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• *BRIEF REPORTS* •

Association of polymorphisms of IL and CD14 genes with acute severe pancreatitis and septic shock

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Abstract

AIM: To investigate IL-1β+3 594 in the 5th intron, IL-10-1 082 and CD14-159 polymorphisms in patients with acute pancreatitis (AP) and septic shock.

METHODS: The study included 215 patients (109 with acute severe pancreatitis (SAP), 106 with acute mild pancreatitis (MAP)) and 116 healthy volunteers. Genomic DNA was prepared from peripheral blood leukocytes. Genotypes and allele frequencies were determined in patients and healthy controls using restriction fragment length polymorphism analysis of PCR products.

RESULTS: The frequencies of IL-1β+3 594T, IL-10-1082G and CD14-159T allele were similar in patients with mild or severe pancreatitis and in controls. Within SAP patients, no significant differences were found in the allele distribution examined when etiology was studied again. Patients with septic shock showed a significantly higher prevalence of IL-10-1082G allele than those without shock ($\chi^2 = 5.921$, $P = 0.015$).

CONCLUSION: IL-10-1082G plays an important role in the susceptibility of SAP patients to septic shock. Genetic factors are not important in determination of disease severity or susceptibility to AP.

Key words: Gene polymorphism; Septic shock; Pancreatitis; Genes

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INTRODUCTION

Acute pancreatitis (AP) is a common disease that normally runs a benign course in the majority of patients. However, in up to 20% of individuals the disease is severe and may have a mortality close to 20%[1]. Two weeks after the onset of acute severe pancreatitis (SAP), sepsis-related complications resulting from systemic inflammatory response syndrome (SIRS) or infection of pancreatic necrosis or bacteria translocation often occur. There is evidence that the production of tumor necrosis factor-α, interleukin (IL)-1β, IL-6, and IL-8 may play a vital role in AP. In addition, anti-inflammatory response, especially IL-10, plays an important role in determining prognosis of AP[2]. Several methods for estimating the complications are widely used in clinic, such as Atlanta classification, Acute Physiology and Chronic Health Evaluation II, Imrie and Ranson scores, Balthazar computed tomographic scoring system, and C-reactive protein. However, these methods have little value in predicting which patients will develop pancreatic infection and SAP-associated septic shock.

It has been hypothesized that there is a correlation between polymorphisms in TNF-α, IL-1β, and IL-10 genes and differential production of respective cytokines[3]. Some of these polymorphisms affect clinical outcome in inflammatory diseases including AP[3-5]. IL-10 is an anti-inflammatory cytokine and plays an important role in downregulating cell-mediated inflammatory responses. Human IL-10 gene is located on chromosome 1 and has been mapped to the junction between 1q31 and 1q32. Three single base pair (bp) substitutions in IL-10 gene promoter at positions -1 082G-A, -819T-C, and -592A-C from the transcriptional start site have been identified. At position -1 082 bp from the transcriptional start site, the presence of G is associated with higher and A with lower production of IL-10 by PBMC cultures[6]. In contrast, IL-1β is a potent proinflammatory cytokine released by macrophages in systemic inflammatory responses. It not only has important biologic effect but also regulates inflammatory reaction and immune response by promoting expression of other cytokines, such as IL-6 and IL-12. IL-1β gene located on chromosome 2 is 7 kb, and has seven exons and six introns. A polymorphism is found at position +3 954 located in the 5th intron of IL-1β gene with a T substitution of C. *In vitro* study demonstrated that IL-1β+3 594 at the 5th exon significantly influences the production of IL-1β[7]. Their polymorphisms may have some association with the development of severe AP and septic shock.

CD14, a 55 ku membrane-anchored protein, is a pattern-recognition receptor for several microbial products,

such as lipopolysaccharide (LPS). It can be expressed on neutrophils, monocytes/macrophages, and fibroblasts, all of which can produce cytokines such as IL-1 and TNF-α in response to LPS stimulation[9]. Recently, a-159 G/A polymorphism in the promoter region of CD14 gene involves a C>T substitution at bp -159 of the 5' flanking region of CD14 gene. Genotypes include CC, CT, and TT alleles. Subjects carrying the T allele have been shown to have significantly higher sCD14 levels than those carrying the C allele[8,9]. Therefore, CD14 polymorphism could be a genetic factor responsible for interindividual differences in the susceptibility to bacterial infection. However, to the best of our knowledge, there were no reports on the linkage of CD14 and pancreatitis.

Our previous studies have shown that some polymorphisms in TNF gene correlate with severe sepsis or SAP-associated septic shock, although no association has been found between TNF gene polymorphisms and SAP[4,5,10]. The purpose of this study was to test the hypothesis that IL and CD14 gene polymorphisms have some correlation with the development of SAP and septic shock.

MATERIALS AND METHODS

Subjects

Patients with a first attack of unequivocal AP from July 2001 to December 2003 were prospectively considered. The diagnosis of AP was based on an increased-amylase activity (enzymatic colorimetric test) in serum and CT verification of pancreatitis. Etiology of AP was gallstones found in radiological and endoscopic retrograde cholangiopancreatography findings, alcoholic if patients were heavy consumers of alcohol (more than 80 g of alcohol per day for over 6 mo)[11], and idiopathic if no other identifiable cause could be discovered. Pancreatitis was classified as severe when APACHE II score ≥ 8[12] and CT severity index ≥ 4[13]. Septic shock was defined according to ACCP/SCCM consensus conference criteria[14]. The control group consisted of 116 healthy volunteers. All subjects gave written informed consent, and the protocol was approved by the local ethics committee.

In order to be eligible for the enrollment, all the subjects from the two groups were yellow Chinese Han. The exclusion criteria were defined as follows: age>75 years, cardiac failure (class>III), liver insufficiency (Child C), patients with evidence suggestive of a diagnosis of chronic pancreatitis and consanguineous mating.

DNA extraction

Genomic DNA was purified from 5 mL of peripheral blood samples using Wizard genomic DNA purification kit (Promega) according to the manufacturer's instructions.

IL-10-1082 G to A substitution

PCR was used to amplify a 377-bp fragment of the IL-10 genomic sequence using primers: upstream, 5'-CCAAGACAACACTACTAAGGCTCCTTT-3; downstream, 5'-GCTTCTTATATGCTAGTCAGGTA-3'[15] (Nanjing Bio Eng Co.). The PCR conditions were at 95 ℃ for 2 min, 35 cycles of 95 ℃ for 40 s, 56 ℃ for 40 s, 72 ℃ for 40 s, 72 ℃ for 7 min using reagents purchased from Promega on a gene cycler (BIO-RAD, Japan). The PCR products were digested directly with 2 U *Xag*I restriction enzyme (Promega) at 37 ℃ for 6 h. Digested DNA was analyzed on 5% polyacrylamide gels. Ethidium bromide staining of the gel demonstrated three fragments of 253, 97, and 27 bp for G/G, four fragments of 280, 253, 97, and 27 bp for G/A, two fragments of 280 and 97 bp for A/A.

IL-1β polymorphism

A 249-bp fragment of the IL-1β genomic sequence including the polymorphic *Taq*I site was amplified using PCR. The following nucleotide sequences were used for PCR amplification: 5'-GTTGTCATCAGACTTTGACC-3', 5'-TTCAGTTCATATGGACCAGA-3'[16] (Nanjing Bio Eng Co.). The PCR conditions were at 97 ℃ for 2 min, 35 cycles of 95 ℃ for 40 s, 55 ℃ for 40 s, 74 ℃ for 30 s, 72 ℃ for 7 min using reagents purchased from Promega on a gene cycler (BIO-RAD, Japan). The PCR products were digested directly with 2 U *Taq*I restriction enzyme (Promega) at 37 ℃ for 4 h. Digested DNA was analyzed on 5% polyacrylamide gels. Ethidium bromide staining of the gel demonstrated the original 249 bp fragment for T/T, two fragments of 135 and 114 bp for C/C, three fragments of 249, 135, and 114 bp for C/T.

CD14-159 C/T polymorphism

PCR was used to amplify a 166-bp fragment of the CD14 genomic sequence using primers: upstream, 5'-TGCCAGGAGACACAGAACCC-3'; downstream, 5'-TGTCATTCAGTTCCCTCCTG-3'[9] (Nanjing Bio Eng Co.). The PCR conditions were at 96 ℃ for 2 min, 35 cycles of 96 ℃ for 40 s, 54 ℃ for 40 s, 72 ℃ for 30 s, and 72 ℃ for 7 min using reagents purchased from Promega on a gene cycler (BIO-RAD, Japan). The PCR products were digested directly with 2 U *Hae*III restriction enzyme (Promega) at 37 ℃ for 4 h. Digested DNA was analyzed on 5% polyacrylamide gels. Ethidium bromide staining of the gel demonstrated the original 166-bp fragment for T/T, two fragments of 86 and 80 bp for C/C, three fragments of 86, 80, and 166 bp for C/T.

In addition, 30% of samples were randomly selected to be genotyped a second time to ensure reproducibility. Genotyping for all subjects was performed with no knowledge of clinical status.

Statistical analysis

Allelic frequencies were determined for statistical significance by χ^2 test. Analysis was made by SPSS 11.0, and $P<0.05$ was considered statistically significant.

RESULTS

Characteristics of the patients

On the basis of the selection criteria, 109 patients (59 females and 50 males) with SAP were studied. Thirty-three developed septic shock (septic shock group), and 76 did not develop septic shock (nonseptic shock group). The APACHE II and CT scores at the time of admission were similar in both septic shock group and nonseptic group.

This study was undertaken in selected patients with acute mild pancreatitis (MAP) (n = 106) as defined by CT severity index and APACHE II score, and matched with SAP for age, sex, and cause of pancreatitis. Patients with MAP had an uneventful recovery. The control group included 116 healthy volunteers (65 females and 51 males).

Polymorphisms of two IL genes

The distribution of IL-10-1 082 and IL-1β polymorphisms in different groups is shown in Tables 1 and 2. The overall IL-10-1 082G and IL-1β+3 594T allele frequencies were similar in patients with mild or severe pancreatitis. Further, no significant difference in allele frequencies studied was noted between patients with AP and control subjects.

Table 1 Comparison of allele frequency between MAP group and SAP group

		MAP (n = 106)	SAP (n = 109)	P
	CC	94	95	
IL-1β	CT	12	14	
+3 594	TT	0	0	
	T allele	12 (5.7)	14 (6.4)	0.740
	GG	0	0	
IL-10	AA	75	81	
-1 082	GA	31	28	
	G allele	31 (14.6)	28 (12.8)	0.592
	CC	62	66	
CD-14	TT	14	14	
-159	CT	30	29	
	T allele	58 (27.4)	57 (26.1)	0.777

Table 2 Comparison of allele frequency between AP and controls

		AP (n = 215)	Controls (n = 116)	P
	CC	189	98	
IL-1β	CT	26	18	
+3 594	TT	0	0	
	T allele	26 (6.0)	18 (7.8)	0.399
	GG	0	0	
IL-10	AA	156	79	
-1 082	GA	59	37	
	G allele	59 (13.7)	37 (15.9)	0.437
	CC	128	71	
CD-14	TT	28	13	
-159	CT	59	32	
	T allele	115 (26.7)	58 (25.0)	0.626

The distribution of IL-10-1082G and IL-1β allele frequencies between septic shock group and nonseptic shock group is shown in Table 3. Patients with septic shock showed a significantly higher prevalence of the IL-10-1082G than those without septic shock (χ^2 = 5.921, P = 0.015). No significant difference in IL-1β+3594T allele frequency was found between septic shock patients and nonseptic shock patients.

CD14-159 C/T polymorphism

The distribution of CD14-159 polymorphism in different groups is shown in Tables 1 and 2. The CD14-159T allele frequency was similar in patients with mild or severe pancreatitis. No significant difference in CD14-159T allele frequency was noted between patients with AP and control subjects.

Comparison of CD14-159 allele frequency between septic shock group and nonseptic shock group is shown in Table 3. No significant difference was found in the allele frequency between septic shock patients and nonseptic shock patients.

Comparisons of polymorphisms in different etiologies of SAP

No significant differences were found in the distribution of allele frequency between any two groups (Table 4).

Table 3 Comparison of allele frequency between septic shock and nonseptic shock groups

		Septic shock (n = 33)	Nonseptic shock (n = 76)	P
	CC	27	68	
IL-1β	CT	6	8	
+3 594	TT	0	0	
	T allele	6 (9.1)	8 (5.3)	0.289
	GG	0	0	
IL-10	AA	19	62	
-1 082	GA	14	14	
	G allele	14 (21.2)	14 (9.2)	0.015
	CC	19	47	
CD-14	TT	5	9	
-159	CT	9	20	
	T allele	19 (28.8)	38 (25.0)	0.559

Table 4 Comparison of allele frequency based on different etiologies of SAP (n)%

Allele	Alcoholic SAP (n = 23)	Gallstone SAP (n = 49)	Idiopathic SAP (n = 34)	P
IL-1β+3 594 T	3 (6.5)	6 (6.1)	5 (7.4)	0.952
IL-10-1 082 G	7 (15.2)	12 (12.2)	9 (13.2)	0.886
CD-14-159 T	11 (23.9)	25 (25.5)	21 (30.9)	0.653

DISCUSSION

In humans, there is increasing evidence that the host's cytokine response is genetically determined[17]. Polymorphic gene sequences of certain cytokines may be potential markers of susceptibility and clinical outcome in different human infectious diseases. In our study, the frequency of well-described variants in IL-1β+3 594 at the 5th exon, IL-10-1 082, and CD14-159 was examined. Our results demonstrated that IL-1β+3594T, IL-10-1082G, and CD14-159T had no correlation with the occurrence or severity of AP. However, the distribution of IL-10-1082G in SAP patients varied, and IL-10-1082G allele was found to be more frequent in septic sock patients than in nonseptic shock patients (P<0.05). The association between septic shock patients and IL-10 polymorphism was restricted to the IL-10-1082G, no such a correlation was seen to either IL-1β+3 594 at the 5th exon or CD14-159 variant.

Before evaluating the role of a cytokine polymorphism played in any disease, three questions need to be answered[18,19]. First, are the subjects homogeneous? To avoid artifact in

population admixture, we selected only Chinese Han people in China. In addition, the consanguineous mating subjects were precluded from our study. Second, does the product of the studied gene play an important role in the pathogenesis of the disease? The central role of IL-1β, IL-10, and CD14 in the occurrence or severity of AP and septic shock has been clearly demonstrated by many studies[20-23]. Third, does the gene polymorphism produce a relevant alteration in the level or function of the gene product? *In vitro*, the *Taq*I polymorphism in human IL-β gene correlates with IL-β secretion[10]. *In vitro* and *in vivo* studies showed that IL-10-1 082 variant significantly influences the secretion of IL-10[24-27]. With regard to CD14-159 polymorphism, there is mounting evidence that the SNP in CD14 genome significantly influences the production of CD14[9].

IL-1 and TNF-α are the most prominent inflammatory mediators and regarded as the "first-line" cytokines. Administration of IL-1β to human beings results in inflammation, tissue injury, and septic shock-like syndrome. Different polymorphisms of the IL-1β gene have been described[7,10], and at least two of them could influence the protein production. One is located within the promoter region, and the other is located in exon 5. In our present study, we examined the frequency of IL-1βA *Taq*I RFLP at the 5th exon and found that it was comparable in patients with mild or severe pancreatitis. Similarly, no significant difference in the allele distribution was noted between patients and controls. In addition, no significant difference in the allele frequency was seen between septic shock patients and nonseptic shock subjects. The results suggest that IL-1βA may not play a principle role in the onset of SAP or SAP-associated septic shock. Our results are in line with the report by Powell *et al*.[28].

In the clinical setting, levels of IL-10 showed a steep increase within the first 24 h from disease onset, and the level of IL-10 on the first day was found to be higher in patients with mild AP than in those with severe AP, suggesting that at position-1082 bp from the transcriptional start site, the presence of G is associated with higher and A with lower production of IL-10[6]. Based on the observation, we postulate that IL-10-1082 polymorphism may have some association with the occurrence or the severity of AP. In our study, no significant difference was found in the frequency of IL-10-1082G between any two groups, but significant difference was found in the distribution of IL-10-1082G between septic shock patients and nonseptic shock patients. The pathophysiology of septic shock is a complex and multifactorial process, involving an imbalance between proinflammatory and anti-inflammatory cytokine release. Our results suggest that in the late stage of SAP, anti-inflammatory cytokine polymorphism likely plays a more important role in the pathopoiesis of septic shock than proinflammatory cytokine polymorphism. The ability to identify patients at high risk for developing septic shock may be a critically important factor that will lead to improvements in the management of septic shock.

CD14, a receptor of LPS, plays a vital role in the mechanism of SIRS and sepsis. Because of the important role of CD14 with respect to LPS binding and signaling, we postulate that the polymorphism in the promoter of CD14 gene might be an important factor in determining the susceptibility to or the severity of AP. In our study, we failed to find an association in CD14-159T frequency between controls and AP or between AP and SAP, indicating that CD14-159T plays no part in disease severity or susceptibility to AP. To the best of our knowledge, there is no report on the association between CD14-159 polymorphism and pancreatitis so far. Our result did not show any difference between patients and controls or between mild pancreatitis and severe pancreatitis. Furthermore, no significant difference was found between CD14-159T frequency and septic shock secondary to severe pancreatitis, indicating that in the late stage of the disease, CD14-159 polymorphism plays little role in the onset of SAP-associated septic shock. This is in line with previous studies[29,30].

Although SAP has many distinct etiologies, the immune system response appears to be almost identical regardless of the cause[1]. In our study, we divided SAP patients into three groups: gallstone pancreatitis, alcohol pancreatitis, and idiopathic pancreatitis according to the etiology of the disease. The observed allele frequency of IL-1β+3594T, IL-10-1082G and CD14-159T was comparable between groups of different etiologies, suggesting that environmental factors may play an important role in the occurrence of SAP.

In conclusion, gene polymorphisms have no association with the occurrence or severity of AP. However, the IL-10-1082G may play an important role in the susceptibility of SAP patients to septic shock.

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