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ARTICLE

Investigation of 1377C/T polymorphism of the Toll-like receptor 3 among patients with chronic hepatitis B

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Abstract: The immunopathogenesis of chronic hepatitis B (CHB) has not been clarified yet. Toll-like receptors (TLR) are a receptor family that initiates immunity with exogenous–endogenous ligands and plays a role in the pathogenesis of infections. In this study, we aimed to investigate the frequency of TLR 3 1377C/T (rs3775290) polymorphism and its role in patients with CHB. We included 50 healthy individuals as control group and 73 active and 43 inactive hepatitis B patients. All DNA samples were isolated from blood samples. For the detection of TLR 3 1377C/T single-nucleotide polymorphism, restriction fragment length polymorphism was used. A statistically significant difference was determined in *Hepatitis B virus* (HBV) DNA levels of CHB patients with the CC, CT, and TT genotypes ($p = 0.013$). The highest levels of HBV DNA were detected in individuals with TT genotypes. Additionally, the frequency of CC genotype was higher in the active CHB patients compared with that of the inactive CHB patients ($p = 0.044$). No statistically significant difference in TLR 3 1377C/T polymorphism was detected between healthy controls and the hepatitis B patients ($p = 0.342$). In conclusion, HBV DNA level was higher in the individuals with TT genotype, and CC genotype was more frequent in the active CHB patients. These results suggest a possible association between CHB and TLR 3 gene (1377C/T) polymorphism.

Key words: chronic hepatitis B, Toll-like receptors, single-nucleotide polymorphism.

Résumé : L'immunopathogenèse de l'hépatite B chronique (HBC) demande toujours à être éclaircie. Les récepteurs de type Toll (« Toll-like receptors », TLR) forment une famille de récepteurs qui déclenchent l'immunité en présence de ligands exogènes ou endogènes et qui jouent un rôle dans la pathogenèse infectieuse. Dans la présente étude, nous avons entrepris d'examiner la fréquence du polymorphisme 1377C/T (rs3775290) du TLR 3 et son rôle chez les patients atteints d'HBC. Nous avons recruté 50 personnes en bonne santé pour le groupe témoin, 73 patients atteints d'hépatite B active ainsi que 43 patients à l'hépatite B inactive. Tous les échantillons d'ADN ont été isolés de prélèvements sanguins. Afin de détecter le polymorphisme mononucléotidique 1377C/T du TLR 3, on a eu recours à l'analyse du polymorphisme de la longueur des fragments de restriction (RFLP). On a relevé des différences statistiquement significatives dans les taux d'ADN du VHB chez les patients HBC de génotypes CC, CT et TT ($p = 0{,}013$). Les taux d'ADN de VHB les plus élevés ont été mesurés chez les personnes dotées de génotypes TT. En outre, le génotype CC était plus fréquent chez les patients HBC actifs par rapport aux patients à la maladie inactive ($p = 0{,}044$). Il n'y a pas eu de différence significative entre les témoins sains et les patients souffrant d'hépatite B eu égard au polymorphisme 1377C/T du TLR 3 ($p = 0{,}342$). On en conclut que les taux d'ADN de VHB étaient plus élevés chez les personnes de génotype TT, et le génotype CC était plus fréquent chez les patients HBC actifs. Ces résultats soulèvent une association possible entre l'hépatite B chronique et le polymorphisme 1377C/T du TLR 3. [Traduit par la Rédaction]

Mots-clés : hépatite B chronique, récepteurs de type Toll, polymorphisme mononucléotidique.

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Introduction

Two billion people encounter hepatitis B virus worldwide. It is estimated that there are 240 million cases of *Hepatitis B virus* (HBV) infection, and 800 000 people die every year from complications associated with HBV (World Health Organization 2015). The immune system plays a critical role in getting the HBV under control in case of viral infection and in making the serological markers evident. Nonetheless, it is considered that the virus does not have direct cytopathic effect on liver; rather, it seems that liver damage depends on immunological mechanisms (Ferrari et al. 2005). One of these mechanisms can be related to Toll-like receptors (TLRs). For example, it has been reported that activation of TLR 3 may cause immune-mediated liver damage (Bertolino and Holz 2007).

The primary response to pathogens in the innate immunity system is triggered by pattern recognition receptors (Werling and Jungi 2003; Tsai et al. 2015). TLRs are the most important family of pattern recognition receptors and play a critical role in both innate and adaptive immunity (Zhang et al. 2013). The TLRs act as sensors of microbial products, start the mechanisms to synthesize the immune and inflammatory genes, and play a role in the activation of adaptive immune system. Currently, a total of 13 TLRs (TLR 1–13) have been identified in mammals, including 11 in humans (Cheng et al. 2007; Boissier et al. 2008). It is known that TLRs 2, 3, 4, 7, 8, and 9 have roles in the pathogenesis of several viral infections, including hepatitis B and C (Wu et al. 2009; Kondo et al. 2011; Yang et al. 2014). The mutations in TLR genes or TLR gene polymorphisms cause the host to be more susceptible to various infectious diseases (Gazzinelli et al. 2004; Schroder and Schumann 2005).

The TLR 3 gene, also called CD283, is located on the 4q35 chromosome (Karimi-Googheri and Arababadi 2014) and is expressed in dendritic cells, Kupffer cells, and hepatocytes (Testro and Visvanathan 2009). Activation of TLR 3 causes stimulation of antiviral responses and inflammatory transcription factors, including interferon regulatory factor 3, activator protein 1, and nuclear factor κB (Li et al. 2010; Tuosto 2011; Szatmary 2012). The plasmacytoid dendritic cells produce type 1 interferon (IFN) using TLR 3 specifically for the response against double-stranded RNA (Carpenter and O'Neill 2007; Onoguchi et al. 2007; Cheng et al. 2014). It has been reported that expressions of TLR 3 and IFN-β were decreased in dendritic cells in chronic hepatitis B (CHB) patients, as compared with healthy volunteers (Li et al. 2009). Additionally, a study revealed that the magnitude of TLR 3 expression was found to be lower in CHB patients than in healthy controls (An et al. 2007). In a recent study, it was found that hepatitis B e antigen (HBeAg) impairs the interaction of some structures that have a role in the TLR signaling pathway (Lang et al. 2011). These studies show that TLR 3 is closely related to the course of HBV infection and may play crucial roles in the development of prolonged hepatitis B forms.

Single-nucleotide polymorphism (SNP) rs3775290 (1377C/T) is located in exon 4 of the TLR 3 gene and affects the receptor–ligand interaction by changing the TLR 3 ectodomain, thereby impairing the receptor function (Pandey et al. 2011). TLR 3 genetic polymorphisms have been reported to be associated with susceptibility to infectious diseases including viral hepatitis (Al-Qahtani et al. 2012; Lee et al. 2013). However, there is not sufficient information regarding the TLR 3 1377C/T polymorphism in HBV-related CHB. In this study, we aimed to detect the frequency of TLR 3 gene polymorphism at the promoter region –1377C/T (rs3775290) among the patients with CHB and to investigate whether this polymorphism has an association with the CHB.

Materials and methods

Study population

A total of 116 CHB patients and 50 healthy subjects who applied to the Ankara Training and Research Hospital Infectious Diseases and Clinical Microbiology Outpatient Clinic between 1 March 2013 and 31 August 2013 were included in the present study. All subjects were living in Ankara Province. Blood samples were obtained with informed written consent. The study was approved by the Ethics Committee of Ankara Training and Research Hospital, Ankara, Turkey (No. 4098/2013).

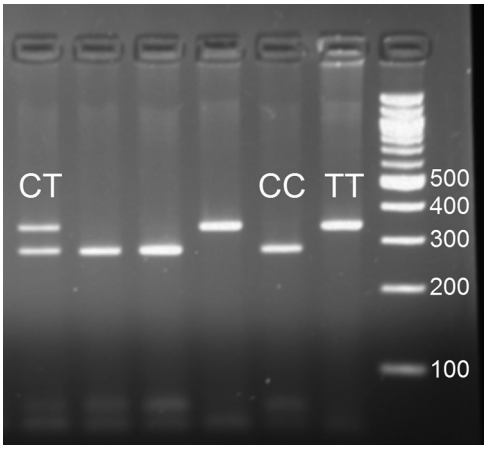
The participants were categorized into 3 groups. The first group included inactive CHB patients who were HBsAg positive for at least 6 months, serum HBV DNA <2000 IU/mL, normal ALT and AST levels with the absence of prominent hepatitis findings in liver biopsy. The second group consisted of active CHB patients who were HBsAg positive for at least 6 months, serum HBV DNA ≥2000 IU/mL, and continuous or intermittent elevation of ALT, with prominent hepatitis findings in liver biopsy. The last group was healthy volunteers without any history or finding in favour of CHB, cirrhosis, and hepatocellular cancer, known genetic diseases, pregnancy, no history of immune system disorders, chronic pulmonary disease, severe heart disease, major organ transplantation or malignancy.

Exclusion criteria were as follows: being under 18 years of age, coinfection with any other virus (such as HCV, HDV, HIV), with other types of liver diseases (e.g., autoimmune, metabolic or alcoholic liver diseases), cirrhosis and hepatocellular cancer, being pregnant and lactating female, immune system disorders.

Genotyping of the TLR 3 (–1377C/T)

Venous blood samples of 10 mL were obtained from all participants, kept in tubes with EDTA, and stored at –20 °C until the time of the DNA isolation. Blood samples were taken from the CHB patients before starting to the antiviral treatment. Genomic DNA was prepared with QIAmp DNA blood kit (Qiagen, Hilden, Germany). For the detection of TLR 3 (1377C/T) genetic polymorphism, polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) was used as described by Noguchi et al. (2004). PCR was performed in a total volume of 25 μL that included

Fig. 1. The image of Toll-like receptor 3 polymerase chain reaction products detected by electrophoresis on a 3% agarose gel. DNA marker, 100 bp.



Thermopol reaction buffer, 200 ng DNA, 0.25 mmol/L (each) deoxynucleotide, 1.25 μmol/L (each) primer, 0.5 U *Taq* DNA polymerase enzyme (New England Biolabs GmbH, Frankfurt, Germany). Primer sequences used for PCR were 5′-CCAGGCATAAAAAGCAATATG-3′ as forward primer and 5′-GGACCAAGGCAAAGGAGTTC-3′ as reverse primer. Amplification was performed using a Px2 Thermo Hybaid thermal cycler (Thermo Electron Co., Massachusetts, USA). PCR conditions were initial denaturation at 94 °C for 5 min, followed by 35 cycles including denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s, and an extension at 72 °C for 60 s. Final extension step was set to 72 °C for 7 min, before the restriction enzyme analysis. PCR products were incubated at approximately 65 °C for 16 h using the *Taq*I restriction enzyme. After cutting with the restriction enzyme, the fragments were separated by agarose gel (3%) electrophoresis; 275 + 62 bp RFLP products for CC genotype, 337 + 275 + 62 bp products for CT genotype, and 337 bp RFLP product for TT genotype were obtained (Fig. 1).

Statistical analysis

Statistical analysis was performed using the SPSS version 18.0 statistical program (SPSS, Inc., Chicago, Illinois, USA). Descriptive statistics (percentage distribution, mean, median, standard deviation) were presented. The χ^2 test for percentage comparisons, Fisher's exact test, Student's *t* test for comparison of normally distributed 2 continuous variables, Mann–Whitney *U* test for the comparison of 2 continuous variables not distributed normally, and Kruskal–Wallis test for comparison 2 or more variables were performed. Bonferroni correction was applied for nonparametric comparison of 2 or more continuous variables. The Kolmogorov–Smirnow test was used to evaluate the normal distribution of variables. A p value of <0.05 was considered statistically significant.

Results

A total of 116 CHB patients and 50 healthy subjects were enrolled in the study. Patients with CHB were divided into 2 groups as active (n = 73) and inactive (n = 43). Age and gender distributions were not statistically different between the 2 groups, as shown in Table 1. The mean HBV DNA value of the patients with CHB was 10^4 IU/mL. The mean (minimum, maximum) ALT and AST levels were 45.6 U/L (10, 512) and 34.5 U/L (12, 256), respectively. The mean AFP was 3.4 ng/L (0.3, 31.8). HBeAg was positive in 9 patients, and negative in 107 patients. The mean HBV DNA values of the patients with active and inactive CHB were 10^4 and 10^3 IU/mL, respectively.

Table 1. Gender and age distribution of the participants.

Characteristic	Patient group	Healthy group	p
Gender			
Male, n (%)	55 (47.4)	24 (48.0)	0.892
Female, n (%)	61 (52.6)	26 (52.0)	
Age, mean±SD (range)	39.0±12.4 years (18–73)	38.2±6.3 years (18–68)	0.460

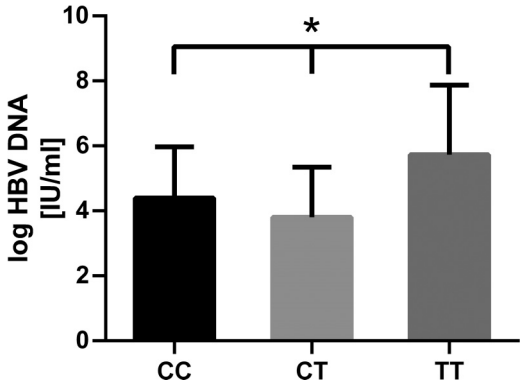
Table 2. The Toll-like receptor 3 (TLR 3) 1377C/T genotype distribution of the patient and control groups.

	Patient group, n (%)	Healthy group, n (%)	p
Genotypes			
CC	60 (51.7)	23 (46.0)	0.342
CT	49 (42.2)	26 (52.0)	
TT	7 (6.1)	1 (2.0)	
Alleles			
C	169 (72.8)	72 (72.0)	0.342
T	63 (27.2)	28 (28.0)	

Among 116 patients with CHB whose TLR 3 (1377C/T) polymorphism was investigated, 51.7% (n = 60) had CC genotype, 42.2% (n = 49) had CT genotype, and 6.1% (n = 7) had TT genotype. In the healthy group, 46% (n = 23) had CC genotype, 52% (n = 26) had CT, and 2% (n = 1) had TT genotypes. No statistically significant difference in terms of genotype distribution was observed between CHB patients and healthy group (p = 0.342). The genotype and allele distribution of the patient and control groups is presented in Table 2. We performed a χ^2 test on the observed and expected values to see if the observational data supports the hypothesis that the population is at Hardy–Weinberg equilibrium for the gene. The p values for the controls and patient groups were 0.25 and 0.83, respectively. Therefore, the distributions of genotypes in both groups were in accordance with Hardy–Weinberg equilibrium.

When the HBV DNA levels of CHB patients were compared according to the CC, CT, and TT genotypes, it was found that patients harboring the TT genotype had higher levels of HBV DNA than the ones with CC and CT genotypes. The lowest HBV DNA levels were observed among patients with the CT genotype (Fig. 2). When the patients with the 3 genotypes (CC, CT, and TT) were compared, a statistically significant difference was found (p = 0.013) (Fig. 2), in terms of HBV DNA levels.

Fig. 2. Comparison of the log *Hepatitis B virus* (HBV) DNA levels between the Toll-like receptor 3, 1377C/T genotype groups (n = 58, 48, 7 for CC, CT, TT, respectively). *, p = 0.013; a p value of <0.017 was considered significant according to a Bonferroni correction.



When the genotype frequencies of CC genotype and variant alleles (T allele including CT+TT genotypes) are compared between active and inactive CHB patients, 58.9% (n = 43) of active patients had CC, and 41.1% (n = 30) of active patients had variant allele (CT+TT) genotypes. Among patients with inactive CHB, 39.5% (n = 17) had CC and 60.5% (n = 26) had variant alleles (CT+TT). When the genotype frequencies are compared between active and inactive CHB patients, the CC genotype was found to be significantly more common in patients with active CHB (p = 0.044) (Fig. 3).

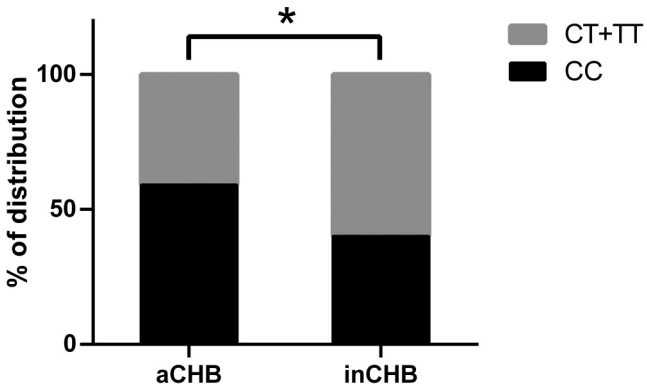
Discussion

In this study, we investigated the effects of the 1377C/T polymorphism of TLR 3 in patients with CHB in a Turkish population. We found that HBV DNA levels were significantly higher among patients harboring the TT genotype, and the CC genotype was more common in active hepatitis B patients.

HBV infection is a major cause of chronic liver disease in the world. TLRs recognize the pathogen-related molecular patterns and play a crucial role in innate and adaptive immune systems. It is well known that the TLR signaling pathway has important role in eradicating viruses, for example, HBV (Chen et al. 2008).

Nucleotide polymorphisms, even a single one, could be important in the susceptibility of the individual against a disease, in the sensitivity to drugs and adverse effects, in the development of personalized treatment strategies, and in determining new therapeutic targets. In recent years, the detection of the relationship between genetic polymorphisms and specific diseases has enhanced the studies in this field. Genetic variants of TLR 3 have been reported to be related to various presentations of viral hepatitis (Al-Qahtani et al. 2012; Lee et al. 2013). Therefore, we hypothesized that the 1377C/T polymorphism (rs3775290) of TLR 3 might be associated with HBV infection in Turkish patients.

Fig. 3. The comparison of genotype frequencies of CC group and variant allele group (CT and TT) between patients with active and inactive chronic hepatitis B (CHB) (n = 43, 30 for CC, CT+TT, respectively, for active CHB group; n = 17, 26 for CC, CT+TT, respectively, for inactive CHB group. *, p = 0.044; a p value of <0.05 was considered significant according to the χ^2 test.



In this study, we investigated the TLR 3 (1377C/T) gene polymorphism that is an important immune system element for hepatocyte damage and development of CHB. To our knowledge, the current study is the first one investigating the relationship between TLR 3 (1377C/T) gene polymorphism and CHB in a Turkish population.

When the TLR 3 gene (1377C/T) polymorphism and genotype frequencies are compared, no statistically significant difference was observed between patients with CHB and healthy controls (p = 0.342), which was compatible with former results of the distribution of genotype frequencies (Table 2). Therefore, our findings should be considered as a preliminary study. More studies on larger cohorts are needed to clarify the effects of TLR 3 polymorphisms in CHB. Etem et al. (2011) investigated the TLR 3 (1377C/T) gene polymorphism among 100 patients with rheumatoid arthritis and 100 healthy subjects and did not state statistically significant difference in terms of frequency distribution (Etem et al. 2011).

Generally, it has been accepted that TLR 3 signaling pathway is active in liver diseases and it might lead to immune-mediated liver damage (Al-Qahtani et al. 2012). Although TLR 3 has an important role in the innate immune system, TLR 3 gene polymorphism has been studied in few diseases (He et al. 2007; Ueta et al. 2007; Askar et al. 2009). Rong et al. (2013) studied 2 TLR 3 gene polymorphisms (C1234T and A952T) in 462 healthy controls and 452 CHB patients. They found 1.4 and 2.3 times higher risk for CHB among the patients carrying CT and TT genotypes, respectively, compared with patients carrying CC genotype in C1234T genotyping (Rong et al. 2013).

Genetic polymorphism in TLR 3 has been linked with susceptibility to infectious diseases, including CHB. A study by Huang et al. (2015) investigated TLR 3 (rs1879026 and rs3775290) polymorphisms among 437 patients with HBV-related diseases and 186 healthy controls in a Chinese Han population. In that study, they found a lower TT genotype

frequency for rs3775290 SNP in CHB patients and concluded that the TLR 3 rs3775290 polymorphism was associated with decreased susceptibility to CHB in the Chinese population (Huang et al. 2015). Al-Qahtani et al. (2012) investigated 9 different TLR 3 gene polymorphisms in 707 patients with CHB and 600 healthy controls in Saudi Arabia. They found a significant difference between patient and control groups for only one polymorphism (rs1879026 (G/T)) (Al-Qahtani et al. 2012). In another study, Sa et al. (2015) investigated TLR 3 gene polymorphism among 35 HBV patients, 74 HCV patients, and 299 healthy volunteers. They did not find a statistically significant difference in distribution of allele, genotype, and haplotype frequencies between the 2 groups (Sa et al. 2015).

In the literature, there are studies that investigated the association between TLR 3 expression with CHB. In Taiwan, Huang et al. (2013) investigated the TLR 3 expression in peripheral mononuclear cells and hepatocytes. They detected TLR 3 expression to be significantly lower in peripheral mononuclear cells and hepatocytes of patients with CHB compared with the healthy subjects (Huang et al. 2013).

Li et al. (2009) found TLR 3 and IFN-β expressions to be lower in monocytic dendritic cells of patients with CHB and acute liver failure following the CHB, compared with healthy controls, in their study of 40 CHB patients, 60 patients with acute liver failure following CHB, and 20 healthy subjects. They noted that TLR 3 and IFN-β expressions were significantly reduced in the patients that died from acute liver failure following CHB, when compared with patients with acute liver failure but alive (Li et al. 2009).

When HBV DNA levels of the patients with CHB were compared, they were found to be significantly higher among patients harboring the TT genotype than patients harboring the CC and CT genotypes. The hypothesis of this study was that TLR 3 (1377C/T) polymorphism might be a risk factor for development of CHB in a Turkish population and it was considered that higher levels of HBV DNA in patients with the TT variant genotype might be a risk factor for CHB.

In this study, active and inactive CHB patients were compared in terms of different genotypes (patients with the CC genotype vs. patients carrying a variant allele; CT and TT). Individuals harboring the CC genotype were more common in the active hepatitis B group and this difference was statistically significant. It is considered that HBV does not have a direct cytopathic effect and the damage to the liver occurs because of immunological etiology. According to our results, the increased activity of the TLR 3 pathway among patients with the CC genotype might have led to an immune response, resulting in hepatocyte damage and active chronic hepatitis.

In conclusion, we detected an association between CHB patients and TLR 3 (1377C/T) gene polymorphism, and the CC genotype seems to be a risk factor for active CHB. However, our study does have some limitations, such as analysis of one TLR polymorphism, and investigation of the polymorphism in one position of the TLR gene. Further studies on large sample groups are required to detect whether there is a relationship between CHB and other SNPs of the TLR 3 gene.

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