RESEARCH ARTICLE

KIR : HLA association with clinical manifestations of HBV infection in Madurai, south India

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Abstract

The antiviral action of natural killer (NK) cells is regulated by a wide repertoire of germ-line encoded membrane receptors which recognize the expression of certain self-molecules on target cells. Among the receptors, killer cell immunoglobulin-like receptor (KIR) which recognizes the expression of human leukocyte antigen (HLA) class I has a predominant role in regulating the effector functions of NK cells, particularly in viral infections. We studied a total of 128 hepatitis B virus (HBV) patients (15 acute, 43 asymptomatic, 27 chronic and 43 with other liver diseases) while attending the Department of Medical Gastroenterology, Government Rajaji Hospital, Madurai, India, and 128 ethnic matched control to find the association between the KIR : HLA genes and differential manifestations of HBV. KIR and its ligand HLA polymorphism were identified by DNA-PCR methods. The activatory receptor KIR-2DS1 was significantly elevated in various disease categories, namely asymptomatic, chronic and other HBV, except acute HBV infection. Whereas, KIR 2DS3 in acute and chronic patients and KIR 2DS5 and 3DS1 in asymptomatic individuals. Among various KIR–HLA combinations, homozygous 2DS2:C1 and individuals with 3DSI:BW4 (OR = 3.23, CI = 1.55–6.7, $Pc = 0.02$) are associated with HBV asymptomatism, while most of the two domain inhibitory receptors with their ligands showed significant risk in other liver diseases. Further, KIR3DL1 : HLA Bw4Iso80 (OR = 3.89, 95% CI = 1.58–9.55, $Pc = 0.004$) is related with higher risk for asymptomatic infection when compared with chronic HBV. Thus, the select KIR : HLA alleles and combinations seem to direct the NK cell activities and immune response in different directions resulting in varied symptoms and manifestations in the subgroups of HBV-infected patients studied.

[Kalyanaraman N., Thayumanavan L. and Jayalakshmi M. 2016 KIR : HLA association with clinical manifestations of HBV infection in Madurai, south India. *J. Genet*. **95**, 13–19]

Introduction

Hepatitis B virus (HBV) infection is a serious health problem in developing and developed countries. It is estimated that around 240 million people live with chronic HBV infection and every year 780,000 people succumb to this disease (WHO 2014, available at: http://www.who.int/mediacentre/factsheets/fs204/en/ (accessed 28 July 2014)). It is known that the HBV sneaks through the innate immune surveillance, infects the hepatocytes and establishes a persistent and chronic infection in susceptible individuals (Guidotti and Chisari 2006). The pathological consequences observed during the course of HBV infection are often associated with host–virus immune interactions (Chang and Lewin 2006; Guidotti and Chisari 2006). This involves both innate and adaptive immunity mediated by natural killer (NK) cells, cytotoxic T cells and antibodies. Of these, NK cells play a key role in both innate and adaptive immune responses to hepatitis and other viral infections through direct killing of infected cells and by cytokine-mediated mechanism (Biron *et al.* 1999; Lodoen and Lanier 2006).

The activation of NK cells is an outcome of a subtle balance between the inhibitory and activatory signal delivered by a wide variety of its cell-surface receptors such as killer cell immunoglobulin-like receptor (KIR) (McQueen and Parham 2002). These germ-line encoded NK cell receptors recognize the expression of either classical major histocompatibility complex (MHC) ligands or certain nonMHC molecules on target cells resulting in the suppression of cytolytic activity of NK cells (Colonna 1996; López-Botet *et al*. 2000). Based on the structure, most MHC class I specific NK cell receptors are categorized as: immunoglobulin

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Keywords. hepatitis B virus; NK cells; KIR genotypes; HLA ligand; immunogenetics; disease association.

superfamily and C-type lectin superfamily (McQueen and Parham 2002). Among them KIRs play a prominent role in the regulation of NK cell functions (Parham 2005). The nomenclature of KIR genes is based on the number of extracellular domains (2D, 2 domains; 3D, 3 domains) and intracellular domain length (short, S; long, L). Upon recognition, KIR genes having short tails deliver activating signal and long tails deliver inhibitory signal with an exception of KIR2DL4 which delivers both types of signals (Kikuchi-Maki *et al.* 2003).

Since it is known that KIR : HLA interactions determine the NK cell function, the allelic diversity of these molecules may alter the course of human diseases. The immune response and outcome of several viral infections are largely influenced by the variability of HLA genes and KIR : HLA genotypes (Bashirova *et al.* 2006). Epidemiological studies have also indicated that specific KIR : HLA genotypes may have definite roles in the outcome of various human diseases (Parham 2005; Khakoo and Carrington 2006). A complete or partial failure in the development of early innate immune response and lack of timely induction of adaptive immune response are considered as crucial factors in the persistence of HBV infection (Chang and Lewin 2006; Guidotti and Chisari 2006). Several studies have suggested the role of host immunogenetic factors such as HLA and cytokines on the outcome of HBV infection (Thursz *et al.* 2011). However, limited information is available on the influence of KIR genes in the clinical outcome of HBV infection (Zhi-Ming *et al*. 2007; Gao *et al*. 2010). Here, we report our study on KIR : HLA genes in HBV patients from south India and found out that KIR : HLA genotype may influence the manifestation of HBV infection.

Materials and methods

Study population

This case–control study included 128 patients attending the Department of Medical Gastroenterology, Government Rajaji Hospital, Madurai, India. All controls were unrelated and randomly selected from south Indian samples. Due institutional ethical clearances were obtained from institutional ethical committees and the blood samples were collected with the informed consents from the volunteers. Clinical categorizations of patients were done on the basis of biochemical test for liver function and serological test for viral markers. The patient group comprises (i) acute HBV (AHBV, $N = 15$) infection positive for HBsAg with clinical onset of liver damage, (ii) asymptomatic carriers (Asy, $N = 43$) positive for HBsAg but without any liver damage, (iii) chronic HBV (CHBV, $N = 27$) remaining positive for HBsAg for more than six months with liver damage, and (iv) other forms of liver diseases (OLD, $N = 43$) without HBV infection. These subgroups served as controls to each other, further validating the study.

DNA isolation and genotyping of KIR–HLA

Genomic DNA was isolated from venous blood by modified salting-out procedure (Miller *et al.* 1988). Genotyping of KIR and HLA ligands was performed by PCR-SSP. KIR genotyping was performed by multiplex PCR, detecting the presence or absence of 14 KIR genes in four groups and each identifying three to four KIR genes (Abalos *et al.* 2011) and a duplex PCR-SSP reaction was employed to detect the presence or absence of two pseudogenes (Ashouri *et al.* 2009). HLA ligand genotyping (HLA B broad specificity and HLA-A3/A11) was performed according to Tonks *et al.* (1999). Subtyping of HLA Bw4 specificities based on polymorphic position, 80 threonine (Thr80)-isoleucine (Iso80) and the HLA A-Bw4 epitope were carried out following the method of Tajik *et al.* (2010). HLA C genotyping was performed according to Frohn *et al.* (1998). Internal control was used to discriminate false negativity and no template negative control to eliminate false positivity. All PCR amplifications were performed with MJ-PTC 100 thermal cycler and amplified products were electrophoresed in agarose-gel prestained with ethidium bromide. The presence of an amplicon at expected molecular weight indicates the presence of relevant gene or genotype in the respective sample.

Statistical analysis

KIR genes: Carrier frequency (CF) for the KIR loci was calculated by direct counting of positively-typed individuals. KIR gene frequencies (GF) were calculated by using the function, frequency for a diploid population (binary data) available in the GenAlEx package (Peakall and Smouse 2012). KIR grouping and KIR genotypes ID were identified according to an open source allele frequency database (Middleton *et al.* 2003). In brief, individuals possessing a limited KIR genes (KIR2DL1-2DL3-2DS4-3DL1) in addition to framework genes were designated as haplotype A. Individuals with more or variable number of activatory KIR genes in addition to frame work genes were designated as haplotype B. KIR genotypes AA or Bx were deduced from the haplotype data where x can be either A or B haplotype. Further, overall KIR genotypes and their relative frequencies were determined by using the haplotype inference function in Arlequin software (v3.5.1.2).

HLA : KIR combinatorial analysis: Frequencies of individual HLA ligands (C1/C2, Bw4/Bw6) were compared between the patient and control population. Further, the presence of Bw4 public epitopes, HLA-A Bw4 specificities, HLA A3/A11 and HLA Bw4 polymorphic position 80 (Thr/Iso) along with KIR genes were analysed. Combinations of iKIR-aKIR : HLA, iKIR : HLA and aKIR : HLA were also assessed based on the genotypic data.

The strength of the association between the patient and control were evaluated by using odds ratio (OR) at 95% confidence interval (CI). Comparison of genetic variables

between patients and control group was performed by Pearson's chi-square test (χ^2) with Yate's continuity correction using the package 'epiR' of *R* statistical computing program. Fisher's exact test was employed wherever necessary. All tests of statistical significance was two-sided and probability value if $P \leq 0.05$ was considered to be significant. The significance of association was further validated with the procedure of false discovery rate (FDR) using the *q* value (according to Benjamini and Hochberg 1995) by *R* statistical program.

Results

Analysis of KIR gene content and genotypes

The frequency of various KIR genes in the control and various subgroups of patients are presented in table 1. Of 16 KIR genes tested, three activatory KIR genes showing high risk in asymptomatic group were 2DS1 (OR = 2.82, 95% CI = 1.27–6.60, *Pc* = 0.009), 2DS5 (OR = 3.04, 95% CI = 1.39–6.98, *Pc* = 0.004) and 3DS1 (OR = 2.89, 95% CI = 1.29–6.94, *Pc* = 0.008) when compared to control and other disease groups. Further, KIR 2DS1 showed high risk for CHBV (OR = 3.81, 95% CI = 1.37–12.33, *Pc* = 0.008) infection and OLD (OR = 3.04, 95% CI = 1.39–6.98, *Pc* = 0.004). Subgroup analysis showed that 2DS5 was significantly associated with high risk for asymptomatic infection when compared with chronic (OR = 3.46, 95% CI = 1.02–11.73, *P* = 0.04) and OLD (OR = 2.65, 95% CI = 1.09–6.42, *Pc* = 0.05). Further, KIR3DL1 : Bw4Iso80 was related with high risk for asymptomatism when compared with chronic HBV (OR = 3.89, 95% CI = 1.58–9.55, *Pc* = 0.004) (not shown in table 1). Distribution of KIR genotypes, genotype ID, number of positive individuals and its relative frequency are tabulated (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet). A total of 67 different KIR genotypes and 21 unassigned genotypes were observed in this study. KIR genotype Bx occurred more frequently.

Analysis of HLA ligand

The frequencies of HLA genotypes and coinheritance of KIR : HLA ligand pairs were compiled (table 2 in electronic supplementary material) and significant associations are reported in table 2. There was a significant association with total HLA C1 and C2 allotypes in the patient group when compared with the control (table 2). With respect to HLA B genotypes, no such significant association was observed. Analysis of HLA Bw4 polymorphic position 80 (HLA Bw4 Thr 80 or Iso 80) showed that Bw4Iso80 was more frequent in asymptomatic patients (*Pc* = 0.002) when compared to the control.

Combinatorial analysis of KIR : HLA pairs

The coexistence of multiple KIR : HLA pairs in a single individual and its association with clinical status showed

Table 1. KIR gene frequencies among controls and various sub groups of patients.

	Control ($n = 128$)		Acute HBV ($n = 15$)		Asymptomatic carriers ($n = 43$)		Chronic HBV ($n = 27$)		Other liver diseases ($n = 43$)	
Gene	CF	GF	CF	GF	CF	GF	CF	GF	CF	GF
Inhibitory										
2DL1	0.99	0.91	1.00	1.00	1.00	1.00	1.00	1.00	0.98	0.85
2DL2	0.88	0.65	0.87	0.63	0.91	0.70	0.93	0.73	0.91	0.70
2DL3	0.77	0.52	0.87	0.63	0.81	0.57	0.81	0.57	**0.91**[h]	0.70
2DL4	1.00	1.00	1.00	1.00	1.00	1.00	0.96	0.81	0.98	0.85
2DL5	0.75	0.50	0.87	0.63	0.86	0.63	0.89	0.67	0.74	0.49
3DL1	0.91	0.71	0.87	0.63	0.93	0.74	0.89	0.67	0.95	0.78
3DL2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3DL3	0.98	0.85	0.93	0.74	1.00	1.00	1.00	1.00	0.98	0.85
Activatory										
2DS1	0.48	0.28	0.53	0.32	**0.72**[b]	**0.47**	**0.78**[e]	**0.53**	**0.74**[g]	**0.49**
2DS2	0.69	0.44	0.73	0.48	0.79	0.54	0.78	0.53	0.79	0.54
2DS3	0.65	0.41	**0.93**[a]	**0.74**	0.79	0.54	**0.89**[f]	**0.67**	0.79	0.54
2DS4	0.80	0.56	0.93	0.74	0.70	0.45	0.74	0.49	0.70	0.45
2DS5	0.43	0.24	0.40	0.23	**0.70**[c]	**0.45**	0.48	0.28	0.47	0.27
3DS1	0.50	0.29	0.60	0.37	**0.74**[d]	**0.49**	0.67	0.42	0.65	0.41
Pseudogene										
2DP1	0.91	0.71	0.87	0.63	0.93	0.74	0.85	0.62	0.91	0.70
3DP1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

CF, carrier frequency; GF, gene frequency. Data with significant *P* value are in bold.
[a]OR: 7.59 (95% CI: 0.96–59.61), *Pc*: 0.05; [b]OR: 2.82 (95% CI: 1.27–6.60), *Pc*: 0.009; [c]OR: 3.04 (95% CI: 1.39–6.98), *Pc*: 0.004; [d]OR: 2.89 (95% CI: 1.29–6.94), *Pc*: 0.008; [e]OR: 3.81 (95% CI: 1.37–12.33), *Pc*: 0.008; [f]OR: 4.95 (95% CI: 1.23–15.19), *Pc*: 0.02; [g]OR: 3.04 (95% CI: 1.39–6.98), *Pc*: 0.004; [h]OR: 2.85 (95% CI: 0.94–8.65), *P*: 0.05.

interesting association (table 2). For instance, KIR2DL2/L3 : HLA C1 combination (OR = 2.82, 95% CI = 1.27–6.60, $q = 0.03$) and KIR2DL1 : HLA C2 (OR = 0.34, 95% CI = 0.14–0.78, $q = 0.03$) were markedly elevated in the OLD group without HBV infection when compared with control. The KIR3DS1 : HLA Bw4 genotype (OR = 3.23, 95% CI = 1.55–6.70, $q = 0.02$) and KIR3DS1 : HLA Bw4Iso80 (OR = 2.99, 95% CI = 1.46–6.09, $q = 0.02$) were associated with asymptomatic infection. Multiple combinations of iKIR and aKIR receptors with corresponding HLA ligands are given in table 2. Individuals with two pairs of KIR : HLA were significantly associated with the patient group when compared with control. Subgroup analysis showed that HLA C1 : C2 (OR = 0.18, 95% CI = 0.04–0.73, $Pc = 0.02$), 2DS1 : C2 (OR = 0.20, 95% CI = 0.04–0.87, $Pc = 0.04$) and 3DS1 : HLA A/Bw4Iso80 (OR = 5.33, 95% CI = 1.09–25.98, $Pc = 0.04$) were significantly related to acute HBV when compared with chronic HBV (not shown in table 2).

Discussion

Current knowledge on the immunological response to human HBV viruses is mostly derived from studies on animal models. Of late, the discovery of various manifestations of the viral infection leads to studies on their underlying immunological mechanisms. The successful containment of HBV infection mediated by NK/NKT cells has been reported in chimpanzee (Guidotti *et al.* 1999). Deficient production of TNF-α and IFN-γ by NK cells is known to lead chronic infection in woodchuck hepatitis model (Menne *et al.* 2002). Presence of early NK/NKT cells-mediated immune response in humans is associated with enhanced viral suppression and timely induction of adaptive immune response leading to acute HBV infection (Webster *et al.* 2000; Fisicaro *et al.* 2009). Further, a functional impairment of these cells such as reduced cytotoxic activity and lowered cytokine production lead to persistent HBV infection and development of hepatocellular carcinoma (Chen *et al*. 2005; Oliviero *et al.* 2009).

Functionally, KIR recognizes the expression of HLA class I molecules based on certain specificities rather than the whole HLA molecule. These specificities are HLA-C1 allotype with asparagine 80 (KIR2DL2/3,2DS2), HLA-C2 allotype with lysine 80 (KIR2DL1,2DS1), HLA Bw4 public epitope (position 77–83) which represent 33 percentage of HLA-B (KIR3DL1), Bw4 motif resembling HLA-A allotypes (KIR3DL1) and HLA-A3/A11 (KIR3DL2). The specific interaction of KIR : HLA follows certain functional hierarchy which determines the effector function of NK

Table 2. KIR:HLA combinations in variable HBV disease outcome.

	AHBV versus control		Asymptomatic versus control		CHBV versus control		OLD versus control	
KIR : HLA ligand	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Section 1								
HLA C1 (total)[a]	2.36 (1.02–5.73)	0.044	1.81 (1.10–2.97)	0.02	2.15 (1.14–4.14)	0.01	2.09 (1.24–3.57)	**0.03**[e]
HLA C2 (total)	0.42 (0.17–0.98)	0.044	0.55 (0.33–0.93)	0.02	0.47 (0.24–0.88)	0.01	0.48 (0.28–0.81)	**0.03**[e]
HLA C1 homozygosity	4.40 (1.22–15.6)	0.01	2.45 (1.02–5.78)	0.04			2.72 (1.14–6.36)	**0.04**[e]
HLA C2 homozygosity					0.17 (0.02–0.74)	0.008[d]	0.35 (0.11–0.92)	0.03
HLA Bw4Ise80[b]			4.56 (1.67–12.37)	**0.01**[e]				
Section 2								
2DL2:C1							3.03 (1.34–6.83)	**0.03**[e]
2DL3:C1					3.78 (1.19–15.9)	0.01[d]		
2DL2/L3:C1 (total)							2.82 (1.27–6.60)	**0.03**[e]
2DL2/L3:C1 homozygosity							3.01 (1.19–7.54)	**0.04**[e]
2DL2/L3:C1-3DL1:A/Bw4[c]			2.26 (1.06–4.88)	0.03			2.49 (1.23–5.05)	**0.04**[e]
2DL1:C2 (total)	0.22 (0.07–0.68)	0.01	0.41 (0.17–0.98)	0.04			0.33 (0.14–0.78)	**0.03**[e]
2DL1:C2 homozygosity					0.16 (0.03–0.75)	0.008[d]	0.35 (0.11–0.92)	0.03
2DL1:C2-3DL1:Bw4					0.29 (0.07–0.94)	0.02[d]	0.30 (0.10–0.74)	0.008[d]
Section 3								
2DS2:C1 (total)					2.60 (1.06–6.39)	0.05	2.26 (1.05–5.10)	0.03
2DS2:C1 homozygosity			3.01 (1.19–7.54)	0.01			2.69 (1.05–6.82)	0.03
2DS2:C1-3DS1:A/Bw4			2.56 (1.14–5.70)	0.01				
3DS1:A/Bw4			2.65 (1.22–5.97)	0.01				
3DS1:Bw4			3.23 (1.55–6.70)	**0.02**[e]				
3DS1:Bw4Iso80[b]			2.99 (1.46–6.09)	**0.02**[e]				

[a]Total represents presence of both homozygous and heterozygous conditions of an allotype. For example, HLA C1 (total) comprised HLA C1/C1 and HLA C1/C2 individuals; [b]belongs to HLA Bw4Iso80 only, excluding HLA Bw4 resembling HLA A motifs; [c]A/Bw4 represents individuals possessing HLA Bw4 and sol or HLA A allele resembling Bw4 motifs; [d]Fisher exact (test two-tailed) *P* value; [e]*q* values calculated according to Benjamini and Holchberg's method (in bold); other unlabelled *P* values were calculated by Pearson's χ^2 test with Yate's continuity correction.

cell upon recognition of target (Vilches and Parham 2002; Hansasuta *et al.* 2004; Parham 2005; Yu *et al.* 2007).

The present observation showed an increased prevalence of diverged KIR B genotype and their skewed distribution in various clinical subtypes of patients suggesting its obvious role in disease process. Previous reports from south India also suggest the predominance of group B haplotype KIR genes in this region (Rajalingam *et al.* 2008; Maruthamuthu and Mariakuttikan 2015). Norman *et al.* (2007) reported that the activatory KIR gene 3DS1 is common in India and is presumably due to the effect of positive selection. We could also observe the higher incidence of activatory KIR genes and specifically 3DS1 and its ligand HLA-Bw4Iso80 in asymptomatic carriers.

Further, HLA C allotype showed an overall increase of HLA C1 in all patient groups and KIR2DS2 : HLA C1 homozygosity frequency was much higher in asymptomatic (OR = 3.01, $P = 0.01$) when compared with control. This combination of KIR2DS2 : HLA C1 is known to deliver much weaker activatory signal than their inhibitory counterparts (Vilches and Parham 2002). Earlier, KIR2DL3 : HLA C1 has been reported to confer protection against CHBV infection (Gao *et al.* 2010). Thus, the interpretation based on KIR2DS2 : HLA C1 genotype with clinical manifestation of HBV infection remains controversial.

Previous investigation conveyed that HLA C2 allotype was more frequent in CHBV patients and KIR2DL1 : HLA C2 genotype conferred susceptibility towards chronic hepatitis in a Chinese population. Further, it was explained that the presence of stronger inhibitory signals leads to the development of CHBV infection (Gao *et al.* 2010). But the present work showed an overall increase in frequency of HLA C2 allotype followed by higher occurrence of KIR2DL1 : HLA C2 genotype in control population. This difference may be a reflection of population-specific distribution of KIR genes and HLA C alleles that has evolved under natural selection pressure (Parham 2005).

Among the KIR : HLA pairs, KIR3DS1 : HLA Bw4 genotype is the most consistently associated with differential outcome of various viral infections (Bashirova *et al.* 2006). In a HIV study by Martin *et al.* (2002), KIR3DS1 : HLA Bw4Iso80 genotype has been implicated in slower progression to AIDS with low viral load and protection against other opportunistic infections (Qi *et al.* 2006). This genotype has also been reported to confer protection against development of the liver cancer among HCV-infected individuals (Bashirova *et al.* 2006). KIR3DS1 gene improves the HCV clearance during antiviral therapy in HIV/HCV coinfected patients (Rivero-Juarez *et al.* 2013). It also develops early immune response against papilloma viral infection (Bonagura *et al.* 2010). To date, there is no report on the role of KIR3DS1 : Bw4 in clinical outcome of HBV infection. A Han Chinese study has indicated that *KIR3DS1* gene might have a role in the clearance of HBV infection (Zhi-Ming *et al.* 2007). The present study showing a higher frequency of KIR3DS1 : HLA Bw4 genotype (OR = 3.23, $q = 0.02$) and HLA-Bw4Iso80 along with KIR3DS1 (OR = 2.99, $q = 0.02$) in HBV-infected asymptomatic group, assigned a clear role for these alleles in asymptomatic status. Analysis based on the number and type of *KIR* genes also reiterated an elevated risk of KIR : HLA combinations, aKIR : HLA (KIR3DS1 : Bw4-KIR2DS2 : C1; $P = 0.01$) and iKIR : HLA pair (KIR2DL2/L3 : C1 – KIR3DL1 : Bw4; $P = 0.03$) for asymptomatic infection.

Interestingly, a lower frequency of two inhibitory ligand iKIR : HLA combinations (2DL1 : HLA C2 – 3DL1 : Bw4) were observed in CHBV (OR = 0.29, 95% CI = 0.07–0.94, $Pc = 0.02$) infection and other liver diseases (OR = 0.30, 95% CI = 0.10–0.74, $Pc = 0.008$) group implicating that the absence of these two iKIR phenotype combinations delivers much stronger inhibitory signal resulting in suppression of NK cell activity on virally-infected cells leading to chronic status. The study thus confirms a definite role for indicated KIR : HLA combinations in determining the asymptomatic, chronic and other clinical manifestations. All these findings supported the assumption that the presence of certain KIR : HLA combinations may confer a level of nonspecific protection against multiple viral infections (Khakoo and Carrington 2006). These combinations alter the disease outcome and this is attributed to reduced expression of MHC by virally-infected cells and the resultant NK cell unresponsiveness: higher the inhibition that restricts the NK cells by KIR, stronger the response towards unhealthy cells (Kim *et al*. 2005; Raulet and Vance 2006).

In conclusion, KIR3DS1 : HLA Bw4Iso80 phenotype may be decisive in receptor-mediated containment of HBV viral replication at the innate/adaptive immunity interface level, thus, tilting and directing the infection towards acute versus asymptomatic or chronic forms of disease.

Acknowledgement

The financial support received from the University Grants Commission (UGC), New Delhi, India, under the schemes major research project (39-585/2010), UGC meritorious fellowship and the ground support offered by the Center for Excellence in Genomic Sciences (CEGS) are acknowledged. We thank Mr P. Maheshkumar for collecting control samples. The support received from physicians and a nontechnical personnel in recruiting the patient for collection of clinical data and spontaneous willingness of volunteers in providing the blood samples are gratefully acknowledged.

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Received 18 February 2015, in final revised form 18 June 2015; accepted 19 June 2015
Unedited version published online: 22 June 2015
Final version published online: 1 February 2016