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ORIGINAL ARTICLE

Association of HLA and TNF polymorphisms with the outcome of HBV infection in the South Indian population

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*The role of host genetic factors in the pathogenesis and outcome of hepatitis B virus (HBV) infection is not well known. We assessed the association of HLA and TNF (rs361525, rs1800629, rs1799724, rs1800630 and rs1799964) polymorphisms with HBV outcome in the South Indian population. Association of HLA polymorphism was analyzed in 90 individuals from each group, that is, spontaneous recovery (SR) and chronic-HBV (C-HBV) infection. The role of TNF polymorphisms was evaluated in 150 subjects with SR and 137 patients with C-HBV infection. After adjusting for age and sex, HLA-DRB1*07:01 was strongly associated with chronicity (corrected P-value (pc) <0.005, odds ratio (OR) 3.76, 95% confidence interval (CI) 1.84–7.68). The rs1800630 genotype was associated with HBV outcome in codominant ($pc<0.01$, $OR=1.99$, 95% CI 1.30–3.05) and dominant ($pc<0.01$, $OR=2.28$, 95% CI 1.35–3.84) analyzing models after adjusting for age and sex. Similarly, the rs1799964 genotype was associated with HBV outcome in codominant ($pc=0.01$, $OR=1.57$, 95% CI 1.09–2.27) and dominant ($pc<0.01$, $OR=2.21$, 95% CI 1.27–3.83) analyzing models. Haplotype analysis (rs1799964/rs1800630/rs1799724/rs1800629/rs361525) revealed that the CACGG haplotype was strongly associated with C-HBV infection ($P=0.0004$). Our study suggests that inheritance of HLA and TNF polymorphisms might explain the outcome of HBV infection in the South Indian population.*
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Keywords: *HLA; TNF-α; HBV outcome*

Introduction

Hepatitis B virus (HBV) infection is a serious public health problem.[1] HBV infects more than 400 million people worldwide, and 75% of them are Asians.[2,3] India harbors the second largest global pool of chronic HBV infection.[4] HBV causes a broad spectrum of diseases from subclinical infection to acute, fulminant and chronic infection. The majority of infection in the adulthood is self-limited, but 5–10% progress into chronic hepatitis. Several lines of evidence indicate that the dichotomy of HBV outcome is largely determined by the host immune response. We have also suspected a strong genetic reason for clustering of chronic HBV carrier in India because of its endogamous population structure.[5,6] In addition, familial clustering of HBV infection has been shown in the Indian population.[7]

HBV is a known noncytolytic hepatotropic virus.[8] Individuals with acute HBV infection who clear the virus elicit strong, polyclonal class 1- and II-restricted cell responses. In contrast, the cellular responses are weak and restricted in patients with chronic HBV infection.[8,9] Thus, immune responses evoked against HBV-encoded antigens are responsible for the liver injury and the associated pathology in the HBV disease. The major histocompatibility complex (MHC) class II molecule function is essential for antibody response to hepatitis B surface antigen (HBsAg). The antibodies to HBsAg (anti-HBs) curtail the spread and clear the circulating virus particles.[10] The MHC class I molecule function is important for cytolytic elimination of HBV-infected hepatocytes.[8] Thus, *HLA* polymorphism is one of the major host determinants that can influence the susceptibility, severity and the outcome of HBV infection.

Tumor necrosis factor-α (TNF-α) is a chief proinflammatory cytokine. It is described as a potential type 1 immunoregulatory cytokine functionally different in modulating the detrimental type 1 immune response.[11] The level of cytokine production varies among individuals, and this may correlate with polymorphisms in the cytokine gene promoters.[12] Many single nucleotide polymorphisms are described in the promoter region of the *TNF* (NM_000594.2).[13–16] These promoter polymorphisms are known to influence TNF-α transcription.[17,18] TNF-α determines host cytolytic and noncytolytic antiviral response to HBV infection.[19] The cytolytic and noncytolytic control of HBV replication is mediated by TNF-α and interferon-γ secreted by natural killer cells, natural killer T cells and $CD8^+$ cells.[20–22]

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The aim of the present study was to ascertain the association of *HLA* polymorphisms and the five single nucleotide polymorphisms located near 5′ of *TNF* (NM_000594.2:c. −238:G>A, −308:G>A, −857:C>T, −863:C>A, −1031:T>C) with the outcome of HBV infection in the South Indian population.

Results

There was a significant difference in median age between spontaneously recovered (SR) group (33.2 ± 9.1) and chronic-HBV (C-HBV) group (36.9 ± 13.4; $P<0.001$). The gender ratio (female/male) was significantly different between SR group (4:146) and C-HBV group (36:101; $P<0.001$). Among the C-HBV carriers, 58.4% were positive for HBV DNA with the median viral load of 402.5 IU ml^{-1} (2–7 × 10^7 IU ml^{-1}). Of C-HBV carriers, 45 (32.8%) showed elevation of serum alanine transaminase levels with the median of 47 U l^{-1} (36–356 U l^{-1}). The histological activity index (HAI) was available for 27 chronic liver disease patients and a majority of them had HAI of ⩽5, indicating mild liver disease except one patient (HAI 8/16). Hepatic fibrosis score (HFS) was available for 9 patients; a majority of them had mild fibrosis (HFS ⩽2), one had HFS of 5/6 indicating severe liver disease and other one had indication of mild steatosis.

HLA *polymorphism and HBV outcome*

No association of class I (A and B) MHC alleles were found between individuals with SR and C-HBV carriers after correction for multiple comparisons (data not shown). However, the allelic frequency (AF) of *HLA-B*44* was higher in the C-HBV carriers than individuals with SR (18.07 vs 3.45%; odds ratio (OR) 6.23, 95% confidence interval (CI) 1.66–23.34, $P=0.007$).

The distribution of class II (*DR*) alleles and association with HBV outcome are shown in Table 1. A significantly higher frequency of *HLA-DRB1*07:01* was observed in patients with chronicity (57.83% of 83) compared with individuals with SR (24.71% of 85) after correction for multiple comparisons (OR 3.76, 95% CI 1.84–7.68, pc<0.006). On the contrary, the allelic frequency of *HLA-DRB1*03:01* was higher in the SR group (12.94% of 85) than the C-HBV group (1.2% of 83; OR 0.08; 0.003–0.64; $P=0.007$). None of the *DQB1* alleles were associated with HBV outcome (data not shown).

TNF polymorphism and HBV outcome

Among the *TNF* polymorphisms only rs1800630 allele was found to be strongly associated with HBV outcome even after correcting for multiple comparison using permutation tests ($P=0.006$; data not shown). The frequency of *TNF* genotypes in the two groups did not violate Hardy–Weinberg equilibrium; data not shown).

Univariate analysis showed a significant association of rs1800630 genotypes with HBV outcome (pc<0.01). We found a strong association between rs1800630 genotypes and HBV outcome in the codominant (OR = 1.99, pc<0.01) and in the dominant (OR = 2.28, pc<0.01) analyzing models after adjusting for age and sex (Table 2). Likewise, univariate analysis revealed a trend between rs1799964 genotypes and HBV outcome ($P=0.025$). After adjusting for age and sex, rs1799964 genotypes showed a significant association with HBV outcome in the codominant (OR = 1.57, pc = 0.01) and the dominant (OR = 2.21, pc<0.01) analyzing models (Table 2).

Linkage disequilibrium coefficients among five *TNF* single-nucleotide polymorphisms are shown in Figure 1. The frequencies of *TNF* haplotypes are shown in Table 3. Among the six haplotypes, the second predominant *TNF* haplotype 2 (rs1799964C; rs1800630A; rs1799724C; rs1800629G; rs361525G) was associated with C-HBV infection ($P=0.0004$). This association is significant even after correction for multiple comparison using permutation test (pc = 0.004; Table 3).

Differential association of TNF genotypes with HLA-DRB1*07:01 *in chronic HBV outcome*

The frequency distributions of rs1800630 and rs1799964 genotypes in *HLA-DRB1*07:01* individuals are shown in Table 4. The frequency of rs1800630 genotypes was not significantly different between *HLA-DRB1*07:01*-positive and -negative individuals. The difference in the frequency

Table 1 Frequency distribution and association of MHC class II *DRB1* alleles with HBV outcome

MHC class II alleles	*HBV outcome*				*OR (95% CI)*	P-*value*
	Spontaneous recovery, total (n = *90*)		*Chronicity, total* (n = *90*)			
DR	n = *85*[a]	*AF %*	n = *83*[a]	*AF %*		
*DRB1*07*	21	24.71	48	57.83	3.76 (1.84–7.68)	<0.0001[b]
*DRB1*15*	36	42.35	36	43.37	0.83 (0.42–1.63)	0.59
*DRB1*04*	25	29.41	16	19.28	0.65 (0.29–1.40)	0.27
*DRB1*14*	17	20	10	12.05	0.76 (0.31–1.88)	0.55
*DRB1*13*	13	15.29	5	6.02	0.34 (0.09–1.16)	0.09
*DRB1*10*	10	11.76	11	13.25	1.23 (0.45–3.33)	0.68
*DRB1*11*	8	9.41	11	13.25	1.72 (0.59–4.97)	0.31
*DRB1*03*	11	12.94	1	1.2	0.08 (0.003–0.64)	0.007[c]
*DRB1*08*	4	4.71	6	7.23	1.91 (0.50–7.34)	0.34[c]

Abbreviations: AF, allele frequency; CI, confidence interval; HBV, hepatitis B virus; MHC, major histocompatibility complex; OR, odds ratio.
Alleles that had frequency <5% in both the groups were not shown in the table (*DRB1 12, *01, *02* and **16*).
[a]Indicates number of samples typed; others failed to amplify.
[b]After correction for multiple comparison, pc<0.006 is significant.
[c]Indicates Yates' correction.

Table 2 Association between *TNF* genotypes and HBV outcome

TNF genotypes	*Spontaneously recovered* (n = *150*)	*Chronic-HBV* (n = *137*)	*Univariate analysis*	*Multivariate analysis after adjusting for age and sex*		
				Analyzing models		
			P-*value*	*Codominant OR (95% CI)* P-*value*	*Dominant OR (95% CI)* P-*value*	*Recessive OR (95% CI)* P-*value*
rs361525	150	137	0.868	1.01 (0.57–1.77) 0.973	1.04 (0.55–1.6) 0.898	0.78 (0.10–5.41) 0.77
GG	122 (81.3%)	108 (78.8%)				
AG	25 (16.7%)	26 (19%)				
AA	3 (2%)	3 (2.2%)				
rs1800629	150	137	0.069	0.43 (0.16–1.11) 0.082	–	–
GG	132 (88%)	129 (94.2%)				
AG	18 (12%)	8 (5.8%)				
AA	–	–				
rs1799724	149[a]	135[a]	0.469	0.75 (0.40–1.42) 0.389	0.72 (0.36–1.42) 0.347	0.98 (0.09–11.15) 0.985
CC	120 (80.6)	116 (86%)				
CT	27 (18.1%)	18 (13.3%)				
TT	2 (1.3%)	1 (0.7%)				
rs1800630	148[a]	135[a]	0.006[b]	1.99 (1.30–3.05) 0.001[b]	2.28 (1.35–3.84) 0.002[b]	2.59 (0.91–7.32) 0.072
CC	91 (61.4 %)	61 (45.1%)				
CA	51 (34.5%)	58 (43%)				
AA	6 (4.1%)	16 (11.9%)				
rs1799964	147[a]	135[a]	0.025	1.57 (1.09–2.27) 0.016	2.21 (1.27–3.83) 0.005[b]	1.36 (0.69–2.66) 0.37
TT	64 (43.5%)	38 (28.1%)				
TC	61 (41.5%)	69 (51.1%)				
CC	22 (15%)	28 (20.8%)				

Abbreviations: CI, confidence interval; HBV, hepatitis B virus; OR, odds ratio; TNF, tumor necrosis factor.
[a]Indicates number of samples typed in each polymorphic site and others not amplified.
[b]After correction for multiple comparison, pc < 0.01 is significant.

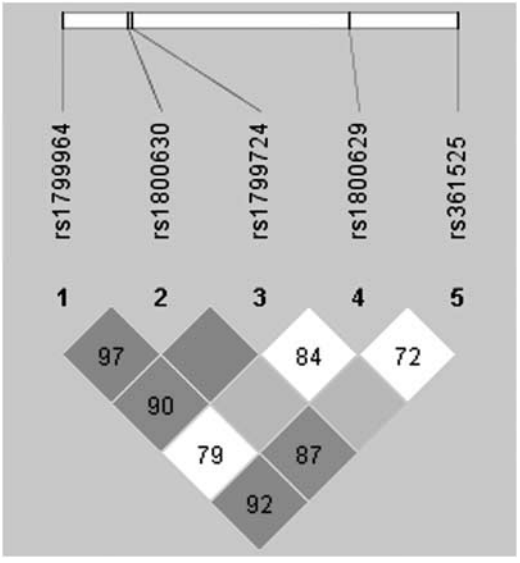

Figure 1 Linkage disequilibrium (LD) as measured by D′ values were assessed using Haploview 4.1. The range of D′ is from −1 to 1, and LD is considered for marker pairs with D′ >0.8. The figures inside the boxes correspond to D′ values × 100. The red square indicates D′ = 1 and logarithm of the odds (LOD) ⩾2, the blue color square indicates D′ = 1 and LOD <2 and white square indicates D′ <1 and LOD <2. LD was detected between rs1799964 and rs1800630; rs1799724 and rs361525; and rs1800630 and rs361525, respectively. A full colour version of this figure is available at the *Genes and Immunity* journal online.

of rs1799964 genotypes was found to be marginally significant between *HLA-DRB1*07:01*-positive and -negative individuals ($P = 0.05$).

Discussion

HLA *polymorphism and HBV outcome*

Polymorphic changes within the MHC regions have been shown to influence the immune response against HBV infection.[23] Our study showed the association of predominant South Indian *HLA* class II allele (*DRB1*07:01*) with C-HBV outcome. This has important biological relevance because individuals who clear the HBV are known to elicit strong, polyclonal class I- and II-restricted cell responses but the cellular responses are weak and restricted in patients with C-HBV infection.[8,9]

Our study revealed that *HLA-B*44* and *DRB1*03:01* alleles were associated with C-HBV and SR ($P = 0.007$) outcomes, respectively. After adjusting for multiple comparisons, these alleles were not associated with HBV outcome. Besides, wide variations in the frequency distribution of these alleles are known to occur in the South Indian castes and tribes.[24,25] We speculate that this variation may impact the epidemiology and outcome of HBV infection in the South Indian population. However, this requires study with larger sample size including various ethnic groups of the South Indian population.

*HLA-DRB1*07:01* allele showed a strong association with C-HBV infection (pc < 0.006). The frequency distribution of this allele was also highest among the *DRB1* alleles in our study population. This observation is concordant with an earlier study by Shankarkumar and Sridharan.[26] Our study also revealed that HBV infection of *HLA-DRB1*07:01*-positive individuals had a 3.8-fold higher risk of progression to chronicity. This OR is higher than what is normally seen (OR 1.2–2) in various genetic association studies.[27] Our study also independently

Table 3 Association of *TNF* haplotypes with the outcome of HBV infection

No. of haplotype	*TNF haplotypes*					*Frequency*	P-*value*	P-*value*[a]
	rs1799964 T>C	*rs1800630 C>A*	*rs1799724 C>T*	*rs1800629 G>A*	*rs361525 G>A*			
1	T	C	C	G	G	0.456	0.22	0.65
2	C	A	C	G	G	0.261	0.0004	0.004
3	C	C	C	G	A	0.100	0.43	0.99
4	T	C	T	G	G	0.088	0.27	0.79
5	T	C	C	A	G	0.044	0.11	0.45
6	C	C	C	G	G	0.036	0.04	0.19

Abbreviations: HBV, hepatitis B virus; TNF, tumor necrosis factor.
[a]Permutation *P*-value (number of permutation = 10 000).

Table 4 Differential association of *TNF* genotypes with *HLA-DRB1*07:01* in chronic HBV outcome

TNF-α genotypes	*DRB1*07:01*[a]		P-*value*
	Positive (n = *48*)	*Negative* (n = *35*)	
rs1800630[b]			
CC	24 (50%)	15 (42.8%)	0.425
CA	17 (35.4%)	17 (48.6%)	
AA	7 (14.6%)	3 (8.6%)	
rs1799964[c]			
TT	9 (18.8%)	15 (42.8%)	0.05
TC	28 (58.2%)	16 (45.7%)	
CC	11 (23%)	4 (11.5%)	

Abbreviations: HBV, hepatitis B virus; TNF-α, tumor necrosis factor-α.
[a]Associated with chronicity in HBV infection (odds ratio (OR) = 3.76, pc < 0.006).
[b]Associated with HBV outcome in the codominant (0.001) and dominant (0.002) analyzing models (refer Table 2).
[c]Associated with HBV outcome in the codominant (0.016) and dominant (0.005) analyzing models (refer Table 2).

replicated the association of *HLA-DRB1*07* with persistent HBV infection as shown earlier by Almarri and Batchelor.[28] Furthermore, studies have shown the association of *HLA-DRB1*07* with nonresponsiveness to HBV vaccination in various populations.[29–32] We speculate that the strong association of *HLA-DRB1*07*:01 with chronicity could be either because of the inability of this allele to present epitopes effectively or because of the inheritance of certain markers linked to other important genes. This warrants further functional and genetic studies to identify the precise role of *HLA-DRB1*07:01* with persistence of HBV infection. This finding may have an impact on the South Indian population as the frequency of *HLA-DRB1*07* have been shown to be higher in this population.[26] Therefore, this observation is more pertinent to the South Indian population than populations where the frequency of this allele is low.

TNF *polymorphism and HBV outcome*

TNF-α determines host cytolytic and noncytolytic antiviral response to HBV infection.[19] Among the *TNF* polymorphisms, only rs1800630 alleles showed a significant trend with the outcome of HBV infection ($P = 0.027$; data not shown). In addition, the rs1800630 genotypes were associated with HBV outcome in the codominant and dominant analyzing models. We speculate that the ORs (1.99 and 2.28) of our study reflect the complex Mendelian trait (HBV disease outcome) and influence of several polymorphic genes on the outcome of HBV infection.[27] However, this association is consistent with the expected OR (1.2–2) that is commonly seen in genetic association studies.[27] According to Thursz,[27] an OR of 1.2–2 requires thousands of cases than hundreds for a valid genetic association studies. Considering this criterion, the potential drawback of our study could be the sample size, which was overcome by using permutation test.[33] Empirical *P*-values were obtained for 10 000 permutation that showed a strong association between rs1800630 polymorphism and the outcome of HBV infection ($P = 0.006$). Our study also independently replicated the earlier findings of a Korean study and a Taiwanese study that showed an association of rs1800630 polymorphism with the outcome of HBV infection.[34,35] This independent replication strengthens the association of rs1800630 polymorphism with HBV outcome.

We believe that the association of rs1800630 polymorphism with HBV outcome should have certain functional basis than serving as a mere marker. Hence, it is reasonable to propose that the *TNF* mutant A-allele (rs1800630) might lead to the production of low level of TNF-α resulting in C-HBV infection. Corroborating this notion, a functional study has shown that the rare variant of rs1800630 (A-allele) exhibited 31% lower transcriptional activity than the wild-type C-allele.[36] Recently, the *TNF* mutant A-allele has been shown to predict lower HBcAg-inducible TNF-α secretion and is also associated with chronicity.[35] We speculate that the transcriptional difference in rs1800630 promoter alleles influence pathogenesis and the outcome of HBV infection in the South Indian population. However, comprehensive functional studies are essential to unravel the underlying mechanism of rs1800630 polymorphism on the outcome of HBV infection.

We also found that rs1799964 polymorphism was associated with HBV outcome. Worldwide, the evidence for association of rs1799964 polymorphism with HBV outcome is highly limited. Till date, only one study has emphasized the importance of rs1799964 promoter wild-type allele-C and rs1800630 allele-A with prognosis of

fulminant hepatitis.[37] The other *TNF* promoter polymorphisms (rs361525, rs1800629 and rs1799724) that are known to influence TNF-α level were not associated with HBV outcome in this study. However, many studies have shown the association of these polymorphisms with diverse HBV outcomes.[37–41] The reason for this non-association in the South Indian population is presently not known.

As *HLA* and *TNF* are closely located in the same chromosome, linkage disequilibrium between these two genes can influence the outcome of HBV infection. To elicit this association, we performed a differential association analysis between *TNF* genotypes and *HLA-DRB1*07:01* that were associated with HBV outcome among the C-HBV carriers. We found that the frequency of rs1799964 genotypes was marginally significant between *HLA-DRB1*07:01*-positive and -negative individuals ($P = 0.05$). We speculate that rs1799964 genotypes and *HLA-DRB1*07:01* may have an additive impact in determining the chronicity in the South Indian population.

In summary, the predominant South Indian *HLA-DRB1*07:01* allele is strongly associated with C-HBV infection. The *DRB1*07:01* allele and rs1799964 genotypes may have an additive effect in determining C-HBV infection. The *TNF* promoter allele associated with lower TNF-α plasma levels, that is, the presence of rs1800630A (C/A and A/A), is strongly associated with C-HBV infection. The rs1799964 promoter genotypes also significantly associated with the outcome of HBV infection. The *TNF* haplotype 2 (rs1799964C; rs1800630A; rs1799724C; rs1800629G; rs361525G) was strongly associated with C-HBV infection. Our findings suggest that MHC-class II polymorphisms and the *TNF* promoter polymorphisms governing the level of TNF-α are important population-specific host genetic factors that may determine the variable outcome of HBV infection. These findings may also provide insights into the molecular mechanism of HBV persistence and open novel therapeutic possibilities to overcome C-HBV infection.

Materials and methods

Power calculations

Power calculations were based on the following assumptions. The OR was fixed at 2.0 and power calculations were made under the log-additive model. Based on an earlier study, the frequency of *TNF* risk allele was at least 10% in the South Indian population.[42] Based on this assumption, a minimum of 140 samples in each group would be required for studying the association between *TNF* polymorphisms and HBV outcome with 80% power at 5% significance level.

At the time of design of our study, the only published Indian study showed *HLA DRB1*15XX* to have the strongest association with HBV outcome in the Western Indian population.[43] The frequency distribution of this allele was 20% in the random South Indian population.[26] Based on this assumption, a minimum of 90 samples in each group would be required for studying the association between HLA polymorphism and the outcome of HBV infection with 80% power at 5% significance level.

Subjects and study design

In this case–control study, subjects were enrolled over a period of 1 year and 8 months (August 2005 to April 2007) from the four South Indian states (Tamil Nadu, Andhra Pradesh, Kerala and Karnataka) at Christian Medical College and Hospital. The HBV-exposed and SR individuals were recruited from volunteer/replacement blood donors. C-HBV carriers were referred from liver clinic of this hospital as a part of routine molecular evaluation of chronic liver diseases. The study was explained to all participants and a written consent was obtained. Institutional Review Board approval was obtained before the commencement of the study. Subjects with human immunodeficiency virus, hepatitis C virus co-infections, alcoholic liver disease, autoimmune hepatitis, primary biliary cirrhosis, Wilson's disease and hematological malignancies were excluded from the study.

Stratification of subjects

The subjects were stratified based on the HBV serological and molecular markers into the following groups.

SR group (n = 150). Subjects in this group were anti-hepatitis B core antibody (anti-HBc-IgG) positive, HBsAg negative, anti-HBs positive and HBV DNA negative.

C-HBV group (n = 137). Subjects in this group were HBsAg positive for >1 year, anti-HBc IgG positive, anti-HBs negative and HBV DNA positive/negative.

Serology

The serological screening of HBV was performed for the following markers: HBsAg (ETI-MAK-4 HBsAg Enzyme Immunoassay Kit, Diasorin, Saluggia, Italy), Anti-HBc IgG (ETI-AB-COREK plus, total Anti-HBc Enzyme Immunoassay Kit, Diasorin) and anti-HBs (BIO-RAD MONOLISA ANTI-HBs 3.0 3, Bio-Rad, Hercules, CA, USA) to stratify the subjects into three groups. Antibody screening for HIV (GENSCREEN PLUS HIV Ag–Ab 3, Bio-Rad and HCV (ORTHO HCV 3.0 ELISA, Ortho-Clinical Diagnostics Inc., Raritan, NJ, USA) were also performed to rule out co-infection.

Detection of HBV DNA

Plasma DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and HBV DNA detection were performed using real-time PCR (Artus HBV RG PCR Kit; Qiagen).

Genomic DNA extraction

Genomic DNA was extracted from the buffy coat by an earlier reported salting-out procedure.[44]

HLA *typing*

The low-resolution typing of class I (*A* and *B*) and II (*DR* and *DQ*) alleles were performed using commercial assay by *AllSet*$^+$ Gold SSP (Invitrogen, Brown Deer, WI, USA). The high-resolution typing was performed for class I (*B*44*) and class II (*DRB1*07* and *DRB1*03*) alleles that showed association with HBV outcome using Olerup SSP AB (Saltsjobaden, Sweden). Manufacturer's instructions were followed. To ensure the validity of HLA typing, 5% of the samples were retyped.

Typing of TNF *promoter polymorphisms*

The rs361525 polymorphism was resolved by amplification refractory mutation system-PCR (ARMS-PCR).[45] To validate the ARMS-PCR results, at least 10% of the samples were typed by PCR-restriction fragment length polymorphism.[46]

The rs1800629 polymorphism was identified by PCR-restriction fragment length polymorphism as described earlier.[47] The results of this method was validated by typing >10% of the samples using tetra-primer ARMS-PCR method.[48]

The rs1799724, rs1800630 and rs1799964 polymorphisms were identified by sequence-specific primer-PCR.[49] To validate the sequence-specific primer-PCR results, 5% of samples were retyped.

Amplification and digestions were performed in a Perkin Elmer GeneAmp PCR system 2700 (Roche diagnostic systems, CA, USA). For all polymorphic sites, negative control (PCR grade water) and previously genotyped samples as controls were included to rule out contamination and validation of results respectively. All PCR products were separated on 2% agarose gels (Sigma, St Louis, MO, USA), stained with ethidium bromide ($0.5\,\mu g\,ml^{-1}$), and visualized by ultraviolet radiation using a gel documentation system (Bio-Rad).

Statistical analysis

The differences in the distribution of age, gender and State of origin between the two study groups (C-HBV and SR) were assessed using either χ^2 test or Student's *t*-test, as appropriate. The allelic and genotypic distributions were represented as simple frequencies and compared between the study groups using χ^2 test (univariate analysis). Hardy–Weinberg equilibrium deviation was tested using χ^2 test. The measures of linkage disequilibrium was calculated between all pairs of biallelic loci, namely, Lewontin's D′ (|D|) and the r^2 measures using Haploview 4.1.[50] Haplotypes and their frequencies were inferred using an accelerated expectation maximization algorithm similar to the partition/ligation method described earlier using Haploview 4.1 (see ref. 51) Multivariable logistic regression analyses were performed for each of the three genetic models (codominant, dominant and recessive) to estimate the independent effect of polymorphisms on disease phenotypes, after adjusting for the effects of age (as a continuous variable) and sex (male = 0, female = 1). ORs with 95% CIs were obtained. To adjust for multiple testing, Bonferroni correction was applied based on the number of single-nucleotide polymorphisms analyzed for *TNF* polymorphisms and the significance level was kept at 0.01. This correction was also applied for *HLA* polymorphisms based on the number of alleles analyzed and the significance levels were kept at 0.005 for *HLA*-class I (*A*), 0.004 for class I (*B*), 0.006 for class II (*DR*) and 0.01 for class II (*DQ*). Permutation test was used and empirical *P*-values for *TNF* polymorphisms were calculated by 10 000 permutations.[33] Statistical analysis was done using STATA 10 (Statacorp, College Station, TX, USA).

Conflict of interest

The authors declare no conflict of interest.

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