

## Messenger RNA expression of human toll like receptors 3, 7 and 9 in Peripheral blood of patients with Hepatitis B infection

Kaushal Yadav\*, Biswajyoti Borkakoty, Dipankar Biswas, Chandra Kanta Bhattacharya and Jagadish Mahanta

Division of Virology, Regional Medical Research Centre, Northeast Region (ICMR), Dibrugarh, Assam, India

QR Code



### \*Correspondence Info:

Mr. Kaushal Yadav,  
Division of Virology,  
Regional Medical Research Centre,  
Northeast Region (ICMR), Dibrugarh, Assam, India

### \*Article History:

Received: 14/02/2017

Revised: 15/03/2017

Accepted: 15/03/2017

DOI: <https://dx.doi.org/10.7439/ijbar.v8i3.3954>

### Abstract

Despite the availability of effective vaccine and antiviral therapy, hepatitis B remains a serious public health problem worldwide. In spite of tremendous work done in the past, some questions pertaining to hepatitis B biology is yet to be understood. For instance the molecular mechanism whereby host fails to eliminate the virus leading to persistent infection is clearly not known. As many works have been directed towards understanding adaptive immune response, the area of innate immunity is poorly explored. The aim of the present study was to elucidate the role of toll-like receptors, an important component of innate immunity, in patients with hepatitis B infection. The mRNA expression of TLRs were studied in a total of 51 hepatitis B patients (45 chronic and 6 hepatocellular carcinoma) and 45 healthy individuals hailing from Arunachal Pradesh of northeastern part of India by quantitative PCR in LC 480 (Roche) system utilizing SYBR green and in house designed primers. Expression of TLR3, TLR7 and TLR9 was increased by 1.09 and 1.11 fold respectively with significant increase in TLR9 mRNA ( $p=0.017$ ) in chronic and HCC patients compared to healthy control. No significant association was found with TLR9 expression and serum HBV DNA load ( $r=0.0033$ ,  $p=0.98$ ). Significant association of TLR9 in hepatitis B patients show that TLR9 does play a key role in persistent HBV infection and hepatocellular carcinoma.

**Keywords:** Hepatitis B, Toll-like receptor, qPCR.

### 1. Introduction

Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family and an enveloped virus with a partially double stranded DNA genome of approximately 3.2 Kb size [1]. It can cause both acute and chronic infection. Despite the availability of safe and effective vaccine as well as antiviral therapy to treat chronic HBV [2], an estimated 240 million people are chronically infected with HBV and over 780000 people die every year due to severe complications such as cirrhosis and liver cancer [3]. Despite being great effort to understand the cause of chronic hepatitis B, the immunologic basis of chronic infection or molecular mechanism whereby the host fails to eliminate the virus and develop chronic infection is yet to be

understood [4]. While many works have been focused towards understanding the adaptive immune response, the role of innate immunity in relation to persistent chronic infection is poorly understood.

The innate arm of immune system works by recognizing gross morphological structures displayed by invading microbial agents collectively known as “pathogen associated molecular patterns (PAMPs)” by means of specialized proteins known as pattern recognition receptors (PRRs). Among the various classes of PRRs, toll like receptors (TLRs), known after their homology with toll protein in *Drosophila*, are of great importance especially for viruses for two reasons. First they recognize virus surface

proteins, nucleic acids and phospholipids. Second, the binding of viral components to specific TLRs leads to activation of a series of downstream signaling molecules ultimately leading to transcription of various cytokines and type-I interferon which provide protection against virus infection [5].

The early evidence about the role of TLRs in eliminating hepatitis B virus came from an experiment where intravenous injection of ligands specific for TLR 3,4,5,7 and 9 inhibited HBV replication in transgenic mice in  $\alpha/\beta$  interferon-dependent manner [6]. Thus, HBV must elicit strategies to modulate TLR expression and the associated pathway to develop persistent infection in the host. Although down-regulation of TLRs such as TLR2, TLR3, TLR7 and TLR9 have been found in chronic hepatitis B patients in a few studies [7] it is difficult to arrive at a treatment regimen based on TLRs due to limited studies in human. As such further studies are required to clearly elucidate the role of TLRs as their involvement in persistent HBV infection.

Thus the aim of the present study was to explicate the role of TLRs associated with chronic hepatitis B infection in HBV patients from northeast India. The prime objectives were to monitor the expression of TLR3, TLR7 and TLR9 which categorically recognize viral RNA and DNA in patients with hepatitis B infection and to compare the results with HBV DNA level.

## 2. Materials and Methods

### 2.1: Study design

Expression of circulating TLRs in peripheral blood was studied in hepatitis B cases relative to healthy individuals. A case of hepatitis B was defined when the patient's sera tested positive for hepatitis B surface antigen (HBsAg). The clinical differentiation between acute and chronic hepatitis B cases were done following CDC case definition [8-9]. Laboratory confirmation of acute HBV was done based on HBsAg and anti-hepatitis B core IgM positivity. Chronic HBV was confirmed based on past clinical history (HBsAg present for at last 6 months). Diagnosis of hepatocellular carcinoma was based on FNAC and ultra sonography test. A convenient sampling procedure was employed to enroll study participants.

Patients and healthy individuals were recruited by conducting screening for hepatitis B surface antigen at some selected places in Arunachal Pradesh between 2012-2015. The patients with known history of hepatitis B as obtained from local medical record were also recruited by inviting them at the screening site. A person found negative for hepatitis B surface antigen and antibody to hepatitis B core antigen with no history of any chronic diseases from the same site served as healthy control.

People of all age group giving written consent for participation in the study were included in the study. Patients receiving any antiviral or surgical treatment in the last one month were excluded from the study.

### 2.2: Specimen collection and processing

5-7ml of venous blood was collected by trained phlebotomist from all the participants. 2.5ml of the collected blood was transferred to PAXgene blood RNA tubes and rest 3.5 ml in sterile K<sub>2</sub>EDTA vials (BD). The plasma was separated and transferred to laboratory (RMRC Dibrugarh) maintaining cold chain conditions. All the collected specimens were kept at +4°C until transferred to laboratory.

### 2.3: Consent

A written informed consent was obtained from all the participants prior to specimen collection. For minors, consent was obtained from their parents. The patient's characteristics were recorded in a structured case investigation form.

### 2.4: Approval & ethics

The study was approved by the human ethics of RMRC Dibrugarh prior to the start of study.

### 2.5: Financial support

The study was supported by the intramural fund of RMRC Dibrugarh.

### 2.6. Laboratory assays

#### 2.6.1: Serology

Presence of hepatitis surface antigen (HBsAg) in patient's sera was confirmed by enzyme immunoassay following kits and procedure after Bio-Rad USA. Other markers of HBV infections such as hepatitis B core antigen (HBeAg), antibody to hepatitis B core antigen (total anti-HBc & anti-HBcIgM), and antibody to hepatitis surface antigen (anti-HBsAg) were done using ELISA kits from DRG, USA.

#### 2.6.2: HBV Viral load

Viral load estimation was done using 500ul of plasma utilizing High Pure extraction system and HBV test kit in Cobas TaqMan 48 Analyzer (Rotkreuz, Switzerland). The assay had a limit of detection 6-100,000,00 IU/ml where 1 IU/ml correspond to 5.8copies/ml

#### 2.6.3: RNA extraction

Total RNA was extracted from blood kept in PAXgene blood RNA tubes using PAXgene Blood RNA extraction kit (PreAnalytix; Hombrechtikon, Switzerland) as per manufacture's protocol. RNA was resuspended in 60 $\mu$ L of elution buffer supplied with the kit. Prior to RNA extraction, tubes were kept at room temperature for 2 hours for complete homogenization. An optical density reading at 260,280 and 320nm was taken by putting 1.5 $\mu$ L of extracted RNA onto nanodrop spectrophotometer (Thermo Scientific, USA) to determine its purity and concentration. The

integrity of RNA was checked by running 8µl of RNA in 1% native agarose gel pre-stained with ethidium bromide.

#### 2.6.4: DNase treatment

In order to remove residual DNA contamination from RNA preparation, 400 ng of RNA was treated with DNase I using RQ RNase free DNase I (Promega, USA) following methodology as mentioned in the kit.

#### 2.6.5: c-DNA synthesis

The DNase treated RNA was reverse transcribed using MMLVRT-enzyme, buffer, dNTPs, MgCl<sub>2</sub> and random hexamer primers from Promega, USA following recommended procedure in the kit. Proper controls such as No-RT (RT-enzyme replaced by equal volume of nuclease free water) and no template controls were also included during c-DNA synthesis.

#### 2.6.6: Primer design

A set of primers targeting genes for human toll like receptors 3,7,9,TNFα-,GAPDH were designed using mRNA & gDNA template downloaded from NCBI-GenBank & using default parameters for SYBR green in “Beacon-Designer” software kindly provided by Premier-Biosoft, USA (details of design and validation given elsewhere).

#### 2.6.7: Quantitative PCR

The cDNA was amplified in 10µl reaction volume containing 2 µL of 2 fold diluted cDNA template, 5µL of 1X SYBR green master mix (Dynamo color Flash, Thermo Scientific) and 0.5µM of newly designed primers. The target genes and housekeeping gene (GAPDH) were amplified in the same plate with two replicates. A pre-denaturation step at 95°C for 7 mins was followed by 40 cycles consisting of denaturation at 95°C for 10 sec and 60°C for 30 sec with single acquisition of fluorescence data during annealing and extension phase. The cyclic steps were followed by melting curve analysis as preset for SYBR green in LC 480 system. Threshold cycles (Ct values) were determined using 2<sup>nd</sup> derivative by LCS480 1.5.0.39 software in LC 480 real time PCR system. Each PCR was accompanied by No-RT & No-Template Control.

#### 2.7: Statistical analysis:

The threshold cycles (C<sub>t</sub>) obtained in real time PCR were converted to 2<sup>-ΔCt</sup> (ΔCt=Ct<sub>gene of interest</sub>-Ct<sub>housekeeping gene</sub>) and fold change calculated as described earlier [10]. Results of mRNA expression were shown relative to healthy control. An unpaired t-test was done for comparing mean difference in expression between cases and control in freely available web tool GraphPad (<http://www.graphpad.com/quickcalcs/ttest2/>). Correlation analysis was done using Spearman’s Rho method in Social Science Statistics software

(<http://www.socscistatistics.com/tests/spearman/default2.aspx>). Statistical significance was set at p<0.05.

### 3. Results

#### 3.1: Patients and controls

A total of 152 hepatitis B cases were enrolled during the period; however blood could be collected in PAXgene blood RNA tubes (for mRNA expression analysis) in 51 only. Among HBV cases, 45 had chronic hepatitis B infection (CHB) while 6 had hepatocellular carcinoma. The age group of such cases ranged from 7-80 years with mean age (SD) 31±18 years. There were 32 male & 19 females. Also 50 of 51 were positive for anti-HBc, 16 with HBeAg and 28 without HBeAg.

Blood specimens were also collected in PAXgene blood RNA tubes from 72 individuals with HBsAg negativity, which comprised of mostly health care workers (Nurses). However, only 45 were negative for both HBsAg and anti-HBc. Thus data for only 45 healthy individuals were included in the gene expression analysis as healthy control. The age group of such individuals ranged from 7-70 years with mean age (SD) 32±16 years where there were 17 male & 28 female.

#### 3.2: Primer-design

The characteristics of primers and their PCR efficiencies are given in Table1 (details of primer design and validation given elsewhere).

#### 3.3: TLR expression

Expression of TLR3 & TLR7 was increased by 1.09 & 1.11 fold respectively in CHB & HCC patients compared to healthy individuals, although not significant (Table2). A significant increase (1.14 fold) in mRNA expression of TLR9 was observed in CHB & HCC patients in comparison to healthy individuals (p=0.017). The mRNA expression of one proinflammatory cytokine (TNF-α) was also studied to monitor the association of TLRs with cytokine expression, which was reduced in HBV cases compared to healthy individuals, although not significant (p=0.2) (Table 2). Expression of TLR9 was found independent of HBeAg status of HBV patients (p=0.8) (Table 3). Activation of TLR9 was not significantly related with serum HBV DNA load (r=0.0033, p=0.98).

#### 3.4 Limitation of the study

The expression pattern of down-stream signaling molecules leading to the transcription of type-1 interferon is not known.

The expression of TLRs and cytokines at protein level is not known.

**Table 1: Primers used in the study along with their genome positions, melting temperature and amplicon length**

Sl No.	GenBank Accession No.	Oligo name	Sequence (5'-3')	Length in bp	Genome position	Melting temperature	Amplicon length
1	NM003265	TLR3F	GCTCTCCTTCACCATTCC	18	1591-1733	60.1	143
		TLR3R	CCGTGCTAAGTTGTTATGC	19	1715-1733	59.8	
2	NM016562	TLR7F	GGTGATGATGACAGCAAGT	19	2701-2719	60.6	113
		TLR7R	CATAGCAACAGTCTGGTGATA	21	2793-2813	60.4	
3	NM017442	TLR9F	AATGTCACCAGCCTTTCC	18	824-841	60.3	101
		TLR9R	TTCCACTTGAGGTTGAGATG	20	905-924	60.3	
4	NM000594	TNF-aF	AGAGGGAGAGAAGCAACT	18	33-50	60.1	101
		TNF-aR	GTCAGTATGTGAGAGGAAGAG	21	113-133	60.0	
5	NG007073	GAPDHf	CAACACAGCCTCCTATACC	19	10096-10114	60.0	105
		GAPDHR	GTCATACACTGAGACCAACA	20	10181-10200	60.0	

**Table 2: mRNA expression of TLRs in patients with hepatitis B infection and healthy control**

Attributes	$2^{-\Delta Ct}$ (TLR3)	$2^{-\Delta Ct}$ (TLR7)	$2^{-\Delta Ct}$ (TLR9)	$2^{-\Delta Ct}$ (TNF-a)
HBV Cases (n=51)	74.8±24.03	45.97±13.33	46.21±11.21	29.19±10.45
Healthy Control (n=45)	68.35±21.57	41.53±10.54	40.62±11.47	32.25±16.98
Fold Change	1.09	1.11	1.14	0.91
P Value (unpaired t-test)	0.175	0.076	0.0178	0.284
Confidence interval at 95%	-2.93-15.83	-0.475-9.35	0.988-10.19	-8.7-2.58

**Table 3: TLR9 expression vs Serum HBeAg status among Hepatitis B positive cases**

TLR9 mRNA expression	Serum HBeAg	
	Positive	Negative
Total No.	16	28
TLR9 expression (Mean±SD)	45.15±14.27	46.06±9.73
Fold change	0.98	
P value	0.8( -8.2 to 6.4)	

#### 4. Discussion

An increased expression of TLRs particularly TLR9 has been observed in the present study. This is in contrast to previous studies where down-regulation of TLRs has been reported. For instance, Chen *et al*[11] have shown a down regulation of TLR7 expression and TLR9 mRNA in PBMC of HBV-infected patients, but an increased TLR9 expression at the protein level. In yet another study [12] expression of TLR9 mRNA and protein was decreased in patients infected with HBV or HCV compared to healthy control and were negatively correlated with serum viral copies of HBV. Similar observations (decreased TLR9 expression) were also made by other studies [13- 14]. Our results are consistent with studies where higher expression of TLR3 and TLR7,TLR9 have been observed in patients with Hepatitis C infection and systemic lupus [15-16] respectively. The contrasting results obtained in the present study may also be explained on the basis of ethnic differences as has been described in patients with systemic lupus where 2 fold higher expression of TLR9 was observed in African American women with lupus when compared to their healthy controls or European American lupus patients [16]. The up regulation of TLRs may be protective in

nature, however, reduced expression of cytokine (TNF $\alpha$ ) gives an indication of inhibitory effect. Thus HBV may elicit factors that may inhibit downstream signaling pathway leading to diminished transcription and thus causing persistence. The TLR based therapies such as agonists of TLR7 [17] and TLR9 [18] which are promising candidates for activating TLRs and thus suppress HBV replication seems to be of limited use in the present setting where already there is activation of TLRs. This may also provide an opportunity to treat with antagonists as suggested in situations where there is increased expression of TLRs such as the case with systemic lupus erythematosus [16].

#### 5. Conclusion

Up-regulation of TLRs specifically TLR9 has been observed in Hepatitis B patients with chronic and HCC in Arunachal Pradesh of northeast-India. This is a new source of information at least in Indian population which not only advances our knowledge in understanding host-pathogen interaction but also demands further studies to be carried out including the downstream signaling molecules, both at transcription and translation level.

## Acknowledgement

I would like to thank Indian Council of Medical Research for providing Senior Research Fellowship grant to carry out the work.

**Conflict of interest:** None declared

## References

- [1] Seeger C, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000; 64:51-68.
- [2] Wang XY, Chen HS. Emerging antivirals for the treatment of hepatitis B. *World J Gastroenterol* 2014; 28:20:24: 7707-17.
- [3] World Health Organization. 2015. Fact Sheet, Hepatitis B, last updated July 2015. <http://www.who.int/mediacentre/factsheets/fs204/en/>
- [4] Liang TJ. Hepatitis B: The Virus and Disease. *Hepatology*; 2009; 49 Suppl5: S13-S21
- [5] Zhang E, Lu M. Toll-like receptor (TLR) -mediated innate immune responses in the control of hepatitis B virus (HBV) infection. *Med Microbiol Immunol* 2015; 204:11-20
- [6] Isogawa M, Robek MD, Furuichi Y, Chisari FV. Toll-like receptor signaling inhibits hepatitis B virus replication in vivo. *J Virol* 2005; 79: 7269-72.
- [7] Pei RJ, Chen XW, Lu MJ. Control of hepatitis B virus replication by interferons and Toll-like receptor signaling pathways. *World J Gastroenterol* 2014; 7: 20:33:11618-29.
- [8] Centers for Disease Control and Prevention, Atlanta USA. Hepatitis B, acute, 2012 case definition. Last updated May 06, 2015.
- [9] Centers for Disease Control and Prevention, Atlanta USA. Hepatitis B, Chronic, 2012 case definition. Last updated May 06, 2015.
- [10] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols* 2008; 3:1101-08.
- [11] Xu N, Yao HP, Sun Z, Chen Z. Toll-like receptor 7 and 9 expression in peripheral blood mononuclear cells from patients with chronic hepatitis B and related hepatocellular carcinoma. *Acta Pharmacologica Sinica* 2008; 29: 239-44.
- [12] Zhou J, Huang Y, Tian D, Xu D, Chen M, Wu H.. Expression of toll-like receptor 9 in peripheral blood mononuclear cells from patients with different hepatitis B and C viral loads. *J Huazhong. Univ Sci Technolog Med Sci* 2009; 29:3:313-17.
- [13] Xie Q, Shen HC, Jia NN, Wang H, Lin LY, An BY. Patients with chronic hepatitis B infection display deficiency of plasmacytoid dendritic cells with reduced expression of TLR9. *Microbes Infect* 2009; 11:4:515-23.
- [14] Sajadi SMA, Mirzaei V, Hassanshahi G, Khorramdelazad H, Daredor HY, Hosseini SMH *et al.* Decreased Expressions of Toll-Like Receptor 9 and its Signaling Molecules in Chronic Hepatitis B Virus-Infected Patients. *Arch Pathol Lab Med* 2013; 137:1674-79.
- [15] Medhi S, Deka M, Deka P, Swargiary SS, Hazam RK, Sharma MP *et al*; 2011. Promoter region polymorphism & expression profile of toll like receptor3 (*TLR3*) gene in chronic hepatitis C virus (HCV) patients from India. *Indian J Med Res* 2011; 134:2: 200-07.
- [16] Lyn-Cook BD, Xie C, Oates J, Treadwell E, Word B, Hammons G, Wiley K. Increased expression of Toll-like receptors (TLRs) 7 and 9 and other cytokines in systemic lupus erythematosus (SLE) patients: ethnic differences and potential new targets for therapeutic drugs. *Mol Immunol* 2014; 61:1:38-43.
- [17] Funk TE, Kottlilil S, Gilliam B, Talwani R. Tickling the TLR7 to cure viral hepatitis. *J Transl Med* 2014; 12:129.
- [18] Bhardwaj N. TLR Agonists: Are They Good Adjuvants? *Cancer J* 2010; 16; 4:382-91.