

Serum Decoy Receptor 3 Is a Useful Predictor for the Active Status of Chronic Hepatitis B in Hepatitis B e Antigen-Negative Patients

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Hepatitis B virus (HBV) infection is a global public health problem, because patients with chronic hepatitis B (CHB) may progress to liver cirrhosis and eventually evolve into hepatocellular carcinoma. Decoy receptor 3 (DcR3) is a soluble receptor of the tumor necrosis factor receptor superfamily, and has been implicated in anti-apoptotic and anti-inflammatory pathways. In this study, we explored the clinical value of serum DcR3 in predicting the active status of CHB in hepatitis B e antigen-negative patients (active HBeAg (-) CHB), which was determined with ELISA. The serum level of DcR3 in active HBeAg (-) CHB patients (1.92 ± 0.68 ng/ml) was higher than that in healthy controls (0.80 ± 0.25 ng/ml, $p < 0.0001$) and that in inactive status of HBeAg (-) CHB (inactive hepatitis B surface antigen carrier, HBsAg-IaC) patients (0.95 ± 0.26 ng/ml, $p < 0.0001$). DcR3 level was correlated with HBV DNA level ($r = 0.819$, $p < 0.0001$) and alanine transaminase level (ALT, $r = 0.704$, $p < 0.0001$) in active HBeAg (-) CHB patients. The area under the Receiver Operating Characteristics curve of DcR3 for detecting the active status of HBeAg (-) CHB patients was 0.914 (95% confidence interval, 0.851-0.977). The optimal cut-off value for DcR3 to predict active HBeAg (-) CHB was 1.22 ng/ml, which had a sensitivity of 87.5% and a specificity of 84.4%. These results suggest that serum DcR3 level may be useful for detecting HBeAg (-) CHB in the active stage, which requires medical treatment.

Keywords: alanine aminotransferase; chronic hepatitis B; decoy receptor 3; hepatitis B e antigen; hepatitis B virus DNA

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Introduction

Hepatitis B virus (HBV) infection is a global public health problem. It is estimated that more than 350 million individuals worldwide are chronically infected with HBV (Benhenda et al. 2009). In infected adolescents or adults, 10% will develop into a chronic carrier state, whereas in infected neonates 90% develop chronicity (Lok and McMahon 2001; Pol 2006). About 20% of chronic HBV patients will progress to liver cirrhosis, and some eventually evolve into hepatocellular carcinoma (HCC) (Ono-Nita et al. 2004). The accurate diagnosis of high-risk chronic

hepatitis B (CHB) is likely to provide timely treatment and prevent progression to cirrhosis and HCC. Hepatitis B e antigen (HBeAg) is the extracellular form of hepatitis B core antigen (HBcAg), translated from the gene of ORF Core and Pre C of HBV. HBeAg is a viral protein released into the blood, which can easily be detected by immunoassay, and hence is widely used as a marker of viral replication (Kimura et al. 2003). In the pathophysiologic process of chronic HBV infection, the "inactive hepatitis B surface antigen carrier (HBsAg-IaC)" status may follow sero-conversion from HBeAg (+) to HBeAg (-), which is characterized by very low or undetectable serum HBV DNA levels

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and normal alanine aminotransferase (ALT). As a result of immunological control of the infection, HBeAg (–) status confers a favorable long-term outcome with a very low risk of cirrhosis or HCC in the majority of patients (Sherman et al. 2007). Patients with the “HBeAg (–) CHB status” demonstrate wild fluctuations in serum ALT and 20%–30% of these patients with pathologically documented chronic hepatitis have normal ALT at the time of presentation (Hadziyannis and Papatheodoridis 2006). Consequently, HBeAg (–) CHB patients with normal liver enzymes may be misdiagnosed as being in the HBsAg-IaC status and thus mistakenly denied treatment. It is difficult and important to distinguish HBsAg-IaC from active HBeAg (–) CHB patients. The former patients have a good prognosis with a very low risk of complications, while the latter patients have active liver disease with a high risk of hepatic fibrosis, cirrhosis and HCC. The most common contradiction in the diagnosis of HBV patients is the differentiation between chronic active cases from inactive carriers, as they share the same serological profile. HBV DNA level measured using polymerase chain reaction (PCR) is a good diagnostic assay for the diagnosis of HBV, however, due to its high cost in many countries and regions, especially low-income countries, ELISA is still used without further confirming viral titer with PCR. Significantly, many HBeAg (–) patients show chronic active HBV in further screening by PCR and vice versa (Nelson and Shan 2008). Therefore, it is important to identify new reliable serum markers as indicators of the active status of HBeAg (–) CHB.

Decoy Receptor 3 (DcR3), a soluble decoy receptor of the tumor necrosis factor receptor family, is known to bind three ligands in the TNF family, namely, FasL, LIGHT and TL1A, and thereby blocks dead signaling and promotes survival, especially under stress (Pitti et al. 1998; Yu et al. 1999; Migone et al. 2002). DcR3 has the potential to be a pleiotropic immunomodulator and biomarker for inflammatory diseases, autoimmune diseases and cancer (Lin and Hsieh 2011). DcR3 is not detectable in most normal tissues, but its expression is up regulated in response to some pathogens or insults. In particular, DcR3 levels are elevated in systemic inflammatory diseases, where serum concentrations are significantly increased in bacterial infections (Kim et al. 2004), active ulcerative colitis (Bamias et al. 2010), systemic lupus erythematosus (Lee et al. 2008), atopic dermatitis (Chen et al. 2004a), rheumatoid arthritis (Bamias et al. 2008), kidney diseases (Ka et al. 2007) and acute respiratory distress syndrome (Chen et al. 2009). It seems that serum DcR3 is a valuable marker in predicting the outcome of inflammatory diseases (Chen et al. 2004b).

The aim of this study was to determine the value of DcR3 in detecting the active status in HBeAg (–) CHB patients by measuring serum DcR3 and correlating this with ALT and HBV DNA levels.

Subjects and Methods

Subjects

Blood samples were collected from 80 HBeAg (–) CHB patients and 96 healthy controls. Consecutive patients with persistent HBV infection were identified at the Songjiang Hospital Affiliated First People's Hospital, Shanghai Jiao Tong University. Inclusion criteria were: HBsAg (+), HBeAg (–) for at least 6 months. Patients were classified into active HBeAg (–) CHB if they had ALT activity > 40 IU/L, HBV DNA > 100,000 copies/ml and histological findings compatible with chronic hepatitis. Active CHB patients met all three criteria. Otherwise, patients with HBeAg (–) were classified as inactive HBsAg carriers (HBsAg-IaC) if they had persistently normal ALT values and HBV DNA < 100,000 copies/ml. Patients with fatty liver, alcohol use, obesity, HCC, hepatitis C, hepatitis D, HIV viral co-infection, any bacterial infection or concurrent immune diseases and autoimmune diseases were excluded. The study was approved by the institutional ethics committee of Songjiang Hospital Affiliated First People's Hospital, Shanghai Jiao Tong University and informed consent was obtained from each subject. Blood samples were collected on admission. Serum samples were separated and kept at –80°C and thawed on ice to minimize degradation upon assay.

Viral assays

HBsAg and HBeAg were determined using a Roche Diagnostic E170 series automatic analyzer. ALT was quantitated using a Roche Diagnostic 7600 series automatic analyzer. Serum HBV DNA was quantitated using a sensitive quantitative PCR assay system (Roche PCR Diagnostic System) with a lower limit of detection (80 copies/ml).

DcR3 Enzyme-linked immunosorbent assay (ELISA)

Reagents for DcR3 ELISA were kindly provided by Dr. Lurong Zhang as previously described (Hou et al. 2012). Briefly, high-affinity polystyrene 96-well microtiter plates (Costar 2592, Corning) were coated with anti-DcR3 monoclonal antibody (2.0 µg/ml, 100 µl/well) overnight at 4°C. After washing with PBS containing 0.05% Tween 20, the plates were incubated with blocking buffer for 1 h at room temperature. Then, 100 µl of serial dilutions of standard DcR3 (0, 0.25, 0.5, 1, 2, 4, 8, 16 ng/ml) or patients' serum was added to the DcR3 antibody-coated plate in duplicate. After incubation at room temperature for 2 h and washing twice, 100 µl of 0.5 µg/ml biotinylated anti-DcR3 monoclonal antibody was added. The plate was further incubated for 2 h at room temperature followed by the addition of 100 µl of 0.2 µg/ml horseradish peroxidase-labeled streptavidin and tetramethylbenzidine (TMB) substrate as standard ELISA procedure (Chen et al. 2004b). After stopping the reaction, the plate was read at A₄₅₀ on a SpectraMax M2 reader. The DcR3 concentration in patients' serum samples was calculated from the standard curve generated from DcR3 standards.

Statistical analysis

Quantitative data were expressed as mean ± s.d. The statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) version 13.0 for Windows. Chi-square tests were applied to evaluate differences in proportions. The Mann-Whitney test for non-parametric data was used for comparisons between the two groups. The Spearman correlation was used to assess the association between two quantitative variables. In order to evaluate the

Table 1. Clinical characteristics of HBeAg (-) patients.

Patient characteristics	HBsAg-IaC (n = 32)	Active HBeAg (-) CHB (n = 48)	P value
Gender (M/F)	20/12	31/17	0.517
Age	34.7 ± 14.1	35.6 ± 12.3	0.192
ALT	27.8 ± 19.2	79.8 ± 34.5	< 0.0001*
DNA(log copies/ml)	3.6 ± 1.1	6.8 ± 1.0	< 0.0001*

The results are shown as mean ± standard deviation. * $p < 0.05$ is significant. M, male; F, female.

predictive value of DcR3, receiver operating characteristic curve (ROC) analysis was applied and the area under the curve (AUC) was compared. All statistical tests were two-sided, and p values of less than 0.05 were considered statistically significant.

Results

Patient's clinical characteristics

A total of 80 HBeAg (-) CHB patients were selected for this study, 32 patients were diagnosed as HBsAg-IaC, while the remaining 48 patients had active HBeAg (-) CHB. Patient data are shown in Table 1. Male patients were predominant in both groups. The age difference between the two groups was not significant ($p = 0.192$). ALT level and HBV DNA level in the active HBeAg (-) CHB group were significantly higher than those in the HBsAg-IaC group (79.8 ± 34.5 vs. 27.8 ± 19.2 IU/L, $p < 0.0001$; $10^{6.8 \pm 1.0}$ vs. $10^{3.6 \pm 1.1}$ copies/ml, $p < 0.0001$).

DcR3 expression in HBeAg (-) CHB patients and healthy controls

The detailed characteristics (sensitivity, specificity, precision, recovery, etc.) of the ELISA-based DcR3 assay were described in our previous publication. The assay had a detection limit of 36 pg/ml with a dynamic range of 0.25-16 ng/ml. The intra-assay CV was less than 10% and the inter-assay CV was less than 15%. The correlation coefficient of the curve was 0.998 (Chen et al. 2004b; Hou et al. 2012). The mean ± s.d. DcR3 concentration was 0.80 ± 0.25 ng/ml in healthy controls, 0.95 ± 0.26 ng/ml in HBsAg-IaC patients and 1.92 ± 0.68 ng/ml in active HBeAg (-) CHB patients. The DcR3 concentration in active HBeAg (-) CHB patients was significantly higher than that in healthy controls and HBsAg-IaC patients ($p < 0.0001$) (Fig. 1).

Correlation between DcR3, ALT and HBV DNA levels in HBeAg (-) CHB patients

In active HBeAg (-) CHB patients, the correlations were as follows: DcR3 vs. DNA ($r = 0.819$, $p < 0.0001$), DcR3 vs. ALT ($r = 0.704$, $p < 0.0001$), and ALT vs. DNA ($r = 0.837$, $p < 0.0001$) (Fig. 2. A, B and C). In HBsAg-IaC patients, the correlations were as follows: DcR3 vs. DNA ($r = 0.099$, $p = 0.590$), DcR3 vs. ALT ($r = -0.250$, $p = 0.167$), and ALT vs. DNA ($r = -0.173$, $p = 0.343$) (Fig. 2. D, E and F) (Table 2).

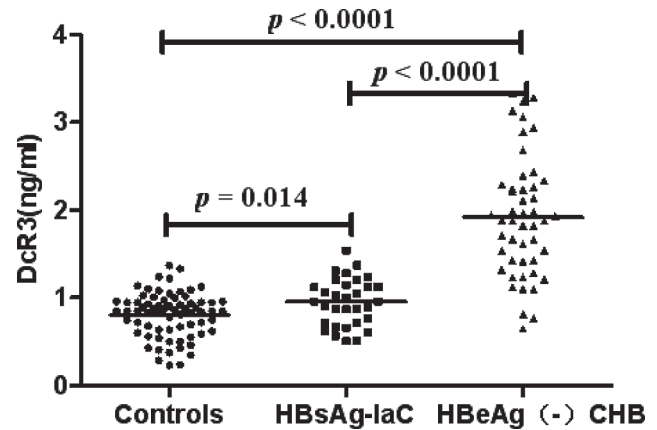


Fig. 1. DcR3 concentrations in controls, HBsAg-IaC and active HBeAg (-) CHB patients. The DcR3 values (mean ± s.d.) were 0.80 ± 0.25 , 0.95 ± 0.26 and 1.92 ± 0.68 ng/ml for controls ($n = 96$), HBsAg-IaC patients ($n = 32$) and active HBeAg (-) CHB patients ($n = 48$), respectively. The Mann-Whitney test was used for comparisons between groups.

Prognostic ability of DcR3 for detecting the active status of HBeAg (-) CHB patients

To evaluate the prognostic ability of DcR3 for discriminating HBsAg-IaC patients and active HBeAg (-) CHB patients, the receiver operating characteristics (ROC) curves of DcR3, ALT and HBV DNA were drawn (Fig. 3). The area under the ROC curves for DcR3 was 0.914 (95% confidence interval, 0.851-0.977), for HBV DNA was 0.978 (95% confidence interval, 0.951-1.004), and for ALT was 0.937 (95% confidence interval, 0.874-1.001) (Table 3). The optimal cut-off value for DcR3 to predict active HBeAg (-) CHB was 1.22 ng/ml, which yielded a sensitivity of 87.5% and a specificity of 84.4%.

Discussion

This study demonstrated, for the first time, that serum DcR3 may identify the active status and monitor the prognosis of HBeAg (-) CHB patients. The evidence for this was as follows: 1) a large portion of HBeAg (-) patients had an elevated level of DcR3; 2) DcR3 was strongly correlated with HBV DNA viral load ($r = 0.819$) and ALT ($r = 0.704$); 3) the area under the ROC curve for DcR3 to discriminate the active status from inactive status in HBeAg (-) CHB patients was 0.914; and 4) the optimal cut-off

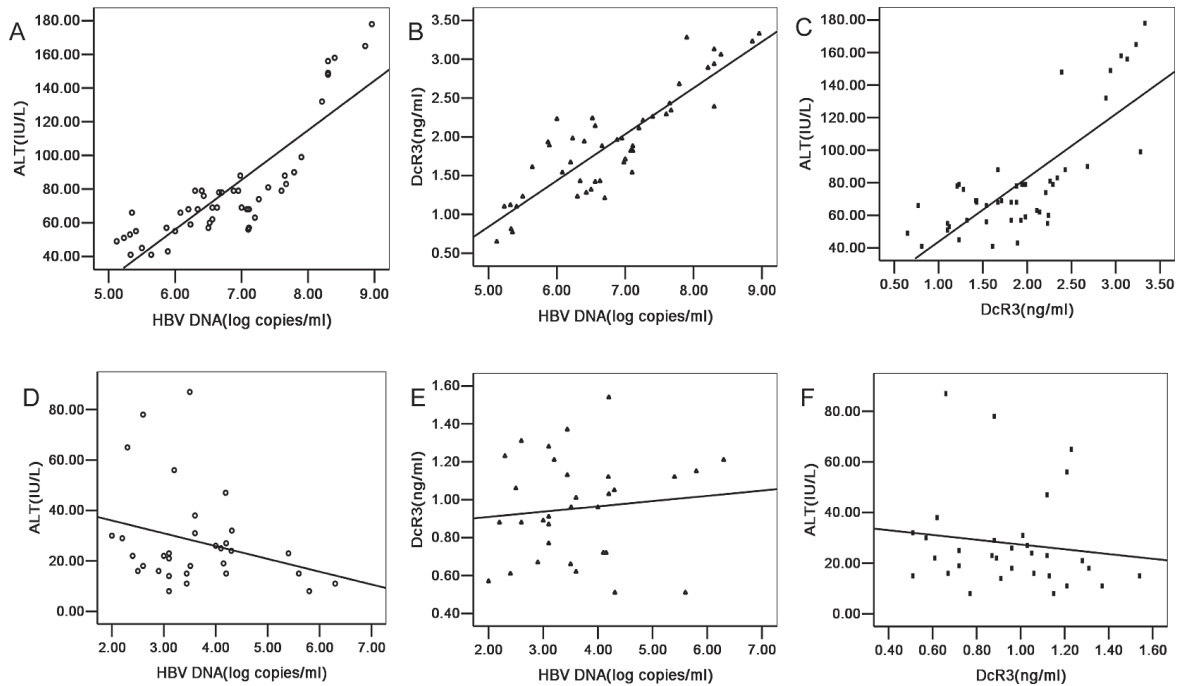


Fig. 2. Correlation between DcR3, ALT and HBV DNA levels in HBsAg-IaC and active HBeAg (-) CHB patients. A, B and C: association between DcR3, ALT and HBV DNA in active HBeAg (-) CHB patients (n = 48); C, D and E: association between DcR3, ALT and HBV DNA in HBsAg-IaC patients (n = 32).

Table 2. Correlation between DcR3, ALT and HBV DNA levels in HBeAg (-) patients.

Variables	HBsAg-IaC (n = 32) SC values	Active HBeAg(-) CHB (n = 48) SC values
DcR3 vs. DNA	$r = 0.099, p = 0.590$	$r = 0.819^{**}, p < 0.0001^*$
DcR3 vs. ALT	$r = -0.250, p = 0.167$	$r = 0.704^{**}, p < 0.0001^*$
ALT vs. DNA	$r = -0.173, p = 0.343$	$r = 0.837^{**}, p < 0.0001^*$

SC, Spearman's correlation. *Correlation is significant at the $p < 0.05$ level (2-tailed), **Correlation is significant at $r > 0.25$ and $r < -0.25$ level (2-tailed).

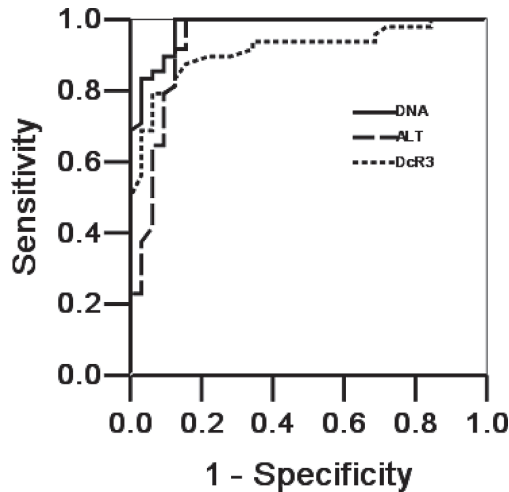


Fig. 3. Prognostic ability of serum ALT, DCR3, HBV DNA viral load for detecting active status in HBeAg (-) CHB patients.

value for DcR3 to predict active status in HBeAg (-) CHB patients was 1.22 ng/ml, which had a sensitivity of 87.5% and a specificity of 84.4%.

Clinically, HBV DNA and ALT are the most commonly used serum biomarkers for the evaluation of viral liver disease (Kim et al. 2009). Studies have been conducted on the value and relationship between HBV DNA and ALT in HBeAg (-) patients. Kim et al. (2003) analyzed the sera from 82 patients with HBeAg (-) CHB, and found that the median serum HBV DNA levels in patients with high ALT levels was significantly higher than that in patients with low ALT levels. The serum ALT level was correlated with serum HBV DNA levels in patients with HBeAg (-) CHB ($r = 0.416, p < 0.001$) (Kim et al. 2003). Sakugawa et al. (2001) discovered that serum HBV DNA level associated with ALT elevation was lower in patients with HBeAg (-) CHB when compared with HBeAg (+) CHB patients, and there was usually no or mild liver disease activity when patients with chronic HBV infection had

Table 3. Prognostic ability of serum ALT, DCR3, and HBV DNA viral load for detecting active status in HBeAg (–) CHB patients.

Test Result Variable(s)	Area	SE	P value	95%CI		Cut-off value (ng/mL)	Sensitivity (%)	Specificity (%)
				Lower Bound	Upper Bound			
DcR3	0.914	0.032	$P < 0.0001^*$	0.851	0.977	1.22	87.5	84.4
DNA	0.978	0.013	$P < 0.0001^*$	0.951	1.004	4.73	100	87.5
ALT	0.937	0.032	$P < 0.0001^*$	0.874	1.001	39.5	100	84.4

* $p < 0.05$ is significant.

serum HBV DNA levels of less than 100,000 copies/ml. Sener et al. (2009) investigated 93 CHB patients and found a positive correlation between viral load and ALT levels ($r = 0.768$, $p < 0.001$). In a recent study, Kim et al. (2011) validated the performance of ALT and HBV DNA, and found that these markers may also be used for discriminating HBeAg (–) active carriers from inactive carriers. In the study by Ijaz et al. (2011), HBV DNA load was five times higher in chronic active patients, and a statistically significant positive correlation was found between HBV DNA levels and ALT in HBeAg (–) CHB ($r = 0.911$, $p < 0.05$), but no such association was observed for ALT in chronic inactive patients. These studies showed that in HBeAg (–) patients, low HBV DNA levels were associated with less liver damage, although this relationship was not observed in other study (Chan et al. 2002). These findings suggest that assessment of HBV DNA load and ALT are the most convenient techniques to predict active status in HBeAg (–) CHB patients, and ALT levels are correlated with HBV DNA. These observations are consistent with our results.

The mechanism of ALT elevation in HBeAg (–) CHB patients is unclear. We speculate that although HBeAg (–) represents non-viral replication, the existing HBV DNA still produces other viral antigens that trigger the host immune system to react with infected hepatocytes and cause cell damage, which releases ALT. Although HBV DNA indicates viral load and ALT reflects cell damage, DcR3 represents a third index as the host response to the viral insult and cell damage. The role of DcR3 in HBV pathogenesis is currently unknown. It is well known that the inflammatory/immune response plays an important role in HBV disease. DcR3 may participate in immune suppression, as shown below. DcR3 inhibits T-cell chemotaxis (Shi et al. 2003) and suppresses the activities of macrophages (Chang et al. 2004) and dendritic cells (You et al. 2008). It was reported that DcR3 attenuated the Th1 response and suppressed cell-mediated immunity, which were important for removing virus infection (Hsu et al. 2005). Researchers demonstrated that DcR3 modulated macrophage activation toward an M2-like phenotype in vitro and downregulated MHC class II expression in tumor-associated macrophages via epigenetic control, which made it difficult to clear the virus (Tai et al. 2012). DcR3 is able to elicit the secretion of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and IL-8 from endothelial cells (Yang et al. 2005), as well as promoting inflammation in Crohn disease

partly by inducing NF- κ B activation (Funke et al. 2009).

For the assay of HBV DNA, the PCR method requires highly well-trained personnel, sophisticated instrumentation and an aerosol-controlled environment. Thus, the ELISA-based DcR3 assay may be easily used with ALT as first-line tests to detect active status in HBeAg (–) CHB patients.

In our future study, more detailed data will be obtained, such as correlations with liver biopsy data, increasing the sample number, performing HBV genotyping, and following up patients for a short period. These factors will be helpful in evaluating the utility of serum DcR3 as a new index for active status in HBeAg (–) CHB patients.

In conclusion, we found that the level of DcR3 was high and strongly correlated with ALT and HBV DNA levels. DcR3 may be an indicator for detecting disease activity in HBeAg (–) CHB patients.

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Conflicts of Interest

The authors declare no conflict of interest.

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