

# Association of Tumor Necrosis Factor Alpha -238G/A and -308G/A Promotor Polymorphisms with Clearance of Hepatitis B Virus Infection in Turkish Population

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## ABSTRACT

**Objectives:** Acute viral hepatitis B may lead to chronic hepatitis in 6% of adult population. We compared the frequency of Tumor necrosis factor alpha promoter polymorphisms in chronic hepatitis B patients and people with natural immunity against hepatitis B.

**Materials and Methods:** Chronic hepatitis B patients and age matched control cases with natural immunity to hepatitis B virus were recruited 1:1 in this study. Tumor necrosis factor alpha -238G/A and -308G/A polymorphisms were studied with PCR-RFLP.  $\chi^2$  test was performed in statistical analysis.

**Results:** A total of 101 volunteers enrolled in two study groups. Thirty-eight men and 12 women constituted the chronic hepatitis B patient group and 40 men and 11 women recruited in natural immunity group. Frequency of -238G allele was 87.5% and 97% in chronic hepatitis B and natural immunity groups, respectively. Frequency of -308G allele was 93% and 92.1% in chronic hepatitis B and natural immunity groups, respectively. Frequencies of polymorphisms at positions -238 and -308 in the promoter of tumor necrosis factor alpha gene were not different between chronic hepatitis B and natural immunity groups.

**Discussion:** Tumor necrosis factor alpha promoter polymorphisms at -238 and -308 positions do not effect the outcome hepatitis B infection in Turkish population. Clearance of hepatitis B virus infection is multifactorial. Thus, further studies needed to identify genetic predisposition to chronic hepatitis B infection.

**Key Words:** Hepatitis B Virus, tumor necrosis factor alpha promoter polymorphism, genetic predisposition to chronic hepatitis B infection.

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## INTRODUCTION

Hepatitis B virus (HBV) is estimated to have infected more than 2 billion people worldwide, of whom, 400 million are chronically infected today and are at an increased risk of liver-related sequelae, including chronic hepatitis B (CHB), cirrhosis, fulminant liver failure, liver transplantation, hepatocellular carcinoma (HCC) and death [1,2].

Outcome of HBV infection is affected by viral and host factors. HBx protein, HBeAg and HBV genotypes are the leading viral factors affecting virus clearance [3, 4]. Age is the most pronounced host factor associated with chronic infection. Infants and children tend to develop CHB after acute HBV infection unlike adults. HBV infection during early

infancy results CHB in 90% patients whereas infection during adulthood leads to chronic infection in less than 5% of patients [5]. Control of HBV infection requires orchestration of innate and adaptive immune systems.

Although immunological data are not available about early intrahepatic events in human infection, data from the animal models of acute HBV infection supports the concept that clearance of HBV DNA mediated antiviral cytokines produced by cells of the innate and adaptive immune response [6, 7]. In particular, interferon-gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and Interferon alpha/beta (IFN- $\alpha/\beta$ ) are believed to trigger several pathways

leading to inhibition of viral replication without direct destruction of infected cells [3].

TNF $\alpha$ , a small protein of 17kDa with immunological, inflammatory and homeostatic activities, after binding its receptor, interferes with viral replication mechanisms and causes viral clearance [8]. High levels of TNF $\alpha$  and its receptor found in infiltrating mononuclear cells and hepatocytes of chronic hepatitis B patients [9]. TNF $\alpha$  kills HBx sensitized cells via apoptosis [10] and inhibits HBV core promoter's transcriptional activity in-vitro [11]. TNF $\alpha$  is capable of degrading HBV mRNA [12]. Reactivation of HBV infection occurs in patients receiving anti-TNF $\alpha$  treatment [13]. These findings suggest that TNF $\alpha$  activity is crucial to control HBV infection and viral replication.

Levels of TNF subjected to transcriptional, translational and posttranslational control [8]. TNF gene mapped to the class III region of MHC between HLA-B and DR loci. Several single nucleotide polymorphisms, at TNF promoter region, effecting TNF transcription levels were previously demonstrated [14]. An A allele at the -308<sup>th</sup> base of the promoter region results in higher serum TNF $\alpha$  levels compared to G allele [15]. Similarly, peripheral blood mononuclear cells of liver recipients with -308AA allele, when challenged with Con-A, produces TNF $\alpha$  thrice the patients with GG allele [16].

TNF $\alpha$  promoter polymorphisms shown to be involved in pathogenesis of several diseases [17]. Conflicting results were present between studies investigating HBV clearance. The aim of the present study was to determine whether certain TNF promoter polymorphisms affect outcome of HBV infection in Turkish population.

## MATERIALS and METHOD

This study carried out at Selçuk University, Meram Medical Faculty Department of Infectious Diseases and Clinical Bacteriology. Study protocol reviewed and approved by ethical committee of Selçuk University Meram Medical Faculty (Decision: 10.02.2006-2006/045).

### Patients

Fifty CHB patients and 51 age-matched controls with natural immunity (NI) against HBV were recruited in this study. All volunteers provided informed consent. All volunteers enrolled in this trial were

coming from central Anatolia. Diagnosis of CHB based on positive HBsAg over 6 months and elevated blood alanine aminotransferase and aspartate aminotransferase levels. All CHB patients were undergone liver biopsy to determine the liver injury. Liver pathology classified according to modified Knodells' score. Patients with hepatitis C, hepatitis D and HIV co-infection were excluded. Patients consuming alcohol 40g/day, patients with cirrhosis and autoimmune hepatitis were also excluded. HBV DNA was measured with real time PCR. Routine follow-ups of patients carried-out at outpatient clinics of Infectious

### Diseases

NI group consisted of volunteers who spontaneously cleared the HBV infection without any intervention. NI determined as negative HBsAg, positive antiHBs and antiHBc total or antiHBe antibodies. All of the NI group were negative for hepatitis C and human immunodeficiency virus antibodies.

### Study Procedures

Two ml venous blood drawn from all recruited subjects and stored at 4°C until DNA isolation. Genomic DNA was isolated from peripheral blood leukocytes using standard phenol-chloroform methods. Isolated DNA kept at -80°C. Gene fragments containing the polymorphic sites were amplified by PCR. PCR was performed in a 20- $\mu$ L reaction mixture containing 0.4  $\mu$ L DNA Taq polymerase (Fermentas, England), 50 ng genomic DNA, 20  $\mu$ L 10 x buffer, and 1.5 mmol/L MgCl<sub>2</sub>, 1.2  $\mu$ L primers and 10 pmol/L dNTPs. The PCR cycles were as follows: 94°C for 3 min, 30 cycles of denaturing at 94 °C for 15seconds, annealing at 57°C for 15seconds, extension at 72 °C for 15 seconds, and a single final extension at 72 °C for 3minutes in an Applied Biosystems GeneAmp PCR System model 2700 thermocycler. After amplification, products were identified under ultraviolet light after electrophoresis in 3% agarose gel stained by ethidium bromide (EB). Then PCR products were digested by allele-specific restriction enzymes (NcoI, Fermentas, England) (MspI Promega, USA) overnight at 37 °C. Cleaved DNA fragments then identified by ultraviolet light after electrophoresis in 3% agarose gel stained by EB. pBR322 DNA/BsuRI (Fermentas, England) DNA ladder was used during analysis (Figure1-2).

Figure 1: PCR-RFLP for TNF $\alpha$  -238 genotype. M, DNA marker, lane 1 PCR product before digestion (152bp), lane 2: genotype: AA (152bp), lanes 3,7,10,11 genotype GA (152+132+20bp), lanes 4,5,6,8,9,12,13 genotype GG (132+20bp).

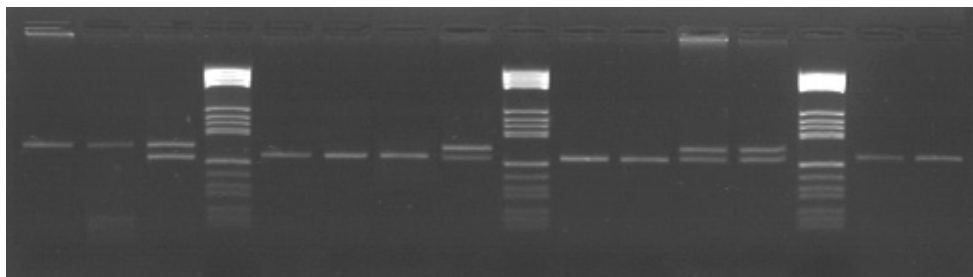
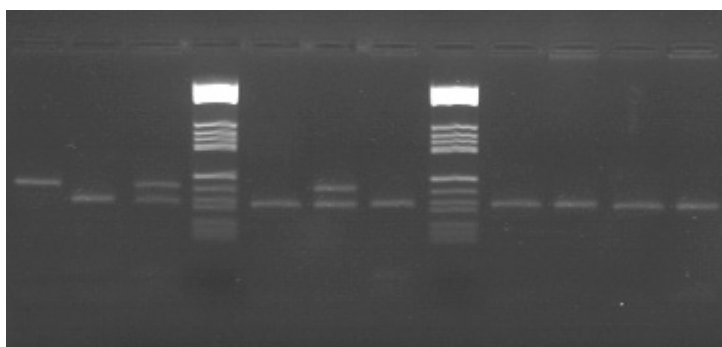


Figure 2: PCR-RFLP for TNF $\alpha$  -308 genotype. M, DNA marker, lane 1 AA allele (107bp), lane 2,4,6,7,8,9,10: genotype: GG (87+20bp), lanes 3,5 genotype GA (107+87+20bp), lanes 4,5,6,8,9,12,13 genotype GG (132+20bp).



of -238GA and -308GA genotypes did not differ between CHB and NI groups.

## Statistical Analysis

Data evaluated by  $\chi^2$  test using SPSS 10.0 programme with  $p \leq 0.05$  as statistical significance.

## RESULTS

### Subject Characteristics

CHB group consisted of 38 men and 12 women. NI groups consisted of 40 men 11 women. Patient characteristics summarized in table 2. Mean age and gender of groups were similar ( $p=0.217$ ).

### Genotype Distributions and Allele Frequencies

Genotypic frequencies of the two groups shown in table 3. We found -238 GG genotype in 42(84%) and 48(94%) of CHB and NI group, respectively. GA genotype was present in 7(14%) and 3(5.8%) of CHB and NI groups, respectively. AA genotype found in one patient from CHB group. Frequency of -238G allele was higher in NI group but did not reach statistical significance. -308 G allele was present in 93% and 92.1% of CHB and NI groups, respectively. Frequency

## DISCUSSION

HBV continues to be public health challenge despite ongoing vaccination programs. HBV is a major cause of morbidity and mortality in high endemicity areas [1, 2]. Outcome of HBV infection has effected by viral and host factors. Among host factors age, is the most prominent factor leading chronic infection. Despite an outstanding interest in the field host factors that are responsible of viral persistence, remain obscure.

The prominent role played by TNF $\alpha$  in inflammation and its relevance to infectious diseases has led to great interest in both the regulation of the TNF $\alpha$  gene, and the possibility that variants of the gene or deregulation of its production may be associated with pathology [17]. The level of TNF production in healthy individuals shows wide and stable variation, with high and low producer phenotypes present in the population, indicating a substantial genetic contribution to TNF regulation [15]. The gene of TNF $\alpha$  is located in the HLA Class III region in the short arm of sixth chromosome. The first 200 bp of

the promoter are highly conserved across a range of species, with the murine, bovine and porcine promoters showing approximately 80% homology with the human promoter, while further upstream there is far less conservation between species. Most of the transcription factors capable of binding and regulating the TNF $\alpha$  promoter shown to bind the proximal 200 bp of the promoter [17]. A putative regulatory box (Y-Box) of the TNF $\alpha$  promoter including a SNP at position -238 was described [8]. This putative regulatory box is strongly conserved among the species and believed to contribute the optimal promoter activity [19].

In this study, we evaluated the role of two SNPs in TNF $\alpha$  promoter in clearance of HBV infection. We found that G allele frequency at -238<sup>th</sup> position of the promoter in 87.5% and 97% of CHB and NI groups, respectively. Difference between groups was statistically insignificant. We also found that 93% of CHB group and 92.1% of NI group had G allele at position -308 of TNF $\alpha$  promoter.

Höhler, found that -238GG genotype was more common in spontaneously recovered subjects than CHB patients [19]. Miyazoe could not demonstrate a relation between -238 genotype and viral clearance [20]. Several investigators from China demonstrated a correlation between -238 GG genotype and CHB infection [21-24]. Niro reported that -238 genotype did not affect history of HBV infection [25]. A large study from Korea could not demonstrate a correlation between -238 genotype and viral clearance [26]. In our study, we could not demonstrate a relation between -238 genotype and viral clearance. This could be explained with ethnical differences between populations. Vertical transmission of HBV infection is common in our society like Japanese [27]. Age of transmission could affect the clearance of HBV rather than the -238 allele in Turkish population. It was previously, shown that, an A allele at the -308<sup>th</sup> base of the promoter region of the TNF $\alpha$  gene results in higher serum TNF $\alpha$  levels compared to G allele [15]. Similarly, peripheral blood mononuclear cells of liver recipients with -308AA allele, when challenged with Con-A, produces TNF $\alpha$  thrice the patients with GG allele [16]. In order to determine a role for -308G/A polymorphisms in chronic hepatitis pathogenesis, some studies were performed and revealed contradictory results. Höhler and Niro found no correlation between -308G/A polymorphisms and chronic hepatic B outcome [19, 25]. Kim, found that -308A allele was strongly associated with clearance of hepatitis B infection [26]. Miyazoe could not

demonstrate a relation between -308 genotype and hepatitis disease progression [20]. In our study, we could not demonstrate an association between TNF -308 promoter and hepatitis virus clearance. Our data is inconsistent with Höhler's and Miyazoe's results. Ethical differences between study groups and modes of transmission might influence the contradictory results.

Wang could not demonstrate a relation between -238 and -308 genotype and response to HBV vaccination [29]. Age, immune-status of the case and HLA DRB1\*07 carriage rather than TNF promoter genotype affect the vaccine response in Wang's cohort. Besides, as TNF loci lie very close to MHC locus, linkage disequilibrium between TNF promoter and MHC loci may exist. Some studies found that TNF-308A allele was in strong linkage disequilibrium with HLA-DRB1\*13 which had been associated with HBV clearance previously [28]. Exact mechanism that is responsible for the association between MHC genotype and disease outcome is obscure.

Kim found that SNP's at position -308 and -863 could affect the outcome individually [26]. However, Kim demonstrated that some TNF haplotypes (-1031T; -863C; -857C; -308G; -238G; -163G and -1031C; -863A; -857C; -308G; -238G; -163G) were significantly associated with HBV clearance, suggesting combination of SNP's rather than a single SNP may affect the outcome.

Former studies reported from Turkey, demonstrated that -238G and -308G allele was present in 73.1% and 68.8-95.2% of control population respectively [30,31]. Our data is consistent with the former Turkish reports.

Data on TNF alpha promoter polymorphisms and clearance of HBV infection is still incomplete and conflicting. Our study has some limitations. Size of the study group was limited which may lead to underestimation of our results. However, Sghaier [32] showed 308GG allele was protective from HBV infection and -308A allele was related to chronic infection with a similar sample size. Ethnic differences might contribute to conflicting results from different studies. Immune modulation for the treatment of HBV infection is coming-of-age. Understanding the natural mechanisms of viral control may ease our way to define new therapeutic targets [33].

As a result, we could not demonstrate a relation between HBV clearance and TNF promoter -238 and -308 polymorphisms. In our population, age and mode of transmission might be effecting the HBV clearance rather than TNF promoter genotype.

Linkage disequilibrium between TNF promoter and MHC loci may also be contributing to HBV clearance in Turkish population.

## CONFLICT OF INTEREST STATEMENT

Authors declare that they have no conflict of interest regarding this manuscript.

Table 1. Outline of TNF $\alpha$  promoter genotyping

Loci	Primer sequences (5' to 3')	PCR Product	Restriction enzyme	Genotype
-	F:AGAAGACCCCCCTCG GAAC	152bp	MspI	GG:132+20bp
238	R:ATCTGGAGGAAGCGG TAGTG			GA:152+132+20bp
				AA:152bp
-	F:AGGCAATAGGTTTTGAGGGCCAT	107bp	NcoI	GG:87+20bp
308	R:TCCTCCCTGCTCCGATTCCG			GA:107+87+20bp
				AA:107bp

Table 2. Characteristics of study population

		CHB group	NI group
Sex,	men	38	40
	women	12	11
Age			
mean $\pm$ STD		36.7 $\pm$ 11.8	39.4 $\pm$ 10.17

Table 3. Genotype distribution and allelic frequencies of study population

Locus	Genotype	CHB group	NI group	P-value
-238	GG	42	48	ns
	GA	7	3	
	AA	1	-	
	G	87.5%	97%	
	A	12.5%	3%	
-308	GG	43	43	ns
	GA	7	8	
	AA	-	-	
	G	93%	92.1%	
	A	7%	7.9%	



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