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• BASIC RESEARCH •

Association of two polymorphisms of tumor necrosis factor gene with acute biliary pancreatitis

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Abstract

AIM: To investigate TNF-α-308 and TNFB polymorphisms in acute biliary pancreatitis (ABP) and to related them to the plasma TNF-α levels.

METHODS: Genomic DNA was prepared from peripheral blood leukocytes. Genotypes and allele frequencies were determined in patients (n=127) and healthy controls (n=102) using restriction fragment length polymorphism analysis of polymerase chain reaction (PCR) products. Reading the size of digested bands from polyacrylamide gel demonstrated the two alleles TNF1 and TNF2, or the two alleles TNFB1 and TNFB2.

RESULTS: The frequencies of TNF2 polymorphism and TNFB2 polymorphism were both similar in patients with mild or severe pancreatitis, so were in pancreatitis patients and in controls. Patients with septic shock showed a significantly higher prevalence of the TNF2 than those without. No significant differences were found in the genotype distribution of TNF-α-308 and TNFB among different groups. Plasma TNF-α levels did not differ significantly in ASBP patients displaying different alleles of the TNF gene studied.

CONCLUSION: Results indicate that TNF gene polymorphisms studied play no part in determination of disease severity or susceptibility to acute biliary pancreatitis; however, TNF2 polymorphism is associated with septic shock from ASBP. Genetic factors are not important in determining plasma TNF-α levels in ASBP.

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INTRODUCTION

In China and most other countries, gallstones are the most common cause of acute pancreatitis. There are reports that gallstones account for between one third and two thirds of cases, with an average of 40 to 50 %[1]. Acute severe pancreatitis (ASP) is a serious disease, with highly persistent morbidity and mortality. Generally speaking, the natural course of severe acute pancreatitis progresses in two phases. The first 14 days are characterized by the systemic inflammatory response syndrome resulting from the release of inflammatory mediators. The second stage, beginning approximately 2 weeks after the onset of the disease, is dominated by septic-related complications resulting from infection of pancreatic necrosis or bacteria translocation. Today, with improvements in the care of the critically ill, many patients with ASP survive over early systemic inflammatory response and enter a second phase of illness dominated by sepsis and the consequences of organ failure. More than two thirds of deaths in ASP are due to late septic organ complications[2]. However, the susceptibility and mechanism of septic shock related to ASP are still unclear.

Tumor necrosis factor-α (TNF-α), the early cytokine to be released, is a principal mediator of immune responses to endotoxin. It can be produced in large amounts in several organs during ASP and is also believed to mediate pathophysiological changes[3,4]. Systemic release of TNF-α is associated with septic shock and fatal outcome. TNF-α levels are increased in patients with ASP and septic shock and appear to correlate with clinical outcome.

Because of its short half-life, the value of TNF-α as a marker of susceptibility or severity for ASP is limited. The production and response of TNF-α are partly regulated at the transcription level, the role of polymorphisms of TNF promoter in determination inflammatory disease susceptibility or as a marker of severity has been the subject of intense research[5]. There are many single nuclear polymorphisms within the TNF-α gene promoter. The TNF-α gene shows a polymorphism at position -308 in the promoter region. This polymorphism results in two allele forms, 1 in which a guanine defines the common allele TNF1 and 1 in which an adenosine defines the uncommon allele TNF2[6]. The TNF2 allele has been associated with a variety of inflammatory disorders, including systemic lupus erythematosus, dermatitis herpetiformis, and celiac disease[7]. Furthermore, TNF2 allele has been found to be a stronger transcription activator than the TNF1 allele[8-12], resulting in higher TNF-α levels. Moreover, TNF2 polymorphism has been associated with morbidity and mortality of severe forms of cerebral malaria[13], mucocutaneous leishmaniasis[14], meningococcal disease[15] and septic shock[16].

A polymorphism is also found at position +252 located in the first intron of the TNFβ gene, with a G in the TNFB1 allele and an A in the TNFB2 allele[17]. In contrast to TNF-α, which is expressed mainly by macrophages, TNFβ is expressed and released by lymphocytes. Genes encoding either cytokine are positioned next to each other within the cluster of human leukocyte antigen class III genes on chromosome 6. With respect to high homology and location in the genome, evolutionary studies suggest a common ancestor for both genes that duplicated during evolution. The TNFB1 allele has been associated with a higher TNFβ response at both the mRNA and the protein levels[17]. Furthermore, some studies have found

that the TNFB2 allele results in a higher TNF-α secretory capacity than the TNFB1 allele[8], and higher plasma TNF-α levels[18], whereas others could not confirm this observation.

The present study was focused on ABP. The aim was to investigate TNF-α-308 and TNFB polymorphisms in ABP patients, and to related the polymorphisms studied to plasma TNF-α levels.

MATERIALS AND METHODS

Subjects

127 consecutive patients with a first attack of unequivocal acute biliary pancreatitis (ABP) were prospectively considered from January 2001 to August 2002. The diagnosis of acute pancreatitis was based on clinical criteria, an increased -amylase activity (enzymatic colorimetric test) in serum and CT verification of pancreatitis. Etiology of acute pancreatitis was gallstones, in the presence of appropriate radiological of endoscopic retrograde cholangiopancreatography (ERCP) findings. Pancreatitis is classified as severe when APACHE II score is ⩾8[19] and CT severity index ⩾4[20]. Septic shock was defined according to ACCP/SCCM consensus conference criteria[21]. The control Group came from 102 healthy volunteers. In order to be eligible for the enrollment, all of the subjects from the two groups had to be yellow Chinese Han. The exclusion criteria were defined as follows: (1) age > 75 years; (2) cardiac failure(class>III); (3) liver insufficiency (Child C); (4) White blood cell counts $<0.4\times10^9$/L; (5) immunosuppression; (6) there was a delay of more than 36 hours from onset of abdominal pain and hospitalization; (7) patients who had clinical, radiological, or ERCP evidence suggestive of a diagnosis of chronic pancreatitis. The study was approved by the local Ethics Committee and informed consent had been obtained from the patient or a close relative.

Measurement of plasma TNF-α concentrations

Peripheral venous plasma samples were collected (EDTA anticoagulation) from only ASBP patients at admission, centrifuged and stored at -70 ℃ before analysis. Plasma TNF-alpha concentrations were measured by enzyme immunoassay kit (Quantikine HS Human TNF-alpha immunoassay. kit, R & D Systems, Inc, Minneapolis, MN). The limit of sensitivity was 2.5 pg/mL.

TNF-α-308 G to A substitution

Each patient's DNA was extracted from whole blood using Wizard Genomic DNA Purification kit (Promega) according to the manufacture's instruction. PCR was used to amplify a 107 basepairs fragment of the TNF-α genomic sequence using primers. Upstream: 5' -AGGCAATAGGTTTTGAGGGCCAT3', downstream: 5' -TCCTCCCTGCTCCGATTCCG 3' (Nanjing Bio Eng Co.Ltd.). The following PCR protocol was used: 94 ℃ for 3 minutes; 35 cycles of 94 ℃ for 45 seconds, 60 ℃ for 45 seconds, 72 ℃ for 45 seconds; 72 ℃ for 5 minutes using reagents purchased from Promega on a Gene Cycler™ (BIO-RAD, Japan). The PCR product was digested directly with 2 U NcoI restriction enzyme (Promega) at 37 ℃ for 6 hours. Digested DNA was analyzed on 5 % polyacrylamide gels. Ethidium bromide staining of the gel demonstrated the original 107 basepairs fragment (homozygous patients for allele TNF2, lacking NcoI site), three fragments of 102, 87 and 20 basepairs (heterozygous patients), or two fragments of 87 and 20 basepairs of size (homozygous patients for the allele TNF1), (Figure 1).

TNF-β NcoI polymorphism

A 782 basepairs fragment of the TNF-β genomic sequence, including the polymorphic NcoI site, was amplified using PCR. The following nucleotide sequences were used for PCR amplification[18]: 5' -CCGTGCTTCGTGCTTTGGACTA 3' and 5' -AGAGGGGTGGATGCTTGGGTTC3' (Nanjing Bio Eng Co.). The following PCR protocol was used: 95 ℃ for 3 minutes; 37 cycles of 95 ℃ for 1 minute, 50 ℃ for 1 minute, 72 ℃ for1 minute; 72 ℃ for 5 minutes using reagents purchased from Promega on a Gene Cycler™ (BIO-RAD, Japan). The PCR product was digested directly with 2 U NcoI restriction enzyme (Promega) at 37 ℃ for 6 hours. Digested DNA was analyzed on 5 % polyacrylamide gels. Ethidium bromide staining of the gel demonstrated the original 782 basepairs fragment (homozygous patients for allele TNFB2), three fragments of 782, 586 and 196 basepairs (heterozygous patients), or two fragments of 586 and 196 basepairs of size (homozygous patients for the allele TNFB1), (Figure 2).

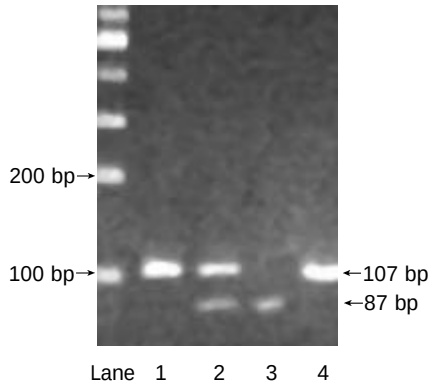

Figure 1 Lane1 and 4, TNF2 homozygote; Lane 2, TNF1/TNF2 heterozygote; Lane 3, TNF1 homozygote.

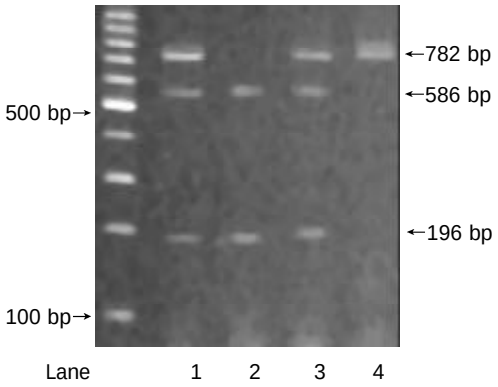

Figure 2 Lane 1 and 3, TNFB1/TNFB2 heterozygote; Lane 2, TNFB1 homozygote; Lane 4, TNFB2 homozygote.

Statistical analysis

Comparison of allelic and genotype frequencies was examined for statistical significance with chi-square test. Descriptive data of continuous variables were tested by Student's *t*-test. Plasma TNF-α levels were reported as median ± SD. Analysis was completed by SPSS 10.0, and a 2-tailed $P<0.05$ was considered statistically significant.

RESULTS

Characteristics of the patients

According to the selected criteria, 61 patients (36 females, males 25) with acute severe pancreatitis were studied. The mean age (± SD) was 54.6±19 years. APACHE II, 11.5±1; CT, 6±1. Of these, 18 had developed septic shock. The APACHE II score and CT score at the time of admission was similar in both septic shock and no septic shock patients. This study was

undertaken in selected patients with acute mild biliary pancreatitis (AMBP, *n*=66) as defined by APOCHE II score[19] and CT severity index[20], and matched with ASBP for age, sex, and cause of pancreatitis. Patients with AMBP had an uneventful recovery. The control group included 102 healthy volunteers, the mean age (± SD) was 44.5±10 years. The distribution of gender was 59 females and 43 males.

Two polymorphisms of tumor necrosis factor gene

The frequency distribution of genotypes for TNF polymorphisms studied is shown in Table 1. There was no significant difference in the TNF-308 or TNFB genotype frequency distributions between patients with mild or severe disease. For the TNF-308 polymorphism, TNF2 was found in 18 (29.5 %) of patients with ASBP compared with 17 (25.8 %) of patients with AMBP (χ^2=0.223, *P*=0.636). Likewise TNFB2 occurred in 42 (68.9 %) of patients with ASBP compared with 44 (66.7 %) of patients with AMBP (χ^2=0.147, *P*=0.702).

Further there were no significant differences in the TNF-308 or TNFB genotype frequency distributions between patients with ABP and controls (Table 1). As to TNF2 frequency, it was found in 35 (27.6 %) of patients with ABP compared with 26 (25.5 %) of controls (χ^2=0.124, *P*=0.725). Likewise TNFB2 occurred in 86 (67.7 %) of patients with ABP compared with 63 (61.8 %) of controls (χ^2=0.882, *P*=0.348 respectively).

TNF2 was found in 9 (50 %) of ASBP patients who developed septic shock compared with 9 (20.1 %) of ASBP patients with no septic shock (χ^2=5.155, *P*=0.023). However, TNFB2 occurred in 13 (72.2 %) of ASBP patients with septic shock compared with 29 (67.4 %) of ASBP patients with no septic shock (χ^2=0.135, *P*=0.713), (Table 2).

Table 1 Comparison of TNF Genotype among different groups

	TNF-308			TNFB		
	G/G	G/A	A/A	1/1	1/2	2/2
ASBP	43 (70.5)	15 (24.6)	3 (4.9)	19 (31.1)	25 (41.0)	17 (27.9)
AMBP	49 (74.2)	14 (21.2)	3 (4.5)	22 (33.3)	26 (39.4)	18 (27.3)
	χ^2=0.040, *P*=0.980			χ^2=0.197, *P*=0.906		
ABP	92 (72.4)	29 (22.8)	6 (4.7)	41 (32.3)	51 (40.1)	35 (27.6)
Control	76 (74.5)	21 (20.6)	5 (4.9)	39 (38.2)	35 (34.3)	28 (27.5)
	χ^2=2.545, *P*=0.280			χ^2=3.594, *P*=0.166		

Note. Comparison by chi-square test. No significant differences were found in the distribution of each genotype frequency [no. (%)] between any of the two groups.

Table 2 Comparison of TNF2 frequency and TNFB2 frequency between septic shock group and no septic shock group

	Septic shock (*n*=18)	No septic shock (*n*=43)	*P*
TNF2	9 (44.4%)	9 (20.9%)	0.023
TNFB2	13 (72.2%)	29 (67.4%)	0.713

Patients with septic shock showed a significantly higher prevalence of the TNF2 than those without. No such association was seen in TNFB2.

Baseline concentrations of TNF-α at inclusion in ASBP

Plasma TNF-α levels at inclusion were detectable in all of the patients with ASBP and shown in Figure 3. At inclusion, among the 61 patients who were admitted for ASBP, 31 (50.8 %) had an increased concentration of TNF-α (normal value <20 pg/mL). There was no significant difference in baseline concentrations of TNF-α, between ASBP patients who developed septic shock and ASBP patients who didn't.

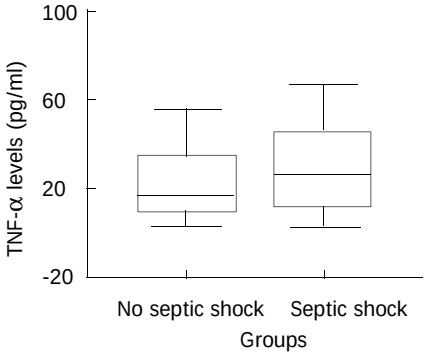

Figure 3 Baseline concentrations of TNF-α at inclusion in ASBP complicated by septic shock or not. *P*=0.643. No significant differences were found in baseline TNF-α levels between septic shock group and no septic shock group.

Association of two polymorphisms of TNF gene with TNF-α levels

In ASBP patients, no association was found in baseline TNF-α levels between TNF2 carrier and TNF1 carrier (30.73±23.05 *vs* 25.65±22.63, *P*=0.430), neither was found between TNFB2 carrier and TNFB1 carrier (25.53±23.71 *vs* 30.73±20.38, *P*=0.412), (Figure 4 and Figure 5).

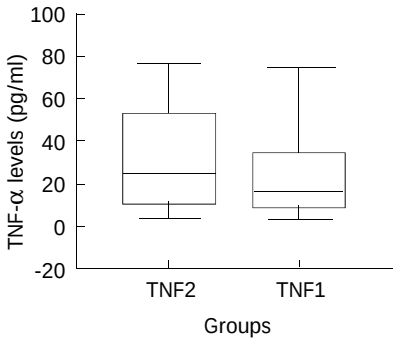

Figure 4 Comparison of TNF-α levels (pg/mL) in ASBP patients based on TNF2 allele. No significant difference was found in TNF-α levels between TNF2 carrier and TNF1 carrier.

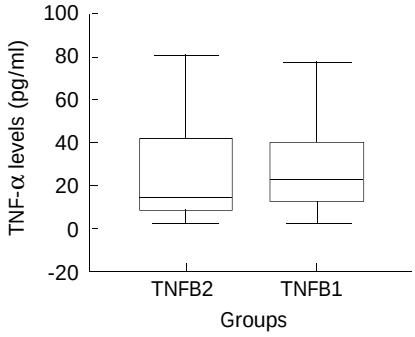

Figure 5 Comparison of TNF-α levels (pg/mL) in ASBP patients based on TNFB2 allele. No significant difference was found in TNF-α levels between TNFB2 carrier and TNFB1 carrier.

DISCUSSION

In the study we have found no association between either TNF-α-308 or TNFB biallelic polymorphism and ASBP or ABP, and thus no evidence that these loci contribute to ASBP susceptibility

or severity. This was in line with previous study[22, 23]. However, the distribution of TNF-α-308 polymorphisms within the ASBP patients varied, and TNF2 allele was found significantly more frequently in the septic sock patients than in no septic shock ones ($P<0.05$). The association between the septic shock patients and TNF polymorphism was restricted to the TNF-α-308 polymorphism (TNF2 allele), no such association being seen with TNFB2. The finding of an apparent association between the TNF-α-308 polymorphism and the septic shock raises the possibility that genetic factors may play a role in controlling the onset of septic shock related to ASBP.

In our study an increased TNF-α value was documented in 50.8 % of ASBP patients at inclusion. This finding confirms two previous clinical studies in which TNF-α was documented in 29 % to 78 % of patients studied[24, 25]. However, there was no significant difference in baseline TNF-α levels between patients who developed septic shock and patients who didn' t. The result suggested that plasma baseline TNF-α level was of little value predicting whether septic shock would occur in ASP.

In sepsis and other diseases TNF polymorphisms have been associated with morbidity and mortality of severe forms[13-16,26]. The present study did not found an association in the distribution of either TNF2 allele frequency or TNFB2 allele frequency between ASBP patients and AMBP patients ($\chi^2=0.223$, $P=0.636$ and $\chi^2=0.147$, $P=0.702$ respectively). The results showed no correlation between the gene polymorphisms studied and disease severity. Comparison of TNF2 allele frequency or TNFB2 allele frequency in patients with ABP and in healthy controls suggested that these polymorphisms studied did not influence disease susceptibility ($\chi^2=0.124$, $P=0.725$ and $\chi^2=0.882$, $P=0.348$ respectively). However, significant difference was found in TNF2 allele frequencies between septic shock patients and non-septic shock patients ($\chi^2=5.155$, $P=0.023$). Indeed, only in severe forms of cerebral malaria[13], mucocutaneous leishmaniasis[14], meningococcal disease[15] and septic shock[16], were morbidity and mortality linked with TNF2 allele or TNFB2 allele. In mild conditions, no such relationship was found between sepsis and the TNF2 allele[27]. As to TNF-α-308 and TNFB genotype, there were no significant difference in the distribution of either type between ASBP patients and AMBP patients, neither was found between ABP patients and controls. It suggested that TNF-α-308 genotype and TNFB genotype were both not related to the susceptibility or severity of ABP.

Although polymorphisms may only be markers of other functionally significant gene polymorphism, at least one of the TNF gene polymorphisms studied is known to have functional significance. It seems that environmental factors trigger cytokine secretion, genetic factors may be important in determining levels of secretion[28]. In vitro studies have identified that individuals may demonstrate consistent differences in leukocyte cytokine secretion[29] and that these difference are probably genetically predetermined[28]. In our study, plasma TNF-α concentrations of ASBP patients with TNF2 allele or TNFB2 allele were not significantly higher than that of patients without TNF2 allele or TNFB2 allele respectively. It suggested that there was no significant correlation between TNF-α concentration and TNF2 or TNFB2 allele carriage. However, many factors could influence plasma TNF-α concentrations. Of these, an important one is its relatively short half-life[30], so we were at great risk of missing the intravascular secretion of this cytokine. Another reason for low plasma TNF-α concentrations may be the breakdown of TNF-α by enzyme released from pancreas into circulation[31, 32]. Furthermore, this detectable level does not take into account the membrane-bound form of TNF-α. In addition, in complex biologic systems, the effect of a single gene polymorphism in determining cytokine production may be minimized through the interaction of other factors[33]. Maybe circulating TNF-α levels do not correspond with the TNF2 and TNFB2 polymorphisms, however, circulating TNF-α levels might be under a multifactoral regulatory process. Local TNF-α levels might be of greater importance and under more control by specific polymorphisms.

To the best of our knowledge, there have two different studies on the association of two polymorphisms of tumor necrosis factor gene with acute severe pancreatitis[23, 35], and our results are in line with theirs. However, they both failed to study the association of two polymorphisms with septic shock due to ASBP. The finding of our study for the first time, to our knowledge, raise the possibility that TNF2 allele may play some role in the susceptibility of septic shock related to ASBP. However, the role, if any, of genetic factors in influencing the occurrence of septic shock awaits confirmation in further prospective studies. If the association between TNF2 allele and septic shock is confirmed, it would have implications not only for understanding of mechanisms of septic shock from ASBP, but also in the clinical management of patients, with the possibility that TNF2 carriers at high risk of septic shock may be identifiable early in the disease course, allowing early and aggressive therapy to be instituted. In addition, the study offers new opportunities for studying intervention with anti-TNF therapies. Determining a patient' s TNF2 genotype before starting the treatment may permit the selection of a TNF2 group of high-risk patients who could benefit from treatment with anti-TNF. Such a possibility deserves further study, since an effective therapy for ASBP patients with septic shock would have important clinical and economic consequences.

In conclusion, our study demonstrated that there was no association between acute biliary pancreatitis and the two polymorphisms of tumor necrosis factor gene studued; however, TNF2 allele were associated with the susceptibility to septic shock related to acute sever biliary pancreatitis. Genetic factors are not important in determining plasma TNF-α levels in ASBP.

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