

Downregulation of Human Toll-like Receptor 2 and 4 Genes in Inactive Carriers of Hepatitis B Virus Infection-A Possible Mode of Viral Persistence

Aparna Jakkampudi, RM Mukherjee, Ramya Kota, Gelli Veena Shravanti, PN Rao, DN Reddy

Aparna Jakkampudi, RM Mukherjee, Ramya Kota, Gelli Veena Shravanti, Asian Health Care Foundation, Somajiguda, Hyderabad, India

PN Rao, DN Reddy, Asian Institute of Gastroenterology, Somajiguda, Hyderabad, India

Correspondence to: RM Mukherjee, Institute of Basic and Translational Research, Asian Health Care Foundation, 6-3-661, Somajiguda, Hyderabad-500082, India.

Email: rathinmukh@gmail.com

Telephone: +914023378888(Ext.742) Fax: +914023324255

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ABSTRACT

AIM: Toll-like receptors (TLRs) although comprise an integral part of innate immune defense mechanism, role of TLRs in hepatitis B virus (HBV) mediated disease categories are sparsely studied. The aim of the present study was to assess host TLR2 and TLR4 gene expressions in various stages of HBV infection in relation to viral replication intermediate pregenomic RNA (pgRNA) in peripheral blood mononuclear cells (PBMCs) and HBV DNA concentration in sera.

METHODS: TLR2 and TLR4 mRNA expression in conjunction with PgRNA level in PBMCs of 65 treatment naïve HBV infected patients grouped in to acute (AHB), inactive carriers (IC), liver cirrhosis (Cirr) and hepatocellular carcinoma (HCC) were evaluated by reverse transcription polymerase chain reaction (RT-PCR) assay. Serum HBV DNA was measured by real time PCR assay. Thirty voluntary blood donors served as controls

RESULTS: Compared to controls, TLR2 gene expression was significantly reduced in AHB ($p=0.003$), IC($p=0.006$) and Cirr($p=0.01$) while expression of TLR4 gene was significantly abridged in IC ($p=0.0005$) group only. A significantly higher elevation in the expression of TLR4 was noted in HCC ($p=0.0001$).

Despite having lower level of HBV DNA, ICs had substantial echelon of pgRNA comparable to other groups.

CONCLUSION: The results showed differential expression of TLR2 and TLR4 mRNA in HBV disease categories. On top, significant downregulation of both TLR2 and TLR4 expression was encountered in ICs only. This observation alongwith the level of viral pgRNA in ICs may well be indicative of dysregulation of one of the crucial arm of innate immune mechanism favoring long term persistence of the virus towards a chronic infection.

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Key words: Toll-like receptors; Gene expression; HBV infection; Inactive carriers; PBMC; pgRNA

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INTRODUCTION

The human hepatitis B virus (HBV) chronically infects near about 360 million people worldwide with an annual mortality rate of about 1 million per year due to HBV-associated liver pathologies^[1]. In spite of the noncytopathic nature of HBV, the spectrum of liver infection frequently proceed from chronic infection (CHB) to liver cirrhosis and development of hepatocellular carcinoma (HCC)^[2]. Increased rate of CHB infection in neonates born from HBV infected mothers than adults indicate the role of a functionally mature immune system. The first line of defense against a pathogen is innate immunity, which slows the progress of infection and allows time for development of adaptive immunity.

At present, HBV has been shown to evade host innate immune

mechanisms while viral clearance is brought about by functional adaptive immune responses^[3]. While a brisk and perpetual polyclonal cytotoxic T lymphocyte (CTL) response to HBV was observed in self resolved acute hepatitis patients, similar responses was significantly lesser or barely discernible in CHB patients^[4,5]. Requirement of intrahepatic HBV-specific CD8+ T cells for viral clearance has been documented during acute HBV infection in chimpanzees^[6]. In CHB, the variable outcome of liver disease has been ascribed either to the poor immune status of the host or HBV induced intonation of cell mediated immune response of the host^[7].

Even though, HBV has been known to thwart the adaptive immune response of the host which is supposed to be activated by the virus itself^[8], the precise role of the innate immune response in HBV infection is still not well understood. Study on the role of effective innate immune response in HBV infected subjects is further complicated because the patients are often diagnosed long after the onset of infection.

The family of Toll-like receptors (TLRs) as evolutionarily conserved pattern recognition receptors (PRRs) form a major component of the innate immune response by engaging themselves with various motifs of the pathogen associated molecular patterns (PAMPs). Despite the fact that TLRs can initiate innate immune cascades through the recognition of exogenous PAMPs, they also recognize endogenous signals released by damaged cells. This put up the possibility that immunopathology, initiated by a TLR mediated response to a PAMP, can protract tissue damage through other TLR-mediated mechanisms^[9]. Induction of the antiviral state and production of type 1 Interferon (IFN α/β) can be mediated by TLRs^[10]. As part of host's antiviral defense mechanism, stimulation of TLRs initiates a MyD88 dependent (TLR2) or MyD88 independent (TLR4) signal transduction pathway leading to the release of inflammatory mediators/cytokines via activation of nuclear factor κ B (NF- κ B)^[11]. Virus induced suppression of TLR mediated immune mechanism, thus, has a role in persistence of viral infection^[12]. This has been corroborated further by the documented ability of HBV precore/hepatitis B secreted e-antigen (HBeAg) to interact and downregulate TLR-2 expression on hepatocytes^[13]. In the presence of HBeAg, HBV has also been shown to down-regulate the expressions of TLR2 on monocytes (CD14+) from peripheral blood^[14]. Significantly lower expression of TLR2 and TLR4 has been reported in PBMCs from CHB patients suggesting a possible interaction between HBsAg, TLR signaling and the innate immune responses^[15].

Regardless of the above informations, no studies has been done so far about the expression of TLR 2 and TLR4 genes in HBV infected subjects having variable spectrum of liver diseases. Henceforth, the purpose of this study was to analyze the expression profile of PBMC derived TLR2 and TLR4 mRNAs from HBV infected subjects belong to different disease categories concerning their respective serum HBV DNA level and amount of HBV pregenomic RNA (pgRNA) as intracellular replication intermediate of the virus.

METHODS

Patients

Sixty five HBsAg positive treatment naïve subjects who attended the Asian Institute of Gastroenterology, Hyderabad, India during the period of January 2012 to September 2012 were enrolled in this study. Patients were further sorted out on the basis of past history, clinical presentations, anti-HBc IgM/IgG status, imaging data, liver

histopathology, Child-Pugh (CP) scores^[16] and as per the American association for the study of liver disease (AASLD) practice guidelines^[17,18] into acute (AHB; $n=14$), inactive carriers (IC; $n=23$), cirrhosis (Cirr; $n=13$) and hepatocellular carcinoma (HCC; $n=15$) respectively. Patients co infected with HIV, HAV, HCV, HDV, or HEV were excluded from this study. A group of 30 healthy voluntary blood donors served as controls. Prior informed consents were taken from all the study subjects and the study protocol was approved by the institutional ethics committee

Serologic and biochemical parameters

Serum aspartate transferase and alanine transferase levels were estimated by an automated clinical biochemistry analyzer (Randox, Oceanside, CA). The serum HBeAg and anti-HBe status of the subjects were ascertained by commercial enzyme-linked immunosorbent assay (ELISA) kits (Amar-EASE, Taiwan) as per the manufacturer's instructions.

HBV DNA quantitation

HBV DNA was extracted from sera by the High Pure System Viral Nucleic Acid Kit (Roche Molecular Systems Inc, USA) as per manufacturer's protocol. Amplification and quantitation of extracted HBV DNA as viral load was performed by Cobas[®] TaqMan[®] 48 Analyzer (Roche Diagnostics, USA) using real time Cobas[®] TaqMan[®] HBV test kit (Roche Molecular System, USA) as per manufacturer's instructions.

Isolation of total RNA from PBMCs

PBMCs were immediately isolated from EDTA containing whole blood using histopaque-1077 (Sigma chemicals, USA) by recommended procedure. Total RNA was extracted from the isolated cells by Trizol (Life Technologies, USA) method and the extracted RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water.

Preparation of cDNA

Extracted RNA was subjected to reverse transcription reaction using random hexamers and MuLV-H reverse transcriptase (Fermentas Life Sciences, Germany) to generate a common cDNA pool. Prior to reverse transcription, 1 μ g of total RNA was treated with 1 U of deoxyribonuclease (DNase I amplification grade, Gibco-BRL, USA) to remove all the contaminating DNA. The possibility of presence of traces of DNA was further ruled out by employing control reactions devoid of reverse transcriptase enzyme. RNA was reverse transcribed (60 min at 37°C) with 200 U of MMuLV reverse transcriptase (Fermentas Life Sciences, Germany) in 20 μ L volume of 5 \times RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂) supplemented with 5 mM dithiothreitol (DDT), 0.5 mM deoxynucleoside triphosphates (dNTPs, Fermentas Life Sciences, Germany), 25 U ribonuclease inhibitor (Promega Corporation, Madison, WI, USA) and 200 ng random hexamers (Fermentas Life Sciences, Germany).

Polymerase Chain Reaction (PCR) amplification of TLR2 and TLR4 genes

Preceded by heating (95°C, 1 min) and quick-chilling on ice, an aliquot of 5 μ L (0.3 μ g) of the cDNA pool was used for PCR amplification in 50 μ L of 10 \times buffer solution (100 mM Tris-HCl pH 9.3, 500 mM KCl, 1% Triton X-100) containing 0.08 mM dNTPs, forward and reverse primers (100 pM each), 1.5 mM MgCl₂ and 2 U of Taq DNA polymerase (Fermentas Life Sciences, Germany). After initial denaturation at 94°C for 5 min, TLR2 and TLR4 cDNA

fragments were amplified by 30 cycles (95°C – 15s, 55°C – 30s & 72°C – 45s per step) followed by a final extension step at 72°C for 5min. For TLR2, the forward and reverse primers used were (5'-3') d (TTC TCA TCT CAC AAA ATT GCA AAT) and (GGA AGG TAA GTC CAG CAA AAT CTT) respectively^[19] which yielded a product of 95bp. TLR4 cDNA fragments were amplified using forward and reverse primers (5'-3') d (CTG CAA TGG ATC AAG GAC CAG) and (TGC CCT GCT TAT CTG AAG GTG)^[19] generating an amplicon of 81bp. Beta-actin as house keeping gene was amplified by 30 cycles (94°C, 55°C & 72°C; 1 min per step) using forward and reverse primers (5'-3') d(TCT ACA ATG AGC TGC GTG TG) and d (GGT GAG GAT CTT CAT GAG GT) generating amplicon of 314 bp^[20]. Blank reactions without cDNA template were performed in all experiments as negative reaction control. Each amplified product (10 µl) was subjected to 2% agarose gel electrophoresis(100 V, 45 min) along with a 100 bp DNA ladder and visualized by UV fluorescence after staining with ethidium bromide.

Upon capturing the gel image by Bio-Capt imager (Vilber Lourmat, France), the integrated density of DNA band was evaluated by using the software Image J 1.42 (Broken Symmetry Software, USA) in respect to the known standard marker as a control with units nanogram permilliliters (ng/mL) and further normalized against the housekeeping gene beta actin used as internal control to define the expression of respective genes by the density of the band^[20].

Polymerase Chain Reaction Amplification of HBV Pregenomic RNA(pg RNA)

In order to ascertain the presence of replicating virus in PBMCs, reverse transcribed PgRNA cDNA fragments obtained from Trizol extracted PBMC derived total RNA were subjected to an amplification protocol of 40 cycles (94°C–30 s, 58°C–30 s & 72°C–30 s per step), having an initial denaturation at 95°C for 5 min and final extension at 72°C for 10 min employing forward and reverse primers (5'-3') d (GCC TTA GAG TCT CCT GAG CA), and d (GAG GGA GTT CTT CTT CTA GG) as mentioned earlier^[21]. The PCR product was finally clarified upon 2% agarose gel electrophoresis to resolve an amplicon of 364 bp after staining with ethidium bromide followed by densitometric evaluation. Corresponding plasma of respective PBMC samples were subjected to identical extraction and amplification procedures as controls to check specificity of PBMC derived pgRNA.

Statistical analyses

Statistical significance for differences in gene expression between different groups was determined by descriptive statistics [mean, median, standard deviations, Inter Quartile Range(IQR)] and Student's *t*-test, as and where applicable using the GraphPad Prism 3.0 software (GraphPad, San Diego, CA, USA). The two-tailed values of *P*<0.05 was considered statistically significant.

RESULTS

All the patients were adults (Mean age±SD=38.4±11.1 years),and consisted of 51 male and 14 females respectively. The detailed demographic, biochemical and virological characteristics of 65 patients assigned to different disease categories are shown in table 1. Control subjects had a mean±SD age of 32.7±10.2 years and consisted of 21 males and 9 females. None of the patients had decompensated cirrhosis. All patients except those belonged to AHB group were negative for HBeAg but positive for anti HBe antibody indicating seroconversion.

Table 1 Baseline characteristics of the patients.

Parameters	Patient (n=65) Categories			
	AHB (n=14)	IC (n=23)	Cirr (n=13)	HCC (n=15)
Age (years)	36.8±11.1	34.7±10.3	37.7±11.5	44.5±11.7
Sex (M: F)	11:3	18:5	11:2	11:4
ALT (IU/L)	1364±1054	27.7±7.16	121.9±102.1	84.72±31.8
HBV DNA (Log Copies/mL)	6.90±1.37	3.08±0.51	6.33±1.59	4.92±1.95

AHB: Acute Hepatitis B; IC: Inactive Carriers; Cirr: Cirrhosis; HCC: HepatoCellular Carcinoma.

Expression profile of TLR2 gene and TLR4 genes in control and patients

No significant changes observed in TLR4 expression between the control and patient groups (Figure 1). The observed median value of TLR4 in controls and patients were 0.538 (mean±SD=0.552±0.159, IQR=0.144) and 0.469 (mean±SD=0.614±0.351, IQR=0.575) ng/mL respectively. Conversely, TLR2 gene expression was significantly reduced (*p*=0.005) in patients having median value of 0.44 (mean±SD=0.53±0.24, IQR=0.456) than the median value of 0.686 (mean±SD=0.69±0.29, IQR=0.314) ng/mL in controls respectively (Figure 1).

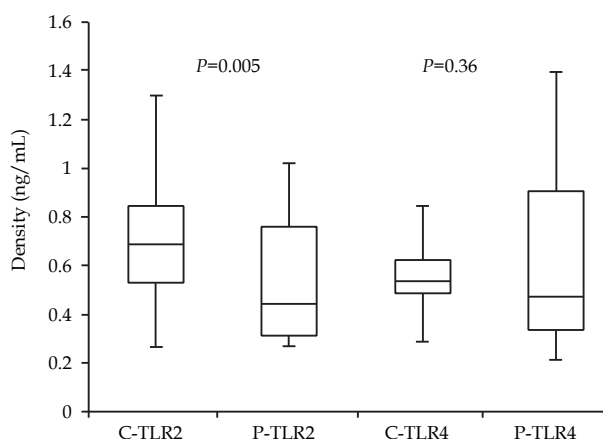


Figure 1 Expression of TLR2 but not TLR4 gene is significantly reduced in patient(P; n=65) than controls (C; n=30) groups. The boxes represent the interquartile ranges(IQR), the whiskers indicate the ranges and the center line in the box indicate median.

Comparative expression of TLR 2 gene in HBV disease categories

In comparison to controls (median=0.686; mean±SD=0.69±0.29, IQR=0.314), expression of TLR2 gene was significantly (*p*=0.003) downgraded in AHB (median=0.41, mean±SD=0.43±0.16, IQR=0.213, *p*=0.003), IC (median=0.33, mean±SD=0.48±0.22, IQR=0.42, *p*=0.006) and cirrhosis group (median=0.40, mean±SD=0.45±0.19, IQR=0.256, *p*=0.01) respectively (Figure 2). However, an otherwise nonsignificant increase of TLR 2 expression was noted in HCC group (median=0.81, mean±SD=0.76±0.23, IQR=0.160, *p*=0.4) than controls (Figure 2).

Comparative expression of TLR 4 gene in HBV disease categories

In respect to controls (median=0.538, mean±SD=0.55±0.15, IQR=0.144), significant downregulation (*p*=0.0005) of TLR 4 expression was encountered in IC (median=0.37, mean±SD=0.39±0.16, IQR=0.197) group only (Figure 3). No significant change in the expression of TLR 4 gene was noticed in AHB

(median=0.454, mean±SD=0.55±0.25, IQR=0.476) and cirrhosis group (median=0.466, mean±SD=0.57±0.26, IQR=0.401) weighed against controls (Figure 3).

On the contrary, a significantly higher ($p=0.0001$) expression of TLR 4 gene was seen in HCC (median=1.17, mean±SD=1.05±0.32, IQR=0.289) than the controls as well as the other disease categories (Figure 3).

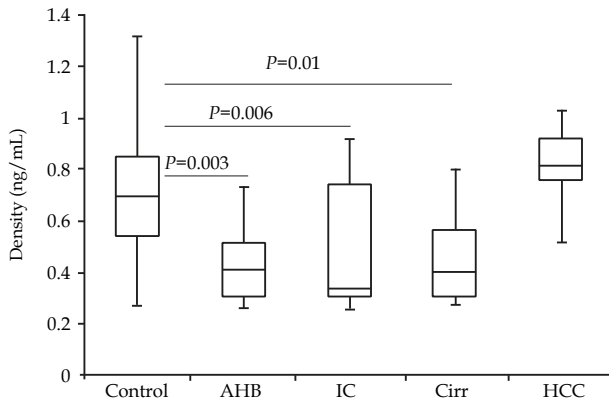


Figure 2 TLR2 gene expression was significantly suppressed in AHB, IC and cirrhosis categories of HBV infection (AHB - acute hepatitis B; IC - inactive carriers; CHB - chronic hepatitis B; Cirrhosis - cirrhosis of liver; HCC - hepatocellular carcinoma). The boxes represent the interquartile ranges, the whiskers indicate the ranges, and the center line in the box indicate the medians.

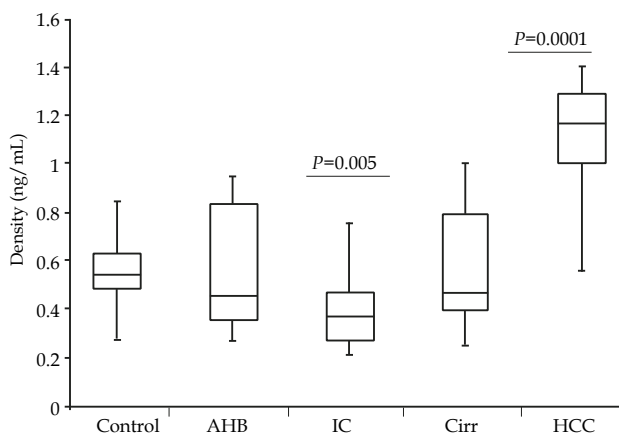


Figure 3 TLR4 gene expression was significantly suppressed in ICs but significantly elevated in HCC than controls (AHB - acute hepatitis B; IC - inactive carriers; CHB - chronic hepatitis B; Cirrhosis - cirrhosis of liver; HCC - hepatocellular carcinoma). The boxes represent the interquartile ranges, the whiskers indicate the ranges, and the center line in the box indicate the medians.

Comparative expression of HBV Pregenomic RNA (pgRNA) in Disease categories

Incongruent to the lowest level of serum HBV DNA in IC group (Table 1), no significant alteration in the PgRNA level was observed among ICs in comparison to other HBV disease categories (Figure 4). The estimated level of PgRNA was comparable in AHB (median=0.543, mean±SD=0.62 ±0.20, IQR=0.352), IC (median=0.632, mean±SD=0.58±0.18, IQR=0.195), cirrhosis (median=0.513, mean±SD=0.52±0.15, IQR=0.245) and HCC (median=0.60, mean±SD=0.54±0.15, IQR=0.30) group respectively (Figure 4). Isolated corresponding plasma samples of respective patients were devoid of detectable level of pgRNA.

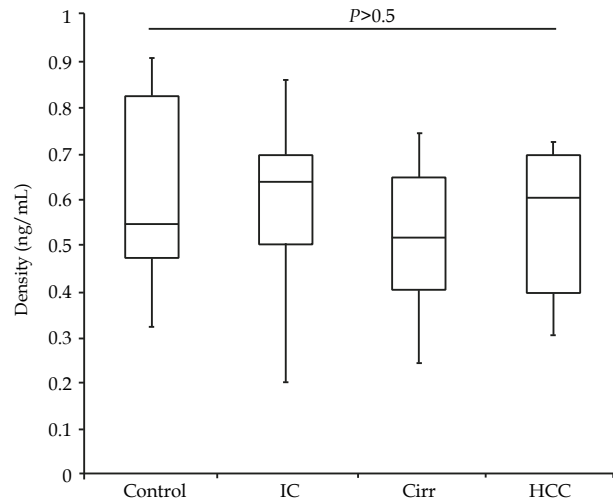


Figure 4 Expression of HBV pgRNA among different disease groups (AHB - acute hepatitis B; IC - inactive carriers; CHB - chronic hepatitis B; Cirrhosis - cirrhosis of liver; HCC - hepatocellular carcinoma). The boxes represent the interquartile ranges, the whiskers indicate the ranges, and the center line in the box indicate the medians.

DISCUSSION

The immunological facets of importunate HBV infection have focused mainly on adaptive immunity, with petite research on the innate immune response to HBV^[22]. The evolutionarily conserved family of pattern recognition toll-like receptors (TLRs) act as one of the crucial components of host mediated innate immune response. It has been described that PBMCs from patients with chronic HBV (CHB) infection expressed significantly lower levels of TLR2 and TLR4 mRNA transcripts compared with those from healthy donors^[15]. TLR2 expressions in peripheral monocytes were shown to reduced in patients with HBeAg+ CHB^[12]. While HBV mediated downregulation of the expression of TLR2 is considered as a means to alter the innate immune response in CHB infection^[23], it has been reported that the expression of TLR2 is regulated by the precore protein in CHB, and that there was significant reduction in cytokine production (TNF-alfa) and phospho-p38 kinase expression in the presence of HBeAg^[23]. Furthermore, co localization of HBeAg with the Toll/IL-1-receptor-(TIR-) containing proteins translocating chain-associating membrane (TRAM) protein, myelin and lymphocyte protein (MAL) and TLR at the subcellular level has been shown to be able to disrupt TIR: TIR interaction critical for TLR-mediated signaling^[24]. Inhibitory effect of HBsAg on TLR2 signalling pathway through selective inhibition of TLR2 ligand Pam3csk4 has also been shown in PBMCs of CHB patients^[25].

Till now, data regarding the expression of TLR2 and TLR4 are mainly available in CHB patients and not been able to address the issue in inactive carriers (IC) of the disease which form the major group of CHB subjects. In this setting, our study for the first time, showed that whilst the expression of TLR2 was manifestly reduced in AHB, IC and cirrhosis group in comparison to controls, TLR4 gene expression was significantly suppressed in IC group only insinuating subsistence of different regulatory mechanism in this arm of innate immunity in diverse phases of HBV infection. It is known that while the TLR2 activation takes place through the instigation of the adaptor protein MyD88, the TLR4 signalling pathway can operate without the involvement of MyD88. In this process, the TLR2 activation leads to the stimulation of NF-κB to produce proinflammatory cytokines like TNFα while augmentation of TLR4 can upshot the generation of

antiviral protein IFN- β through escalation of IRF-3^[13]. It appears that in AHB, IC and cirrhotics, the viral modulation of proinflammatory cytokines through suppression of TLR2 is more pronounced to inhibit inflammatory process which might not be favourable for the virus. In this milieu, noteworthy repression of TLR4 genes observed only in IC group in our study might have implications since it can inhibit the production of specific anti viral proteins like interferon favoring viral persistence. Despite having lower level of serum HBV DNA, the IC group depicted substantial amount of pgRNA comparable to other groups indicative of intracellular replication activity of the virus. This observation is in accord with our recent pronouncement of higher level of pgRNA alongwith downregulation of DNA damage repair genes raising the possibility of diversion of the viral replication pathway towards integration of viral DNA in to host genome^[21]. Since the IC group in our study were devoid of HBeAg and seroconverted to anti HBe positivity, involvement of other HBV proteins/epitopes antagonizing TLR4 expression has to be worked out. Based on the notions that TLR agonists were strong candidates to activate and modulate the impaired immune responses instigated by HBV proteins^[10,26], early therapeutic intervention using such agonists might appear beneficial to impede viral persistence towards the establishment of chronic infection.

In our study, the TLR4 expression was seen to be drastically enhanced in HCC but not in any other disease categories. Similar observations has been reported by others in HCC, although, not mediated by HBV, suggesting the role of TLR2 and TLR4 signaling in the course of HCC development^[27]. Role of TLRs promoting liver tumorigenesis through expression of cytokine and hepatomitogens mainly in myeloid cells has also been implicated^[28]. Furthermore, a marked decrease in the incidence, size, and number of chemically induced liver cancer tumors was noticed in mice deficient in TLR4 and MyD88, but not with TLR2, thus, indicative of a strong input of TLR4 signaling in development of HCC^[29]. Our finding of reduced TLR4 expression in ICs also has some bearing with the previous report that the liver tissues from patients with severe CHB expressed higher level of TLR4 than those from patients with mild CHB^[30]. Since it is known that in absence of HBeAg, HBV replication is associated with activation of the TLR2 pathway, leading to increased TNF α production, while suppression of TLR4 results in a decreased production of proinflammatory cytokines, thus, demonstrating a potentially important interaction between HBV and the innate immune response^[31]. Although the mechanism of HBV mediated repression of TLR4 in IC and elevation of the same in HCC is yet to be unravelled, further studies identifying the interfacing HBV proteins or the candidate TLR ligands regulating TLR4 expression ought to determine whether upregulation of TLR4 in ICs has the potential to be pondered as an overriding strategy to control HBV replication.

In conclusion, our data showed for the first time that the host TLR4 gene expression was drastically reduced in ICs of HBV infection but highly elevated in HCC where as TLR2 gene expression was markedly suppressed in AHB, IC and cirrhosis groups. Despite the lower viral load, level of HBV replication intermediate pgRNA in ICs was comparable to other disease groups. In view of the magnitude of TLR4 signaling as a part of innate antiviral defense mechanisms, repression of TLR4 gene in ICs may perhaps favor viral persistence in the host to expand chronic disease.

CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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Peer reviewers: Limin Chen, MD, PhD Professor and Director, Center for Transfusion-transmitted Infectious Diseases, Institute of Blood Transfusion, Chinese Academy of Medical Sciences and, Peking Union Medical College, Chengdu, Sichuan 610041, China; Tianhua Huang, Professor, Director, Research Center for Reproductive Medicine, Shantou University Medical College, 22 Xinling Road, Shantou, 515041, China; Hsien Hui Chung, Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan City, 70101, Taiwan.