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doi:10.3748/wjg.14.4923

World J Gastroenterol 2008 August 21; 14(31): 4923-4927
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

OCTN and *CARD15* gene polymorphism in Chinese patients with inflammatory bowel disease

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Supported by Doctoral Natural Science Fund of Guangdong Province, China, No. 04300361
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Received: May 20, 2008 Revised: July 19, 2008
Accepted: July 26, 2008
Published online: August 21, 2008

Abstract

AIM: To investigate the single nucleotide polymorphism (SNPs) distribution of NOD2/CARD15 (R702W, G908R), OCTN1 1672C/T and OCTN2-207G/C in Chinese patients with inflammatory bowel disease (IBD).

METHODS: A total of 61 patients with Crohn's disease (CD), 151 patients with ulcerative colitis (UC), and 200 unrelated healthy controls were genotyped. Genotyping was performed by sequence specific primer polymerase chain reaction (PCR-SSP) or by restriction fragment length polymorphism (PCR-RFLP) analysis.

RESULTS: Among the subjects in our study groups, including patients with CD, UC and healthy controls, none had OCTN and CARD15 variants and very rare IBD family history was found in our patients with the percentage of 0 (0/61 with CD) and 1.3% (2/151 with UC).

CONCLUSION: Our results indicate that although OCTN or CARD15 variation is associated with susceptibility to IBD in Western populations, these might be rare and may not be associated with susceptibility to IBD in Chinese patients.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; CARD15; Carnitine/organic cation transporter gene

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Li M, Gao X, Guo CC, Wu KC, Zhang X, Hu PJ. *OCTN* and *CARD15* gene polymorphism in Chinese patients with inflammatory bowel disease. *World J Gastroenterol* 2008; 14(31): 4923-4927 Available from: URL: http://www.wjgnet.com/1007-9327/14/4923.asp DOI: http://dx.doi.org/10.3748/wjg.14.4923

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), the two common forms of idiopathic inflammatory bowel disease (IBD), are chronic, relapsing inflammatory disorders of the gastrointestinal tract. CD and UC are very common in developed countries with a prevalence of 0.7-11.6 per 100 000 and 2.0-14.3 per 100 000, while it is relatively uncommon in Asian countries with a prevalence of 0.08 per 100 000 and 0.5 per 100 000 in Japan[1]. However, the incidence of IBD has been increasing in some Asian countries in recent years, and China is one of the notable countries[2].

The precise etiology of the disease is unknown, but interplay of environmental risk factors and immunologic changes will trigger the onset of the disease in a genetically susceptible host. Epidemiological studies in the past suggested a genetic susceptibility that has been confirmed by total genome scans and candidate gene studies. After the IBD1 locus in the chromosome 16 was identified as a CD locus by Hugot *et al*[3], fine mapping of the IBD1 locus and following candidate gene approach led people to identify the *CARD15* (previously NOD2) as a susceptibility gene of CD. The *NOD2/CARD15* gene product is expressed in monocytes. It is involved in the binding of bacteria lipopolysaccharides and peptidoglycans so that it played an important role in activation of nuclear transcription factor kappa-B (NF-κB) in inflammatory response. Two missense mutations Arg702Trp (2104C→T), Gly908Arg (2722G→C) and one frameshift mutation (3020insC) of the *NOD2/CARD15* gene affecting the function of binding microbial pathogens are independently associated with the development of CD[4].

Numerous genome-wide scans and replication studies have identified IBD susceptibility loci since the initial

study was published in 1996 by Hugot *et al*[3]. Recent studies suggested that *OCTN* (Carnitine/organic cation transporter gene) 1 and 2 in the IBD5 locus on chromosome 5 both encoded organic cation transporters and revealed significant associations with CD. The OCTN family is a family of transporter proteins for organic cations, and may also transport carnitine, an essential cofactor of the metabolism of lipids. OCTNs are therefore important in the maintenance of intracellular homeostasis and play an important role in the energy production of the cell. A C1672T substitution in exon 9 of the *OCTN1* gene and a G-207C in the *OCTN2* promoter region were indicated as functional and causative mutations to increase susceptibility to CD[5].

The aim of the present study was to investigate the single nucleotide polymorphism (SNPs) distribution of *NOD2/CARD15* (R702W, G908R), *OCTN1* 1672C/T, *OCTN2*-207G/C and its association with IBD in Chinese patients.

MATERIALS AND METHODS

Study population

Blood samples from 61 patients with CD and 151 patients with UC were prospectively collected at the IBD Outpatient Clinic of the first affiliated hospital of Zhongshan University (Guangzhou, Guangdong Province, China) and Xijing Hospital of the Fourth Military Medical University (Xi'an, Shaanxi Province, China) between March 2005 and June 2006. All patients were followed up at least for one year and registered with an integrated clinical and epidemiological registry. A total of 212 healthy controls matched for age, sex and geography were healthy physical examinees in the two hospitals. All patients and healthy controls were of unrelated Chinese Han nationality. The diagnosis of either CD or UC was in accordance with previously established international criteria[6] based upon clinical, endoscopic, radiological and histopathological findings. All patients gave informed consent to participate in the study that was approved by the Ethics Committee of Zhongshan University and the Fourth Military Medical University.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the spin column technique (TIANamp Blood DNA kit, Tiangen Biotech, China).

Gene polymorphisms(R702W,G908R, *OCTN1* 1672C/T, *OCTN2*-207G/C) were determined using the sequence specific primers by polymerase chain reaction (SSP-PCR). Primer sequences and methods are depicted in Table 1. The PCR cycling parameters were a denaturing step at 94℃ (3 min); 5 cycles of 94℃ (30 s), 70℃ (45 s), 72℃ (30 s); 18 cycles of 94℃ (30 s), 65℃ (50 s), 72℃ (30 s); 10 cycles of 94℃ (30 s), 55℃ (1 min), 72℃ (1 min); and a final elongation step of 72℃ (10 min). The PCR products were electrophoresed on 2% agarose gels containing ethidium bromide and viewed under ultraviolet light.

Restriction fragment length polymorphism (RFLP) and sequencing were performed as means of verifying the PCR results. Five samples were chosen from each allele (including homozygous wild-type, heterozygous SNP and homozygous SNP) to perform RFLP and sequencing. Sequencing was performed by AuGCT Corporation of Beijing. The primers of sequencing were the same with RFLP. RFLP was performed as follows: (1) PCR amplification: initially a denaturing step at 96℃ (1 min); 25 cycles of 96℃ (30 s), 70℃ (40 s), 72℃ (30 s); 10 cycles of 96℃ (30 s), 65℃ (30 s), 72℃ (30 s); and a final elongation step of 72℃ (10 min). (2) RFLP: 10 μL of the PCR products mixed with 2 μL 10 × buffer and 2 μL restriction enzyme, then adding water to 30 μL, incubated for 12 h at 37℃ and electrophoresed on 20% non-denaturing polyacrylamide gels, finally viewed under ultraviolet light after stained with ethidium bromide solution for 30 min. Primer sequences and restriction enzymes are depicted in Table 2.

The results of gene sequence and RFLP are identical. The result of SSP-PCR about G908R and *OCTN2* -207G/C was consistent with sequence and RFLP, so we chose 30 samples to perform RFLP and obtained the same result. But the result of SSP-PCR about R702W and *OCTN1* 1672C/T was not consistent with sequence or RFLP, so we changed all samples to perform RFLP.

Table 1 Primer sequences for SSP-PCR genotyping

SNP	Primers	PCR product (bp)
OCTN1 1672C/T	W: TCTGACTGTCCTGATTGGA ATCC	Allel C: 518 bp
(rs1050152)	S: TAGTCTGACTGTCCTGATT GGAATCT C: TTTTGAGACGGAGTTT TGCTCTTGT	Allel T: 520 bp
OCTN2 -207G/C	W: GCACGACCAGGGAAGGTTG	Allel G: 493 bp
(rs2631367)	S: GCACGACCAGGGAAGGTTC C: TCCCAGCCTCTCTAC TAGGGTAGTT	Allel C: 493 bp
R702W 2104C/T	W: CTGAGAAGGCCCTGCTCC	Allel C: 396 bp
(rs2066844)	S: CATCTGAGAAGGCCCTGCTCT C: CAATGCCCAGTAAC ACTCACTACAG	Allel T: 399 bp
G908R 2722G/C	W: TGGCCTTTTCAGATTCTGGG	Allel G: 308 bp
(rs2066845)	S: TGGCCTTTTCAGATTCTGGC C: TGTATCAAAACCCTG AGAGGACAA	Allel C: 308 bp

Statistics analysis

Comparison between cases and controls was made using the Chi-square test for categorical data with the SPSS software ver.13.0.

RESULTS

In this study, we first performed genotyping by SSP-PCR with less cost and time than RFLP or sequencing. But the shortage of SSP-PCR is easy to result in false positivity. So after we completed the SSP-PCR, we used

Table 2 Primer sequences and restriction enzymes used for RFLP genotyping

SNP	Primers	Restriction enzyme	Length of restriction fragments
OCTN1 1672C/T	F: CGTCATGGGTAGTCTGACTGTCCTGATTGGGATC	*Bam*H I	Allel C: 30 + 88 bp
(rs1050152)	R: TCCTACTTACCATTTCACTTTCTGCATCTGCTCTAAGG		Allel T: 118 bp
OCTN2 -207G/C	F: GCGCCGCTCTGCCTGCCAG	*Msp* I	Allel G: 44 + 83 bp
(rs2631367)	R: AGGGTAGGCTCGCGAGCTGACACC		Allel C: 127 bp
R702W 2104C/T	F: TGGGGCCTGCTGGCTGAGTG	*Msp* I	Allel C: 76 + 45 bp
(rs2066844)	R: GTGCAGCTGGCGGGATGGAG		Allel T: 121 bp
G908R 2722G/C	F: TCTGGCTGGGACTGCAGAGG	*Bst*U I	Allel G: 131 bp
(rs2066845)	R: CCCCTCGTCACCCACTCTGTCGC		Allel C: 109 + 22 bp

Table 3 Demographics and phenotype of IBD patients

	CD patients (n = 61)		UC patients (n = 151)
Sex (M:F)	40:21		91:60
Median age (yr, mean ± SD)	36.9 ± 13.7		43.8 ± 13.4
Patients with relative(s) who have IBD	0		2 (1.3%)
Location of CD		Location of ulcerative colitis	
Small bowel only (%)	25 (41.0)	Rectum sigmoid colon	86 (57.0)
Colon only (%)	7 (11.5)	Left hemicolon	18 (11.9)
Small bowel & colon (%)	29 (47.5)	Extensive	47 (31.1)
Behaviour of Crohn's disease (%)		Severe criteria of ulcerative colitis	
Non-stricturing, non-penetrating (%)	26 (42.6)	Mild	74 (49.0)
Penetrating (%)	15 (24.6)	Moderate	57 (37.7)
Stricturing (%)	19 (31.1)	Severe	20 (13.2)
Stricturing & penetrating (%)	1 (1.6)		

Table 4 Genotype and allele results

SNP	Group	Cases	Genotype			Allele	
OCTN1			C/C	C/T	T/T	C	T
1672C/T	CD	61	61	0	0	122	0
(rs1050152)	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0
OCTN2			G/G	G/C	C/C	G	C
-207G/C	CD	61	61	0	0	122	0
(rs2631367)	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0
R702W			C/C	C/T	T/T	C	T
2104C/T	CD	61	61	0	0	122	0
(rs2066844)	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0
G908R			G/G	G/C	C/C	G	C
2722G/C	CD	61	61	0	0	122	0
(rs2066845)	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0

RFLP and sequencing to verify the result. The SSP-PCR results of G908R and *OCTN2*-207G/C were consistent with RFLP or sequencing so that the SSP-PCR is successful in genotyping these alleles. On the contrary, we failed to genotype alleles of R702W and *OCTN1* 1672C/T by SSP-PCR, we therefore changed to use RFLP which had been used to detect polymorphism for a long time with reliable result.

We found very rare IBD family history in our patients with the percentage of 0 (0/61 with CD) and 1.3% (2/151 with UC). The demographics and phenotype of the IBD patients are described in Table 3.

As shown in Table 4, we found that the four SNPs, *OCTN1* 1672C/T, *OCTN2*-207G/C, R702W and G908R were completely absent in the Chinese Han nation population, in either the IBD patients or the control group. These results demonstrated that *OCTN* and *CARD15* variations might be rare and may not be associated with susceptibility to IBD in Chinese patients of Han nation.

DISCUSSION

In the present study, we found that the polymorphism of C1672T in exon 9 of *OCTN1*, G-207C in the *OCTN2* promoter region, and 2104C/T(R702W), 2722G/C(G908R) in *CARD15* were completely absent in Chinese patients with IBD and healthy controls. The study suggested that the four SNPs might not play a role in susceptibility of IBD in Chinese patients, thereby differing from the case of Western populations, but consistent with the results in Asian population. In our study, there were 212 IBD patients (61 CD, 151 UC) and 200 healthy controls. Compared with the studies in the Chinese population before, we had the largest number of cases. In Asia, it was the first study to detect the polymorphism of OCTN in UC patients.

In the last ten years, there have been tremendous researches on genetic susceptibility of inflammatory bowel diseases (IBD) and over 10 chromosomal regions have been identified by genome-wide scanning. The regions on chromosomes 16, 12, 6, 14, 5, 19 and 1 have been renamed IBD 1-7, respectively[7]. Further fine mapping as well as candidate gene studies have already led to the identification of a number of susceptibility genes including CARD15, DLG5, *OCTN1* and 2, NOD1, HLA, TLR4, TNF-α, IL-1RA, and ICAM-1[8]. The *CARD15* gene is undoubtedly replicated most widely at present. The three *NOD2/CARD15* variant alleles, Arg702Trp, Gly908Arg and 3020insC were found to increase the risk of CD in Caucasians, including those from Germany, England[9], Australia[10] and America[11]. When *OCTN1* and 2 were reported to be associated to IBD, the west-

ern countries such as Canada[5], England[12], German[13], Greek[14], Spain[15] and New Zealand[16] carried out experiments and proved that the SNPs of *OCTN1* and 2 independently or the haplotype OCTN-TC (SNPs of OCTN1 and 2 create a two-allele risk haplotype, TC) were positively associated with IBD (with CD only in most studies). On the contrary, studies performed in Asian population differed from the case in Caucasians. The three NOD2 mutations were proved to be totally absent in the studies of Yamazaki *et al*[17] in Japan with 483 CD patients, Lee *et al*[18] in Korea with 128 CD and 47 UC by sequencing, and Leong *et al*[19] in Hong Kong with 65 CD and 63 UC, Gao *et al*[20] in Zhejiang University of China with 32 CD and 110 UC by SSP-PCR. Guo QS in Wuhan University of China found Two heterozygotes of the 3020insC mutation in 74 UC patients and one in 15 CD, and only one in healthy controls by SSP-PCR. So they concluded that the NOD2 3020insC mutation was not associated with CD or UC in Hubei Han population[21]. Similar to the *CARD* gene, Yamazaki *et al*[22] in Japan found the SNPs of OCTN1 and 2 were completely absent with 484 CD patients and 345 healthy control by means of sequencing. The studies above showed that there was apparent genetic heterogeneity among Caucasians and Asians, so there should be a presence of ethnic differences in susceptibility to IBD in Chinese population.

In our study, familial clustering was rare in IBD patients. Although Chinese, Korean and Japanese races differ, the familial aggregation was similarly rare in IBD patients from the three countries. Does low prevalence of familial clustering and absence of NOD2/CARD15 and OCTN gene variants suggest that genetic factors may play a less important role in the development of IBD in the Asian population? The study of Kim *et al*[23] in Korea did not support this point. He found that although a positive family history [21 of 1043 (2.01%) with UC and 6 of 397 (1.51%) with CD] is much lower than that with Western patients, the population relative risk was 13.8 in first-degree relatives, indicating that a positive family history is an important risk factor for IBD in Koreans. Montgomery *et al*[24] found that young Asians who were born in Britain are at a significantly higher risk of developing IBD than the indigenous European population with relative odds of 6.1. This may reflect a greater genetic predisposition to IBD when uncovered by exposure to environmental factors. Undoubtedly, genetic susceptibility plays a most important role in the etiology of IBD. Recently, IL23R has been regarded as a milestone in unraveling etiology of CD and SNPs of IL23R was reported to be associated with CD[25]. We are recruiting more cases and controls to investigate whether SNPs of IL23R would also play a protective role in Chinese CD patients. Since there is great genetic heterogeneity between Chinese and the Caucasians, further studies including total genome-wide scans among Chinese patients are warranted to identify genes susceptible to IBD which would shed more light on the etiology of this disease in our own country.

COMMENTS

Background

Inflammatory bowel disease (IBD), including two clinical subtypes: Crohn's disease (CD) and ulcerative colitis (UC), has been increasing in some Asian countries in recent years, and China is one of the notable countries. Previous epidemiological studies suggested a genetic susceptibility in IBD which has been confirmed by molecular biology techniques. CARD15 (previously NOD2) was first confirmed to be a susceptible gene of CD. Two missense mutations Arg702Trp (2104C→T), Gly908Arg (2722G→C) and one frame-shift mutation (3020insC) of the NOD2/CARD15 gene affecting the function of binding microbial pathogens are independently associated with the development of CD. In recent years, A C1672T substitution in exon 9 of the OCTN1 gene and a G-207C in the OCTN2 promoter region were indicated as functional and causative mutations to enhance the susceptibility to IBD.

Research frontiers

In the last ten years, there have been tremendous researches on genetic susceptibility of IBD and over 10 chromosomal regions have been identified by genome-wide scanning. Further fine mapping as well as candidate gene studies have already led to the identification of a number of susceptible genes. When CARD15 was first confirmed to be a susceptible gene of CD, its three variant alleles, Arg702Trp, Gly908Arg and 3020insC were found to increase the risk of CD in many western countries but completely different in Asian countries. Similar to the CARD gene, many western countries have proved that the SNPs of OCTN1 and 2 were positively associated with IBD but absent in Japanese. These studies showed that there was apparent genetic heterogeneity between Caucasian and Asian as well as Chinese population.

Innovations and breakthroughs

The study suggested that the four SNPs, C1672T, G-207C, 2104C/T and 2722G/C, might not play a role in susceptibility of IBD in Chinese patients, thereby differing from the case of Western populations, but consistent with the results in Asian population. Compared with the studies in the Chinese population before, we had the largest number of cases. In Asia, it was the first study to detect the polymorphism of OCTN in UC patients.

Applications

With great genetic heterogeneity between Chinese and the Caucasian, further studies including total genome-wide scans among Chinese patients are warranted to identify genes susceptible to IBD which would shed more light on the etiology of this disease in China.

Peer review

In this study, the authors found that of the Chinese patients with IBD as well as the healthy controls none had OCTN and CARD15 variants. These results suggest that OCTN and CARD15 are rare in the Chinese population and may not be associated with susceptibility to IBD in Chinese patients. This was an interesting study with an aim that was well justified.

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