

## Short Communication

# Association of TNF- $\alpha$ promoter polymorphisms with the outcome of persistent HBV infection in a northeast Chinese Han population

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**Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) plays an important role in the pathogenesis and clinical outcome of chronic hepatitis B virus (HBV) infection. The objective of this study was to evaluate the relationship between functional polymorphisms of TNF- $\alpha$  and different outcomes of persistent HBV infection in a northeast Chinese Han population. Here 189 HBV spontaneously recovered subjects (SR), 571 HBV-infected patients including 180 chronic hepatitis B (CHB), 196 liver cirrhosis (LC), and 195 hepatocellular carcinoma (HCC) individuals were enrolled in this study. All the samples were genotyped for TNF- $\alpha$  –857C/T and –863C/A using the polymerase chain reaction-restriction fragment length polymorphism method. The frequency of –857CC genotype was significantly higher in CHB and LC individuals compared with that of SR subjects ( $P = 0.03$ , OR = 1.57, 95% CI 1.04–2.39 and  $P = 0.03$ , OR = 1.57, 95% CI 1.04–2.35, respectively). A significant difference in the distribution of the allele –857C was observed for both CHB vs. SR ( $P = 0.01$ , OR = 1.52, 95% CI 1.08–2.13) and LC vs. SR ( $P = 0.02$ , OR = 1.47, 95% CI 1.06–2.04) cohorts. In addition, the frequency of –863AA genotype was significantly higher in CHB and LC patients than that of SR subjects ( $P = 0.01$ , OR = 3.90, 95% CI 1.35–11.23 and  $P = 0.01$ , OR = 3.83, 95% CI 1.34–10.96, respectively), and allele –863A frequency was significantly more common in CHB, LC, and HCC cohorts than that of SR controls ( $P = 0.004$ , OR = 1.72, 95% CI 1.19–2.50;  $P = 0.001$ , OR = 1.81, 95% CI 1.26–2.61 and  $P = 0.001$ , OR = 1.90, 95% CI 1.33–2.73, respectively). Our data also revealed that haplotype CA was strongly associated with persistent HBV infection. These results suggest an association between the TNF- $\alpha$  promoter variants and different outcomes of persistent HBV infection in the studied population.**

**Keywords** TNF- $\alpha$ ; polymorphism; HBV infection; Chinese Han population

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## Introduction

It is estimated that more than 400 million people have been chronically infected by hepatitis B virus (HBV) worldwide, and nearly one million deaths were recorded as a consequence of the diseases due to persistent HBV infection globally each year. There is a high prevalence of HBV infection in China, accounting for over one-third of infected populations of the world [1,2]. Previous studies have shown that hepatocellular injuries are mainly caused by immune response to HBV infection [3–5]. Although the majority of HBV is cleared after infection in adults, about 5%–10% of them become persistently infected and develop chronic liver disease that might progress to chronic hepatitis, cirrhosis, or primary hepatocellular carcinoma (HCC) [6]. The exact mechanism of this progression to chronicity and development is currently unclear. However, it has been confirmed to be a multistage process with the complex interactions of immunological factors and multiple genetic variants [7,8].

Cytokines play important roles in defending against viral infection, both through inhibition of viral replication and through determination of the predominant pattern of the host response. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), one of the most important cytokines, has been proved to take part in the viral clearance and the progression of cirrhosis and primary HCC [9]. Thus, TNF- $\alpha$  may be a susceptibility gene for different outcomes of persistent HBV infection.

Several single-nucleotide polymorphisms (SNPs) have been found in the promoter region of *TNF- $\alpha$*  gene, which can influence TNF- $\alpha$  expression at the transcriptional and post-transcriptional levels [10]. There have been a number of reports validating the associations between these genetic variants and HBV infection diseases in various ethnic groups. Interestingly, a trend in the opposite association direction was observed among different studies. Kim *et al.* [11] found that TNF- $\alpha$  -863C allele strongly increased susceptibility to chronic hepatitis B in Korea population, whereas Kummee *et al.* [12] concluded that allele -863 A was the susceptible allele to HCC after HBV infection in a Thai population. Similarly, Li *et al.* [9] revealed that genotype -857CC was associated with CHB in a Chinese population. However, another group reported that genotype -857TT was associated with chronic severe hepatitis B in Chinese population [13].

The major reason for these discrepancies may be related to ethnicity and/or environmental factors. Population substructure was also a potentially important cause of different results in case-control genetic studies [14]. While Chinese Han constitute the largest ethnic group in the world, spurious associations due to population structure pose a challenge to genetic studies. One approach to counteract this problem may be to study a greater number of populations to obtain a broader spectrum of data. In this study, we used a case-control design to investigate the association of TNF- $\alpha$  polymorphisms with different outcomes of persistent HBV infection in a northeast Chinese Han population and to accumulate evidence regarding the potential role of TNF- $\alpha$  in the diseases.

## Materials and Methods

### Study subjects and samples

Total 571 HBV-infected subjects and 189 HBV spontaneously recovered subjects were included in the present study. Subjects were recruited constitutively from Heilongjiang Province Hospital, and the Third Affiliated Hospital of Harbin Medical University. All patients included in this study were positive for HBsAg. Cases were categorized into three clinical groups including the chronic hepatitis B group (CHB;  $n = 180$ ), liver cirrhosis group (LC;  $n = 196$ ), and hepatocellular carcinoma group (HCC;  $n = 195$ ). The control group consisted of HBV spontaneously recovered subjects who matched for age, sex, and ethnic with case group (SR;  $n = 189$ ). The diagnosis of different outcome of HBV infection was made on the basis of the criteria issued by the Association of Infectious Diseases and Parasites Diseases of China in 2000. Patients and SR subjects had to satisfy the following criteria. The SR subjects for this study were confirmed by the serological examination. Spontaneously recovered is

defined as two positive tests for anti-HBs and anti-HBc IgG, and one negative test for HBsAg. The subjects who were positive for anti-HBs but negative for anti-HBc were excluded because of the possible history of vaccination. The CHB patients were confirmed by being HBsAg positive, HBcAb positive, and HBeAg or HBeAb positive for at least 6 months. The subjects with HBV infection will be excluded from this group if he or she fits the criteria for diagnosis of the LC or HCC. The diagnosis of LC was based on the typical morphologic finding from computed tomography (CT) or ultrasound, and the corresponding laboratory features or evidence of portal hypertension (such as esophageal varices or ascites), sometimes combined with liver biopsy if needed. The diagnosis of HCC was based on histological, combined with at least one positive HCC image on enhanced CT or magnetic resonance imaging (MRI), sometimes combined with serum AFP analysis ( $>400$  ng/ml). All patients were with raised alanine aminotransferase (ALT) and quantifiable HBV DNA, while SR subjects were with the normal ALT. Patients who had any other types of liver disease such as infection with hepatitis C virus, hepatitis D virus or, human immunodeficiency virus, autoimmune liver disease and alcoholic liver disease were excluded from the study. After this vigorous selection process, we believe that the existence probability of liver cirrhosis or tiny HCC in the SR and CHB groups is close to zero.

All subjects were exclusively enrolled from Harbin, northeast of China. The study individuals must be stable residents in the areas; the participants should not be genetically related for at least three generations. A standard informed written consent procedure was included in the protocol, and was reviewed and approved by the Ethics Committee of Harbin Medical University, China. Participants gave their consent after the nature of study had been fully explained. A sample of approximately 2–3 ml of venous blood was collected from each participant. Genomic DNA was extracted from peripheral blood leukocytes using QiaAmp DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and stored at  $-20^{\circ}\text{C}$  until assay.

### TNF- $\alpha$ -857C/T and -863C/A genotyping assay

The -857C/T (rs1799724) and -863C/A (rs1800630) polymorphisms in the promoter region of *TNF- $\alpha$*  gene were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The PCR primer sequences for the amplification are as follows: the forward primer was 5'-AAGTCGAGTATGGGGACCCCCGTTAA-3' and the reverse primer was 5'-CCCCAGTGTGTGGCCATATCTTCTT-3' for -857C/T; and the forward primer was 5'-ATGTAGCGGCTCTGAGGAATGGTTACA-3' and the reverse primer was 5'-CTACATGCCCTGTCTTCGCCAAG-3' for -863C/A.

PCR conditions were as follows: pre-denaturation at 94°C for 5 min, followed by 30-step cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s, with a final extension at 72°C for 8 min. *HincII* and *StyI* (TaKaRa, Dalian, China) are the restriction endonuclease for the position of -857 and -863, respectively. Ten microliters of PCR products were digested with restriction enzyme for the whole night at 37°C. The digestion products were separated on 2% agarose gel and visualized directly under ultraviolet light with ethidium bromide staining.

### Statistical analysis

Allele and genotype frequencies of each population were calculated by direct counting. Then the Hardy–Weinberg equilibrium was evaluated using Pearson's  $\chi^2$  test separately for cases and controls. Differences between cases and controls in demographic characteristics and risk factors were evaluated by  $\chi^2$  test (for categorical variables) or Student's *t*-test (for continuous variables). We used Haploview 3.2 software (<http://www.broad.mit.edu/mpg/haploview/>) to reconstruct haplotypes and estimate haplotype frequencies in the cases and controls. The allele, genotype, and haplotype frequencies for the persistent HBV-infected patients and control individuals were statistically compared using  $\chi^2$  test, measured by the odds ratios (ORs) and 95% confidence intervals (CIs). Statistical analyses were performed using SPSS for Windows software (version 17.0; SPSS, Chicago, USA). We analyzed the data using two-sided *P* values. All associations with *P*-values <0.05 are referred to as nominal.

## Results

### Subjects characteristics

Total 571 patients with persistent hepatitis B infection and 189 spontaneously recovered subjects were studied. The case–control cohort used in this investigation was matched for ethnicity, culture, and geographical locations. The baseline characteristics of the subjects in the study groups are as follows: the mean age at enrollment was not significantly different between cases and controls ( $56.24 \pm 12.71$  vs.  $55.17 \pm 11.93$ ,  $P > 0.05$ ); the ratio of male/female was not significantly different between cases and controls (428/143 vs. 141/48,  $P > 0.05$ ). A slightly higher proportion of males than females were observed in both groups, which may be due to a participation bias.

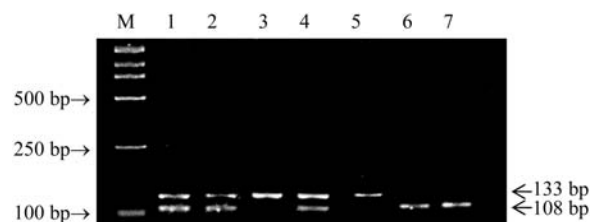
### TNF- $\alpha$ -857C/T and -863C/A polymorphisms

The allele -857C with *HincII* produced two fragments (108 and 25 bp). The allele -857C lacking *HincII* site yielded a 133-bp fragment. Heterozygote contained three bands, corresponding to 133, 108, and 25 bp (**Fig. 1**).

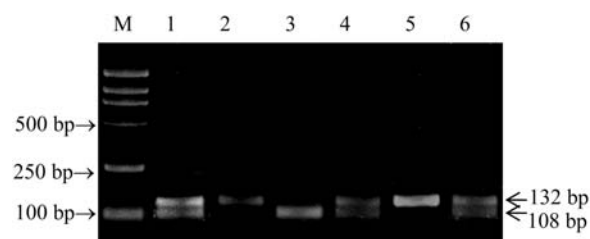
When *StyI* restriction site (allele -863C) was present, two fragments (108 and 24 bp) were generated. The allele -863A was not cleaved by *StyI* with a single 132 bp band. Heterozygote included three bands, corresponding to 132, 108, and 24 bp (**Fig. 2**). Twenty percent of the samples were duplicated as internal quality control to avoid sample or reading errors. The concordance percentage of twice genotyping was 100%.

### Association of TNF- $\alpha$ -857C/T and -863C/A with persistent HBV infection

The genotype distribution and allele frequencies of the TNF- $\alpha$  -857C/T polymorphism in case and control groups are shown in **Tables 1** and **2**. The genotype frequencies of -857CC, CT, and TT were 63.89%, 30.56%, and 5.55% in CHB patients; 63.78%, 29.59%, and 6.63% in LC patients; 53.85%, 38.97%, and 7.18% in HCC patients and 52.91%, 37.04%, and 10.05% in SR subjects, respectively. The genotype frequency of -857CC were significantly higher in persistent HBV-infected individuals (CHB and LC) when compared with that of SR subjects (OR = 1.57, 95% CI 1.04–2.39,  $P = 0.03$ ; OR = 1.57, 95% CI 1.04–2.35,  $P = 0.03$ ). The frequency of allele -857C was 79.17% in CHB patients, 78.57% in LC patients, 73.33% in HCC patients, and 71.43% in SR subjects, respectively. The allele -857 C showed strong association with persistent HBV infection (CHB and LC) in the study population (OR = 1.52, 95% CI 1.08–2.13,  $P = 0.01$ ; OR = 1.47, 95% CI 1.06–2.04,  $P = 0.02$ ).



**Figure 1** The typical genotypes of TNF- $\alpha$  -857C/T M: marker (DL2000); 1, 2, 4: TNF- $\alpha$  -857CT (133, 108, and 25 bp); 3, 5: TNF- $\alpha$  -857TT (133 bp); 6, 7: TNF- $\alpha$  -857CC (108 and 25 bp).



**Figure 2** The typical genotypes of TNF- $\alpha$  -863C/A M: marker (DL2000); 1, 4, 6: TNF- $\alpha$  -863CA (132, 108 and 24 bp); 2, 5: TNF- $\alpha$  -863AA (132 bp); 3: TNF- $\alpha$  -863CC (108 and 24 bp).

**Table 1 Association of TNF- $\alpha$  –857CC genotype with different clinical outcomes of persistent HBV infection**

Group	Sample number ( <i>n</i> )	Genotype (%)			Contrast	<i>P</i> value	OR (95% CI)
		CC	CT	TT			
CHB	180	63.89	30.56	5.55	CHB vs. SR	0.03	1.57(1.04–2.39)
LC	196	63.78	29.59	6.63	LC vs. SR	0.03	1.57(1.04–2.35)
HCC	195	53.85	38.97	7.18	HCC vs. SR	0.85	1.04(0.69–1.55)
SR (control)	189	52.91	37.04	10.05			

**Table 2 Association of TNF- $\alpha$  –857C allele with different clinical outcomes of persistent HBV infection**

Group	Sample number ( <i>n</i> )	Allele (%)		Contrast	<i>P</i> value	OR (95% CI)
		C	T			
CHB	180	79.17	20.83	CHB vs. SR	0.01	1.52(1.08–2.13)
LC	196	78.57	21.43	LC vs. SR	0.02	1.47(1.06–2.04)
HCC	195	73.33	26.67	HCC vs. SR	0.55	1.10(0.80–1.51)
SR (control)	189	71.43	28.57			

**Table 3 Association of TNF- $\alpha$  –863AA genotype with different clinical outcomes of persistent HBV infection**

Group	Sample number ( <i>n</i> )	Genotype (%)			Contrast	<i>P</i> value	OR (95% CI)
		CC	CA	AA			
CHB	180	61.67	30.55	7.78	CHB vs. SR	0.01	3.90(1.35–11.23)
LC	196	59.70	32.65	7.65	LC vs. SR	0.01	3.83(1.34–10.96)
HCC	195	53.85	42.56	3.59	HCC vs. SR	0.39	1.72(0.50–5.90)
SR (control)	189	72.49	25.40	2.11			

**Table 4 Association of TNF- $\alpha$  –863A allele with different clinical outcomes of persistent HBV infection**

Group	Sample number ( <i>n</i> )	Allele (%)		Contrast	<i>P</i> value	OR (95% CI)
		C	A			
CHB	180	76.94	23.06	CHB vs. SR	0.004	1.72(1.19–2.50)
LC	196	76.02	23.98	LC vs. SR	0.001	1.81(1.26–2.61)
HCC	195	75.13	24.87	HCC vs. SR	<0.001	1.90(1.33–2.73)
SR (control)	189	85.19	14.81			

The genotype distribution and allele frequencies of the TNF- $\alpha$  –863C/A polymorphism in case and control groups are shown in **Tables 3** and **4**. The genotype frequencies of the –863CC, CA, and AA were 61.67%, 30.55%, and 7.78% in CHB patients; 59.70%, 32.65%, and 7.65% in LC patients; 53.85%, 42.56%, and 3.59% in HCC patients; 72.49%, 25.40%, and 2.11% in SR subjects, respectively. The genotype frequency of –863AA were

significantly higher in persistent HBV-infected individuals (CHB and LC) when compared with that of SR subjects (OR = 3.90, 95% CI 1.35–11.23, *P* = 0.01; OR = 3.83, 95% CI 1.34–10.96, *P* = 0.01). The frequency of allele –863A was 23.06% in CHB patients, 23.98% in LC patients, 24.87% in HCC patients, and 14.81% in SR subjects, respectively. The frequency of allele –863A was significantly higher in patients with persistent HBV infection



**Table 5** The frequencies of haplotypes in the TNF- $\alpha$  gene promoter in case and control cohorts

Group	Sample number ( <i>n</i> )	Haplotype (%)			Contrast	<i>P</i> value*
		CC	TC	CA		
CHB	180	58.1	18.9	21.1	CHB vs. SR	0.003
LC	196	57.6	18.4	20.9	LC vs. SR	0.002
HCC	195	54.2	20.9	19.1	HCC vs. SR	0.011
SR (control)	189	59.0	26.0	12.8		

\**P* value of haplotype CA for each pair of contrasts.

(CHB, LC, and HCC) than that of SR controls (OR = 1.72, 95% CI 1.19–2.50, *P* = 0.004; OR = 1.81, 95% CI 1.26–2.61, *P* = 0.001; OR = 1.90, 95% CI 1.33–2.73, *P* < 0.001).

### TNF- $\alpha$ haplotype analysis

Haplotypes were constructed on the basis of the genotype data from TNF- $\alpha$  –857C/T and –863C/A using Haploview, and the results of haplotype analysis were shown in **Table 5**. In the four cohorts, the most common TNF- $\alpha$  haplotype was CC, followed by TC or CA. Among them, the frequencies of the TNF- $\alpha$  haplotype CA in CHB, LC, and HCC cohorts (21.1%, 20.9%, and 19.1%) were higher than that in SR subjects (12.8%; *P* = 0.003, *P* = 0.002 and *P* = 0.011, respectively). However, there were no differences in the frequencies of the other haplotypes between cases and controls and no significant association with persistent HBV infection (*P* > 0.05) (**Table 5**).

## Discussion

Chronic HBV infection is a major risk factor for CHB, LC, and HCC, which are threatening people's health in China, where HBV infection is more common than in the rest of the world. Although the underlying pathogenesis of the chronic HBV infection are incompletely understood, it is becoming increasingly apparent that HBV persistence is not only associated with the viral factor but also with the host immune and genetic factors, and the latter factors have been drawing more and more attention.

TNF- $\alpha$  may be a determinant of pathogenesis and disease progression in persistent HBV infection. The accumulating evidence implicates the role of TNF- $\alpha$  in inflammatory pathways that facilitate fibrosis [15] and increase tumorigenesis [16]. In humans, several lines of evidence suggested the importance of TNF- $\alpha$  in HBV persistence [17] and HCC [18,19]. Animal studies also showed that gradual increase occurred in the levels of TNF- $\alpha$  during hepatic fibrogenesis [20]. Data from the TNF- $\alpha$  receptor type 1 knockout mice model have provided evidence of the essential role of TNF- $\alpha$  in hepatocarcinogenesis [21]. An

appropriate expression of TNF- $\alpha$  may regulate the immune and metabolic response of the body, which plays a significant role in maintaining internal stability and resisting various diseases, while overexpression can cause a variety of pathological changes.

Previous studies have described extensive polymorphisms within the TNF- $\alpha$  promoter region at positions –1031, –863, –857, –308, and –238. Some of these SNPs appear to influence TNF- $\alpha$  expression at the transcriptional and post-transcriptional levels [10], and to be associated with the susceptibilities to various human diseases including autoimmune diseases, infectious diseases, and many cancers [22–25]. Therefore, we hypothesized that gene polymorphism of TNF- $\alpha$  promoter at position –857C/T, and –863C/A may lead to different levels of its production, which can lead to a different host immune response to HBV. This may affect the HBV infection and therefore its progression.

In the present study, we found that both CHB patients and LC patients carried more –857CC genotype than that of SR controls. Furthermore, the frequency of allele –857C was significantly more common in persistent HBV infection patients (CHB and LC) than that of SR controls. All these results suggested that allele –857C was associated with CHB and LC in this studied population. However, there was no statistically significant association between this SNP and the presence of HCC in our data. In former studies, Li *et al.* [9] revealed that genotype –857CC was associated with CHB in a Chinese population, which is in consistent with our results. Hohjoh *et al.* [26] revealed that TNF- $\alpha$  –857C/T was associated with higher transcriptional activity of TNF- $\alpha$ , which may affect the response to HBV infection and result in different outcomes after persistent HBV infection. In contrast, Li *et al.* [13] reported that genotype –857 TT was associated with chronic severe hepatitis B. The possible reason for this inconsistency could be due to genuine genetic heterogeneity existing among Han Chinese, because of no declaration of detail geographic locations in these two studies. The Chinese Han population, although seemingly homogeneous, exhibits a complicated substructure as the genetics

of different Chinese Han populations differ greatly [27]. Accordingly, a previous study suggests that the significant differences among northern and southern Chinese Han sub-populations should be carefully examined, especially when sample sources are diverse, as they may influence association studies [28]. In the current sample of northeastern Han Chinese, false-positive or false-negative associations owing to population substructure are less likely to exist. Our carefully ascertained, relatively homogeneous case-control sample of northeastern Chinese Han belongs to a single geographic location of Harbin.

It has been reported that the allele -863C is associated with CHB in a Korean population [11], whereas the allele -863A was found to be significantly increased in the HCC group compared with healthy controls in a Thai population [12]. It is well known that the difference in genetic background, lifestyle and environment exposures may lead to inconsistent association results for complex diseases among different populations. In our study, we find that the genotype frequency of -863AA in patients of persistent HBV infection (CHB and LC) was significantly higher than that in SR subjects. In addition, the frequency of allele -863A was more common in persistent HBV infection patients (CHB, LC, and HCC) than that in SR controls. In a word, genotype -863AA and allele -863A could increase the risk of persistent HBV infection and played an important role in the disease progression. Allele -863A has been demonstrated to be associated with elevated circulating TNF- $\alpha$  levels [29,30]. TNF- $\alpha$  can activate NF- $\kappa$ B, which will then stimulate proinflammatory genes that are associated with hepatic inflammation and hepatic fibrosis. Therefore, we can propose that carriers of allele-863A are associated with increased levels of TNF- $\alpha$  in the liver in response to HBV infection, which can induce inflammatory damage of hepatocyte that promotes fibrogenesis and tumorigenesis.

The interactions of multiple polymorphisms within a haplotype could affect disease phenotypes [31]. Therefore, we further performed association studies between TNF- $\alpha$  haplotypes and persistent HBV infection. The frequencies of the haplotype CA in several case cohorts were higher than that in SR controls, and the association between this haplotype and persistent HBV infection was shown to be significant by  $\chi^2$  test. No association was observed between the other haplotypes and persistent HBV infection. Thus, the haplotype CA seems to be involved in a mechanism that determines susceptibility to persistent HBV infection and may have a role in persistent HBV infection.

In the present study, TNF- $\alpha$  polymorphisms at position -857 and -863 were found to be associated with the development of persistent HBV infection. Our results suggest that TNF- $\alpha$  gene polymorphism is associated with not only the susceptibility to HBV infection, but also the

inflammation progress, advanced fibrogenesis and cancer development. These results will help us better understand the pathogenesis of HBV persistence, how to improve diagnosis, risk prediction, and clinical management of diseases associated with persistent HBV infection. Further well-designed investigations with larger sample sizes, more polymorphic sites and representing different ethnicities are warranted to confirm our findings.

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