

Original Article

Association of angiotensin-converting enzyme gene polymorphisms with Crohn's disease in a Chinese Han population

Jie Zhou^{1*}, Sichang Zheng^{1*}, Zhengting Wang¹, Rong Fan¹, Jieliu Yuan², Jie Zhong¹

¹Department of Gastroenterology, Ruijin Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200000, China; ²Department of Gerontology, Minhang District Central Hospital, Shanghai 201100, China. *Equal contributors.

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Abstract: Objective: To investigate whether Angiotensin-converting enzyme (ACE) gene polymorphisms alter the susceptibility of a Chinese Han population to Crohn's disease (CD). Methods: Blood samples were collected from patients with CD and from healthy control subjects for analyzing SNP rs4291 (promoter, A262T), SNP rs4343 (exon 16, A11860G), and rs4646994 (intron 16, Alu insertion/deletion). Allele and genotype frequencies were compared, and pairwise linkage disequilibrium and haplotypes were analyzed in patients with CD. Results: Both rs4343 A/G and rs4646994 I/D allele frequencies differed significantly between patients with CD and control subjects (rs4343: OR=1.438, 95% CI=1.099-1.882, P=0.008; rs4646994: OR=1.559, 95% CI=1.191-2.039, P=0.001). There were also significant associations between the risk of CD and both rs4343 AA/(AG+GG) and rs4646994 II/(ID+DD) genotype frequencies (P=0.039 and P=0.019). The frequency of the G-D haplotype was significantly lower in patients with CD than control subjects (31.7% vs. 40.4%, P=0.010). Conclusions: The results suggest that ACE rs4343G and rs4646994D alleles protect against CD, while rs4343AA and the I allele in the dominant genetic model are risk alleles for CD. The association between the G-D haplotype and CD was significant, suggesting a protective role in the pathogenesis of CD.

Keywords: Crohn's disease, ACE gene, genetic polymorphisms

Introduction

Crohn's disease (CD) is a chronic and incurable inflammatory bowel disease (IBD). While the etiology is not fully understood, CD appears to develop through interactions among environmental factors, susceptibility genes, and gut microbes [1]. The incidences of both CD and ulcerative colitis (UC, the other major form of IBD) are increasing globally, particularly in regions with historically low rates such as Asia [2, 3]. These observations strongly implicate a Westernized diet and industrialization in the risk of IBD. Vascular endothelial dysfunction is also significantly higher in patients with IBD than healthy individuals [4], indicating that an interplay between abnormalities in vascular and immune systems contribute to the initiation and perpetuation of CD [5, 6].

Family and twin concordance studies have confirmed an important role for genetics in the

development and progression of CD [7, 8]. Genes linked to the risk of CD include the autophagy-associated gene ATG16L and inflammation-associated genes IL23R and NOD2 [9]. Angiotensin II is a potent physiological vasoconstrictor, and more recent studies have revealed additional roles in immune regulation and tissue remodeling [10-12]. Angiotensin I converting enzyme (ACE), expressed mainly in endothelial cells, converts angiotensin I to angiotensin II as part of the renin-angiotensin system (RAS). Mice lacking the angiotensin II precursor (angiotensinogen, AGT) gene are more resistant to trinitrobenzene sulfonic acid-induced colitis, a mouse model of IBD, possibly by shifting the balance from pro- to anti-inflammatory cytokines in the colon [13]. Circulating levels of angiotensin can also be controlled by regulated expression of ACE. In humans, a 287-bp insertion/deletion (I/D) polymorphism in intron 16 of the ACE gene (17q22-q24, 26 exons and 25

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Table 1. ACE gene SNPs

SNP	Location (bp)	Primers	Allele	Effect
rs4291	61445194	F: CACGGTGGGCAGGCTCGGGTG R: GGGGTGGCGGGATGGGGCTGG	A262T	Promoter
rs4343	61566031	F: TGCTTCCCTTGCTCCTGGTTC R: GGGGGCAAGGTCTGTTAGTGG	A11860G	Coding exon
rs4646994	61565904	F: AGGACTGCTGAGGCCCTGCAG R: GACGTGGCCATCACATTCGTC	del/ins	Intron

Table 2. Participant demographic and clinical characteristics

	CD (n=156)	Control (n=302)
Age (Y)	34.3±12.9	46.4±9.8
Course of disease (Months)	50.4±50.0	
Sex (male/female)	103/53	208/94
Type of disease		
Ileitis	98	
Ileocolitis	44	
Colitis	12	
Upper gastrointestinal	2	
Complication		
Fistula	46	
Perforation	45	
Perforation	28	

introns) may regulate the levels of ACE in serum. Individuals homozygous for the rs4646994 D allele have higher levels of ACE, which can lead to multiple diseases, particularly diseases affecting the cardiovascular and renal systems [14]. In addition to rs4646994I/D, there are many single-nucleotide polymorphisms (SNPs) such as T5941C, A262T, T93C, T1237C, C4656T, and A11860G [15, 16], in linkage disequilibrium (LD) with the I/D polymorphism that are known to influence serum ACE [17]. Among these, A262T and A11860G may have the largest effects on serum ACE [16], resulting in vascular remodeling [18].

Several SNPs in the ACE gene may affect the risk of certain autoimmune diseases such as rheumatoid arthritis [19, 20], spondylarthropathies [21], systemic lupus erythematosus [22, 23], immunoglobulin A nephropathy [24], and Kawasaki's disease [25]. In addition, one group reported that the rs4646994D allele increases the risk of extra-intestinal manifestations in patients with UC [26]. Asians with a specific genotype are at heightened risk of renal and

cardiovascular diseases associated with RAS dysfunction [14]. It was speculated that Asian individuals harboring specific ACE polymorphisms that alter the expression or activity of ACE would be at altered risk for CD, and hence compared ACE polymorphism frequencies between Chinese patients with CD and matched control subjects. It was reported that the risk of CD is indeed modulated by two specific ACE polymorphisms, rs4343 (exon 16, A11860G) and rs4646994 (intron 16, Alu ins/del).

Materials and methods

Subjects

A total of 156 patients with CD were recruited from RuiJin Hospital (Shanghai, China) between January 2010 and June 2011. The diagnosis of CD was based on clinical symptoms as well as radiological, endoscopic, and pathological evidence based on the Chinese consensus criteria for the diagnosis and treatment of IBD. The 302 control subjects were selected from a group of individuals who underwent a health examination at the hospital during this same period. Control subjects had no history of rheumatism or immunological diseases and no abnormal clinical examination results. Ethics approval for this study was obtained by the institutional research ethics committee, and procedures conformed to the Declaration of Helsinki. Written informed consent was obtained from all participants.

Genotyping of the SNPs of the ACE gene

SNP information of the ACE gene is shown in **Table 1**. Genomic DNA was isolated from peripheral blood samples using the phenol-chloroform method. The ACE rs4291 and

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Table 3. Differences in ACE allele and genotype frequencies between CD patients and controls

Polymorphism	Genotype	CD n (%)	Control n (%)	χ^2 (P)	OR (95% CI)
rs4291	AA	62 (39.8)	94 (31.1)	3.401 (0.065)	1.459 (0.976-2.183)
	AT	71 (45.5)	155 (51.3)	1.390 (0.238)	0.792 (0.538-1.167)
	TT	23 (14.7)	53 (17.6)	0.585 (0.444)	0.812 (0.477-1.384)
	A allele	195 (62.5)	343 (56.8)	2.769 (0.096)	1.268 (0.958-1.678)
	T allele	117 (37.5)	261 (43.2)	2.769 (0.096)	0.798 (0.596-1.043)
rs4343	AA	68 (43.6)	102 (33.8)	4.245 (0.039)	1.515 (1.019-2.252)
	AG	71 (45.5)	156 (51.7)	1.533 (0.213)	0.782 (0.531-1.152)
	GG	17 (10.9)	44 (14.5)	1.201 (0.273)	0.717 (0.395-1.302)
	A allele	207 (66.3)	360 (59.6)	3.967 (0.046)	1.336 (1.004-1.778)
	G allele	105 (33.7)	244 (40.4)	3.967 (0.046)	0.748 (0.562-0.996)
rs4646994	II	68 (43.6)	98 (32.5)	5.524 (0.019)	1.609 (1.181-2.394)
	ID	70 (44.9)	154 (51)	1.543 (0.214)	0.782 (0.531-1.153)
	DD	18 (11.5)	50 (16.5)	2.409 (0.152)	0.657 (0.359-1.171)
	I allele	206 (66)	350 (57.9)	5.628 (0.018)	1.410 (1.061-1.875)
	D allele	106 (34)	254 (42.1)	5.628 (0.018)	0.709 (0.533-0.942)

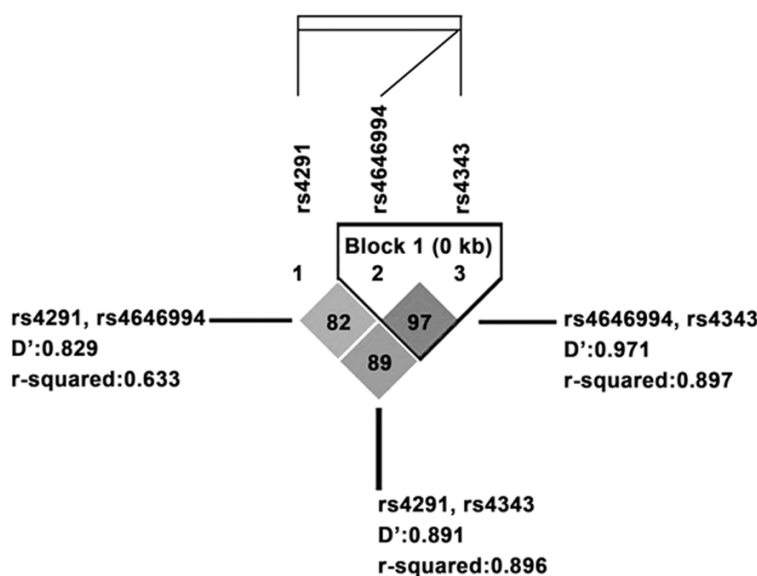


Figure 1. Linkage disequilibrium analysis for rs4291, rs4343 and rs4646994 polymorphisms: The D' values suggest that rs4646994 and rs4343 constitute a haplotype block. The two most frequent haplotypes, A-I and G-D, complemented each other at the two loci.

rs4343 polymorphisms were assessed by polymerase chain reaction-ligase detection reaction (PCR-LDR) as previously described [27-29]. The I/D polymorphism in intron 16 of the ACE gene was assessed by PCR electrophoresis of agarose gels.

The PCR reactions were performed in a 15 μ L reaction volume containing DNA (1 μ L), 10 \times PCR buffer (1.5 μ L), magnesium chloride (1.5

μ L), dNTP and NEB (10 mM, 0.3 μ L), Primer F (10p, 0.25 μ L), Primer R (10p, 0.25 μ L), and Taq polymerase (5 U/ μ L, 0.2 μ L, NEB). The amplification protocol consisted of an initial denaturation at 94 $^{\circ}$ C for 2 min, 35 cycles of denaturation at 94 $^{\circ}$ C for 20 s, annealing at 56 $^{\circ}$ C for 20 s, and extension at 72 $^{\circ}$ C for 40 s, followed by a final extension at 72 $^{\circ}$ C for 3 min. The primer sequences were listed in **Table 1**. LDRs were performed in a 10 μ L reaction volume containing PCR products (2 μ L), 10 \times Taq DNA ligase buffer (1 μ L), Taq DNA ligase (40 U/ μ L, 0.125 μ L), and probes (10p, 0.01 μ L). The LDR protocol consisted of 35 cycles of 94 $^{\circ}$ C for 30 s and 56 $^{\circ}$ C for 3 min. The LDR

products were sequenced with an ABI 3730XL sequencing instrument (Applied Biosystems, Foster City, CA). The rs4646994 product from the original PCR reaction was digested and separated by 2% agarose gel electrophoresis.

Statistical analysis

Descriptive statistics were used to express demographic and clinical information (**Table 1**).

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Table 4. Comparison of ACE haplotype frequencies between CD patients and controls

Haplotype	Frequency	Group n (%)		2 (P)	OR (95% CI)
		CD	Control		
A-I	0.600	200 (64.1)	350 (57.9)	3.251 (0.071)	1 1.296 (0.977-1.718)
G-D	0.374	99 (31.7)	244 (40.4)	6.594 (0.010)	0.686 (0.650-0.949)
G-I	0.619	7 (2.2)	10 (1.7)	0.385 (0.535)	1.363 (0.514-3.617)

The associations between CD and both allele and genotype frequencies were analyzed by the Chi-square test and logistic regression using SPSS13.0 software (SPSS Inc., Chicago, IL). Deviation from the Hardy-Weinberg equilibrium (HWE) was tested for all SNPs in patients and control subjects with one degree of freedom. As these ACE gene loci were tightly linked in a LD block, a haplotype analysis was conducted using Haploview 4.2 software (Broad Institute, MIT, Cambridge, MA). The relative risks of alleles were estimated using odds ratios (ORs) and 95% confidence intervals (CIs). $P < 0.05$ was considered statistically significant.

Results

Demographic and clinical characteristics of participants

To examine whether the ACE gene polymorphisms SNP rs4291 (promoter, A262T), SNP rs4343 (exon 16, A11860G), and/or rs4646994 (intron 16, Alu ins/del) affect the risk of CD, allele and genotype frequencies were compared between patients with CD and a control group matched for mean age and sex ratio (Table 2).

Differences in allele and genotype frequencies between patients and control subjects

The expected frequency of the genotypes under the assumption of HWE did not differ from the observed genotype frequencies ($P > 0.05$). There were significant differences in rs4343 A/G and rs4646994 I/D allele frequencies between patients with CD and control subjects (rs4343: OR=1.438, 95% CI=1.099-1.882, $P=0.008$; rs4646994: OR=1.559, 95% CI=1.191-2.039, $P=0.001$). Significant associations were observed between the risk of CD and both rs4343 AA/(AG+GG) genotypes ($P=0.039$) and rs4646994 II/(ID+DD) genotypes ($P=0.019$). There was no significant difference in rs4291A/T allele or genotype fre-

quency between patients with CD and control subjects ($P > 0.05$) (Table 3).

LD and associations between haplotypes and the risk of CD

These polymorphisms were in tight but not complete LD (Figure 1). The D' values suggest that rs4646994 and rs4343 constitute a haplotype block. The two most frequent haplotypes, A-I and G-D, complemented each other at the two loci. The frequency of the G-D haplotype was significantly lower in patients with CD than control subjects (31.7% vs. 40.4%, $P=0.010$; Table 4).

Discussion

The incidence of CD in Asia is increasing rapidly [2, 3]. Abnormal vascular signaling contributes to the pathogenesis of CD [10-12] and ACE acts as a critical vascular regulator by controlling serum levels of angiotensin II. A significant association was demonstrated between the risk of CD and two ACE polymorphisms, SNP rs4343 (exon 16, A11860G), and rs4646994 (intron 16, Alu ins/del). Specifically, the rs4343G and rs4646994D alleles and their haplotype combination (G-D) significantly reduce the risk of CD in Chinese Han patients, possibly by effecting angiotensin activity under conditions that trigger CD.

Endothelial cells of the intestinal microcirculation regulate innate immunity by modulating leukocyte recruitment, barrier permeability, and cytokine signaling, in addition to traditional roles in coagulation, vasoconstriction, and angiogenesis [30]. Pathologically, CD is characterized by the appearance of sarcoid-like granuloma and vascular endothelial-associated lesions, particularly granulomatous vasculitis and gastrointestinal infarction [31, 32]. Indeed, these vascular endothelium-associated lesions are the most prominent and earliest feature of CD. In patients with CD, macrophage and lym-

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phocyte aggregates can be observed on the surface of the gastrointestinal mucosa, especially around the lesions, and perpetuate formation of granuloma. A growing body of evidence suggests that vascular endothelial damage may be the trigger for IBD [10-12, 32]. ACE impacts hemodynamics through regulation of angiotensin II and inflammatory processes by regulation of angiotensin II as well as degradation of bradykinin and substance P. However, there is no consistent relationship between activity of ACE and CD, with some studies reporting reduced ACE [33, 34] and other studies reporting unaltered ACE in patients with CD [35, 36]. However, CD follows a relapse-remission pattern [1], hence it is necessary to examine the activity of ACE during these different phases. Moreover, many additional factors influence the levels of ACE aside from ACE genotype [1, 33, 34, 37, 38] including zinc absorptive capacity, cytokines [39], and steroids [32].

The SNPs chosen for this study were those most strongly linked to the risk of autoimmune diseases [19, 20]. Protective roles for 11860G and rs4646994D alleles were found, while the 11860AA genotype and the I allele in the dominant genetic model enhanced the risk of CD. The G-D haplotype conferred protection against CD in the Chinese Han population. Routine genotyping of ACE may identify individuals at risk for CD, thereby facilitating closer clinical monitoring. Such patients should be advised to avoid known environmental risk factors such as smoking [2].

The present study has several limitations. The control subjects were recruited from a hospital and hence may not accurately reflect the local community. Also, the patient sample was relatively small due to the limited time for enrolling patients. Third, environmental factors were not documented or controlled. Finally, the effects of these polymorphisms on the activity of ACE in the gut during different phases of pathogenesis and expression of CD have not yet been examined. Nonetheless, the association between ACE SNPs and progression of CD warrants further study in larger multicenter patient groups.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jie Zhong, Department of Gastroenterology, Ruijin Hospital, School of Medicine, Shanghai Jiaotong University Shanghai 200025, China. Tel: 86-21-64370045 Ext. 600907; E-mail: Jimmyzj64@hotmail.com

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