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Toll-like receptor 7 and 9 expression in peripheral blood mononuclear cells from patients with chronic hepatitis B and related hepatocellular carcinoma

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Key words

hepatocellular carcinoma; peripheral blood mononuclear cells; hepatitis B; innate immunity; receptors; viruses; Toll-like receptor 7; Toll-like receptor 9

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Abstract

Aim: The aim of the present study was to investigate the expression of Toll-like receptors (TLR) 7 and 9 in peripheral blood mononuclear cells (PBMC) of patients with chronic hepatitis B virus (HBV) infection and related hepatocellular carcinoma. **Methods:** The study group was comprised of 52 patients: 41 with chronic hepatitis B and 11 healthy controls. The protein and mRNA levels of TLR7 and TLR9 were evaluated using real-time PCR, Western blot analysis, and flow cytometry. We also detected the serum viral load of HBV in the patients and analyzed the correlation between HBV–DNA copies and the TLR expression. **Results:** Our results demonstrated a lower TLR7 expression in all HBV infection groups compared to the controls. We found that HBV infection led to a decreased expression of TLR9 mRNA, but an increased expression of the TLR9 protein compared to the healthy group. The TLR protein levels are related to serum HBV–DNA ($P<0.01$). **Conclusion:** There are downregulations of TLR7 expression and TLR9 mRNA in PBMC of HBV-infected patients, but an increased TLR9 expression at the protein level.

Introduction

Hepatitis B virus (HBV) infection is a major cause of chronic liver inflammation worldwide. Many chronic HBV carriers suffer from progressive liver diseases, such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC) during their lifetime. The outcome of hepatitis B patients is closely linked to their immune status. Immunity plays a decisive role in host-virus interactions, and greatly influences viral replication and the clinical outcome of infection[1]. Chronic HBV infection is associated with T cell hyporesponsiveness or tolerance[2]. Thus, innate and/or adaptive immune responses are likely to be either absent or diminished when viral persistence follows HBV infections.

Toll-like receptors (TLR) 7 and 9 are members of the Toll-like family of receptors[3,4], and sense infection by detecting molecular structures of invading microbial pathogens and initiate innate immune responses[5]. These receptors mediate adaptive immune responses by activating immune cells, such as dendritic cells (DC)[4,6,7]. TLR7 and TLR9 are present on both immune and non-immune cells[8]. TLR9 recognizes unmethylated deoxycytidyl-phosphate-deoxyguanosine dinucleotides, which are common in bacterial and some viral nucleic acids[9], whereas TLR7 recognizes single-stranded RNA in the cytoplasm of infected cells during viral replication[10]. High expression levels of TLR9 were recently detected in clinical samples of lung and breast cancer and corresponding cell lines[11,12], suggesting a relationship between TLR9 and carcinogenesis.

Recent studies on TLR9 subfamily members describe the relationship between viral pathogens such as hepatitis C virus (HCV), herpes simplex virus, HIV, and these TLR[13–16]. It was demonstrated that the secretion of type I interferon (IFN) in response to these viruses is mediated by the TLR7/TLR9 pathway. However, the expression profiles of TLR7 and TLR9 in the peripheral blood mononuclear cells (PBMC) of patients with chronic HBV infection and related HCC have not been evaluated. Information about the regulation of

TLR by viruses is also limited[10,17,18]. The current study was undertaken to investigate the expressions of TLR7 and TLR9 in the PBMC of chronic HBV and related HCC patients, and to analyze any relationship between the TLR expression and the disease states.

Materials and methods

Study patients The study patients included 11 healthy volunteers (normal controls, NC) and 41 patients at various stages of chronic HBV infection, including 19 with chronic hepatitis B (CHB), 11 with HBV-related LC, and 11 with HBV-related HCC. The patients were enrolled at the First Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China) from February 2006 to May 2006. Informed consent was obtained from each patient, and the study protocol was approved by the Ethics Committees of the School of Medicine, Zhejiang University. The clinical parameters of the patients are shown in Table 1.

The diagnostic criteria conformed to "The guideline of prevention and treatment for chronic hepatitis B"[19]. The patients had not received any surgical treatment and had not received any antiviral treatment in the last month. All LC patients were in Child class B and C by Child-Pugh score evaluation. HCC patients were confirmed by liver tissue biopsy. All healthy volunteers were negative for HCV, HBV, and HIV.

Preparation of PBMC The PBMC were separated from 5 mL heparinized whole blood by centrifugation on a Lympholyte-H system (Cedarlane, Hornby, Canada) by gradient centrifugation. The PBMC were resuspended in ACK lysis buffer (0.15 mol/L NH_4Cl, 1 mmol/L $KHCO_3$, 0.1 mmol/L Na_2 EDTA, pH 7.4) to lyse the red blood cells.

RNA isolation, reverse transcription, and quantitative real-time PCR Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) from 1×10^6~2×10^6 PBMC. Reverse transcription was performed using the RevertAid first-strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania). Real-time PCR was performed with the FastStart DNA SYBR premix ex *Taq* kit (Gene Home Biotechnology, Hangzhou, China) using an ABI 7500 system (Applied Biosystems, Foster, CA, USA). The primers were synthesized by Shanghai Yingjun Biotech (Shanghai, China) as follows: TLR7 forward, 5'-TGGAAATTGCCC TCGTTGTT-3', reverse, 5'-GTCAGCGCATCAAAAGCATT-3'; TLR9 forward, 5'-CCCGCTACTGGTGCTATCC-3', reverse, 5'-CCTTCCTCTTTCCACTCCC-3'; and β-actin forward, 5'-CCG-CCATGTAGGTCGCTAT-3', reverse, 5'-TGACACGCCATCA-CCAGAGT-3'. Thermocycling was performed at 95 °C for 20 s, followed by 40 cycles at 95 °C for 15 s, 58 °C for 10 s, and 72 °C for 40 s to measure the fluorescence signal. The dissociation stages, melting curves, and quantitative analyses of the data were performed using 7500 system SDS software v1.2.3 (Applied Biosystems, USA). The relative quantitation of target gene expression was evaluated using the comparative CT method as described by Ross *et al*[20].

Western blot analysis The cytoplasmic extracts were prepared by lysis in an lysis buffer containing 150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.9), 0.5% Triton X-100, 0.6% NP-40, and protease inhibitors (1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 2 μg/mL aprotinin). The protein contents were determined using the DC protein assay kit (Bio-Rad, Richmond, CA, USA). The PBMC lysates were mixed with 2×SDS sample buffer. In total, 40 μg of protein was separated in a 10% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% skim milk powder in TBST, the membrane was incubated with primary antibody, mouse anti-TLR9 mAb or rabbit anti-TLR7 polyclonal antibodies (Imgenex, San Diego, CA, USA) in TBST overnight at 4 °C. Subsequently, the membranes were washed in washing buffer (phosphate-buffered saline with 0.1% Tween-20) incubated with horseradish peroxidase-conjugated secondary antibody, rabbit antimouse, or goat anti-rabbit (Pierce, 1:10000 in blocking buffer) for 1 h at room temperature. The reactive bands were visualized with an EZ-ECL kit (Bioind, Kibbutz, Israel). A monoclonal anti-β-actin antibody was used as a control at

Table 1. Clinical parameters of patients.

Parameters	Number	Age (years)	Gender (male/female)	WBC (10^9/L)	ALT (U/L)	HBV DNA load (10^6 copies/mL)	Serologies HBeAg (+/-)	HBsAg (+/-)	Anti-HBc (+/-)
NC	11	31±2.1	8/3	6.05±1.1	12.09±6.49	0	0/11	0/11	0/11
CHB	19	42±2.4	14/5	6.96±1.1	116.6±50.2	57±25	18/1	5/14	19/0
LC	11	53±2.1	11/0	5.08±1.1	66.7±11.4	5.3±4.1	10/1	3/8	9/2
HCC	11	59±4.6	10/1	7.11±1.4	153.6±47.3	5.2±5.1	10/1	0/11	10/1

a dilution of 1:400 (Santa Cruz, CA, USA).

Intracellular TLR staining and flow cytometry analysis A total of 6×10^6–10×10^6 PBMC were fixed and permeabilized with IntraPrep permeabilization reagent (Beckman Coulter, Fullerton, CA, USA). The cells were then stained with fluorescein-isothiocyanate-conjugated anti-TLR9 mAb or an unconjugated rabbit anti-TLR7 polyclonal antibody (Imgenex, San Diego, CA, USA). PE-conjugated goat antirabbit immunoglobulin G (Molecular Probes, Eugene, OR, USA) was used as a secondary antibody for TLR7 staining. Stained PBMC were analyzed on a FACSCalibur machine (BD Biosciences, San Jose, CA, USA). Data were analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA)

Serum HBV–DNA assay The viral load of HBV–DNA in the serum samples was quantified using a high sensitivity fluorescent real-time PCR kit (PG biotech, Shenzhen, China) and amplified using a Light Cycler 2.0 instrument (Roche Applied Science, Basel, Switzerland). The detection sensitivity of the PCR assay was 4×10^2 copies/mL.

Statistical analysis Statistical analyses (non-parametric tests, Mann-Whitney *U*-test) were performed using SPSS software version 13.0 (SPSS, Chicago, IL, USA). Correlation analyses were calculated according to the Spearman-Rho method. A *P*-value of less than 0.05 was considered to be significant.

Results

Decreased expression of TLR7 and TLR9 mRNA in PBMC of patients As indicated in Figure 1, the patient group with the lowest TLR7 and TLR9 expressions in PBMC was the CHB group. The expressions of TLR7 and TLR9 mRNA decreased more than 23.2- and 7.6-fold, respectively, compared with the NC group. Between the NC and HCC groups, the decrease of the TLR7 mRNA expression was not significantly different compared with the NC group ($P>0.05$). The decrease in the TLR7 mRNA expression in the LC and HCC groups differed 2.8- to -0.34-fold, respectively, compared to the NC group ($P>0.05$). A similar but weaker trend was also observed in the decrease of TLR9 mRNA expression among the groups. The expression of TLR9 mRNA decreased in the LC and HCC patients by more than 6- and 1.7-fold, respectively, compared with the NC group.

Western blot analysis of TLR7 and TLR9 in PBMC There was a significant decrease in the TLR7 expression of the CHB group compared with controls, and an increased TLR7 expression in various patient stages (Figure 2). In contrast, the TLR9 expression significantly increased in the 3 patient groups compared with the NC group. An upward

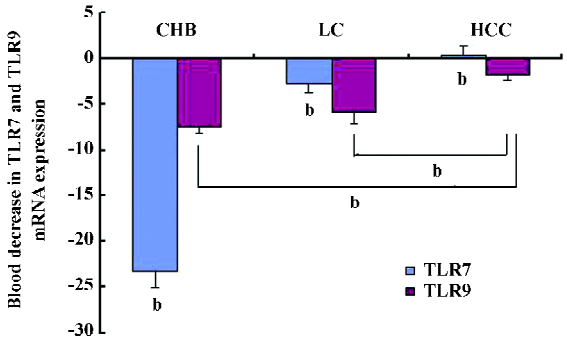

Figure 1. Decrease in TLR7 and TLR9 mRNA in patients relative to the control. Decreased levels of TLR7 and TLR9 mRNA were determined by quantitative real-time PCR in RNA isolated from PBMC. We obtained the average data of 3 independent analyses for each sample. TLR mRNA expression decreased in the patient groups compared with the NC group ($P<0.05$), with the greatest decrease in the CHB group. Data are shown as mean±SEM. $^bP<0.05$.

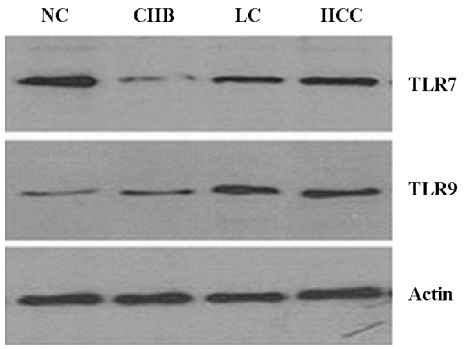

Figure 2. Western blot analysis of TLR7 and TLR9. Western blot analysis was performed on PBMC lysates; bands were observed at approximately 120 kDa and normalized by β-actin. There was a significant decrease in the TLR7 expression in the CHB group, but a significant increase in the TLR9 expression of the patient groups compared with the controls. From CHB to HCC, an increase in the TLR expression was observed.

tendency was observed in the CHB, LC, and HCC groups.

Flow cytometry analysis of TLR7 and TLR9 expressions in PBMC Mean fluorescence intensity (MFI) values corresponding to TLR7 were downregulated in all patient groups compared with the control group ($P<0.05$, Figure 3). The MFI±SEM values were: NC=196.6±14.2, CHB=70.1±3.9, LC=79.7±3.7, and HCC=83.8±3.2. The results showed no significant differences among the 3 patient groups ($P>0.05$).

The MFI values corresponding to TLR9 increased in all the patient groups compared with the NC group ($P<0.05$). The MFI±SEM values were: NC=76.2±6.2, CHB=183.7±15.1, LC=240.8±34.4, and HCC=250.2±45.5. The TLR9 expression

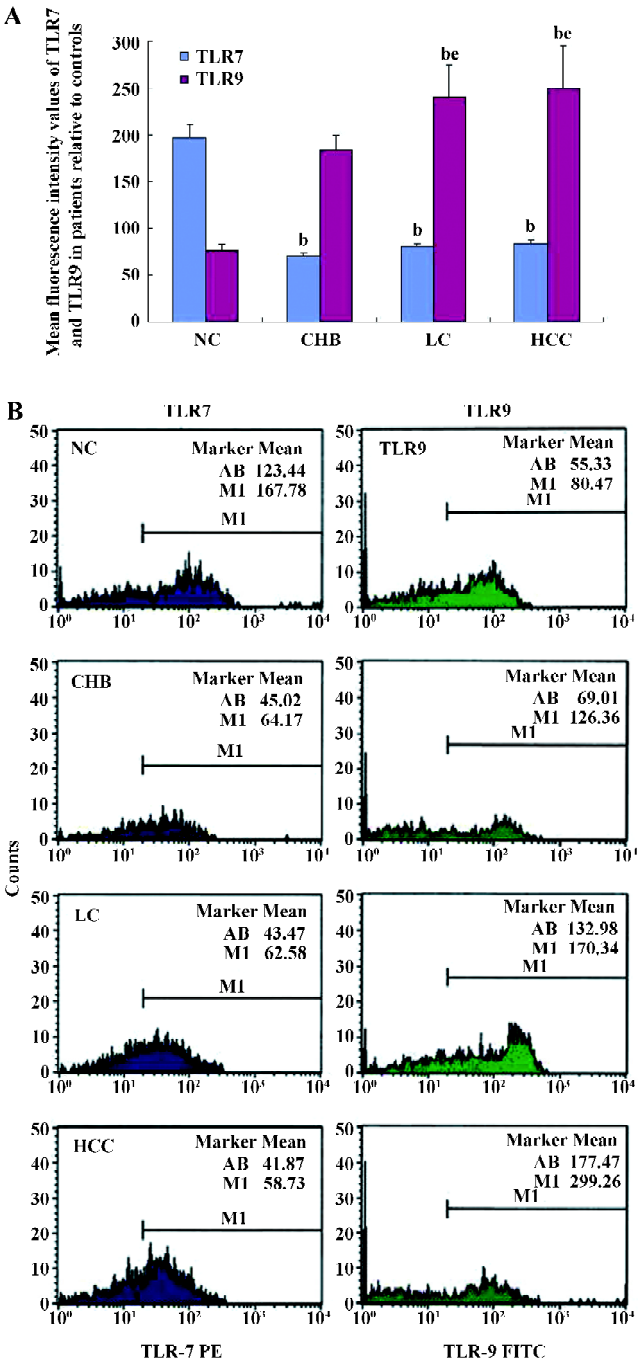

Figure 3. Flow cytometry analysis of TLR7 and TLR9 expressions. TLR7 and TLR9 were detected by intracellular staining methods. To evaluate the efficacy of the experiment, an isotype-matched control was used. At least 3000 cells were assessed to calculate the median value. (A) statistical analyses of TLR7 and TLR9 MFI. Data are shown as mean±SEM. $^{b}P<0.05$ compared with the NC group. $^{e}P<0.05$ compared with CHB TLR9 group. (B) expression of TLR7 and TLR9 using flow cytometry analysis.

in the HCC patient group was upregulated ($P<0.05$) compared to the CHB patient group.

Correlation between the TLR protein expression and serum HBV–DNA levels The expression of the TLR7 protein was negatively correlated with the serum copies of HBV–DNA ($r=-0.669$, $P<0.01$), and the upregulation of the TLR9 protein was positively correlated with the serum copies of HBV–DNA ($r=0.563$, $P<0.01$).

Discussion

After recognizing particular microbial molecules, subsets of immune cells in PBMC equipped with the corresponding set of TLR release immunological repertoire, such as IFN-α, TNF-α, and interleukin-6, which directly regulate immunocompetent cells[8]. TLR7 and TLR9 are members of the TLR9 subfamily[21,22]. Recent research indicates that the ligands for TLR3, TLR4, TLR5, TLR7, and TLR9 can inhibit HBV replication in the livers of HBV transgenic mice[23], suggesting that TLR7 and TLR9 may play a role in regulating HBV. The high expression of TLR9 was recently detected in clinical samples of lung and breast cancer and corresponding cell lines[11,12], suggesting a possible role in carcinogenesis. If TLR7 and TLR9 are associated with the recognition of specific viral components, it is important to characterize TLR7 and TLR9 expression in the PBMC of patients with chronic HBV infection and HBV-related HCC to further understand pathogen-host interactions and predicted outcomes.

The results of our study clearly show decreased TLR7 at the mRNA and protein levels, and TLR9 expression at the mRNA level in patient PBMC compared with the healthy controls. These results are different from the upregulated TLR after other virus infections, as shown in previous studies[24,25]. HBV may elicit a factor that inhibits TLR7 expression and TLR9 mRNA in the PBMC of patients, resulting in immune escape and even immunological tolerance. Because the same decreased TLR7 and TLR9 expressions of plasmacytoid DC were also found in further studies, the alteration of TLR7 and TLR9 expressions even impair the IFN-α production of pDC (unpublished data).

The regulation of eukaryotic gene expression is the result of complex, multi-level processes; mRNA levels do not always correspond to the actual expressed protein levels[26–28]. The increase of the TLR9 protein might be related to post-transcriptional processes and might be involved in the immune surveillance of HCC. This also may reflect negative feedback inhibition from the TLR9 protein to the mRNA or other regulatory influences which are not currently under-

stood[29].

TLR9 is mostly localized in the endoplasmic reticulum[30] and traffics to endosomal and lysosomal compartments after cellular activation[31,32]. Some studies also showed that a fraction of the TLR9 expresses on the cell surface, in the gastric epithelium[33], intestinal epithelial cells[34], and some peripheral blood mononuclear cells[35]. It was no doubt that FACS data indicated the functional cellular expression of TLR while the Western blot analysis showed the total TLR expression, including the superficial pool. These results reflect another level of regulation that is protein trafficking. The TLR9 expression in the HCC group was the highest, which may be directly related to carcinogenesis of HCC. The statistical analysis indicated no difference in the TLR9 levels among the HCC and LC groups. If the sample size was enlarged, the results may be different. The expression of TLR7 was not different among the groups of patients, suggesting that unlike TLR9, TLR7 has no correlation with HCC.

Finally, we were interested in understanding whether changes in TLR7 and TLR9 expressions were correlated with the levels of serum HBV–DNA. Positive results suggested that the TLR expression was related to the state of HBV replication. We conclude that HBV might produce an inhibitor when it is replicating. However, the expression of TLR9 was influenced by chronic HBV infection and the carcinogenesis of HCC, which may further complicate the situation.

In summary, this study is the first investigation of the expression of TLR7 and TLR9 of PBMC in CHB, LC, and HCC patient groups. A larger scale clinical investigation should be undertaken to determine whether TLR7 and TLR9 expressions may be used to evaluate patient immunity and predict HCC outcomes.

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