0.1–1.7%, and did not attain statistically significant levels (data not shown). However, upon separate association analyses for CD and UC, six SNPs showed opposite risk alleles and the SNPs associated with CD and UC were different (Tables 2 and 3).

Association analysis of *CARD8* polymorphisms with UC in Koreans

The genotype distributions of the seven SNPs in UC are summarized in Table 2. Results are expressed as adjusted ORs (aOR) using logistic regression analysis with adjustment for gender and age.

Among the seven SNPs genotyped, rs2043211, a nonsynonymous SNP of *CARD8* (C10X), showed the most significant difference in allele frequency (4.9%) between UC patients and controls (*P* = 0.011). However, the difference between control and CD groups was 1.4%, in the opposite direction (Table 3). The difference in stop allele frequency between CD and UC patients was 6.3% and statistically significant (*P* = 0.001). The aOR of rs2043211 for the TT genotype in comparison with the combined genotype (TA and AA) was 1.50 (95% CI = 1.12–2.00, *P* = 0.006).

Next, we analyzed association by gender. The only marker showing a statistically significant frequency difference between men and women was rs2043211. The difference was 6.8% in men and 2.8% in women, and statistical significance was thus evident in men only (*P* = 0.011). The aOR of rs2043211 for the TT genotype in comparison with the combined genotype (TA and AA) was 1.70 (95% CI = 1.16–2.50, *P* = 0.007) in men and 1.27 (95% CI = 0.81–1.99, *P* = 0.302) in women, suggesting that men predominantly bore the increased UC risk.

Association analysis of *CARD8* polymorphisms with CD in Koreans

The genotype distributions of the seven SNPs in CD patients are summarized in Table 3. Results are expressed as aORs using logistic regression analysis, with adjustment for gender and age.

A χ^2 -test comparing allele and genotype frequencies between CD patients and controls revealed that rs1972619 located in intron 1 of *CARD8* was of a marginal significance (*P* = 0.033, *P* = 0.022, respectively). The difference in allele frequency between controls and CD

patients was 3.9%. However, the difference between control and UC groups was 2.6% in the opposite direction (Table 2). Therefore, the difference in rs1972619 allele frequency between UC and CD patients (6.5%) was statistically significant (*P* = 4.0×10^{-4}). The aOR of rs1972619 for the GG genotype in comparison with the AA genotype was 2.00 (95% CI = 1.23–3.26, *P* = 0.005). Another SNP in intron 8, rs4389238, showed a 3.3% difference in allele frequency between controls and CD patients. The minor allele of rs4389238 showed a marginal association with CD under the recessive model (aOR = 2.00, 95% CI = 1.17–3.41, *P* = 0.012). In contrast, the nonsynonymous SNP, rs2043211, which was significantly associated with UC, failed to show any association with CD, and the frequency of the Cys allele was higher in CD patients than in controls (0.590 vs 0.576).

The only marker showing a weakly significant difference in allele frequency between men and women was rs4389238; specifically, the difference was 4.6% in men and 2.6% in women, indicating marginal significance in men (*P* = 0.041). The aOR of rs4389238 for the TT genotype in comparison with the CC genotype was 3.04 (95% CI = 1.33–6.93, *P* = 0.008) in men and 1.35 (95% CI = 0.63–2.89, *P* = 0.439) in women, suggesting an increased risk of CD for men.

CARD8 polymorphisms and clinical phenotypes of UC or CD

The relationship between UC clinical phenotypes and rs2043211 was next investigated. No significant differences were found in the frequencies of alleles or genotypes between the UC subgroups classified by maximal endoscopic extent. We also explored relationships between clinical phenotypes of CD, and rs4389238 and rs1972619. No significant differences in allele or genotype frequencies were evident among CD subgroups stratified by disease location, behavior and presence of perianal fistula.

Association analysis of *NALP3* and *NOD* polymorphisms with IBD in Koreans

We genotyped five SNPs from *NALP3* in Korean samples. Among these SNPs, rs76291085 was not polymorphic in Koreans. The allele frequency of the *NALP3* nonsynonymous SNP rs35829419 (Q705K) was 0.002 in CD and 0.003 in UC patients, but 0 in controls. In association analyses, the four SNPs did not show a significant association with either CD or UC (Supplementary Table 1). Additionally, none of the four SNP genotypes from *NOD2* was associated with CD (Supplementary Table 2).

Measurement of serum IL-1 β concentrations

To explore the functional significance of the *CARD8* nonsynonymous SNP rs2043211 (C10X) on the activity of caspase-1, we measured serum levels of IL-1 β in 70 controls, 107 CD and 106 UC patients, to assess correlation with genotype. IL-1 β levels were significantly higher in CD (mean $\pm$ s.d., 7.0 ± 17.3 pg ml⁻¹) and UC patients (4.9 ± 8.4 pg ml⁻¹) than in controls (1.0 ± 6.8 pg ml⁻¹, *P* = 0.010 for both CD and UC patients). However, the difference in mean IL-1 β level between UC and CD patients was not statistically significant (*P* = 0.265). Upon analysis of variance between IL-1 β level and genotype in CD and UC patients, serum IL-1 β levels did not differ significantly among UC or CD patients with the three rs2043211 genotypes (TT, 6.6 ± 11.9 pg ml⁻¹; TA, 5.3 ± 4.1 pg ml⁻¹; AA, 2.8 ± 4.8 pg ml⁻¹, *P* = 0.116 for UC patients, TT, 4.1 ± 9.7 pg ml⁻¹; TA, 12.9 ± 29.5 pg ml⁻¹; AA, 5.5 ± 10.1 pg ml⁻¹, *P* = 0.114 for CD patients). As shown in Table 4, a combined analysis of heterozygotes and homozygotes revealed a positive correlation between the presence of the stop allele of rs2043211 and higher serum IL-1 β levels in UC patients only (TT+TA 6.1 ± 9.8 pg ml⁻¹, AA, 2.8 ± 4.8 pg ml⁻¹,

Table 2 Summary of association of CARD8 between UC cases and controls

ID	Position ^a	Genotype of UC cases						Genotype of controls				Allele 1 vs 2		Genotype 11 vs 22		Genotype 11+12 vs 22		Genotype 11 vs 12+22	
		Allele ^b		11 (%)		12 (%)		Sum		11 (%)		12 (%)		Sum		aOR (95% CI)		P-value	
		1	2	11 (%)	12 (%)	22 (%)	Sum	11 (%)	12 (%)	22 (%)	Sum	χ ² (P-value)		aOR (95% CI)	P-value	aOR (95% CI)	P-value	aOR (95% CI)	P-value
rs1965759	53399393	C	T	341 (53.7)	252 (39.7)	42 (6.6)	635	302 (51.0)	249 (42.1)	41 (6.9)	592	0.696 (0.404)		1.229 (0.745–2.026)	0.419	1.140 (0.701–1.856)	0.598	1.192 (0.935–1.519)	0.157
rs1062808	53403288	G	A	173 (27.4)	314 (49.7)	145 (22.9)	632	143 (24.2)	305 (51.5)	144 (24.3)	592	1.294 (0.255)		1.239 (0.876–1.752)	0.225	1.102 (0.828–1.467)	0.505	1.208 (0.915–1.594)	0.184
rs4389238	53412251	C	T	388 (61.2)	221 (34.9)	25 (3.9)	634	353 (59.7)	214 (36.2)	24 (4.1)	591	0.226 (0.634)		0.991 (0.527–1.864)	0.978	0.987 (0.528–1.842)	0.966	1.010 (0.788–1.294)	0.938
rs2288877	53429363	A	G	377 (57.8)	242 (37.1)	33 (5.1)	652	388 (57.1)	244 (35.9)	48 (7.1)	680	0.689 (0.407)		1.584 (0.961–2.611)	0.071	1.583 (0.969–2.586)	0.067	1.068 (0.847–1.346)	0.577
rs2043211	53429518	T	A	150 (22.9)	318 (48.6)	186 (28.4)	654	112 (16.4)	354 (51.9)	216 (31.7)	682	6.411 (0.011)		1.554 (1.114–2.169)	0.010	1.179 (0.919–1.514)	0.195	1.499 (1.122–2.002)	0.006
rs2288876	53429576	T	C	371 (57.4)	241 (37.3)	34 (5.3)	646	387 (57.1)	243 (35.8)	48 (7.1)	678	0.420 (0.517)		1.512 (0.921–2.482)	0.102	1.516 (0.932–2.465)	0.094	1.055 (0.836–1.330)	0.652
rs1972619	53442741	A	G	59 (9.5)	273 (44.0)	288 (46.5)	620	52 (8.8)	237 (40.3)	299 (50.9)	588	1.838 (0.175)		1.176 (0.758–1.825)	0.469	1.151 (0.902–1.470)	0.258	1.104 (0.724–1.685)	0.646

Abbreviations: CI, confidence interval; aOR, adjusted odds ratio for age and sex.

^aChromosome position.

^bAllele 1 is a risk allele.

Table 3 Summary of association of CARD8 between CD cases and controls

ID	Allele ^a	Genotype of CD cases						Genotype of controls						Allele 1 vs 2 χ^2 (P-value)				Genotype 11 vs 22				Genotype 11+12 vs 22				Genotype 11 vs 12+22			
		11 (%)		12 (%)		22 (%)		Sum		11 (%)		12 (%)		22 (%)		Sum		aOR (95% CI)		P-value		aOR (95% CI)		P-value		aOR (95% CI)		P-value	
		1	2	11 (%)	12 (%)	22 (%)	Sum	11 (%)	12 (%)	22 (%)	Sum	11 (%)	12 (%)	22 (%)	Sum	Allele 1 vs 2 χ^2 (P-value)	aOR (95% CI)	P-value	aOR (95% CI)	P-value	aOR (95% CI)	P-value	aOR (95% CI)	P-value	aOR (95% CI)	P-value			
rs1965759	C	T	330 (53.1)	244 (39.3)	47 (7.6)	621	302 (51.0)	249 (42.1)	41 (6.9)	592	0.167 (0.683)	0.964 (0.607–1.530)	0.875	0.944 (0.602–1.481)	0.803	0.777	1.034 (0.819–1.305)	0.777											
rs1062808	A	G	169 (27.2)	316 (50.9)	136 (21.9)	621	144 (24.3)	305 (51.5)	143 (24.2)	592	1.606 (0.205)	1.326 (0.949–1.852)	0.098	1.216 (0.922–1.604)	0.165	0.194	1.193 (0.914–1.557)	0.194											
rs4389238	T	C	45 (7.3)	226 (36.4)	350 (56.4)	621	24 (4.1)	214 (36.2)	353 (59.7)	591	3.582 (0.058)	1.995 (1.167–3.410)	0.012	1.166 (0.921–1.476)	0.202	0.014	1.938 (1.144–3.284)	0.014											
rs2288877	G	A	44 (6.8)	249 (38.4)	355 (54.8)	648	48 (7.1)	244 (35.9)	388 (57.1)	680	0.352 (0.553)	1.002 (0.638–1.575)	0.991	1.134 (0.905–1.421)	0.273	0.801	0.945 (0.607–1.470)	0.801											
rs2043211	A	T	230 (35.4)	306 (47.2)	113 (17.4)	649	216 (31.7)	354 (51.9)	112 (16.4)	682	0.528 (0.467)	1.042 (0.746–1.454)	0.809	0.933 (0.692–1.257)	0.648	0.217	1.161 (0.916–1.470)	0.217											
rs2288876	C	T	44 (6.8)	246 (38.1)	355 (55.0)	645	48 (7.1)	243 (35.8)	387 (57.1)	678	0.277 (0.599)	1.001 (0.637–1.573)	0.997	1.125 (0.898–1.410)	0.307	0.808	0.947 (0.609–1.473)	0.808											
rs1972619	G	A	335 (54.7)	248 (40.5)	30 (4.9)	613	299 (50.9)	237 (40.3)	52 (8.8)	588	4.569 (0.033)	2.000 (1.226–3.262)	0.005	1.970 (1.222–3.177)	0.005	0.280	1.138 (0.900–1.437)	0.280											

Abbreviations: CI, confidence interval; aOR, adjusted odds ratio for age and sex.

^aAllele 1 is a risk allele.

Table 4 Mean serum IL-1 β levels according to genotypes of rs2043211

ID	Allele ^a			UC									
	1	2		11 (N)	12 (N)	22 (N)	11+12 (N)	12+22 (N)	11 vs 12 vs 22 P	12 vs 22 P	11 vs 22 P	11+12 vs 22 P	11 vs 12+22 P
rs2043211	T	A	Total	6.639 (43)	5.254 (24)	2.793 (39)	6.143 (67)	3.731 (63)	0.116	0.041	0.055	0.048	0.133
			Male	3.997 (21)	4.990 (11)	5.176 (15)	4.338 (32)	5.097 (26)	0.824	0.936	0.592	0.658	0.535
			Female	9.161 (22)	5.479 (13)	1.303 (24)	7.793 (35)	2.770 (37)	0.027	0.003	0.025	0.004	0.064

^aAllele 1 is a risk allele.

$P=0.048$). Moreover, we observed a more significant association in analysis by gender in UC patients only. Serum IL-1 β levels among female UC patients with the three genotypes of rs2043211 were markedly different ($P=0.027$).

DISCUSSION

Of the seven SNPs of *CARD8* tested in this study, rs2043211 and rs1972619 showed statistically significant associations with UC and CD, respectively. Interestingly, SNPs associated with UC and CD did not overlap in Koreans. In addition, the risk alleles of six of the seven SNPs in UC and CD patients were opposite to each other. The differences in allele frequencies of rs2043211 and rs1972619 between UC and CD patients were 6.3% and 6.5%, respectively, and were statistically significant ($P=0.001$, $P=4\times 10^{-4}$, respectively). Our data of the opposite direction of the six SNPs between UC and CD patients suggest distinct roles for *CARD8* in these related diseases. Notably, the fact that the effects were opposite resulted in absence of an association signal upon analysis of combined CD and UC samples, suggesting that previous negative reports on genetic associations with IBD should be reexamined with caution. To the best of our knowledge, this is the first report to show that alleles associated with UC and CD are distinct in effect pattern, and this is the first analysis of *CARD8* genotype in an Asian population.

McGovern *et al.* found that the common allele A (Cys) was associated with CD,²⁵ whereas Fisher *et al.* demonstrated an association of the rare allele T (stop) with IBD.²⁶ Subsequent studies on German and Norwegian IBD patients,²⁷ and on three independent European cohorts, German, Dutch and Hungarian,²⁸ failed to replicate the association of rs2043211 with CD or UC. Recent studies suggested that these conflicting results might be because of an interaction of rs2043211 with loss-of-function variants in *NOD2* and gain-of-function SNP (rs35829419, Q705K) in *NALP3*. A Swedish report showed that men carrying both the C10X and Q705K alleles in *CARD8* and *NALP3*, and wild-type *NOD2*, were at increased risk specifically for CD.³² Another study performed in New Zealand found protective effects of the minor *CARD8* allele rs2043211 or *NALP3* rs35829419 against CD in the context of major allele homozygosity of the other SNPs.³³ Moreover, the effects were more intense in the absence of *NOD2* mutations.³³ *NOD2* on chromosome 16 was the first CD susceptibility gene identified in patients of European origin.^{42–44} However, this finding has not been replicated in Asian populations.^{45–47} Examination by our group of four SNPs across *NOD2* found no evidence of association with CD in Koreans (Supplementary Table 2). In addition, we observed no association of *NALP3* genotype with CD or UC in Koreans (Supplementary Table 1). The frequency of the *NALP3* risk allele, rs35829419 (Q705K), in the control population was higher in Caucasians than in Koreans (0.04–0.07 vs 0.00),^{31–33} whereas the frequency of the stop allele of rs2043211 in controls was higher in Asians than in Caucasians (0.42 vs 0.32–0.34).^{25–28} As we identified only four and three heterozygotes among UC and CD patients, respectively, it was not possible to examine the

combined effects of *NALP3* and *CARD8* genotype in Koreans. These data further emphasize the differences in the genetic background of IBD patients in Asian and Western countries.

Another interesting finding of this study is the gender-related difference in association of rs2043211 and rs4389238 with UC and CD, respectively, in Koreans. One well-known difference between Asian and Caucasian CD patients is male predominance among Asians. Specifically, a male-to-female ratio of around 2:1 is evident in Korea and Japan, compared with about 1:1 in Western countries.^{48–50} In contrast, the incidence of UC in Koreans is comparable between men and women.⁴⁹ It is not known whether this gender difference in IBD incidence is caused by environmental factors, genetic influences or a combination of these parameters. We observed gender differences in UC susceptibility associated with rs2043211 and CD susceptibility with rs4389238. In both instances, the risk was higher in men (aOR=1.70, $P=0.007$ for rs2043211, aOR=3.04, $P=0.008$ for rs4389238). The earliest report on gender differences in IBD susceptibility among Western populations showed an increased risk of developing CD in men with both the C10X and Q705K alleles of *CARD8* and *NALP3*, respectively, and wild-type *NOD2*.³² Although the SNPs involved in CD in Koreans are distinct from those in Caucasian CD patients, the *CARD8* variants showed gender susceptibility differences to CD in both Caucasian and Korean populations.

Given the role played by *CARD8* in assembly of the *NALP3* inflammasome and *CARD8* interactions with *NOD2*, we also determined whether UC- or CD-associated *CARD8* SNPs affected IL-1 β processing, by measuring serum IL-1 β levels in IBD patients and controls. Serum IL-1 β levels were significantly higher in UC ($P=0.010$) and CD ($P=0.010$) patients than in controls. However, the differences between IL-1 β levels in UC and CD patients were not statistically significant ($P=0.265$). IL-1 β levels sorted by gender were not significantly different in either UC ($P=0.742$) or CD patients ($P=0.071$). An analysis of variance between IL-1 β levels and genotypes showed that the stop allele of rs2043211 appeared to be correlated with higher serum IL-1 β levels in female UC patients ($P=0.027$). However, no association was evident between the rs2043211 or rs1972619 genotypes and serum IL-1 β levels in CD patients. Although the association of rs2043211 with UC was more significant in males than in females, the correlation between serum IL-1 β levels and genotype appeared more significant in female patients with UC. This discrepancy may be because of polymorphisms present in genes associated with other functions of *CARD8*.

Our study was limited by an absence of information on disease activity in UC and CD patients during the period of serum collection. We cannot rule out the possibility of bias in serum IL-1 β levels, attributable to various levels of disease activity in patients at the times of sampling. In addition, we do not know whether the UC- and CD-associated SNPs exhibit loss-, or gain-of-function effects. CD patients carrying the stop allele of rs2043211 express an immunoreactive isoform of *CARD8* and a somewhat reduced level of its mRNA.²⁹ However, the functional significance of this stop allele in UC patients,

as well as the role played by rs1972619 in Korean CD patients, remain to be determined.

In summary, we confirmed previously described associations between *CARD8* polymorphisms and UC or CD in the Korean population. Interestingly, the SNPs associated with the two diseases did not overlap. Although the biological functions of UC- or CD-associated *CARD8* SNPs remain to be established, the nonoverlapping alleles between UC and CD patients suggest that these clinically related diseases have distinct pathogenic mechanisms. Further studies are essential to elucidate the specific roles played by *CARD8* variants in UC and CD.

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