Tumor necrosis factor–alpha gene promoter polymorphisms in Chinese patients with nonalcoholic fatty liver diseases

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Abstract

Background : **Nonalcoholic Fatty Liver Disease (NAFLD) encompasses a histopathological spectrum of clinical conditions such as simple fatty liver (steatosis), nonalcoholic steatohepatitis (NASH), and a variant that has degrees of fibrosis. Tumor Necrosis Factor–alpha (TNF-α) is considered essential for NAFLD. Therefore, we investigated the correlation between TNF-α gene promoter polymorphism and NAFLD in this human study.**

Patients and methods : **The TNF-α gene polymorphisms at position -238 and -308 were analyzed in 189 Chinese patients with NAFLD and 138 healthy controls by using polymerase chain reaction and restriction fragment length polymorphism assay. The serum levels of TNF-α in both patient and control groups were measured by ELISA. The associations of TNF-α polymorphism and serum TNF-α, and/or insulin resistance, and/or clinical features were analyzed.**

Results : **The carrier frequencies of TNF-α gene polymorphism with G/A mutation at -238 were significantly higher in the patients with NAFLD than those in the control subjects ($p < 0.05$). However, there were no significant differences between the NAFLD patients and control subjects in the polymorphisms at -308 ($p > 0.05$). In addition, the serum level of TNF-α was markedly higher in the patients with NAFLD than in the control subjects ($p < 0.05$). There were significant associations between TNF-α gene polymorphism in the -238 A allele and increased serum TNF-α, insulin resistance, as well as increased body mass index in the NAFLD patients.**

Conclusions : **The results indicate that the TNF-α gene polymorphism at position -238 is associated with susceptibility of nonalcoholic Fatty Liver Disease in a Chinese population.** (Acta gastroenterol. belg., **2009**, 72, **215-221**).

Key words : NAFLD ; NASH ; TNF-alpha Gene polymorphism ; TNF-alpha protein.

Introduction

Nonalcoholic Fatty Liver Disease is a very common disorder occurring in individuals who have minimal or no alcohol consumption, and who have no other etiology for liver disease. It refers to a wide spectrum of liver disease ranging from simple steatosis, to nonalcoholic steatohepatitis (NASH), and cirrhosis (scarring of the liver) as well. The accumulation of excess fat in the liver cells happens in all of the stage of NAFLD (1, 2).

NAFLD affects up to 20 percent of adults and nearly 5 percent of children in USA. Recently, it was found that the increasing rate of NAFLD in China was due to China's gradually westernized eating habit (3). The exact cause of NAFLD remains unclear. Strong evidence, however, supports the hypothesis that the metabolic syndrome, including insulin resistance, obesity, hypertension, and dyslipidemia, is associated with the disease process (4, 5). A number of other factors may be involved, such as release of toxic inflammatory mediators and oxidative stress (6, 7). In addition, some reports have shown that hereditary predisposition plays an important role in the development and progression of NAFLD (8-10).

Tumor Necrosis Factor–alpha (TNF-α) is a pleiotropic inflammatory cytokine produced mainly by macrophages, but also by a variety of other cell types including lymph cells, mast cells, fibroblasts, adipose cells, endothelial cells, and hepatocytes. TNF-α is a trimeric protein, which was first identified in its 17 kd secreted form, but further research then has shown that a 27kd precursor existed in a transmembrane form (11). TNF-α can bind either directly to TNFR-55 and TNFR-75 receptors or through cleavage in its soluble form. TNF-α gene lies in the class III region of the major histocompatibility complex (MHC) on human chromosome 6p21.3 (9).

In physiologic conditions, TNF-α is an important inflammatory mediator of homeostatisis through its ability to regulate inflammatory pathways. It also plays a major role, however, in modulating insulin resistance and obesity by 1) modulating insulin-mediated glucose uptake in skeletal muscle and 2) increasing circulating free fatty acids in the pathogenesis of the diseases (12). The TNF-α gene promoter contains single nucleotide polymorphism (SNP) that is able to cause different transcriptions of TNF-α gene and protein synthesis of TNF-α. Recent reports have extensively described two polymorphisms in the TNF-α promoter region at position -238 (TNFA allele) and -308 (TNF2 allele). Both TNFA and TNF2 alleles are able to induce the release of the cytokine leading to the pathophysiologic conditions.

Increasing evidence indicates TNF-α gene polymorphisms are associated with the susceptibility of different

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Submission date : 26/08/2008
Acceptance date : 04/03/2009

hepatic diseases, such as steatohepatitis and NAFLD (13-15). However, the genomic variants in Chinese NAFLD patients remain unclear. In this study, we analyzed the TNF-α gene promoter polymorphisms at position -238 and -308 in189 Chinese patients with NAFLD and 138 healthy controls. We also determined the serum level of TNF-α. Finally, we analyzed the associations of the TNF-α gene polymorphisms with insulin resistance, and/or the clinical features in NAFLD patients. The goal for this study is to investigate the role of TNF-α gene polymorphisms in the pathogenesis of NAFLD in Chinese patients.

Patients and methods

1. *Patients*

189 consecutive Chinese patients were diagnosed with NAFLD by physical examination, biochemical test, ultrasonographic images, and histology of 44 patients (liver biopsies were taken when the diagnosis was suspected), according to the American Gastroenterological Association Medical Position Statement : Nonalcoholic fatty liver disease (16). Briefly, we diagnosed NAFLD basing on the combination of patient's medical history, symptoms, biochemical results and ultrasonographs. Fatty liver disease was diagnosed and staged by ultrasonography. When a subject fulfilling the fatty liver disease criteria and the alcohol consumption was less than 20-40 g (male) or 10-20 g (female), NAFLD was diagnosed. These patients were seen between October 2005 and October 2006 at the Department of Hepatic Diseases, Guangzhou No. 8 People's Hospital in China. One hundred ninety were men, and seventy were women, and the average age of NAFLD patients was 53.6 ± 13.7 years.

All patients received a complete physical examination and underwent blood test, liver function laboratory test, total cholesterol, triglyceride, and hepatitis B and C analysis. Viral hepatitis and other chronic liver diseases were excluded. Body mass index (BMI) was calculated as follow : weight (kg)/height (m^2). Hyperlipidemia was diagnosed in patients with above-normal fasting levels of total cholesterol and /or triglycerides. For diagnosis of hypertension, the patient's systolic blood pressure was ≥ 140 mm Hg or diastolic blood pressure was ≥ 90 mm Hg. The criteria for diagnosis of type II diabetes mellitus (DM) was a level of random glucose over 200 mg/dL on two different occasions. Because 44 of the 189 NAFLD patients had persistent abnormalities of their liver function tests, liver biopsies were performed for diagnosis of NASH. According to Kleiner (17), NASH was defined as steatosis plus lobular inflammation, plus either ballooning of hepatocytes or abnormal fibrosis (stages 1-4). The method for determining a homeostasis model assessment-insulin resistance (HOMA-IR) was a fasting serum insulin μU/mL × fasting glucose mg/dL/405.

2. *Controls*

138 healthy Chinese volunteers (90 were men and 48 were women) were recruited from the same geographical origin as the control group. The average age in this group was 52.9 ± 14.1 years. All control subjects were considered normal by blood test, biochemical test, liver function test, and ultrasonography. None of the control subjects were alcoholics or had a diabetic disease, obesity, kidney disease, and viral hepatitis and other chronic liver diseases. There were no significant differences between the NAFLD patients and the control subjects in age, gender, or nationality.

All patients and healthy controls signed informed consent before the study. The study protocol was approved by Institution's Human Research Committee.

3. *Samples*

Peripheral blood samples (3 mL of each) were taken from both the NAFLD patients and the control subjects, centrifuged, and separated into blood cells and serum and stored at -80 °C.

4. *Methods*

Analysis of TNF-α gene promoter polymorphisms : According to the manufacturer's protocol, Genomic DNA was isolated from peripheral blood cells of the patients and controls using Invisorb Spin Blood Mini Kit (Invite Co., Berlin, Germany). The PCR primers were designed and synthesized by the Shanghai Biochemical Company (Shanghai, China). PCR-RFLP was performed with primers for the human TNF-α gene promoter with 152 base pairs, containing the -238 site (forward : 5-AGAAGACCCCCCTCGGAACC-3 ; reverse : 5-ATCTGGAGGAAGCGGTAGTG-3), and with 116 bp, containing the -308 site (forward : 5-AGGCAATAG-GTTTTGAGGGCCAT-3 ; reverse : 5-ACACTCCC-CATCCTCCCTGCT-3). PCR was carried out using 1 U r of Taq polymerase (Promega, Madison, WI, USA), 200 mM of dNTPs (Promega, Madison, WI, USA), and 0.4 mM of primer at each reaction. PCR was run for 35 cycles (melting at 94°C for 45 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min per cycle). Restriction digests were performed on unpurified PEC products by adding specific restriction enzyme (Msp I or Nco I). The polymorphisms of the TNF-α promoter region at -238 and -308 were analyzed under UV light and compared with standard DNA 100 bp ladders.

Measurement of TNF-α protein level : The protein level of TNF-α in serum was measured by ELISA kit (DIA clone Co., Besançon, France) with TNF-α antibody according to the manufacturer's protocol. The results were shown as mean ± SD from at least three independent experiments.

Table 1. — **Clinical features of the NAFLD patients and the control subjects**

Characteristics	NAFLD (n = 189)	Control (n = 138)	*P*-Value
Gender (men)	119	90	NS
Age (year, mean ± SD)	53.6 ± 13.7	52.9 ± 14.1	NS
Alcohol intake (M/F, 30/20 g/day)	0	0	NS
BMI (>25)	152	0	< 0.05
Hyperlipidemia	68	0	< 0.05
Hypertension	36	0	< 0.05
Diabetes mellitus	56	0	< 0.05

NS, not significant.

5. *Statistical analyses*

The frequencies of polymorphisms were analyzed by frequency counting and Hardy-Weinberg Equilibrium Law. The frequencies were compared by $\chi 2$ test. Results were shown as mean ± standard deviation (SD). Mean values were analyzed by Student's *t*-test for unequal variances. Values of $P < 0.05$ were considered to be statistically significant.

Results

Clinical features

The general clinical features of the NAFLD and control subjects are shown in Table 1. There was no significant difference between Chinese patients with NAFLD and Chinese healthy control subjects with regards to age, gender, and alcohol comsumption (Table 1, $P > 0.05$). However, the BMI in the NAFLD group was significantly higher than that in the control group (Table 1, $P < 0.05$). In addition, metabolic diseases (hyperlipidemia, hyper tension, and diabetes mellitus) were markedly associated with NAFLD (Table 1, $P < 0.05$), as compared to the control group.

The comparison of TNF-α gene promoter polymorphisms between the NAFLD patients and the healthy control subjects

After digestion of -238 or -308 primer PCR products with Msp I or Nco I respectively, we found several different genotypes at -238 allele, as well as at -308 allele. The fragment of 133 bp represents -238G/A genotype, and the fragments of 152 bp and 133 bp represent -238G/G genotype. Similarly, there were two genotypes for -308 alleles as follow : -308 G/G genotype including 96 bp, and -308G/A genotype including 116 bp and 96 bp. By using Hardy-Weinberg Equilibrium Law, the allele frequencies and genotype ratios of TNF-α show homozygous distributions and random mating in both patient's and healthy control's population.

When comparing frequency distribution of the -238 allele and -308 allele of TNF-alpha gene polymorphisms in 189 NAFD patients and 138 control subjects, we found that there were no AA genotype in either the patient or the control groups (Table 2, 3). This data may result from the very low frequencies and small sample number in this study. However, wild type (G/G) and heterozygote (G/A) genotypes were expressed in NAFLD patients and control subjects (Table 2, 3).

We further analyzed the genotype and allele frequencies of TNF-alpha gene at -238 and -308 sites by c^2 test. The results show that G/A genotype and A allele frequency at the -238 site of TNF-alpha gene in the patients are significantly different compared to the genotype and allele frequency in the control subjects (Table 2). The genotype distribution (G/A) of the -238, but not of the -308, TNF-alpha gene polymorphism was significantly higher in the patient group than that in the control group ($P < 0.05$). The frequencies of the A allele of the -238 G/A genotype in the patient group was also significantly higher than that in the control group ($P < 0.05$). The data indicates that TNF-alpha gene polymorphism at the -238 site was associated with NAFLD susceptibility (RR = 2.19), but there was no significant association between the -308 site of TNF-alpha gene polymorphism and NAFLD in this study.

Relationship between TNF-α gene Polymorphism and the serum levels of TNF-α in the NAFLD patients

In order to determine whether NAFLD is related to TNF-α gene polymorphism via increasing serum level of TNF-α, we analyzed the relationship between the serum levels of TNF-α protein and two different sites of TNF-α gene polymorphism in patients with NAFLD. Table 5 shows that the serum level of TNF-α of NFALD patients carrying the -238 A allele was significantly higher than that of patients without this allele ($P < 0.05$). There was no association between -308 A allele and serum level of TNF-α in NAFLD patients.

Association between TNF-α gene Polymorphism and insulin resistance in the NAFLD patients

Because high serum level of TNF-α is related to insulin resistance (18, 19) we measured HOMA-IR as an index of insulin resistance in the 189 Chinese patients with NAFLD. The association between TNF-α gene Polymorphism and HOMA-IR is shown in Table 6. HOMA-IR was significantly higher in the NAFLD patients with -238 A allele (5.91 ± 3.6) than in those without this allele (3.27 ± 2.3, $P < 0.05$). No significant association between TNF-α gene polymorphism in -308 A and insulin resistance was found ($P > 0.05$) in our study, even though HOMA-IR in the patients carrying -308 A (4.86 ± 3.3) was higher than in those with -308 G/G (3.8 ± 2.6). The data indicates that there is an

Table 2. — **Polymorphism of the -238 site in TNF-alpha gene promoter in patients with NAFLD and the control subjects**

Groups	n	Genotype (%) *			Allele frequency (%)	
		G/G	G/A	A/A	G	A
NAFLD	189	137 (72.7%)	52 (27.3%)	0	326(86.3%)	52 (13.7%)
Control	138	116 (84.4%)	22 (15. 6%)	0	254 (92.2%)	22 (7.8%)

* indicated $P < 0.05$ (comparison between the NAFLD patients and the control subjects).

Table 3. — **Polymorphisms of the -308 site in TNF-alpha gene promoter in the patients with NAFLD and in the control subjects**

Groups	n	Genotype			Allele frequency	
		G/G	G/A	A/A	G	A
NAFLD	189	163 (86.2%)	26 (13.8%)	0	352 (93.1%)	26 (6.9%)
Control	138	123 (89.0%)	15 (11.0%)	0	221 (94.5%)	15 (5.5%)

$P > 0.05$ (comparison between the NAFLD patients and the control subjects).

Table 4. — **The serum levels of TNF-alpha in the NAFLD patients and the control subjects**

	Number	Age	Gender (M/F)	TNF-alpha * (pg/mL)
NAFLD	189	53.6 ± 13.7	119/70	197.3 ± 83.4
Control	138	52.9 ± 14.1	90/48	95.2 ± 46.8

* Indicated $P < 0.05$ (comparison between the NAFLD patients and the control subjects).

Table 5. — **The relationship between TNF-alpha gene Polymorphism and the serum levels of TNF-alpha in the NAFLD patients**

Polymorphism	Number	TNF-alpha (pg/mL)	*P*-Value
TNF-a			
-238 G/A	52	247.9 ± 38.6	0.02
-238 G/G	137	164.7 ± 52.3	
-308 G/A	26	203.8 ± 48.6	0.06
-308 G/G	163	172.8 ± 66.6	

Table 6. — **The relationship between TNF-alpha gene Polymorphism and HOMA-IR in the NAFLD patients**

Polymorphism	Number	HOMA-IR (mean ± SD)	*P*-Value
TNF-a			
-238 G/A	52	5.91 ± 3.1	0.03
-238 G/G	137	3.27 ± 2.1	
-308 G/A	26	4.86 ± 3.3	0.07
-308 G/G	163	3.8 ± 2.6	

association between TNF-α gene polymorphism in -238 site and insulin resistance in NAFLD patients.

Relationship between TNF-α gene Polymorphism and clinical features in NAFLD patients

Since our data indicated that TNF-α gene polymorphism in -238-site is associated with NAFLD patients, we investigated the relationship between TNF-α gene polymorphism of -238 site and patient's clinical features. A total of 44 patients out of the 189 NAFLD patients had liver biopsies. Fig 1B shows the representative histological sections of NASH. The photo was taken from a case scored as steatosis 2 plus ballooning injury. There was a contiguous patch of hepatocytes showing prominent ballooning injury, sharply contrasted against the normal hepatocytes in the field. Fig 1A shows simple steatosis with easily identified megamitochondria. Table 7 shows the analysis of several clinical features the patients with -238 G/A or -238 G/G genotypes. The NAFLD patients carrying the -238A allele show significantly lower levels of cholesterol, higher triglycerides, and BMI compared to those patients are not carriers of this allele. However, no significant differences were seen in patients with or without -238A in regards to age, disease progression (NASH or simple steaosis), serum levels of alanine amiontransferase and glutamyltransferase, or hypertension.

The protein levels of TNF-α in NAFLD patients and healthy control

It is well known that TNF-α protein level in serum has been implicated in the pathogenesis of NAFLD and other inflammatory diseases (20, 21). Therefore, we measured the protein levels of TNF-α in serum of all patients with NAFLD and in healthy control subjects by ELISA. The results are presented in Table 4. The level of TNF-α in the patients with NAFLD was significantly higher than that in the control subjects ($P < 0.05$). The data suggests

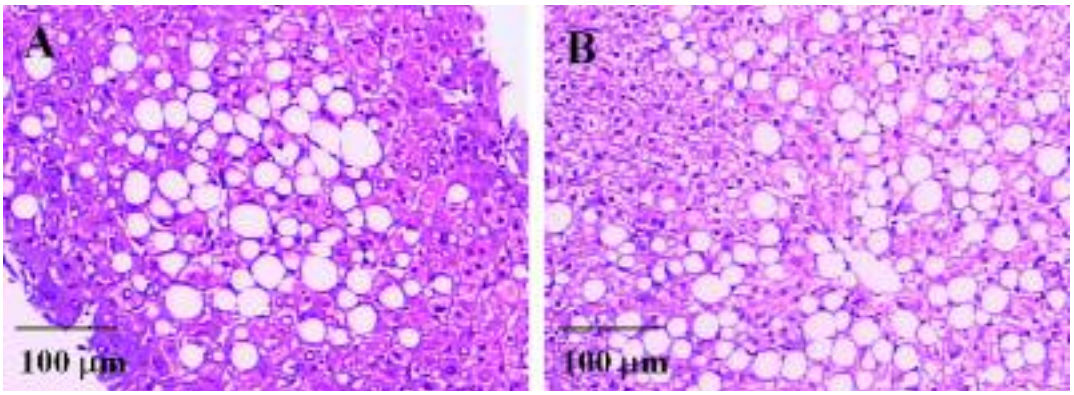

Fig. 1. — Histology of patients with NAFLD. Liver sections were obtained from NAFLD patients by tissue biopsy, processed for routine light microscopy, and stained with H&E. The images were captured from (A) patient's liver with simple steatosis and (B) patient's liver tissue with NASH (H&E, 20×).

Table 7. — **Analysis of clinical data and TNF-alpha gene Polymorphism of the -238 site in the NAFLD patients**

Characteristics	-238 G/A (52)	-238 G/G (137)	*P*- Value
Mean age (yr)	54.6 ± 18.3	52.8 ± 14.2	NS
NASH	14/18 (22%)	21/26 (19%)	NS
Simple steatosis	4/18 (78%)	5/26 (81%)	NS
Alanine aminotransferase (U/L)	78.2 ± 37.3	69.3 ± 39.2	NS
γ-glutamyltransferase(U/L)	50.7 ± 21	48.4 ± 23.6	NS
Cholesterol (mg/dL)	170.2 ± 39	228.3 ± 46	0.02
Triglycerides (mg/dL)	219.1 ± 51	166.3 ± 62	0.04
BMI (kg/m^2)	29.4 ± 5.2	26.2 ± 4.1	0.04
Hypertension	14 (26.9%)	4 (22.7%)	NS

NS, not significant.

that increased TNF-α protein contributes to the pathphysiology of NAFLD.

Discussion

The cause and pathogenesis of Nonalcoholic Fatty Liver Disease are complex and remain poorly understood. In this study, we investigated the relationship between the TNF-alpha gene polymorphisms and the NAFLD and found the TNF-alpha gene polymorphism is associated with susceptibility of NAFLD (RR = 2.19). Specifically, we showed that : (i) there were A alleles in both -238 and -308 of TNF-alpha gene polymorphisms in Chinese NAFLD patients and healthy control subjects ; (ii) the TNF-alpha gene polymorphism of -238 A allele frequency was significantly increased in Chinese patients with NAFLD compared to control subjects ; (iii) the serum TNF-alpha was significantly higher in the NAFLD patients when compared with control subjects, and analysis involving all patients revealed a significant correlation between TNF-alpha gene polymorphism of -238 A allele and serum TNF-α ; and (iv) there were significant associations between TNF-α gene polymorphism in -238 A allele and insulin resistance and increased BMI in the NAFLD patients.

In this study, patients were included in the order that they come to our department. The researchers did not select the patients in any particular way that might influence the results of this study.

Studies have reported the association of TNF-α gene polymorphism with autoimmune diseases, such as rheumatoid arthritis, and infectious diseases. This may be due to the specific MHC haplotypes encoded different TNF-α phenotype. For example, DR3 and DR4 haplotypes produce higher levels of TNF-α (22), while DR2 haplotypes lead to low production of TNF-α (8). At position -238 or -308 of TNF-α gene promoter, the guanine could mutate to adenine and form three different genotypes : wild type (GG), heterozygote (GA), and homozygote (AA). Wilson *et al.*, identified a G to A transition polymorphism located at -308 in the TNF promoter, which defined the TNF2 (A) alleles. The TNF2 allele leads to high TNF-α production and is associated with insulin-dependent diabetes mellitus (23). Gordon *et al.* have shown that the polymorphism was responsible for the association of TNF2 with high TNF-α phenotype and more severe disease in infections such as malaria and leishmaniasis (14). Our study demonstrates an association between -238 A allele (TNFA) and increasing serum TNF-α in Chinese patients with NAFLD. However, we

have shown the absence of a correlation between TNF2 and increasing serum TNF-α.

The role of increased serum TNF-α in pathogenesis of NAFLD is not fully clear. Scientists have hypothesized that TNF-α plays a role in the pathogenesis of disease related to insulin resistance and metabolic dysfunctions. TNF-α can directly attract inflammatory cells (leucocytes and macrophages) to the liver that cause hepatitis, and/or induced SREBP-1c (sterol regulatory element binding protein 1c) expression that regulates lipid metabolism and leads to intrahepatic lipid deposition (24). Indeed, elevated serum TNF-α and overexpression of TNF-α in adipose tissue correlated with body mass index (BMI) and the level of hyperinsulinemia (25-27). The absence of TNF-α resulted in a dramatic increase insulin sensitivity in obese mice by targeting null mutation in the gene encoding TNF-α and the two TNF-α receptors (28). By inducing insulin resistance in pancreatic b cells and releasing toxic mediators, TNF-α leads to impair insulin release (29, 30). Our data shows that the serum level of TNF-α increased significantly in the NAFLD paitents, emphasizing the potential role of TNF-α in NAFLD. Additionally, we analyzed the associations of TNF-α gene polymorphism with insulin resistance index, metabolic tests, and liver functions. This study confirms reported findings (31) that TNF-α polymorphism at -238A is associated with insulin resistance and increased BMI in the NAFLD patients, contributing to the pathogenesis of NAFLD. However, we did not observe a significant relationship between polymorphism and NAFLD progression in this study.

In conclusion, our data indicates that the TNF-α gene promoter polymorphism at position -238 A allele is associated with susceptibility of nonalcoholic fatty liver disease in the Chinese population. This finding might be due to increasing serum TNF-α, insulin resistance, or BMI by the gene polymorphism. Our studies also suggest that TNF-α is crucial for the pathogenesis of NAFLD. Therefore, targeted inhibition of TNF-α may provide a novel approach to prevention and treatment of NAFLD.

Acknowledgements

We are thankful to Dr. Zak Zheng, Ms. Margaret Shewood, and Mr. Yao Su for critical reading of this manuscript. This work was supported by the GuangDong Science Program Funding (2004B36001039).

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