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ORIGINAL ARTICLE

Confirmation of three inflammatory bowel disease susceptibility loci in a Chinese cohort

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Abstract
Purpose Recent genome-wide association studies have identified a number of inflammatory bowel diseases (IBD) susceptibility loci in White populations. The aim of our study was to evaluate whether these susceptibility loci also existed in a Chinese Han IBD population.
Methods Peripheral blood DNA samples from groups of patients with Crohn's disease (CD) (n=48), ulcerative colitis (UC) (n=49), and healthy controls (n=50) were genotyped for eight genes. Then, an extended analysis of the relationship between genotype and phenotype was performed.
Results *NOD2*-P268S (P=0.025) was found to contribute susceptibility to CD in the Chinese population. *IL23R*-rs11805303 was detected to confer a strong protective effect against UC (P=0.010), whereas *PTPN2*-rs2542151 was significantly associated with an increased risk of UC (P=0.001). Further phenotype–genotype analysis revealed that P268S was associated with early age of onset (P=0.028), ileal disease (P=0.003), and enteric cavity narrowing (P=0.007).
Conclusions The study indicates that *IL23R*-rs11805303 and *PTPN2*-rs2542151 might contribute to the development of UC and *NOD2*-P268S might be involved in the etiology of CD in the Chinese Han population.

Keywords Inflammatory bowel diseases · Nucleotide-binding oligomerization domain containing 2 · Interleukin 23 receptor · Protein tyrosine phosphatase N2

Chaolan Lv and Xiaoming Yang equally contributed to the work.

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Introduction

Inflammatory bowel diseases (IBD) are common chronic relapsing inflammation disorders of the gastrointestinal tract with two main pathological entities: Crohn's disease (CD) and ulcerative colitis (UC). Though the pathogenesis of IBD remains elusive, numerous epidemiological investigations strongly suggested that genetic factors may play a prominent role in the development of IBD [1].

With the help of genome-wide association techniques, our knowledge of the genetic basis of IBD advanced greatly [2]. The discovery of the first CD susceptibility gene nucleotide-binding oligomerization domain containing 2 (*NOD2*), also known as caspase recruitment domain family member 15 (*CARD15*), created a new era in IBD research. Since then, more and more single nucleotide polymorphisms (SNPs) have been identified to be associated with IBD. These included rs11209026 and rs11805303 in *IL23R* (which encodes a crucial subunit for the interleukin 23 receptor), rs2241880 in *ATG16L1* (which encodes autophagy-related 16-like 1 gene), rs2542151 in *PTPN2* (which encodes protein tyrosine phosphatase N2), rs10761659 in 10q21, rs13361189 and rs4958847 in *IRGM* (which encodes immunity-related GTPase family M), rs1050152 in *OCTN1* (which encodes organic cation transporter gene cluster 1), and rs2631367 in *OCTN2* (which encodes organic cation transporter gene cluster 2) [3–12].

Approximately 3.6 million people in the west are affected by IBD every year. In the past, few cases were reported in Asia. But in these years, the incidence of IBD increased continuously. Genetic studies of IBD carried out in China were limited and the major part of the genetic background of IBD in the Chinese population remains unclear [13, 14]. Therefore, we aimed to evaluate the contribution of those confirmed IBD susceptibility loci mentioned above to the

Chinese population and also examine their possible phenotype–genotype relationships.

Materials and methods

Patient specimens

A total of 97 IBD patients (48 CD and 49 UC) of Chinese Han ethnicity from the departments of gastroenterology and general surgery and 50 control samples from healthy persons under routine health screening in the outpatient clinics of Nanfang Hospital, Guangzhou, China were consecutively recruited from January 2007 to January 2009.

All patients were unrelated and their medical histories were obtained from clinical records. The diagnosis of IBD was conducted by senior physicians based on standard clinical, endoscopic, radiologic, and histological criteria. Severity of IBD was classified according to the criteria of the modified Williams DAI standard and the Chinese Association of Digestive Diseases. The institutional ethics committee approved all protocols and all enrolled subjects gave their informed consents.

Genotyping and sequencing analysis

Genotypic DNA was obtained from peripheral blood leukocyte of CD, UC, and normal controls. DNA fragments were amplified by polymerase chain reaction (PCR) using specific primers (Table 1). The PCR conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturing at 94 °C for 45 s, annealing at the temperature (Table 1) for 45 s, extension at 72 °C for 1 min, and final incubation at 72 °C for 10 min. The PCR productions were confirmed by purification. DNA sequencing for P268S, R702W, G908R, 3020insC, rs11209026, rs11805303, rs2241880, rs1050152, and rs2631367 was carried out using an ABI377 automated sequencer by Invitrogen. Gene sequences from UC, CD, and normal controls were aligned with sequence in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST). If a mutation was found, the corresponding exon will be reamplified and resequenced using both upstream and

Table 1 PCR primers and conditions

Gene	Variant	Sequence (5′–3′)	*T* (°C)	Size (bp)	Methods/enzyme
NOD2	P268S	F TGCCTCTTCTTCTGCCTTCC R AGTAGAGTCCGCACAGAGAG	59	425	DNA sequencing and RFLP/*Bam*HI
	R702W	F TTCTTTGCCGCGTTCTACCTG R CCACACTTAGCCTTGATGGTG	58	698	DNA sequencing
	G908R	F CTGTTGACTCTTTTGGCCTT R ACATTTCCAAGTCACCCAGA	53	294	DNA sequencing
	3020insC	F CAAGAGGAAAACCAAGAATC R CTCCTAACCTGTGTAATCTC	53	185	DNA sequencing
IL23R	rs11209026	F AGACCTTTGCTTTGAGCAGAGT R CATGTAGTCTAAATCAGAAAACAGAAA	55	241	DNA sequencing
	rs11805303	F CTTTCACCACCCATCATCATTC R TCCTCTTAGTCGGAGCTTTGTCT	60.5	319	DNA sequencing
ATG16L1	rs2241880	F ATTTGATGAGCAGTAAACCTCTG R GGGGCTGAAGCATACTTACG	55	193	DNA sequencing
OCTN1	rs1050152	F AGTCCTCCTATCTGATTGATGTTCT R TCTCCCTAAGGCATTTTGGTAT	55	226	DNA sequencing
OCTN2	rs2631367	F TGAGTTCCTTGGGTTGTATTGTT R GGAGCGGATGGCGTTCT	58.5	286	DNA sequencing
PTPN2	rs2542151	F TCCGTGAGTTCCTGCTGTCTTGTC R AGCCTGGGCGATGGGGTGAG	68.7	445	RFLP/*Bmy*I
10q21	rs10761659	F GCTTAGGAAACAAAGGAATCAC R AGTCAAAGAGGAGGGCGTT	59	262	RFLP/*Tsp*4CI
IRGM	rs13361189	F GGTTTTCTGCTGACCTCCCA R CTTTACCATTGTACTCCTTGTGCC	62	252	RFLP/*Tsp*EI
	rs4958847	F GATGACAACTAAGAAATGGGT R TTCTCCTGTTAGTATTCCAAAA	53	206	RFLP/*Alu*I

T annealing temperature, *Size* size of PCR product, *F* forward primer, *R* reverse primer, *RFLP* restriction fragment length polymorphisms, *NOD2* nucleotide-binding oligomerization domain containing 2, *IL23R* interleukin 23 receptor, *ATG16L1* autophagy-related 16-like 1 gene, *PTPN2* protein tyrosine phosphatase N2, *IRGM* immunity-related GTPase family M, *OCTN1* organic cation transporter gene cluster 1, *OCTN2* organic cation transporter gene cluster 2

downstream sequencing primers for confirmation. Restriction fragment length polymorphisms (RFLP) assay was used for rs2542151, rs10761659, rs13361189, and rs4958847. The P268S variant was also confirmed by RFLP.

Table 2 Genotype distributions and allele frequencies of gene variants in IBD patients compared with healthy controls

Locus	Genotype	Allele	CD		UC			Control
			n (%)	*P*[a]	*n* (%)	OR (95 % CI)	*P*[b]	*n* (%)
NOD2								
P268S	CC		43 (89.5)		49 (100.0)			50 (100.0)
	CT		2 (4.2)		0 (0.0)			0 (0.0)
	TT		3 (6.3)	0.025[c]	0 (0.0)		NS	0 (0.0)
		T	(38.5)	<0.001[d]	(0.2)		NS	(6.0)
IL23R								
rs11209026	GG		44 (91.7)		42 (85.7)			43 (86.0)
	GA		4 (8.3)		7 (14.3)			7 (14.0)
	AA		0 (0.0)	NS	0 (0.0)		NS	0 (0.0)
		A	(4.2)	NS	(7.1)		NS	(7.0)
rs11805303	CC		8 (16.7)		17 (34.7)			8 (16.0)
	CT		30 (62.5)		26 (53.1)			25 (50.0)
	TT		10 (20.8)	NS	6 (12.2)		0.010[c]	17 (34.0)
		T	(52.1)	NS	(38.8)	0.27 (0.10–0.74)[d]	0.004[d]	(59.0)
ATG16L1								
rs2241880	AA		8 (16.7)		11 (22.4)			9 (18.0)
	AG		36 (75.0)		34 (69.4)			36 (72.0)
	GG		4 (8.3)	NS	4 (8.2)		NS	5 (10.0)
		G	(45.8)	NS	(42.9)		NS	(46.0)
PTPN2								
rs2542151	TT		19 (39.6)		12 (24.5)			28 (56.0)
	TG		29 (60.4)		33 (67.3)			22 (44.0)
	GG		0 (0.0)	NS	4 (8.2)		0.001[c]	0 (0.0)
		G	(30.2)	NS	(41.8)	3.92 (1.76–8.75)[d]	0.003[d]	(22.0)
10q21								
rs10761659	GG		24 (50.0)		37 (75.5)			29 (58.0)
	GA		19 (39.6)		12 (24.5)			21 (42.0)
	AA		5 (10.4)	NS	0 (0.0)		NS	0 (0.0)
		A	(30.2)	NS	(12.2)		NS	(21.0)
IRGM								
rs13361189	CC		14 (29.2)		17 (34.7)			18 (36.0)
	CT		29 (60.4)		25 (51.0)			28 (56.0)
	TT		5 (10.4)	NS	7 (14.3)		NS	4 (8.0)
		T	(40.6)	NS	(39.8)		NS	(36.0)
rs4958847	AA		19 (39.6)		21 (42.9)			22 (44.0)
	AG		29 (60.4)		28 (57.1)			28 (56.0)
	GG		0 (0.0)	NS	0 (0.0)		NS	0 (0.0)
		G	(30.2)	NS	(28.6)		NS	(28.0)

The other five variants are wild type

OR odds ratio, *CI* confidence interval, *NS* no significance, *IBD* inflammatory bowel diseases, *CD* Crohn's disease, *UC* ulcerative colitis

[a] CD vs. control

[b] UC vs. control

[c] Under the genotypic model

[d] Under the allelic model

Statistical analysis

All analyses were performed using the software of SPSS13.0. Case–control analysis was performed by Fisher's exact. Odds ratios (OR) were calculated with the corresponding chi square distribution test and 95 % confidence intervals (95 % CI). $P<0.05$ was considered significant. The genotype frequencies for each variant were tested for deviation from the Hardy–Weinberg equilibrium by means of the chi square test.

Results

Identification of mutations associated with Chinese IBD patients

All the genotypic and allelic distributions analyzed in this study were in accordance with the Hardy–Weinberg equilibrium. The involvements of *NOD2*/*CARD15* mutations in the etiology of CD were commonly seen in the west. To examine their possible associations with IBD in Chinese patients, all 12 exons of the *NOD2*/*CARD15* gene were amplified by PCR. Then, the PCR products were subjected for both direct DNA sequence and RFLP analysis. However, no significant associations between three common SNPs (Arg702Trp, Gly908Arg, and Leu1007fsinsC) and CD were detected in our cohort (data not show). A novel P268S mutation was observed in 5 out of 48 CD patients, with codon changed from CCC to UCC (Table 2; Figs. 1 and 3a, b). This novel P268S mutation was only found in CD patients, but not in UC patients and the healthy control group (Table 2; $\chi^2=5.49$, $P<0.05$).

Another mutation, *IL23R*-rs11805303 (C → T), was detected in 32 out of 49 UC compared with 42 out of 50 in controls. Carriage of the T allele of *IL23R*-rs11805303 was found to have a decreased risk of UC ($P=0.010$, under the genotypic model; $P=0.004$, under the allelic model). The OR for the protective T allele was 0.27 (95 % CI, 0.10–0.74). Alternatively, the wild-type CC genotype may be considered as the risk genotype with an OR of 3.70 (Fig. 2). Moreover, the presence of a G allele at *PTPN2*-rs2542151 was significantly associated with an increased risk of UC ($P=0.001$, under the genotypic model; $P=0.003$, OR GG/TG vs. TT, 3.92; 95 % CI, 1.76–8.75, under the allelic model) (Fig. 3c, d). Other previously identified IBD-associated SNPs were not detected in the Chinese population (Table 2).

Identification of association between genotype and clinical features

To examine whether the above-identified SNPs have any relations with clinical features, the occurrence of each SNP was compared with age at onset, gender, lesion location, disease severity, and so on. Intriguingly, the lesions of the five CD patients with P268S mutation were all located to the terminal ileum ($P=0.003$) and accompanied with enteric cavity narrowing ($P=0.007$). Four of these five patients were under 20 years of age at onset ($P=0.028$), suggesting that P268S in Chinese CD patients might correlate with younger disease occurrence. However, P268S carriers were not related to gender ($P=0.521$) or disease severity ($P=0.289$). No significant associations between phenotype and genotype of both rs11805303 and rs2542151 were observed in Chinese patients with UC (Table 3).

Discussion

Identification of IBD susceptibility genes will provide key insights into pathogenic mechanisms and be beneficial to patients by proper interventions. During the past decades, a

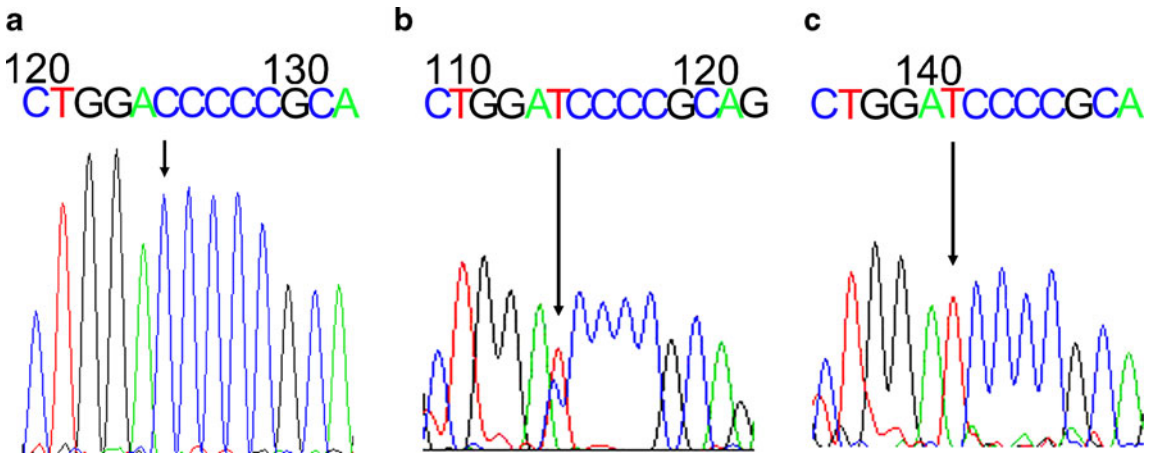

Fig. 1 Forward sequence atlas of the PCR product which contains the P268S variant (indicated by *arrows*). **a** Wild-type CC, **b** heterozygote TC, **c** homozygote TT

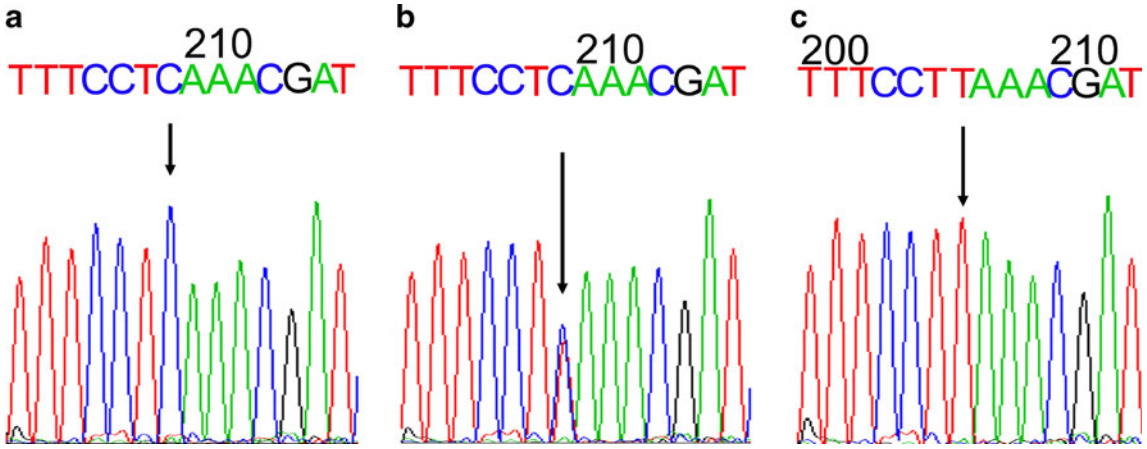

Fig. 2 Forward sequence atlas of the PCR product which contains the rs11805303 variant (indicated by *arrows*). **a** Wild-type CC, **b** heterozygote CT, **c** homozygote TT

number of genes have been found to contribute to IBD susceptibility in White populations [2–12]. Here, we selected a total of eight genes and assessed the contribution of these established risk variants to the genetic susceptibility to IBD in a Chinese cohort.

Among all the IBD susceptibility genes, *NOD2*/*CARD15* is the most well studied and has been shown to give rise to CD with a strong dissimilarity among different ethnicities [5]. Here, we genotyped the coding region of *NOD2*/*CARD15* but failed to replicate associations of CD with the three SNPs (G908R, 1007fsCins, and R702W) which were commonly seen in White populations [10]. Our finding provides additional support for the limited role of these three SNPs in Asian CD patients [8, 14, 15].

Instead, our study confirmed that P268S was involved in the predisposition to CD in the Chinese population. Similar presence of P268S was also observed in Chinese Tu, Indian, and Pakistani populations, indicating it to be a common risk factor of CD among Asians [16]. Genetic heterogeneity of P268S exists among different ethnic groups, too. For instance, a North Indian UC study even observed significant association for P268S [17]. Moreover, P268S usually occurred in phase with the three SNPs and was regarded as a background polymorphism of *NOD2*/*CARD15* in European ancestry [9]. P268S was also found to be in linkage disequilibrium (LD) with other mutations, such as IVS8-158(JW1) which acted as a potential "risk-conferring" variant in Jewish and Asian patients with CD [10, 11]. This raises the question whether P268S in LD with additional *NOD2*/*CARD15* polymorphisms contributes to CD susceptibility in the Chinese Han population. Further studies are needed to explore the possible yet unidentified Chinese-specific variants in *NOD2*/*CARD15* in a larger cohort.

CD is known to have a bimodal age distribution of disease onset, and recent studies revealed that P268S was associated

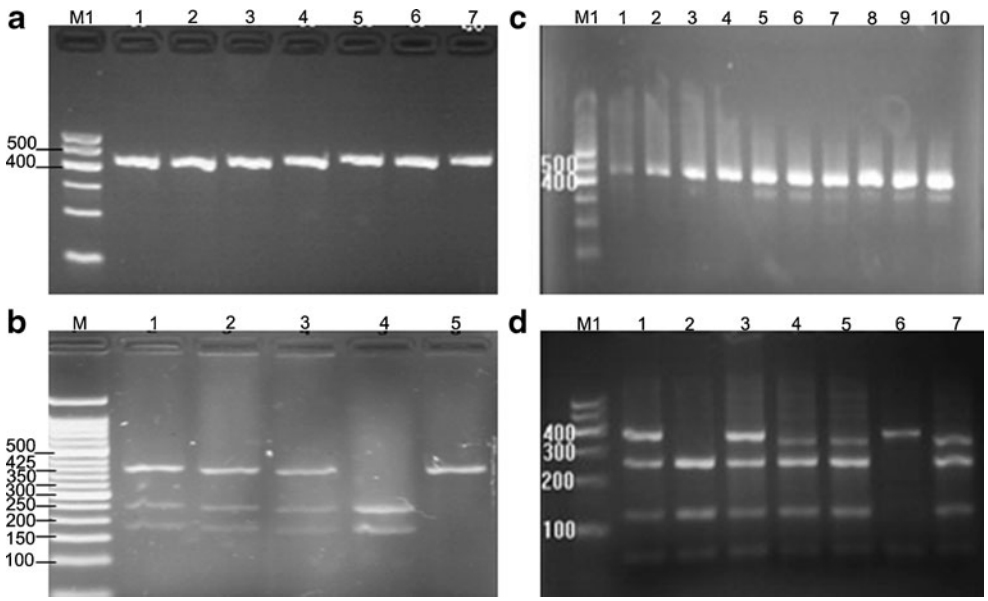

Fig. 3 Electrophoresis of PCR products (**a**, **c**) and products incised by enzymes (**b**, **d**). *Lane M1* DNA marker I, *lane M* 1.5-kb DNA marker (50 bp ladder). **a**, **b** Analysis of P268S. **b** *Lanes1–3* heterozygote CT, *lane 4* homozygote TT, *lane 5* wild-type CC. **c**, **d** Analysis of rs2542151. **d** *Lane 2* wild-type TT, *lane 6* homozygote GG. The other lanes: heterozygote TG

Table 3 Relations between three variants and IBD clinical features

	CD (n=48)	UC (n=49)	P268S in CD		rs11805303 in UC		rs2542151in UC	
			n	P	n	P	n	P
Gender (M/F)	32/16	29/20	3/2	0.521	20/12	0.517	23/14	0.456
Age (years)[a]	30.4±11.9	32.2±14.3						
Age at disease onset (years)[a]	23.7±6.5	26.9±10.5		0.028				0.708
≤20	15	11	4		8	0.418	9	
>20	33	38	1		24		28	
Duration (years)[a]	6.7±7.3	7.1±7.7						
Location				0.003				
Ileum	16		5					
Small intestine and colon	20		0					
Colon	11		0					
Rectum	1		0					
Stenosis of enteric cavity				0.007				
Yes	19		5					
No	29		0					
Severity				0.289				
Mild	16		0					
Moderate	26		4					
Severe	6		1					
Maximum extent						0.922		0.66
							0	
Proctitis		7			5		6	
Left-sided		13			8		9	
Right-sided		8			6		5	
Extensive		21			13		17	
Activity						0.482		0.69
							0	
Paracmasis		10			6		7	
Active phase		39			26		30	

[a] Values are expressed as the mean±SD

F female, *M* male

with age onset of CD [18]. Our confirmation of CD patients carrying P268S polymorphism with a tendency of young disease occurrence adds to this predisposition. Furthermore, it has been recognized that patients with a younger age at onset or ileal involvement usually have a stricturing phenotype more frequently than other patients [7, 19]. And in our present study, P268S also conferred susceptibility to ileal lesions and enteric cavity narrowing. We, therefore, speculate that P268S may be used as a marker to represent specific clinical subgroups in Chinese CD patients. *NOD2*/*CARD15* mutations may result in an impaired activation of NF-κB by altering the recognition of the bacterial lipopolysaccharide [5]. But a recent study revealed that P286S was not involved in the regulation of NF-κB signaling pathway [20]. As the exact mechanism by which P268S contributes to the development of CD remains unclear, further functional studies of P268S should be conducted to achieve a better understanding of NOD2-mediated CD pathogenesis.

Similar to previous reports, no statistically significant associations between UC and *NOD2*/*CARD15* mutations were detected, extending the hypothesis that *NOD2*/*CARD15* mutations which lead to defects in bacterial antigen processing are likely less important in UC than in CD [21].

CD and UC are thought to be distinct but related disorders. Unlike *NOD2*/*CARD15*, which is unique to CD, some susceptibility genes are common to both types of IBD [22–24]. *IL-23R* and *PTPN2* are two of them.

IL-23 is believed to promote the differentiation of native CD4+ T cells into Th17 cells and production of several inflammatory mediators from both T cell and non-T cell sources. This cytokine can also restrain the activity of regulatory T cells. Its corresponding ligand IL-23R is thus of great

importance for intestinal inflammation. *IL23R*-rs11209026 was found to be a risk factor for both CD and UC in the non-Jewish White population, and a magnitude association with UC was observed in a Jewish White cohort [4]. However, this SNP appears to be absent or very rare in Asians, including Japanese, Korean, and Indian [25, 26]. Similarly, we also failed to replicate the association of rs11209026 with neither UC nor CD in the Chinese population. Compared with other *IL23R* mutations, current studies focused on rs11805303 are limited. We found rs11805303 had a protective effect on UC susceptibility in our study. Moreover, this SNP was identified as a risk factor for CD, suggesting a common genetic background for these two diseases [27]. Although IL-23R signaling has a key role in the adaptive immune response, it remains unclear whether additional *IL-23R* variants are associated with IBD in the Chinese patients, and if so, how exactly these *IL-23R* polymorphisms modulate IBD susceptibility.

A defective PTPN2 function could lead to an enhanced production of proinflammatory mediators and elevated activity of pathways in the intestinal epithelium. The association between *PTPN2*-rs2542151 and CD was detected by multiple studies analyzing cohorts of European ancestry [12, 21]. Additionally, rs2542151 also showed an association with UC in German patients, as well as in ours [23, 25]. Pooling our data with previous studies, we conclude that *IL-23R* and *PTPN2* may have a limited role in CD patients of East Asia. We further analyzed the interaction of *PTPN2*-rs2542151 with UC subphenotypes. However, we did not find any specific group of clinical features associated with rs2542151. One possible explanation may be that we overlooked gene–environmental interactions, since IBD are multifactorial diseases and can also be affected by environmental factors. For example, genetic studies carried out among Netherlands and New Zealand cohorts noticed that patients should be stratified according to their smoking behavior; otherwise, the moderate association between CD and rs2542151 can be missed [28]. Thus, environmental factors may need to be taken into account in future studies.

As for CD, a recent study demonstrated a novel role for rs2542151 in the pathogenesis of the disease by increasing the permeability of the intestinal epithelium [29]. Although epithelial barrier dysfunction was observed in both UC and CD, it is still unknown whether rs2542151 could also regulate intestinal epithelial permeability of UC. Biological studies of rs2542151 are required to assess its role in the development of UC.

Except for *NOD2*-P268S, *IL23R*-rs11805303, and *PTPN2*-rs2542151, we did not find other susceptibility loci related to IBD in Chinese people. Similarly, most of those possible associations of the variants with IBD reported in White populations were not detected in Asians, such as Japanese and Korean [13, 27].

A limitation of this study is that our sample size was relatively small. It is likely that the observed SNPs showing evidence of association may be explained by chance finding because studies with small sample size may decrease statistical power or increase the possibility of a false-positive finding. The limited sample size may also contribute to the lack of ability to discern significant differences in allele frequencies between patients and controls. Moreover, some susceptibility variants with low allele frequencies in the Chinese population may be missed. Thus, the results of our exploratory study should be viewed as preliminary findings and further replicated studies in larger cohorts will be required to determine the true interaction between genetic variants and IBD in the Chinese population.

In summary, we identified a novel P268S polymorphism in *NOD2*/*CARD15* associated with Chinese CD patients. We also confirmed associations of *PTPN2*-rs2542151 and *IL23R*-rs11805303 with UC for the first time in the Chinese Han population. We conclude that P268S, rs11805303, and rs2542151 are possible candidates for conferring susceptibility to IBD in Chinese people. Our findings complement prior studies and suggest that the genetic background of IBD may vary among different ethnical groups. Further functional studies investigating the role of these genetic polymorphisms in the development of IBD are needed.

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