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Original Article

Association of tumor necrosis factor-α gene polymorphisms with susceptibility to helicobacter *pylori*-associated gastroduodenal diseases in the Chinese population

Wenxian You[2*], Xiaofei Lai[1*], Jing Lv[3], Pu Chen[1], Jirong Xia[1], Fang Cui[1], Yu Xiang[1]

Departments of [1]Laboratory Medicine, [2]Gastroenterology, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China; [3]Department of Dermatology, Traditional Chinese Medicine Hospital of Chongqing, Chongqing, China. [*]Equal contributors.

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Abstract: An association between the genetic variants of cytokine genes and various clinical outcomes in subjects infected with *Helicobacter pylori* (*H. pylori*) are not completely defined. We explored the relationship between *TNF-α* polymorphisms and *H. pylori*-associated gastroduodenal diseases. 114 *patients with* chronic gastritis, 90 *patients with* duodenal ulcer, 117 *patients with* gastric ulcer, 105 *patients with* gastric cancer and 140 healthy adults were enrolled, and all of participants were infected with *H. pylori. TNF-A-308* and *TNF-A-857* were genotyped by PCR-restriction fragment length polymorphism analysis and confirmed by DNA sequencing. Compared with the control group, a significant difference in the frequency distribution of *TNF-A-308* was found in the chronic gastritis group, the gastric ulcer group and the gastric cancer group. Additionally, we found a significant difference in the genotype frequency distribution of *TNF-A-857* between the duodenal and gastric ulcer groups. Logistic regression analysis revealed that, compared with the *TNF-A-308 G/G* genotype, the odds ratio (OR) of patients with the *TNF-A-308 A/A* genotype of developing chronic gastritis was 22.70, of gastric ulcer was 21.62 and of gastric cancer was 16.41. Meanwhile, compared with the *TNF-A-308 C/C* genotype, the OR of patients with the *TNF-A-308 T/T* genotype of developing duodenal ulcer was 6.73 and gastric cancer was 5.37. *TNF-α* polymorphisms are associated with the onset of *H. pylori*-associated gastroduodenal diseases.

Keywords: Gastroduodenal disease, gene polymorphism, helicobacter pylori, tumor necrosis factor-α

Introduction

Helicobacter pylori have a high infection rate around the world. In 1994, the International Agency for Research on Cancer classified *H. pylori* as belonging to the first category of carcinogenic factors [1]. Chronic gastritis develops in virtually all infected individuals, but only a small percentage (20%) experience clinically significant diseases, including peptic ulcer (PU), gastric cancer (GC), or mucosa-associated lymphoid tissue lymphoma [2, 3]. A large number of studies have revealed that the majority of people infected with *H. pylori* do not develop to peptic ulcers or gastric cancer, and this phenomenon was related to the type of *H. pylori* strain, the host genotype and environmental factors [4, 5]. Recent studies have shown that host genetic factors may play a decisive role in the outcome of *H. pylori* infection [6]. After infection, neutrophils and mononuclear cells infiltrated in the gastric mucosa secrete inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-10, which are important factors resulting in gastric mucosal injuries. Recent studies linked *IL-1B*, *IL-1RN*, and *TNF-α* gene polymorphisms to an increased risk of developing *H. pylori*-related diseases [7-10]. Due to *TNF-α* polymorphisms difference in *TNF-α* expression may impact the outcome of *H. pylori* infection.

The gene encoding TNF-α is located on chromosome 6, and the base replacements of G/A and

Table 1. Sequence of primers

Gene	Primer sequence 5'-3'
TNF-A-308	5'-AGGCAATAGGTTTTGAGGGCCAT-3'
	5'-TCCTCCCTGCTCCGATTCCG-3'
TNF-A-857	5'-AAGTCGAGTATGGGGACCCCCCGTTAA-3'
	5'-CCCCAGTGTGTGGCCATATCTTCTT-3'

C/T leads to the polymorphisms at sites -308 and -857 in the promoter region [11]. It has been found in the Japanese population that *TNF-α* variants are associated with the risk of gastric ulcer and cancer, such that patients carrying the *TNF-A-857* T allele had an increased risk of gastric ulcer and cancer, while the *TNF-A-308* polymorphism was not associated with an increased risk of these diseases.

We initiated the present study to genotype the TNF-A-308 and *TNF-A-857* variants in order to validate the association of *TNF-α* polymorphisms with susceptibility to *H. pylori*-associated gastroduodenal diseases in the Chinese population. We can search risk factors of gastroduodenal diseases and provide new ideas for the prevention and control of *H. pylori* infection-related diseases.

Materials and methods

Subjects

In this study, all selected cases were enrolled from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) from 2011 to 2014. Healthy persons with *H. pylori* infection served as the control group, while hospitalized patients with gastroduodenal diseases or gastric cancer accompanied with *H. pylori* infection served as the disease group. All subjects were unrelated. Clinical data on all subjects was collected by questionnaires and by reviewing medical records and informed consent was obtained from all subjects. All procedures of this study were approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. The control group included 84 males (60.0%) and 56 females (40.0%), with a mean age of 35.0 ± 8.9 years (the age range of the group: 20-62 years), without any history of gastroduodenal diseases. Meanwhile, the disease group included 114 cases of chronic superficial gastritis and chronic atrophic gastritis, 90 cases of duodenal ulcer, 117 cases of gastric ulcer and 105 cases of gastric cancer. Of these, 252 cases (59.2%) were male while 174 cases (40.8%) were female, with a mean age of 55.7 ± 14.8 years (the age range of the group: 16-86 years). Final diagnoses were made by gastroscopy and histopathology.

Reagents

The *H. pylori* antibody detection kit was purchased from Shanghai Touching Technology Co, (Shanghai, China). Rapid detection reagent papers were purchased from Guangzhou Beisiqi Reagent Co, (Guangzhou, China). Primers for *TNF-A-308* and *TNF-A-857* were synthesized by Shanghai Invitrogen Biotechnology Co, (Shanghai, China). Premix Taq, DNA fragment length standard DL2000, 20 bp DNA Ladder; and the restriction enzymes *NcoI* and *HincII* were purchased from Dalian TaKaRa Co, (Dalian, China). The remaining reagents were products of analytical pure.

Detection of H pylori infection

Serum *H. Pylori* antibodies were detected using an immunoblotting assay and a urease reagent paper assay. All procedures were done according to the manufacturer's instructions, and when both assays were positive, the sample was considered to be positive for *H. pylori* infection.

Detection of cytokine polymorphisms

Preparation of DNA template: Venous blood (2 ml) was collected from all subjects, and was then anticoagulated with EDTA-K2. Using the sodium iodide method, the genomic DNA of leukocytes was extracted and then stored at -20°C.

Primer sequences: TNF-A-308 and *TNF-A-857* primers were designed referring to the previous literature (Carlo, *et al*., 2005). The primer sequences are listed in **Table 1**.

PCR condition: The PCR system included 10 μl Premix Taq (dNTP, Taq DNA polymerase and Mg^{2+}), 4 μl template DNA and 5 pmol primers, and 20 μl total volume was reached with deionized water. The PCR detection conditions of both genes are listed in **Table 2**.

Table 2. PCR conditions for both variants

Gene	Initial denaturation	Denaturation	Annealing	Extension	Cycle number
TNF-A-308	94°C 5 min	94°C 1 min	58°C 1 min	72°C 1 min	35 cycles
TNF-A-857	95°C 10 min	95°C 1 min	58°C 1 min	72°C 1 min	38 cycles

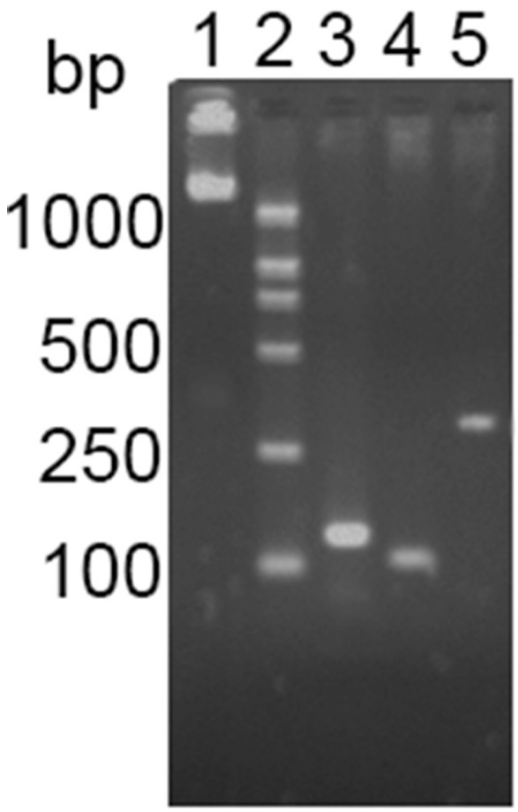

Figure 1. *TNF-A-308* and *TNF-A-857* PCR products: Lane 1 Genomic DNA; Lane 2 Marker (DL2000); Lane 3 TNF-A-857 PCR Product; Lane 4 TNF-A-857 PCR Product.

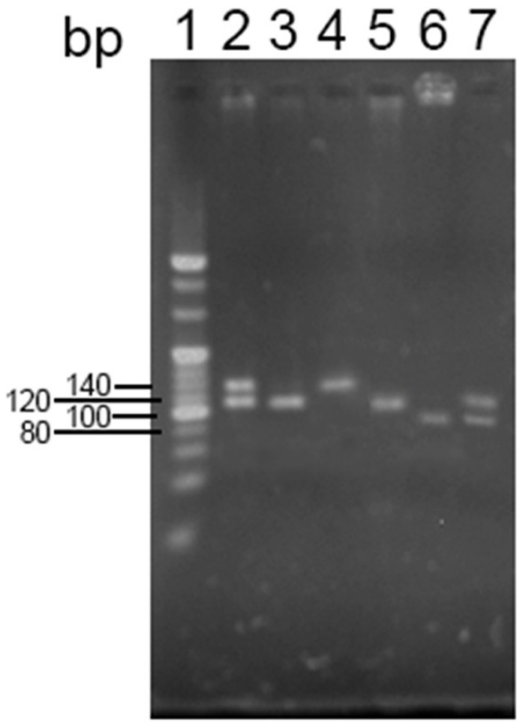

Figure 2. *TNF-A-308* and *TNF-A-857* polymorphism. Lane 1 Marker (20 bp DNA Ladder); Lane 2 *TNF-A-857 C/T* genotype; Lane 3 *TNF-A-857 C/C* genotype; Lane 4 *TNF-A-857 T/T* genotype; Lane 5 *TNF-A-308 A/A* genotype; Lane 6 *TNF-A-308 G/G* genotype; Lane 7 *TNF-A-308 G/A* genotype.

Detection and analysis of restriction enzyme digestion products: The PCR amplification products were digested by restriction enzymes in a water bath at 37°C, and the digestion products were separated using 3% agarose gel electrophoresis. After Goldview staining, DNA bands were observed and analyzed by a gel imager.

Statistical analyses

All analyses were performed using SPSS software for Windows, version 13.0 (IBM-SPSS, Inc., Chicago, IL, USA). All genotype and allele frequencies were calculated by the frequency counting method and then verified to be in Hardy-Weinberg equilibrium. The difference of each genotype frequency between the disease group and the control group was compared by the χ^2 test, and $P<0.05$ was considered statistically significant. The risk of subjects with each genotype developing peptic ulcer and gastric cancer is expressed with the odds ratio (OR) and 95% confidence interval (CI). OR values were calculated by logistic regression analysis. All statistical tests were bilateral probability tests.

Results

PCR amplification products

Agarose gel electrophoresis revealed that the DNA bands corresponding to PCR amplification products of *TNF-A-308* and *TNF-A-857* PCR were 107 bp and 133 bp, respectively (**Figure 1**).

Table 3. Genotype and allele frequencies of *TNF-α* polymorphisms in chronic gastritis, duodenal ulcer, gastric ulcer and gastric cancer cases and the control group

Genotype	Control *n*=140	Chronic gastritis *n*=114	Duodenal ulcer *n*=90	Gastric ulcer *n*=117	Gastric cancer *n*=105
TNF-A-308					
G/G	126 (0.90)	57 (0.50)	66 (0.73)	18 (0.15)	15 (0.14)
G/A	13 (0.09)	36 (0.32)	18 (0.20)	36 (0.31)	30 (0.29)
A/A	1 (0.01)	21 (0.18)	6 (0.07)	63 (0.54)	60 (0.57)
Alleles					
G	265 (0.94)	150 (0.66)	150 (0.83)	72 (0.66)	60 (0.29)
A	15 (0.06)	78 (0.34)	30 (0.17)	162 (0.34)	150 (0.71)
TNF-A-857					
C/C	100 (0.71)	66 (0.58)	39 (0.43)	48 (0.41)	93 (0.89)
C/T	32 (0.23)	33 (0.29)	30 (0.33)	42 (0.36)	12 (0.11)
T/T	8 (0.06)	15 (0.13)	21 (0.23)	27 (0.23)	0 (0.00)
Alleles					
C	232 (0.83)	165 (0.72)	108 (0.60)	138 (0.59)	198 (0.94)
T	48 (0.17)	63 (0.28)	72 (0.40)	96 (0.41)	12 (0.06)

Table 4. Association of *TNF-α* polymorphisms with the development of chronic gastritis and peptic ulcer among gastroduodenal disease patients and healthy controls

Genotype	Risk for Chronic Gastritis OR (95% CI)	Risk for Gastric ulcer OR (95% CI)	Risk for duodenal ulcer OR (95% CI)
TNF-A-308			
G/G	1.00	1.00	1.00
A/A	22.70 (2.51-205.40)*	21.62 (2.07-226.13)&	8.86 (0.74-106.82)
G/A	7.48 (2.32-23.95)	5.07 (1.61-15.99)	2.13 (0.56-8.13)
TNF-A-857			
C/C	1.00	1.00	1.00
C/T	1.01 (0.34-3.01)	2.73 (0.99-7.48)	2.40 (0.89-6.52)
T/T	2.40 (0.47-12.12)	5.37 (1.28-22.50)$	6.73 (1.71-26.53)#

*P=0.005; #P=0.006; &P=0.010; $P=0.021.

Detection results of gene polymorphisms

Electropherotyping was performed after digestion with the restriction enzyme *NcoI*, and we found that the genotypes of *TNF-A-308* included the A/A type (107 bp), the G/G type (87 bp) and the G/A type (107 bp and 87 bp). After digestion with the restriction enzyme *HincII* and we found that the genotypes of *TNF-A-857* included the C/C type (108 bp), the C/T type (108 bp and 133 bp) and the T/T type (133 bp; **Figure 2**).

Frequency distribution of each genotype

The χ^2 tests revealed that the six genotypes and allele frequencies were consistent with Hardy-Weinberg equilibrium, with group representativeness. Compared with the control group (G/G, 90%; G/A, 9%; A/A, 1%), there was significant difference in the *TNF-A-308* genotype frequencies (G/G, 50%; G/A, 32%; A/A, 18%; χ^2=22.614, P<0.001) in the chronic gastritis group. Meanwhile, compared with the control group (C/C, 71%; C/T, 23%; T/T, 6%), there was significant difference in the *TNF-A-857* genotype frequencies (C/C, 44%; C/T, 33%; T/T, 23%; χ^2=9.444, P=0.009) in the duodenal ulcer group. Compared with the control group, there was a significant difference in the *TNF-A-308* genotype frequencies (G/G, 15%; G/A, 31%; A/A, 54%; χ^2=19.317, P<0.001) and *TNF-A-857* genotype frequencies (C/C, 41%; C/T, 36%; T/T, 23%) in the gastric ulcer group (χ^2=11.702, P=0.003). Furthermore, there was a significant difference in the *TNF-A-308* genotype frequencies (G/G, 14%; G/A, 29%; A/A, 57%; χ^2=16.062, P<0.001) in the gastric cancer group compared with the control group. Detailed data are presented in **Table 3**.

Risk analysis of each genotype for development of gastroduodenal diseases and gastric cancer

A logistic regression model was established to analyze the risks of subjects with various *TNF-A-308* and *TNF-A-857* genotypes of developing gastroduodenal diseases and gastric cancer (**Tables 4** and **5**).

In the disease and control group, compared with the *TNF-A-308 G/G* genotype, the OR of subjects with the *TNF-A-308 A/A* genotype of developing chronic gastritis was 22.70 (95% CI: 2.51-205.40), while the OR of carriers of the *TNF-A-308 A/A* developing gastric ulcer was

Table 5. Association between *TNF-α* polymorphisms and the development of gastric cancer among the disease and control groups

Genotype	Risk for Gastric cancer OR (95% CI)
TNF-A-308	
G/G	1.00
A/A	16.41 (1.62-116.55)[#]
G/A	4.89 (1.54-15.53)
TNF-A-857	
C/C	1.00
C/T	0.54 (0.15-1.90)
T/T	0

[#]P=0.018.

21.62 (95% CI: 2.07-226.13). Compared with the *TNF-A-857 C/C* genotype, the OR of *TNF-A-857 T/T* carriers developing duodenal ulcer was 6.73 (95% CI: 1.71-26.53) of developing gastric ulcer was 5.37 (95% CI: 1.28-22.50). Compared with the *TNF-A-308 G/G* genotype, the OR of carriers of the *TNF-A-308 A/A* genotype of developing gastric cancer was 16.41 (95% CI: 1.62-116.55).

Discussion

H. pylori induce a variety of inflammatory cytokines in the gastric mucosa, and then aggravate inflammatory responses in the stomach and duodenum, leading to mucosal damage and abnormal gastric acid secretion. Host genetic factors play an important role in the development of *H. pylori*-related gastroduodenal diseases. Physiological characteristics of the host including genetic polymorphisms, HLA genotype, blood type, and other factors impact susceptibility to *H. pylori*-associated gastroduodenal diseases. Recently, the effect of cytokine gene polymorphisms on the outcome of *H. pylori* infection has become a focus of research focuses. TNF-α, an important cytokine participating in inflammatory reactions, can inhibit gastric acid secretion, which is beneficial to the implantation of *H. pylori* in the stomach, resulting in the occurrence of gastroduodenal diseases. TNF-α also activates neutrophils in gastric mucosal injury.

Genetic polymorphisms can be referred to as single nucleotide polymorphisms (SNPs) which refer to single nucleotide replacements at specific genomic sites, as the major manifestation of disease-susceptible genes. Therefore, searching for SNPs is an important way to find disease-susceptibility genes. However, the relationship between TNF-α polymorphisms and *H. pylori*-associated gastroduodenal diseases has not yet been completely explained.

Variation in the coding region of *TNF-α* affects the activity of the protein, resulting in a relationship between *TNF-α* genotype or allele and susceptibility to *H. pylori*-associated gastroduodenal diseases. Carlo, *et al*. [12] conducted a correlation study of *H. pylori*-related diseases and cytokine gene polymorphisms, and found that the majority of duodenal ulcer patients carried the *TNF-A-857 T/T* allele, which was correlated with gastric cancer. It was also found that *TNF-α* variants were associated with an increased risk of gastric ulcers and gastric cancer [13]. In this study, compared with the *TNF-A-308 G/G* genotype, the OR of people with the *TNF-A-308 A/A* genotype of developing chronic gastritis was 22.70 (95% CI: 2.51-205.40). Meanwhile, the OR of subjects carrying the *TNF-A-308 A/A* allele of developing gastric ulcer was 21.62 (95% CI: 2.07-226.13). In addition, the allele frequencies of these genotypes in the chronic gastritis and gastric ulcer group were also significantly increased, which might result in up-regulated *TNF-α* expression, which then aggravates the inflammatory reaction in the gastric mucosa, resulting in inflammatory cell infiltration and the formation of gastric ulcers. Our results indicate that the *TNF-A-308 A/A* genotype might be a susceptibility variant for gastric ulcer. Additionally, we observed that, the OR of people harboring the *TNF-A-857 T/T* allele of developing duodenal ulcer was 6.73 (95% CI: 1.71-26.53) while the OR of people with the *TNF-A-857 T/T* genotype of developing gastric ulcer was 5.37 (95% CI: 1.28-22.50) compared with the *TNF-A-857 C/C* genotype. Meanwhile, the allele frequencies of these variants in the peptic ulcer group were also significantly increased, which might also result in up-regulated *TNF-α* expression which then aggravates the inflammatory reaction in the gastric mucosa, resulting in inflammatory cell infiltration and the formation of gastric and duodenal bulb ulcers. Finally, our results also indicated that the *TNF-A-308 A/A* allele might be a susceptibility variant for gastric ulcer. The aforementioned results all show that *H. pylori*-related gastroduodenal disease susceptibility depends on the additive effects of multiple genetic factors as well as interaction with environmental factors.

However, it has been found in Japanese subjects with *H. pylori* infection that carriers of the *TNF-A-857 C/C* and *1031 C/C* alleles had the lowest serum *H. pylori*-positive rates in the population, while carriers of the *TNF-A-857 T/T* and *TNF-B-1031 T/T* genotypes had the highest serum *H. pylori*-positive rates [6]. Yea, *et al*. found that *TNF-A-308A* was closely related with the *H. pylori* cagA subtype infection in Koreans with gastric diseases [14]. Kunstmann, *et al*. also found that the *TNF-A-308 G/G* genotype was a risk factor for duodenal ulcers in females with *H. pylori* infection [7]. Differences between this study and related studies in western countries may result from the different genetic background of different ethnicities.

Due to the complicated etiology of gastric cancer, its development involves multiple stages. *H. pylori*-induced gastric disease may be caused by the cumulative effects of damage resulting from long-term *H. pylori* infection, host genetic factors and environmental factors. During process of *H. pylori* infection, the immune response of the host is complex, and *H. pylori* may induce the inflammatory cells in the gastric mucosa to secrete inflammatory factors such as TNF-α, which eventually result in various kinds of epithelial damage. We also observed that, compared with the *TNF-A-308 G/G* genotype, the OR of subjects with the *TNF-A-308 A/A* allele of developing gastric cancer was 16.41 (95% CI: 1.62-116.55), and its allele frequency was also significantly increased in gastric cancer patients. This genotype might result in up-regulated *TNF-α* expression that inhibit gastric acid secretion, which benefits *H. pylori* implantation into the gastric mucosa, and subsequently, the mucosa atrophy further, eventually promoting the occurrence of gastric cancer. Thus, our results show that *TNF-α* variants are closely related to the occurrence of gastroduodenal diseases and the susceptibility of gastric cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Yu Xiang, Department of Laboratory Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China. Tel: +86 23-89012513; Fax: +86 23-89012513; E-mail: 8051226@163.com

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