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Association of toll-like receptor (TLR) 2, 7 and 9 gene polymorphism with hepatitis A virus infection risk in population of Assam, India

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ABSTRACT

Hepatitis A is one of the most common causes of acute viral hepatitis worldwide. Hepatitis A infection can also result in a cholestatic hepatitis or a relapsing course or fulminant liver failure. TLR polymorphisms have been implicated in susceptibility, pathogenesis and progression of viral hepatitis.The aim of the present study was to investigate the role of TLR 2 (rs5743708), TLR7 (rs179009) and TLR9 (rs5743836) polymorphism with association of HAV infection risk in population of Assam. The SNP study was performed by genomic DNA extraction followed by PCR–Restriction Fragment Length Polymorphism (RFLP) and the genotypic and allelic frequency distributions were analyzed.Statistical analysis was performed by SPSSv13.0 software. The presence of variant allele A of TLR2 rs5743708 significantly increased the risk of cholestasis compared to controls (p=0.004) [OR=2.5 (1.328- 4.808) at 95%CI]. The TLR7 polymorphism rs179009 was not found to be significantly associated with HAV infection and sisease severity. The presence of the mutant allele T significantly increased the risk of acute HAV [p<0.0001; OR=10.07 (5.242- 19.97) at 95%CI] , HAV with cholestasis [p<0.0001; OR=5.05 (2.784- 9.324) at 95% CI] and FHF (P<0.0001; OR=8.5(4.542- 16.64) at 95% CI). Our results provide first evidence for the TLR2 rs5743708 polymorphism as a host factor affecting HAV infection and thus the disease severity. TLR9 rs5743836 was found to be a significant risk factor for HAV disease severity.

KEY WORDS: CHOLESTASIS, FULMINANT, HEPATITITS A, POLYMORPHISM, TOLL LIKE RECEPTOR.

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INTRODUCTION

Hepatitis A is one of the most common causes of acute viral hepatitis worldwide (MMWR.,1996). Globally, an estimated 1.4 million cases of hepatitis A virus (HAV) infection occur annually. The liver injury from hepatitis A infection is usually mild, mostly in children, where it is frequently subclinical. But in adults, infection with hepatitis A can result in acute or fulminant liver failure. Hepatitis A infection can also result in a cholestatic hepatitis or a relapsing course (Glikson *et al.*,1992). The reported overall case-fatality rate of hepatitis A is between 0.01% to 0.03%; however, it is believed to be much higher in adults. Hepatitis A virus (HAV) belongs to the genus Hepatovirus within the family Picornaviridae and contains a positive-sense, single-stranded RNA genome of approximately 7.5 kb in length (Yokosuka., 2000 and Buggs 2016).

TLRs recognize a wide variety of pathogen associated molecular patterns (PAMPs) from a wide range of host like bacteria, viruses, and fungi, as well as some other host molecules (Aderem and Ulevitch.,2000). TLR's play a key role in the pathophysiology of a range of human diseases, including cancer. TLR2 gene is located on chromosome 4 and recognizes conserved molecular patterns (e.g. lipopolysaccharide (LPS) on bacteria which are major inducers of the inflammatory response. It is involved in the innate immune system (Medzhitov.,2001) and TLR2 is capable of detecting the widest repertoire of pathogen-associated molecular patterns from a large variety of pathogens, including gram-positive or gram-negative bacteria, myco bacteria, fungi, viruses, and parasites.

Genetic studies on the TLR2 gene have identified a number of polymorphisms which have been shown to affect host defense and disease progression (Chen et al.,2010; Takeda *et al.*, 2003). TLR 2 SNP rs5743708 is associated with tuberculosis, staphylococcal septic shock, heart restenosis, Lyme disease and cytomegalovirus (CMV) disease after liver transplantation (Ogus *et al.*, 2004;Lorenz *et al.,* 2000; Schro¨der *et al.*, 2005; Kijpittayarit *et al.*, 2007). Also it correlates with transplant rejection and higher mortality of chronic hepatitis C patients (Eid *et al.,* 2007).

TLR7 is mainly expressed in the endosome-lysosome membrane of plasmacytoid dendritic cells (pDCs), hepatic natural killer cells, and B lymphocytes. TLR7 recognises ss viral RNA which are released upon phagocytosis when phagocytes take up a virus or virus-infected apoptotic cell.

TLR9 is localized intracellularly mostly in the endocytic compartments and TLR9 recognizes unmethylated CpG motifs present in bacteria and viruses (Pandey *et al.,* 2006). TLR9 functions through the MyD88 dependent pathway leading to NF-kappa-B activation, cytokine secretion and the inflammatory response. TLR9 rs5743836 polymorphism has been demonstrated to predispose to Hodgkin lymphoma, as well as to several autoimmune and chronic inflammatory diseases like asthma and Crohn's disease (Hong et al.,2007; Lachheb *et al.*, 2008; Lazarus *et al.,* 2003; Mollaki *et al.*, 2009; Torok *et al.,* 2004).

The role of host genetic factors in determining susceptibility to infections has become more evident; some persons may be predisposed to certain infections, whereas others are protected (Hill, 2001). Evidence suggests that single nucleotide polymorphisms (SNPs) in the TLR genes impair the ability to respond to different type of infection (Weltzler., 2003). And so we hypothesise that a better understanding of the HAV disease infection may be achieved by studying the TLR2, TLR7 and TLR9 gene polymorphism of the susceptible and resistant persons may contribute to the HAV infection severity. The aim of the present study was to investigate the role of TLR 2 (rs5743708), TLR7 (rs179009) and TLR9 (rs5743836) polymorphism with association of HAV infection risk in population of Assam.

MATERIAL AND METHODS

Study subjects: A total of 179 patients with HAV infection were included in the study between 2012 and 2014 with prior written informed consent. An almost equal number of age- and sex- matched healthy controls (voluntary blood donors) who had no history of hepatitis, hepatobiliary diseases, negative for HAV IgM and autoimmune hepatitis, were also included in the study. Patients were enrolled from general OPD and Medicine Ward of Gauhati Medical College and Hospital (GMCH), Guwahati, Assam on the basis of clinical investigation, positive for HAV IgM using 3rd generation ELISA kit and confirmed by HAV detection PCR spanning a region of 154 b.p. for VP1 region. Patients who were positive for other hepatotropic virus, HIV, autoimmune hepatitis, alcoholic and drug abuse were excluded. The study was approved by the institutional ethical committee of Gauhati University, Guwahati.

In this study, 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 there would be evidence of coagulation abnormality, usually a prolongation of PT by 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).

DNA EXTRACTION

Blood samples (5.0 ml) from HAV patients and control subjects were collected in EDTA vials and stored at 4 °C until required. Genomic DNA was extracted from whole blood by standard Proteinase-K digestion and phenol/chloroform extraction procedure.

GENOTYPING

TLR2 (rs rs5743708), TLR7 (rs179009) and TLR9 (rs5743836) genotypes were determined by a polymerase chain reaction (PCR) method, and were genotyped by PCR–Restriction Fragment Length Polymorphism (RFLP). The PCR product of 152 b. p. of TLR2 rs5743708 was amplified using the set of primers 5'-GCTGCCATTCT-CATTCTTCTG-3'and5'-CGCAGCTCTCAGATTTACCC-3' and digested with the restriction enzyme Pst1 (MBI Fermentas, USA) at 37°C overnight, and then analyzed on 2.5% agarose gel electrophoresis. A single band of 152 b. p. characterised wild type G allele, while the presence of three bands at 152, 85 and 67 b.p. or two bands at 85 and 67 b.p. only characterises heterozygous and homozygous variant respectively (A allele is the mutant allele).

The PCR product of 190 b. p. of TLR7 rs179009 was amplified using the set of primers 5'- ATTTGCTGC-CTCTCTTTTGC-3' and 5'-GCTGCTTCTACCCTCTCGAA-3' and digested with the restriction enzyme Hin1 (MBI Fermentas, USA) at 37°C overnight, and then analyzed on 2.5% agarose gel electrophoresis. A single band of 190 b. p. characterised mutant type C allele, while the presence of three bands at 190, 104 and 86 b.p. or two bands at 104 and 86 b.p. only characterises heterozygous and homozygous variant respectively (T allele is the wild allele).

The PCR product of 164 b. p. of TLR9 rs5743836 was amplified using the set of primers 5'-GCAAA-GGAGCTCAGGAGTG-3' and 5'-GCCTTGGGATGTGCT-GTTC-3'digested with the restriction enzyme BstN1 (MBI Fermentas, USA) at 37°C overnight, and then analyzed on 2.5% agarose gel electrophoresis. A single band of 164 b. p. characterised mutant type T allele, while the presence of three bands at 164, 136 and 28 b.p. or two bands at 136 and 28 b.p. only characterises heterozygous and homozygous wild variant respectively (C allele is the wild allele). Positive and negative controls were used in the genotyping protocol and 5% of randomly selected samples were re-genotyped by other lab member with 100% concordance.

STATISTICAL ANALYSIS

All statistical analyses were performed by the standard methods using SPSS computer software (Version 13, SPSS Inc., Chicago, IL, USA). Results were expressed as means $\pm$ standard deviations (SD). A two-tailed p value < 0.05 was considered statistically significant. Chi-square (χ^2) analysis was used to assess deviation from Hardy–Weinberg equilibrium (HWE) and to compare the genotype and allele frequency between patients and controls. Odds ratio (OR) were obtained by unconditional logistic regression analysis.

RESULTS AND DISCUSSION

DEMOGRAPHIC DATA OF THE STUDY SUBJECTS

A total of 179 HAV confirmed cases were included in the study. The male and female ratio was found out to be 17:9 for all the HAV cases together and so the male gender was predominant (65%) (Table I). It was seen that the confirmed cases of HAV infection belonged mostly to the young adults in this region and so Hepatitis A frequently occurred in the young adults where mean age was calculated to be 25 years. Fulminant hepatic failure developed in 2.8% of the Hepatitis A patients (Table II). Among the 179 HAV patients, 11.2% patients had prolonged cholestasis during admission.

Table 1: Clinical and laboratory characteristics of hepatitis A cases (n = 100) [ALT – alanine transaminase; AST –amino serum transaminase]			
Parameter	Acute Hepatitis A (n=154)	HepatitisAwith cholestasis (n=20)	Fulminant Hepatic Failure (FHF) (n=5)
Age	25 $\pm$ 7.12	28 $\pm$ 12.41	34 $\pm$ 10.44
Sex ratio	7:4	7 :3	3:2
ALT(IU/L) ≤ 40	468 $\pm$ 171.24	923 $\pm$ 192.21	4763.5 $\pm$ 744.58
AST(IU/L) ≤ 40	252.33$\pm$ 220.94	747.7 $\pm$ 168.34	4416 $\pm$ 642.05
Prothrombin time(INR) (2.0 – 3.0)	1.20 $\pm$ 0.60	2.36 $\pm$ 2.12	8 $\pm$ 0.56
Total Bilirubin(mg/dl) 0.4-1.2	5.37 $\pm$ 0.84	16.24 $\pm$ 11.22	18.40 $\pm$ 11.30
Total protein (gm/dl) 6.5-8	7 $\pm$ 1.98	7.95 $\pm$ 1.16	8.1 $\pm$ 0.42

Table 2: Stratification of the Hepatitis A cases based on their clinical profile	
Cases	Frequency (case/total)
Acute hepatitis A	86 (154/179)
Hepatitis A with cholestasis	11.2 (20/179)
Fulminant Hepatic Failure (FHF)	2.8 (5/179)

ASSOCIATION OF TLR2 (RS5743708), TLR7 (RS179009) AND TLR9 (RS5743836) GENE VARIANTS WITH HAV INFECTION RISK

The observed genotype frequencies in the study subjects were in agreement with Hardy Wienberg Equilibrium. To analyse the association between genetic variant with risk of HAV infection, the genotype frequency distribution of TLR2, 7 and 9 in HAV cases and control group was compared and shown in Table III.

The genotype and allele frequencies observed at this rs site 5743708 were in agreement with Hardy-Weinberg Equilibrium. There was no significant difference between the allelic and genotypic frequencies among the HAV cases and controls (p=0.4) [OR=1.2 (0.7063- 2.276) at 95% CI]. TLR2 rs5743708 wild variant GG was found more in controls compared to cases (37.6% vs 33%) but

overall the heterozygous variant GA was most prevalent in both control and HAV cases (52.9 % and 34 % respectively). The mutant variant AA was more prevalent in cases than controls (33% vs 9.5%). The variant allele A was found overall more in HAV cases compared to controls (67% vs 62.4 %) (Table III).

The wild variant TT of TLR7 rs179009 frequency was higher in controls compared to HAV cases (68.9% vs 67.6%). The wild allele T was dominant in our study cohort (78% and 74.6% in controls and HAV cases respectively). The mutant variant CC was comparatively less in both HAV cases and controls (18.4% vs 12.9%). There was no significant difference between the allelic and genotypic frequencies among the HAV cases and controls (p=0.9). The presence of mutant genotype CC did not significantly increased the risk of HAV infection compared to controls [OR=1.0(0.5619- 1.864) at 95%CI] (Table III).

The wild variant CC frequency was higher in controls compared to HAV cases (69.4% vs 17.3%). The mutant variant TT prevalence was very high in HAV cases compared to controls (76.6% vs 2.4%). There was significant difference between the allelic and genotypic frequencies among the HAV cases and controls (p<0.0001). The presence of mutant genotype TT significantly increased the

Table 3: TLR2, TLR7 and TLR9 gene variants and susceptibility to HAV infection risk [Bold values represent significant risk ; OR Odds ratio; CI confidence interval]				
	Controls n=170	Patients n=179	OR (95%CI)	p value
TLR2rs5743708				
GG	64(37.6)	59(33)	Ref	Ref
GA	90(52.9)	61(34)		
AA	16(9.5)	59(33)		
GA+AA	62.4	67	1.2((0.7063- 2.276)	0.4
Allele G	218(64.1)	179(50)	Ref	
Allele A	122(35.9)	179(50)	1.78(1.011- 3.156)	0.04
TLR7rs179009				
TT	117(68.9)	121(67.6)	Ref	Ref
CT	31(18.2)	25(14)		
CC	22(12.9)	33(18.4)		
CT+CC	31.1	32.4	1.0(0.5619- 1.864)	0.9
Allele T	265(78)	267(74.6)	Ref	Ref
Allele C	75(22)	91(25.4)	1.1(0.5981- 2.234)	0.6
TLR9rs5743836				
CC	118(69.4)	34(17.3)	Ref	Ref
CT	48(28.2)	93(6.1)		
TT	4(2.4)	52(76.6)		
CT+TT	30.6	82.7	10.2(5.30- 20.29)	<0.0001
Allele C	284(83.5)	161(45)	Ref	Ref
Allele T	56(16.5)	197(55)	6.1(3.202-12.1)	<0.0001

Table 4: Genotypic frequency of TLR2 gene variants and susceptibility to different HAV complications [Bold values represent significant risk;OR Odds ratio;CI confidence interval].

Groups	Genotype distribution (%)			Allele frequencies (%)		Less common allele frequency	χ2 value (p value)	Odds ratio (95% CI)
	GG	GA	AA	G	A			
Control (n=170)	64(37.6)	90(52.9)	16(9.5)	218(64.1)	122(35.9)	62.4%	ref	
Acute HAV(n=154)	55(35.7)	55(35.7)	44(28.6)	165(53.6)	143(46.4)	64.3%	0.6	1.1(0.6296-2.005)
HAVwith cholestasis (n=20)	4(20)	4(20)	12(60)	12(30)	28(70)	80%	0.004	2.5 (1.328-4.808)
HAV caused FHF (n=5)	0(0)	2(40)	3(60)	2(20)	8(80)	100%	<0.0001	0

Table 5: Genotypic frequency of TLR7 gene variants and susceptibility to different HAV complications [Bold values represent significant risk; OR Odds ratio;CI confidence interval].

Groups	Genotype distribution (%)			Allele frequencies (%)		Less common allele frequency	χ2 value (p value)	Odds ratio (95% CI)
	TT	CT	CC	T	C			
Control (n=170)	117(68.9)	31(18.2)	22(12.9)	265(78)	75(22)	31.1%	ref	
AcuteHAV (n=154)	108(70.1)	21(13.7)	25(16.2)	237(77)	71(23)	29.9%	0.8	0.9 (0.52-1.749)
HAVwith cholestasis (n=20)	13(65)	3(15)	4(20)	29(72.5)	11(27.5)	35%	0.5	1.1 (0.6589-2.162)
HAVcaused FHF (n=5)	0(0)	1(20)	4(80)	1(10)	9(90)	100%	<0.0001	0

risk of HAV infection compared to controls [OR=10.2 (5.30- 20.29) at 95%CI] by several folds (Table III).

ASSOCIATION OF TLR2(RS5743708), TLR7(RS179009) AND TLR9(RS5743836) GENE POLYMORPHISM WITH DIFFERENT HAV COMPLICATIONS

The genotypic frequencies of the TLR2, TLR7 and TLR9 SNP's were correlated with the two HAV case groups ie HAV with cholestasis and HAV caused FHF to investigate whether any possible association is implicated between the polymorphisms and the HAV complications.

In case of different HAV case groups, the presence of variant allele A of TLR2 rs5743708 significantly increased the risk of cholestasis compared to controls (p=0.004) [OR=2.5 (1.328- 4.808) at 95%CI] and non significantly compared to Acute HAV (p=0.6) [OR=1.1(0.6296- 2.005) at 95% CI) and HAV caused FHF (p<0.0001) [OR=0 at 95% CI) . The variant allele was most prevalent in FHF cases (100%) compared to controls and other HAV case groups (Table IV).

When the distribution of *TLR7 rs179009* was studied in the different HAV case groups, the data showed that

presence of the wild variant TT was higher in acute HAV cases (70.1%) compared to the controls (68.9%) and HAV with cholestasis (65%), but was not found in the FHF cases (0%) . The mutant variant allele C was most prevalent in FHF cases (90% vs 22%) compared to controls. The mutant variant CC was associated significantly with HAV caused FHF (p<0.0001) and was not found to be a risk factor (OR=0) . This polymorphism is also not significantly associated with acute HAV (p=0.8) and HAV with cholestasis (p=0.5), and did not significantly increase the risk of acute HAV [OR=0.9 (0.52- 1.749) at 95%CI] and HAV with cholestasis [OR= 1.1 (0.6589- 2.162) at 95% CI] (Table V).

The distribution of *TLR9 rs*5743836 in the different HAV case groups showed that presence of the wild variant CC was higher in controls (69.4%) compared to the acute HAV (17.5%) and HAV with cholestasis (30%), and FHF cases (20%) . The heterozygous mutant variant CT was most prevalent in FHF cases (80% vs 28.2%) compared to controls and other case groups. The overall mutant variant (CT+TT) was more prevalent in the HAV case groups rather than the controls. The presence of the mutant allele T significantly increased the risk of acute HAV [p<0.0001; OR=10.07 (5.242- 19.97) at 95%CI] ,

HAV with cholestasis [p<0.0001; OR=5.05 (2.784- 9.324) at 95% CI] and FHF (P<0.0001; OR=8.5(4.542- 16.64) at 95% CI) (Table VI).

There is ever increasing evidence suggesting the role of host genetic factors on the different outcomes of hepatitis infection (Thurz., 2001; Lin *et al.,* 1989). The TLR pathways have been implicated to play important roles in innate immunity against different types of pathogens in humans (Isogawa *et al.,* 2005; Hoebe *et al.,* 2003; Kurt-Jones *et al.,* 2004).

Understanding the host immune factors and its interaction with the virus may improve the Hepatitis A disease management. Genetic studies on the TLR2 gene have identified a number of polymorphisms which have been shown to affect host defense and disease progression (Chen *et al.,* 2010; Takeda *et al.,* 2003).

Studies have shown the possible involvement of TLR9 in the pathogenesis of human hepatitis (Jiang *et al.,* 2009). The TLR7 gene is located on the X-chromosome, covering a region of three exons (Du *et al.,* 2000).TLR7 have been thought to play a pivotal role in the immune response against HAV infection as it can bind ssRNAs leading to the production of large amounts of the antiviral cytokine interferon-α via dendritic cells (Colonna *et al.,* 2002).

This is the first study of its kind in this region which shows that TLR2 rs5743708 mutant allele A was significantly associated with Hepatitis A infection and the mutant variant 'A' was prevalent in our study cohort ie in the polpulation of this region (both control and patient groups).The genotypic variants AA and GA+AA was also found to be risk factor for HAV caused cholestasis. The mutant variants (TT+CT) was most prevalent for TLR9 rs5743836 in our study cohort and the mutant variant T was found to be a significant risk factor for HAV infection and was significantly associated with acute HAV, Cholestasis and FHF. TLR7 rs179009 mutant variant CC was highly prevalent in the study and patient groups and the mutant genotypes CC and TC was not significantly associated with HAV infection and was not a risk factor for the same.

There was certain limitation in our study that needs to be addressed. The sample number in this study was moderate and so other large population-based studies are needed to validate the findings.In summary, our results provide first evidence for the TLR2 rs5743708 polymorphism as a host factor affecting HAV infection and thus the disease severity. Ultimately, such studies may also offer an opportunity to find out how TLR-mediated signals might be used as future therapeutic targets.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest and also no financial interests relevant to the subject of the manuscript.

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