Acta virologica **62**: 58 – 67, 2018 doi:10.4149/av_2018_107

Association of Toll-like receptor 4 with hepatitis A virus infection in Assam

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Received March 23, 2017; revised June 26, 2017; accepted January 10, 2018

Summary. – Hepatitis A virus (HAV) which causes liver disease is recognized by Toll-like receptors (TLRs) through the viral nucleic acid, initiating the host defense response. The study aims to analyze the role of TLR4 rs11536889 polymorphism in the pathogenesis of hepatitis A cases from Assam. There was significant correlation between TLR4 SNP G/C (rs11536889) and between acute viral hepatitis (AVH) A cases and controls. The correlation of the 3 different genotypes GG, GC and CC of TLR4 rs11536889 with the TLR4 mRNA expression level in all the HAV cases groups have been found to be statistically significant ($p < 0.001$). TLR4 expression was most significantly upregulated in the acute HAV cases, HAV with cholestasis cases and even the HAV caused fulminant hepatitis failure (FHF) cases with the CC genotype of TLR4 rs11536889. The upregulation is mostly seen in the cases with the CC genotype of TLR4 rs11536889 and thus indicates that the mutant variant of TLR4 rs11536899 (CC) may have an effect on the expression of TLR4 at the transcription level. Our study did not show any significant association between AVH and HAV caused FHF ($p = 0.32$, OR = 0; $p = 0.59$, OR = 2.06 at 95% CI) among the genotypes GG, GC and CC. Our data suggest that *TLR4* gene polymorphism rs11536889 may play a prominent role in HAV disease susceptibility and TLR4 expression in population from Assam.

Keywords: polymorphism; real-time PCR; Toll-like receptor; hepatitis A infection; cholestasis; fulminant hepatic failure

Introduction

Hepatitis A virus (HAV) infection is the leading worldwide cause of acute viral hepatitis (AVH). Hepatitis A is a very common form of viral liver disease which leads to serious health and economic problems with its epidemiologic pattern changing over time (Koopmans and Duizer, 2004; Vaughan *et al*., 2014; Pereira *et al*., 2014). Globally, an estimated 1.4 million cases of HAV infection occur annually resulting in fulminant hepatitis and death in a very small proportion of patients (0.1–2.1%) (Bell *et al*., 1998). HAV is present in a worldwide distribution. The highest prevalence of infection occurs in many regions of Central and Southeast Asia, Africa, and Central and South America (Hadler, 1991). HAV was found to be the most common cause of acute hepatitis followed by hepatitis E viral infection (17.97% cases) in North Indian population (Jain *et al*., 2013). Study by Tandon *et al.* (1984) have shown that the HAV prevalence has came down to 50–60% in India (Tandon *et al*., 1984) and recent study by Jain *et al.* (2013) have reported that HAV (26.96%) was identified to be the most common cause of AVH. The frequency of HAV induced AVH has been reported to be 8% by Batra *et al.* (2002) while Jain reported that the percentage of AVH in adults is 26.96% (Jain *et al*., 2013).

HAV, first identified in 1973, is a non-enveloped, spherical, positive stranded RNA virus with 7.5 kb genome classified within the genus *Hepatovirus* of the *Picornaviridae* family. Transmission of HAV is typically by the fecal-oral route. Acute

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Abbreviations: ALT = alanine transaminase; AST = aspartate transaminase; AVH = acute viral hepatitis; FHF = fulminant hepatic failure; HAV = hepatitis A virus; MyD88 = myeloid differentiation factor 88; PT = prothrombin time; SNP = single nucleotide polymorphism; TLR(s) = Toll-like receptor(s); TNF-α = tumor necrosis factor α

HAV infection cannot be clinically distinguished from other causes of AVH as the infection is often asymptomatic in young children, whereas in older children and adults it presents in a range of clinical manifestations from mild, anicteric infection to fulminant hepatic failure (FHF). Different clinical variants range from prolonged to relapsing to cholestatic forms.

Toll-like receptors (TLRs) are pattern recognition receptors (PRR) which recognize molecules that are broadly shared by pathogens like viral nucleic acids (dsRNA, ssRNA) but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). Toll-like receptors are innate sensors that recognize both microbial and endogenous ligands, initiating the host defense response. The different types of TLRs together as homodimeric or heterodimeric receptor complexes in association with accessory proteins recognize microbial components representing almost the entire spectrum of the microbial world (Akira *et al*., 2001). The first of (currently) 12 human TLRs was described in 1997 as a human homolog of the Drosophila Toll protein, later to be designated as TLR4 (Medzhitov *et al*., 1997). TLR4 is expressed in hepatocytes and recognizes not only bacterial components but also viral envelope proteins like the fusion (F) protein from Rous sarcoma virus (RSV) and envelope protein of mouse mammary tumor virus (MMTV). Activation of TLR4 can cause inflammation by promoting the secretion of inflammatory cytokines such as TNF-α and IL-6 through the MyD88 dependent pathway, and anti-virus effects by promoting the secretion of IFN-β through the MyD88 independent pathway (Andreakos *et al*., 2004). The TLR4 rs11536889 single nucleotide polymorphism (SNP) has been found to be significantly associated with hepatitis type B virus recurrence after liver transplantation (Zhou *et al*., 2011). TLR4 SNPs have been associated with the risk of developing advanced fibrosis or cirrhosis (Yonghong *et al*., 2009). The SNP in the 3'-untranslated region (UTR) of TLR4 rs11536889 has been found to regulate the expression of TLR4 and has some influence on the response to lipopolysaccharides and therefore polymorphism rs11536889 could be an excellent genetic marker for the diseases caused by TLR4-ligands (Sato *et al*.,2012).

TLR4 is up-regulated in peripheral blood monocytes (PBMCs) and hepatocytes in patients with chronic hepatitis C (CHC) and so may also play an important role in the pathogenesis of hepatitis A in different stages of infection and may also have a possible role in its clearance. Enhanced TLR4 expression leads to enhanced production of IFN-β and IL-6 in RNA virus induced hepatitis (Yonghong *et al*., 2009) and so TLR4 may also be an important factor in HAV pathogenesis. The presence of SNP rs11536889 in the 3'UTR of TLR4 gene suggests its possible role in TLR4 expression (Sato *et al*., 2012). Therefore, we attempted to analyze the association of the SNP rs11536889 and expression of TLR4 among the HAV cases from Assam.

Materials and Methods

Patient enrollment. HAV cases (n = 104) with an age ranging from 12–40 years and sex ratio (3:1; male female) were enrolled from general Outward Patient Department and Medicine Ward of Gauhati Medical College and Hospital (GMCH), Guwahati, Assam for a period of 1.5 year from 2012 April to 2013 December. Age/sex-matched healthy controls (n = 90) were also collected from GMCH. The study was approved by the Institutional Ethical Committee of Gauhati University and a written informed consent was taken from all subjects enrolled in the study (GUEC-09/2015). All the patients were selected on the basis of clinical investigation and biochemical profile including ALT (alanine transaminase), AST (aspartate transaminase), PT (prothrombin time), TB (total bilirubin), total protein and bilirubin and positivity for HAV IgM. The patients' histories including age, sex, personal hygiene, etc parameters were investigated and recorded.

Inclusion and exclusion criteria and sample source. Five ml of blood were collected from each patient with their informed consent with the help of a registered practitioner in GMCH. The plasma or serum was separated and stored at -20°C. To avoid repeated freezing and thawing of the serum or plasma, aliquots were prepared and stored at -70°C. The whole blood was stored at 4°C. The patients who were clinically diagnosed as acute viral hepatitis, fulminant hepatitis, cholestasis on the basis of diagnostic criteria in GMCH were included in the present study. The liver disease patients, who were associated with high-risk group like intravenous drug abusers, chronic renal failure, thalassemia, haemophilia, diabetes mellitus, psychiatric illness, and confection with other viruses, were excluded from the study. Almost an equal number of non-related voluntary healthy age and sex matched individuals (n = 90) who had no prior history of liver disease and any systemic illness were enrolled in the present study with all clinical history and dietary habits details and was used as comparative control group.

Study design. The collected cases were analyzed by age, sex, other hepatotropic viral infections like hepatitis B or hepatitis C, significant alcohol intake, duration of hospital stay, serum biochemistry, and coagulation test at admission and most severe clinical course. In this study, acute hepatitis A was diagnosed by typical symptoms of acute hepatitis, by the presence of anti-HAV IgM positive serum associated with an elevation of serum amino transferase levels five times above the upper normal limit. Prolonged cholestasis was defined as that with total bilirubin level ≥ 5 mg/dl lasting for more than 4 weeks after admission (Jung *et al*., 2010). Acute liver failure according to the widely accepted definition of acute liver failure that states that there is evidence of coagulation abnormality, usually a prolongation of PT by International normalized ratio (INR) ≥1.5 or prothrombin time >15 seconds, and any degree of mental alteration (encephalopathy) in a patient without pre-existing cirrhosis and with an illness with duration <26 weeks (Polso and Lee, 2005).

Total RNA isolation and cDNA preparation. The total RNA from whole blood was isolated by using the Trizol RNA reagent (Invitrogen Life Technologies, USA) (Chomczynski and Mackey,

1995). The quality of RNA was checked in Genei Nanovalue plus instrument (GE Healthcare life sciences, USA). Absorbance was measured at 260 nm, 230 nm and 280 nm. RNA solution with 260/280 ratio ~2.0 and 260/230 ratio >2 was considered for study. The extracted RNA was converted to cDNA with random primers and reverse transcriptase enzyme using the cDNA synthesis kit (Applied Biosystem, USA). The cDNA was stored at -20°C.

Differential mRNA expression analysis of TLR4 by real-time PCR. Real-time PCR (qPCR) was performed in the Qiagen Rotor Gene detection system (Rotor-Gene Q 2plex HRM Platform, Qiagen, Germany) using the SYBR® Premix Ex Taq™ (Tli RNase H Plus) (Takara, Japan) according to the manufacturer's instructions. Briefly, 2 µl of template cDNA was used for each reaction in a total volume of 25 µl. PCR amplification with the help of a pair of primers was carried out with initial denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. A single cycle for template extension was done at 72°C for 7 min. All PCR reactions were performed in triplicates. The relative quantification of target gene expression was evaluated using the comparative CT method (Livak and Schmittgen, 2001). The expression of mRNA was calculated on the basis of difference of threshold cycle between gene of interest (GoI) and house-keeping gene (HKG) among cases and controls. The relative change in expression, fold change in gene expression of GoI was calculated by ΔCt method (Livak and Schmittgen, 2001).

Statistical analysis. Statistical analysis was performed by the standard methods using SPSS computer software (Version 16, SPSS Inc., USA). The results were presented as mean ± standard deviation or number of instances (percent). Fisher's exact test was used to compare clinical features according to the presence of complications. Student's *t*-test was used for continuous variables. Associations between clinical features and HAV disease severity were calculated as odds ratio (OR) with 95% confidence intervals (CIs). P-values of <0.05 were considered statistically significant.

*DNA extraction and polymorphism analysis by direct sequencing***.** Blood samples (5 ml) were collected in 0.5 mol/l ethylene diamine tetra acetic acid (EDTA) and genomic DNA was extracted by a standard protocol used routinely (phenol-chloroform method) (Sambrook *et al.*, 1989). PCR was performed using the primer pair F: 5' TCCAGAAACATATGGGCTGA 3'and R: 5'TGTTTC TGAGGAGGCTGGAT3'generating an amplicon of 345 bp for the rs11536889 of TLR4 for 35 cycles (95°C for 30 s; 58.8°C for 30 s and 72°C for 45 s) with an initial denaturation at 95°C for 5 min and a final extension at 72°C for 7 min. Polymorphism for the SNP site rs11536889 of TLR4 was analyzed by direct sequencing of PCR products using ABI Prism Big Dye terminator Cycle sequencing system (PE Applied Biosystem, USA) and compared with the TLR4 gene sequence from the National Centre for Biotechnology Information (NCBI) database.

Statistical analysis. Allele frequencies were compared in controls and HAV patients using 2x2 contingency table. Genotype frequencies were compared using 2x3 contingency table by Fisher's exact test. The Hardy-Weinberg equilibrium at individual locus was assessed by chi-square ($\chi 2$) statistics using OpenEpi software.

Results

Demographic data of hepatitis A patients

Viral hepatitis cases were tested in the OPD and Medicine ward of GMCH. The cases that were collected were

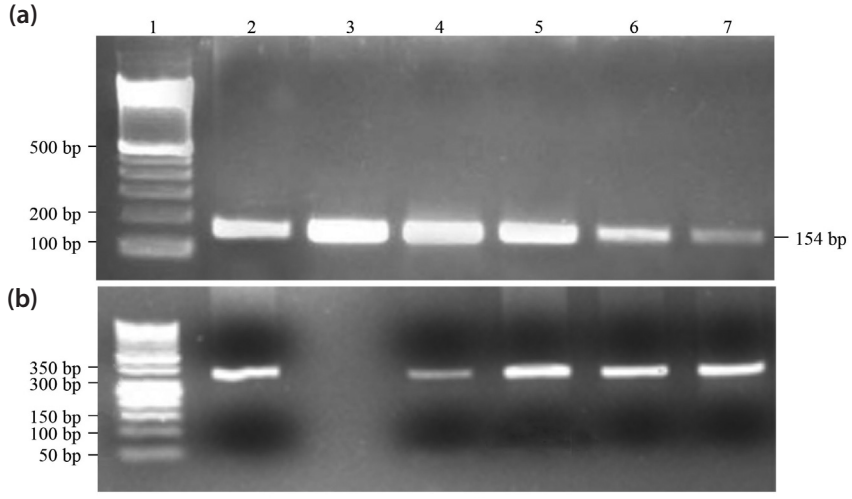

Fig. 1

Electrophoresis of HAV positive samples and TLR4 rs11536889

(a) representative agarose gel showing bands for HAV positive samples (lane1: 100 kbp ladder, lane 2,4,5,6 and 7: HAV positive samples) **(b)** representative agarose gel showing the PCR bands (345 bp) of TLR4 rs 11536889.

Table 1. Stratification of the hepatitis A cases based on their clinical profile

Cases	Frequency % (case/total)
Acute hepatitis A	82 (85/104)
Hepatitis A with cholestasis	14 (15/104)
Fulminant hepatic failure (FHF)	4 (4/104)

Table 2. Demographic and etiological characteristic of the HAV cases

Parameter	Acute hepatitis A (n = 85)	Hepatitis A with cholestasis (n = 15)	Fulminant hepatic failure (FHF) (n = 4)
Age	25 ± 7	28 ± 12	34 ± 8
Sex ratio	29:6	7:3	2:1
ALT(IU/l) ≤40	468 ± 17.12	923 ± 1.92	4763.5 ± 12.58
AST(IU/l) ≤40	252.33±9.9	747.7 ± 1.68	4416 ± 6.05
Prothrombin time (INR) 2.0–3.0	1.20 ± 0.60	2.36 ± 2.12	8 ± 0.56
Total Bilirubin (mg/dl) 0.4–1.2	5.37 ± 0.84	16.24 ± 11.22	18.40 ± 11.30
Total protein (gm/dl) 6.5–8.0	7 ± 1.98	7.95 ± 1.16	8.1 ± 0.42

confirmed by PCR to be HAV induced hepatitis (Fig.1a). The HAV cases were categorized (Table 1).

The demographic and etiologic characteristics of the HAV cases are summarized in the Table 2. The male and female ratio was found to be 3:1 where the male gender was predominant (76%). From the confirmed cases of HAV infections it was seen that mostly the young adults in this region were infected. The mean age was calculated to be 25 years. Among the laboratory characteristics, ALT mean value was 468 IU/l, with a total bilirubin value of 5.37 mg/dl in the acute HAV cases. Fulminant hepatic failure developed in 0.03% of the hepatitis A patients (Table 1). Among the 104 patients, 0.14 % of patients had prolonged cholestasis in time of admission. The travel histories of the patients were also recorded (Table 5). The comparison of the clinical characteristics among the HAV cases groups was analyzed (Table 3 and 4). Patients with prolonged cholestasis were older but there was no significant correlation with patients without cholestasis. They also had significantly prolonged PT (INR) ($p < 0.0002$) and PT (INR) >1.5 at the time of admission. The patients with prolonged cholestasis had significantly higher ALT and AST values than HAV patients without cholestasis ($p < 0.0001$).

Patients with FHF had a mean age of 28 years and were older compared to acute HAV patients. FHF patients had significantly lower hemoglobin level and significantly higher prothrombin time, total bilirubin, AST, ALT and creatinine levels in comparison to acute HAV patients in time of admission and also during peak time.

Polymorphism analysis

TLR4 rs 11536889 was detected by PCR as 345 bp band (Fig. 1b). Figure 2 depicts the partial chromatograms representing the homozygous G/G, C/C genotype and heterozygous G/C genotype at rs11536889 in the TLR4

Table 3. Comparison of clinical characteristics of acute HAV infection patients with the presence of prolonged cholestasis (bold values represent significant risk)

Variable	Prolonged cholestasis (n = 15)	Noncholestasis (n = 89)	p-value
Age	28 ± 8.34	25 ± 7.12	**0.03**
Sex	7:3	29:6	
Laboratory values at admission			
Haemoglobin (g/dl)	12.9 ± 2.1	12.32 ± 2.65	0.37
Prothrombin time (INR)	2.36 ± 2.12	1.20 ± 0.60	**<0.0002**
Total Bilirubin (mg/dl)	16.24 ± 11.22	5.37 ± 0.84	**<0.0001**
AST (IU/l)	747.7 ± 168.34	252.33±220.94	**<0.0001**
ALT (IU/l)	923 ± 192.21	468 ± 171.24	**<0.0001**
Creatinine (mg/dl)	0.90 ± 0.26	0.98 ± 0.38	0.28
Laboratory values at peak time			
Haemoglobin (g/dl)	12.9 ± 2.1	12.32 ± 2.65	0.35
Prothrombin time (INR)	3.2 ±2.8	1.5 ± 0.9	**<0.0001**
Total Bilirubin (mg/dl)	23.64 ± 12.18	12.07±2.90	**<0.0001**
AST (IU/l)	3230 ± 121.23	3100 ± 324.1	0.07
ALT (IU/l)	4017 ± 781.4	2670 ± 564.3	**<0.0001**
Creatinine (mg/dl)	1.2 ± 0.35	0.9 ± 0.4	**0.0019**

Table 4. Comparison of clinical characteristics of acute HAV infection patients with the FHF patients ((bold values represent significant risk)

Variable	HAV caused FHF (n = 4)	Acute HAV (n = 85)	p-value
Age	28 ± 10.44	25 ± 7.12	0.47
Sex	2:1	29:6	
Laboratory values at admission			
Haemoglobin (g/dl)	7.1 ±2.95	12.32 ± 2.65	**0.001**
Prothrombin time (INR)	8 ± 0.56	1.20 ± 0.60	**<0.0001**
Total Bilirubin (mg/dl)	18.40 ± 11.30	5.37 ± 0.84	**<0.0001**
AST (IU/l)	4416 ± 642.05	252.33±220.94	**<0.0001**
ALT (IU/l)	4763.5 ± 744.58	468 ± 171.24	**<0.0001**
Creatinine (mg/dl)	2.4± 0.7	0.98 ± 0.38	**<0.0001**
Laboratory values at peak time			
Haemoglobin (g/dl)	7.1 ±2.95	12.32 ± 2.65	**0.001**
Prothrombin time (INR)	8 ± 0.56	1.5 ± 0.9	**<0.0001**
Total Bilirubin (mg/dl)	26.4 ± 0.8	12.07 ± 2.90	**<0.0001**
AST (IU/l)	4628 ± 612.8	3100 ± 324.1	**<0.0001**
ALT (IU/l)	4798 ± 621.7	2670 ± 564.3	**<0.0001**
Creatinine (mg/dl)	2.4± 0.7	0.9 ± 0.4	**<0.0001**

Table 5. Demographic characteristics and travel histories for HAV infected subjects (n = 104)

Factor	No (%) of Subjects			p-value	
	Acute HAV (n = 85)	Acute HAV with cholestasis (n = 15)	HAV caused FHF (n = 4)	Acute HAV vs. HAV with cholestasis	Acute HAV vs. FHF
Reported exposure risk:					
Contaminated food or water	62 (72.9)	8 (57)	2 (0.5)	Ref	Ref
Contact with patient infected with HAV	6 (7.0)	0 (0)	0 (0)	0.5	0.8
Blood transfusion	0(0)	0 (0)	0 (0)	<0.005	<0.005
Health profession	3 (3.5)	0 (0)	0 (0)	0.7	0.9
Herbal medication	4(4.7)	1 (7)	0 (0)	0.5	0.8
History of travel	35 (41.1)	4 (28.5)	0 (0)	0.3	0.3
Course of disease:					
Hospitalization	82 (96.4)	14 (1.0)	4 (1.0)	Ref	Ref
No hospitalization	2 (2.0)	0 (0)	0 (0)	0.7	0.9

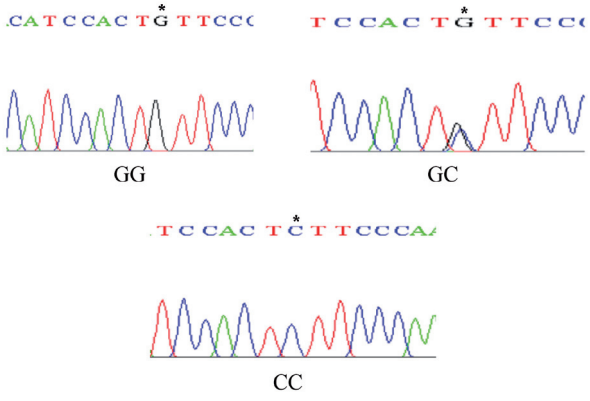

Fig. 2

Chromatograms of HAV polymorphism

Partial chromatograms representing the homozygous G/G, C/C genotype and heterozygous G/C genotype at rs11536889 in the TLR4 promoter region depicting G > C variation.

Table 6. Genotypic distribution and allele frequencies of TLR4 rs11536889 (G/C) polymorphisms in the control and HAV case groups

Groups	No.	Genotype distribution (%)			Allele frequencies (%)	
		GG	GC	CC	G	C
Control	90	49(54.4)	29(32.2)	12(13.4)	127(70.5)	53(29.5)
HAV Cases	90	18(20)	32(35.5)	40(44.5)	68(37.8)	112(62.2)

Groups	No.	Genotype distribution (%)			Allele frequencies (%)	
		GG	GC	CC	G	C
Control	90	49(54.4)	29(32.2)	12(13.4)	127(70.5)	53(29.5)
Acute HAV	72	16(22.2)	23(32)	33(45.8)	55(38.2)	89(61.8)
HAV with cholestasis	14	2(14.2)	8(57.2)	4(28.6)	12(42.9)	16(57.1)
HAV caused FHF	4	0(0)	1(25)	3(75)	1(12.5)	7(87.5)

Table 7. Comparison between the TLR4rs11536889 genotypes and fold change of TLR4 expression in the different HAV case groups

Genotype	Fold change in acute HAV	Fold change in HAV with cholestasis	Fold change in HAV caused FHF
GG	0.685 ± 0.135	–	–
GC	2.748 ± 0.913	0.481 ± 0.050	–
CC	4.23 ± 0.92	13.306 ± 4.606	0.18 ± 0.11

In acute HAV cases p value = 0.003 (statistically significant) CI: 3.26 to 0.86 in GG vs. GC; Acute HAV cases p-value <0.0001 (statistically significant) CI: 4.61 to 2.47 in GG vs. CC; HAV cases with cholestasis p value = 0.0005 (statistically significant) CI: 8.79 to 6.85 in GC vs. CC.

Table 8. TLR4 gene variants and susceptibility to HAV risk (bold values represent significant risk; OR odds ratio; CI confidence interval; p<0.005 is considered statistically significant)

	Controls n = 90	Patients n = 90	OR (95%CI)	p-value
TLR4rs11536889				
GG	49 (54.4)	18(20)	Ref	Ref
GC	29 (32.2)	32 (35.5)	**2.97 (1.42, 6.33)**	**0.003**
CC	12 (13.4)	40 (44.5)	**8.87 (3.8, 21.3)**	**<0.0001**
Allele G	127 (70.5)	68 (37.8)	Ref	Ref
Allele C	53 (29.5)	112 (62.2)	**3.93 (2.53, 6.13)**	**<0.0001**

Table 9. Genotype frequency and odds ratio (OR) of the TLR4 gene polymorphism rs11536889 in different HAV case groups

	Acute HAV (n = 72)	HAV with cholestasis (n = 14)	HAV caused FHF (n = 4)	Acute HAV vs. HAV with cholestasis		Acute HAV vs. FHF	
				p-value	OR	p-value	OR
GG	16 (22.2)	2 (14.2)	0 (0)	0.94	0.97	0.32	0
GC	23 (32)	8 (57.2)	1 (25)	0.12	0.35	0.59	**2.06**
CC	33 (45.8)	4 (28.6)	3 (75)	Ref	Ref	Ref	Ref
G	55 (38.2)	12 (42.9)	1 (12.5)	0.6	0.82	0.15	**4.29**
C	89 (61.8)	16 (57.1)	7 (87.5)	Ref	Ref	Ref	Ref

promoter region depicting G > C variation. Polymorphism study of total of 90 cases shows that CC genotypes are more prevalent in the HAV cases compared to controls (445% vs. 13.4%). There was significant correlation between TLR4 SNP G/C (rs11536889) and AVH between HAV cases and controls (Table 8). Genotype GG, GC [OR = 2.97 (1.42;

6.33), p = 0.003; OR = 8.87 (3.8; 21.3), p <0.0001] and allele C was found to be a significant risk factor [OR = 3.93 (2.53; 6.13), p <0.0001] for HAV infection. However, there was no significant correlation between the TLR4 polymorphism and different HAV cases groups (Table 9).

TLR4 expression

TLR4 expression was most significantly upregulated in cases of HAV with cholestasis (fold increase 10.74 ± 1.45), followed by acute HAV cases (fold increase 4.93 ± 0.92), and HAV caused FHF (fold increase 0.18 ± 0.11) (Fig. 3). The amplification plot and melt curve analysis plot are illustrated in Fig. 4.

Correlation between TLR4 SNP rs11536889 and TLR4 expression in the HAV cases groups

The CC genotype of TLR4 rs11536889 is the most prevalent in the HAV infected cases from Assam. The correla-

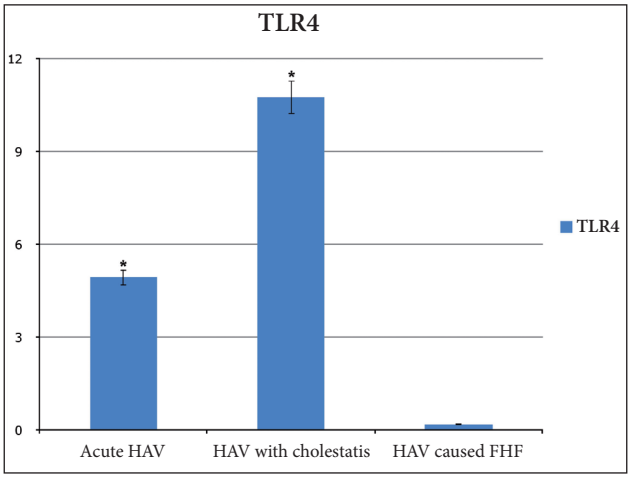

Fig. 3

Bar diagram showing the relative TLR4 mRNA expression in all the HAV cases groups

P-value <0.05 were considered to be statistically significant.

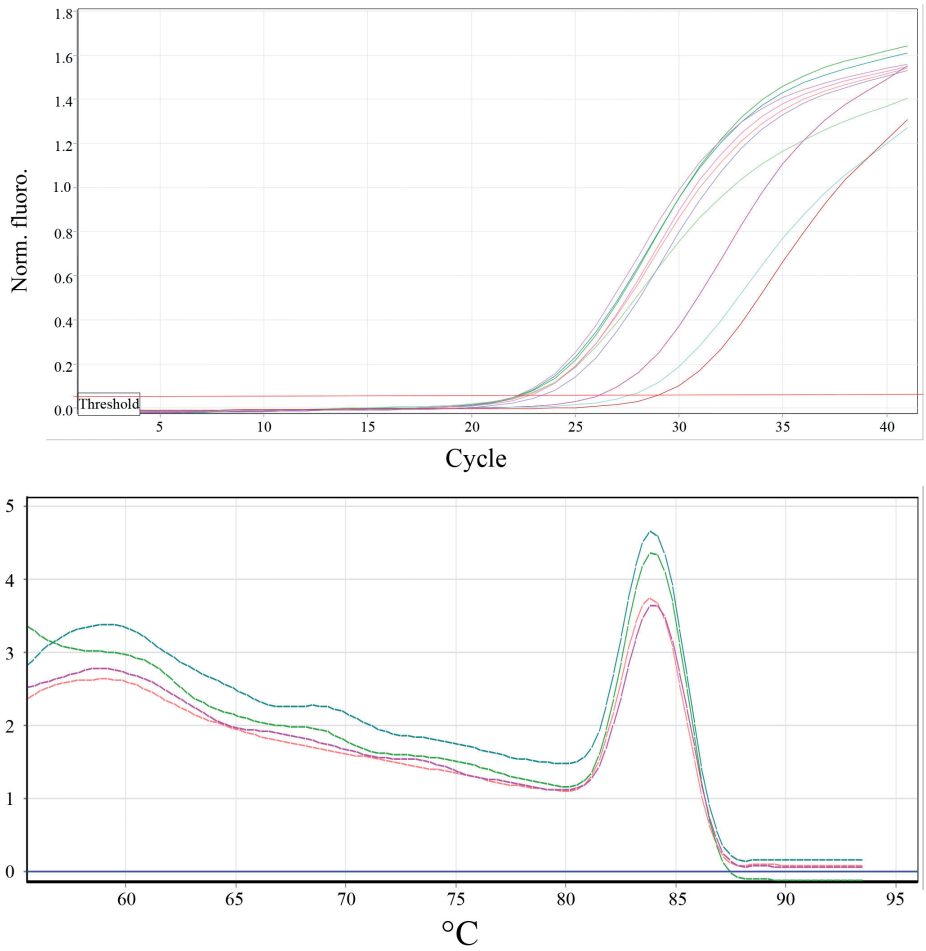

Fig. 4

Representative amplification plot and melt curve of TLR in 9 HAV cases

tion of the 3 different genotypes GG, GC and CC of TLR4 rs11536889 with the TLR4 mRNA expression level in all the HAV cases groups have been found to be statistically significant (Table 7). Our study indicates that TLR4 expression was upregulated in the HAV cases with cholestasis followed by AVH cases and HAV caused FHF cases. The upregulation is mostly seen in the cases with the CC genotype of TLR4 rs11536889 and thus indicates that the mutant variant of TLR4 rs11536899 (CC) may have an effect on the expression of TLR4 at the transcription level.

Association of TLR4 rs11536889 gene polymorphism with different HAV cases groups

The comparison between AVH and HAV cases with cholestasis did not show any significant association ($p = 0.94$; OR = 0.97 at 95% CI) between the genotype GG vs. GC for TLR4 rs11536889. Our study did not show any significant association between AVH and HAV caused FHF ($p = 0.32$, OR = 0; $p = 0.59$, OR = 2.06 at 95% CI) among the genotypes GG, GC and CC (Table 9).

Discussion

TLR4 is unique in being able to signal via both the MyD88-dependent and -independent pathways. This is possible due to the use of two different adaptors, TRIF related adaptor molecule (TRAM) and MyD88 adaptor like molecule (Mal). TLR4 uses the adaptor TRAM to recruit TRIF and induce a type-1 IFN response via the MyD88-independent pathway (Akira *et al*., 2006). Activation of TLR4 causes inflammation by promoting the secretion of inflammatory cytokines, such as TNF-α and IL-6, and anti-viral effects by promoting the secretion of IFN-β through the MyD88-independent pathway. Studies suggest that TLR4 has been associated with the pathogenesis of multiple diseases (Sato *et al*., 2012; Schwartz, 2002; Werner *et al*., 2003; Miedema *et al*., 2011). Studies clearly suggest a role of TLR4 in the pathogenesis of HCV-related liver damage (Gentilucci *et al*., 2012).

Earlier studies on the association of TLR4 SNP rs11536889 with TLR4 expression have demonstrated that C/C genotypes expressed significantly higher levels of TLR4 protein on their surfaces than those from the G/G and G/C genotypes even though no significant change in the expression level was noticed in the TLR4 mRNA expression levels in case of patients associated with periodontitis (Sato *et al*., 2012; Zheng *et al*., 2004). This suggested that TLR4 protein expression was down-regulated in the G/G subjects or up-regulated in the C/C subjects. In this study it can also be seen that patients with CC genotype have increased expression of TLR4 in all the HAV cases groups.

Our study also shows that TLR4 mRNA expression was upregulated in the subjects with CC genotype and was down-regulated in the subjects with GG genotype in both study groups, AVH and HAV cases with cholestasis even though TLR4 expression did not show any upregulation in the HAV caused FHF cases of the three genotypes (GG, GC, CC) thus indicating a role of TLR4 signaling in the clearance of HAV. Our study clearly shows that the CC genotype is related to disease severity as the HAV caused FHF cases and cholestatic group cases have CC genotype.

Study by Machida *et al*. (2006) shows that almost no TLR4 RNA was detectable in healthy individuals, however TLR4 showed an altered expression (a three to seven-fold up-regulation) after HCV infection (Machida *et al*., 2006). Peripheral blood mononuclear cells from HCV-infected individuals showed a higher expression level of TLR4 compared with those of healthy individuals. HCV being a RNA virus like HAV might have a similar response to TLR4 expression as HAV. In the HAV cases, it was found that there is an upregulation of TLR4 expression, mostly in the HAV caused cholestatic cases (thirteen-fold).

Studies also show that TLR4 was up-regulated in the hepatocytes in patients with chronic hepatitis B and TLR4 expression may play an important role in the pathogenesis of chronic hepatitis B (Zhou *et al*., 2011; Wei *et al*., 2008) indicating a potentially important interaction between TLR4 expression and the pathogenesis of viral hepatitis. Zhou *et al*. found that the TLR4 rs11536889 SNP is significantly associated with hepatitis type B virus recurrence after liver transplantation (Zhou *et al*. 2011).

Other studies have also shown quantification of HAV RNA by PCR in Kerala reported by Arankalle *et al.* (2006) and also in Shimla by Chobe and Arankalle (2009) from blood, water and sewage samples.

Hepatitis A infection can cause severe liver disease in adults with a higher morbidity and mortality leading to fulminant liver failure. The HAV caused FHF cases collected in our study show an upregulation in the TLR4 expression but only with the CC genotype. There is no TLR4 upregulation in the FHF cases with the GG and GC genotype thus clearly indicating that CC genotype of TLR4 rs11536889 can be correlated with the HAV disease severity. Also, it is seen that in the FHF cases TLR4 is not much upregulated as compared to the acute HAV and cholestatic cases.

To the best of our knowledge, this is the first investigation evaluating this putative regulatory polymorphism in the key innate immune receptor TLR4 in HAV infected patients, showing a significant association between the TLR4 rs11536889 CC genotype and the severity of the disease.

In conclusion, our data suggest that TLR4 gene polymorphism rs11536889 plays a prominent role in determining susceptibility to HAV infection severity. The present study assesses the possible association of TLR4 SNP rs11536889

and TLR4 expression in the acute viral hepatitis A cases indicating the role of CC genotype of TLR4 rs11536889 in the upregulation of TLR4 expression in the HAV cases. Our studies also show a significant upregulation of TLR4 expression indicating a potentially important interaction between TLR4 expression and the pathogenesis of hepatitis A infection. It is seen that TLR4 rs11536889 mutant variant CC shows an enhanced expression indicating a strong host immune response through TLR4 signaling in HAV disease severity leading to conclusion that TLR4 rs11536889 genetic variation may influence the HAV disease severity and pathogenesis.

According to these results, it would be worthwhile to further assess the TLR4 rs11536889 polymorphism for its relevance to HAV infection in larger and independent cohorts.

Acknowledgment. The work was supported by research grants from the Department of Biotechnology, India. The first author thanks the Department of Science and Technology, New Delhi, India for DST-INSPIRE fellowship.

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