Investigation of the 1377C/T Polymorphism of Toll-like Receptor 3 among patients with Chronic Hepatitis B

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Investigation of the 1377C/T Polymorphism of 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 initiate immunity with exogenous-endogenous ligands and play 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 the 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 (SNP), restriction fragment length polymorphism (RFLP) was used. Statistically significant difference was determined in 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 to 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). As a 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 chronic hepatitis B and TLR 3 gene (1377 C/T) polymorphism.

Keywords: Chronic hepatitis B, Toll-like receptors, single nucleotide polymorphism
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 due to the complications associated with HBV every year (World Health Organization 2015). The immune system plays a critical role in getting the HBV under control in case of viral infection and 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 C 2005). One of these mechanisms can be related with 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 (PRRs) (Tsai et al. 2015; Werling and Jungi 2003). TLRs are the most important family of pattern recognition receptors (PRRs) 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 role in the activation of adaptive immune system. Currently, a total of 13 TLRs (TLR 1-13) were identified in mammalians, including 11 in humans (Boissier et al. 2008; Cheng et al. 2007). It is known that TLR 2, 3, 4, 7, 8, and 9 have role in the pathogenesis of several viral infections including the hepatitis B and C (Kondo et al. 2011; Wu et al. 2009; 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).

TLR 3, also called as CD283, is located on the 4q35 (Karimi-Googheri and Arababadi 2014) and 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 (IRF3), activator protein 1 and nuclear-factor(NF)-κB (Li et al. 2010; Szatmary 2012; Tuosto 2011). The plasmacytoid dendritic cells produce type 1-IFN using the TLR 3 specifically for the response against double-stranded RNA (Carpenter and O'Neill 2007; Cheng et al. 2014; Onoguchi et al. 2007). It has been reported that expression of TLR 3/IFN-β was decreased in...
dendritic cells in chronic hepatitis B (CHB) patients as compared to 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 from healthy controls (An et al. 2007). In a recent study, it was found that HBeAg impairs the interaction of some structures that have role in TLR signaling pathway (Lang et al. 2011). These studies show that TLR 3 is closely related with the course of HBV infection and may play crucial roles in the development of prolonged hepatitis B forms.

Single-nucleotide polymorphism (SNP) rs3775290 (1377 C/T) is located in exon 4 of TLR 3 gene and affects the receptor–ligand interaction by changing the TLR 3 ectodomain and 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 1377 C/T polymorphism in HBV related CHB. In this study, we aimed to detect the frequency of TLR 3 gene polymorphism at the promoter region -1377 C/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 the 1st of March and 31st of 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 three groups. The first group included inactive CHB patients who were HBsAg positive for at least six months, serum HBV DNA <2,000 IU/ml, normal ALT/AST levels with the absence of prominent hepatitis findings in liver biopsy. Second group consisted of active CHB patients who were HBsAg positive for at least six months, serum HBV DNA ≥2,000 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 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 (-1377 C/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 (1377 C/T) genetic polymorphism, the methods of polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) was used as described by Noguchi et al. (Noguchi et al. 2004). PCR was performed in a total volume
of 25µl that included Thermopol reaction buffer, 200 ng DNA, 0.25 mM of each deoxynucleotide, 1.25 µM of 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 and -5’ GGACCAAGGCAAAGGAGTTC-3’ as reverse primers. Amplification was performed using a Px2 Thermo Hybaid thermal cycler (Thermo Electron Co., MA, USA). PCR conditions were initial denaturation at 94°C for 5 min., followed by 35 cycles including denaturation at 94°C for 60 sec., annealing at 60°C for 60 sec. and an extension at 72°C for 60 sec. Final extension step was set to 72°C for 7 min., before the restriction enzyme analysis. PCR products were incubated approximately at 65°C for 16 hours using the Taq I restriction enzyme. After cutting with the restriction enzyme, the fragments were separated by agarose gel (3%) electrophoresis, 275bp+62bp RFLP products for CC genotype, 337bp+275bp+62bp products for CT genotype, and 337bp RFLP product for TT genotype were obtained (Fig. 1.)

**Statistical analysis**

Statistical analysis was performed using the SPSS 18.0 statistical program. Descriptive statistics (percentage distribution, mean, median, standard deviation) were presented. The chi square test for percentage comparisons, Fisher’s exact test, Student’s t test for comparison of normally distributed two continuous variables, Mann-Whitney U test for the comparison of two continuous variables not distributed normally, Kruskal-Wallis test for comparison two or more variables, were performed. Bonferroni correction was applied for nonparametric comparison of two or more continuous variables. The Kolmogorov-Smirnov test was used to evaluate the normal distribution of variables. A p<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 two groups as active (n=73) and inactive (n=43). Age and gender distributions were not statistically different between the two 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 value of the patients with active and inactive CHB were $10^4$ IU/ml and $10^3$ IU/ml, respectively.

Among 116 patients with CHB whose TLR 3 (1377 C/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 was presented in Table 2. We performed chi-square 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. 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 CT genotype (Fig. 2). When the patients with three genotypes (CC, CT and TT) were compared, statistically significant difference was found (p=0.013) (Fig. 2), in terms of HBV DNA levels.

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; 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 1377 C/T polymorphism of toll-like receptor 3 (TLR 3) in patients with chronic hepatitis B in a Turkish population. We found that HBV DNA levels were significantly higher among patients harboring TT genotype, and CC genotype were 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 crucial role in innate and adaptive immune system. It is well known that TLR signaling pathway has important issue in eradicating viruses; for example, HBV (Chen et al. 2008).

Nucleotide polymorphisms even a single one could be important for considering the susceptibility of the individual against a disease, sensitivity to drugs and adverse effects, development of personalized treatment strategies, and determining new therapeutic targets. In recent years, the detection of the relationship between genetic polymorphisms and specific diseases enhanced the studies in this field.

Genetic variants of the TLR 3 have been reported to be related with 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.

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. The current study is the first one investigating the relationship between TLR 3 (1377C/T) gene polymorphism and CHB in Turkish population, as far as we are concerned.

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 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 was accepted that TLR 3 signaling pathway was active in liver diseases and it might lead to immune mediated liver damage (Al-Qahtani et al. 2012). Although TLR 3 has important role in innate immune system, TLR 3 gene polymorphism has been studied in few diseases (Askar et al. 2009; He et al. 2007; Ueta et al. 2007). Rong et al. (2013) studied two TLR 3 gene polymorphisms (C1234T and A952T) on 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 to ones carrying CC genotype in C1234T genotyping (Rong et al. 2013).

Genetic polymorphism in TLR 3 have been linked with susceptibility to infectious diseases, including CHB. A study by Huang et al. (2015) investigated TLR 3 (rs1879026 and rs 3775290) polymorphisms among 437 patients with HBV related diseases and 186 healthy controls in Chinese Han population. In that study, they found a lower TT genotype frequency for rs 3775290 SNP in CHB patients and concluded that the TLR 3 rs3775290 polymorphism was associated with decreased susceptibility to CHB in Chinese population (Huang et al. 2015). Al-Qahtani et al. (2012) investigated 9 different TLR 3 gene polymorphisms on 707 patients with CHB and 600 healthy controls in Saudi Arabia. They found significant difference between patient and control groups for only one polymorphism (rs1879026 (G/T)) (Al-Qahtani et al. 2012). In another study, Sá et al. (2015) investigated TLR 3 gene polymorphism among 35 HBV patients, 74 HCV patients and 299 healthy volunteers. They did not found statistically significant difference in distribution of allele, genotype and haplotype frequencies between the two groups (Sa et al. 2015).

In the literature, there are studies that investigated the association between TLR 3 expression with CHB. Huang et al. (2013) from Taiwan, 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 to 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 to healthy controls; in their study on 40 CHB patients, 60 patients with acute liver failure following CHB, and 20 healthy subjects. They noted that TLR 3 and IFN-β expressions reduced significantly in the patients that died due to acute liver failure following CHB, when compared to ones with acute liver failure but alive (Li et al. 2009).

When HBV DNA levels of the patients with CHB were compared; it was detected that HBV DNA levels were significantly higher among patients harboring TT genotype compared to ones harboring 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 TT variant genotype might be a risk factor for CHB.

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

As a conclusion, we detected an association between CHB patients and TLR 3 (1377 C/T) gene polymorphism and CC genotype seems to be a risk factor for active CHB. However, there are some limitations of our study 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 single nucleotide polymorphisms of the TLR 3 gene.

Compliance with ethical standards and Conflict of interest

E Firat Goktas, C Bulut, MT Goktas, EK Ozer, O Karaca, S Kinikli, AP Demiroz, and A Bozkurt declare that they have not conflict of interest.


Table 1. Gender and age distribution of the participants

<table>
<thead>
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<th>Characteristics</th>
<th>Patient Group n (%)</th>
<th>Healthy Group n (%)</th>
<th>p value</th>
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<td>Gender</td>
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<tr>
<td>Male</td>
<td>55 (47.4)</td>
<td>24 (48.0)</td>
<td>0.892</td>
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<tr>
<td>Female</td>
<td>61 (52.6)</td>
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<td>Age (mean ± SD, range)</td>
<td>39.0±12.4 years (18-73)</td>
<td>38.2±6.3 years (18-68)</td>
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Table 2. The Toll-like receptor 3,1377C/T genotype distribution of the patient and control groups

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<th>Healthy Group n (%)</th>
<th>p value</th>
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<td>Genotypes</td>
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</tr>
<tr>
<td>CC</td>
<td>60 (51.7)</td>
<td>23 (46.0)</td>
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<tr>
<td>CT</td>
<td>49 (42.2)</td>
<td>26 (52.0)</td>
<td>0.342</td>
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<tr>
<td>TT</td>
<td>7 (6.1)</td>
<td>1 (2.0)</td>
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<tr>
<td>Alleles</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>169 (72.8)</td>
<td>72 (72.0)</td>
<td>0.342</td>
</tr>
<tr>
<td>T</td>
<td>63 (27.2)</td>
<td>28 (28.0)</td>
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Figure Legends

Figure 1: The image of TLR 3 PCR products treated with 3% on agarose gel electrophoresis. Indicated with 100 bp DNA marker.

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

Figure 3: The comparison of genotype frequencies of CC group and variant allele group (CT and TT) between patients with active and inactive CHB (n=43, 30; CC, CT+TT respectively for active CHB group and n=17, 26; CC, CT+TT respectively for inactive CHB group. *p=0.044, p<0.05 was considered significant according to Chi square test. aCHB, active Chronic Hepatitis B; inCHB, inactive Chronic Hepatitis B).
Figure 1: The image of TLR 3 PCR products treated with 3% on agarose gel electrophoresis. Indicated with 100 bp DNA marker.

113x103mm (192 x 192 DPI)
Figure 2: Comparison of the Log HBV DNA levels between the Toll-like receptor 3,1377C/T genotype groups (n=58, 48, 7; CC, CT, TT respectively. *p=0.013, p<0.017 was considered significant according to Bonferroni correction).

69x50mm (600 x 600 DPI)
Figure 3: The comparison of genotype frequencies of CC group and variant allele group (CT and TT) between patients with active and inactive CHB (n=43, 30; CC, CT+TT respectively for active CHB group and n=17, 26; CC, CT+TT respectively for inactive CHB group. *p=0.044, p<0.05 was considered significant according to Chi square test. aCHB, active Chronic Hepatitis B; inCHB, inactive Chronic Hepatitis B).