ORIGINAL ARTICLE

Gut and Liver, Vol. 7, No. 4, July 2013, pp. 462-468

Hepatitis B Core Antigen Expression in Hepatocytes Reflects Viral Response to Entecavir in Chronic Hepatitis B Patients

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Background/Aims: Hepatitis B core antigen is known to be a major target for virus-specific T cells and also reflects the progression of liver dissease and viral replication. Hepatitis B core antigen expression in hepatocytes leads to altered histological activity, viral replication, and immune response. The purpose of this study is to evaluate whether the topographical distribution of hepatitis B core antigen expression can predict the viral response to entecavir in patients with chronic hepatitis B. **Methods:** We enrolled 91 patients with treatment-naïve chronic hepatitis B. All the patients underwent liver biopsy, and the existence and pattern of hepatitis B core antigen evaluated by immunohistochemistry. All patients received 0.5 mg of entecavir daily following a liver biopsy. We checked the viral response at 3, 6, and 12 months during antiviral therapy. **Results:** Of the 91 patients, 64 (70.3%) had hepatitis B core antigen expression. Of the subcellular patterns, the mixed type was dominant (n=48, 75%). The viral response was significantly higher in the hepatitis B core antigen-negative group than in the hepatitis B core antigen-positive group (88.9% and 54.7%, respectively; p=0.001) after 12 months of entecavir therapy. **Conclusions:** Chronic hepatitis B patients who are hepatitis B core antigen-negative have a better response to entecavir therapy than do hepatitis B core antigen-positive patients. **(Gut Liver 2013;7:462-468)**

Key Words: Hepatitis B virus; Chronic hepatitis B; Hepatitis B core antigen; Hepatitis B e antigen; Entecavir

INTRODUCTION

Hepatitis B virus (HBV) is a type of noncytopathic virus which can cause various degrees of damage to liver tissue, by host immune response. In particular, when this response occurs incompletely in virus-infected hepatocytes, a necroinflammatory process with viral persistence develops, ultimately resulting in end-stage liver disease.[1] Hepatitis B surface antigen and hepatitis B core antigen (HBcAg) are known to be important markers for the proliferation of HBV.[2] In particular, HBcAg, which is produced from precore and core lesions of viral genes, protects HBV DNA and is essential to viral replication.[3] According to immunochemical staining patterns, HBcAg can be classified as cytoplasmic expression (cHBcAg), nuclear expression (nHBcAg), cytoplasmic and nuclear mixed expression (mHBcAg), or negative expression. Negative type shows no HBcAg expression in either the nucleus or cytoplasm of the hepatocytes, cytoplasmic type shows HBcAg expression only in the cytoplasm, but not in the nucleus of hepatocytes, and mixed type shows HBcAg expression in both the nucleus and cytoplasm of the hepatocytes (Fig. 1). The distribution of HBcAg expression in the hepatocyte nucleus and cytoplasm reflects the level of viral replication and histological activity in chronic HBV infection.[2-4] In the viral replicative stage, HBcAg is localized primarily in the nucleus and has minimal liver injury and high HBV-DNA load. However, HBcAg is found in the nucleus and/or cytoplasm of hepatocytes in the viral clearance phase and suggests severe liver cell injury and low viral load.[5,6] Uzun *et al*.[7] suggested that the absence or low levels of HBcAg expression at baseline may be an important predictor in the response to lamivudine and interferon treatment, especially in hepatitis B e antigen (HBeAg)-negative patients. Negative HBcAg staining likely results from a more

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Received on September 18, 2012. Revised on November 16, 2012. Accepted on November 30, 2012. Published online on June 11, 2013.
pISSN 1976-2283 eISSN 2005-1212 http://dx.doi.org/10.5009/gnl.2013.7.4.462

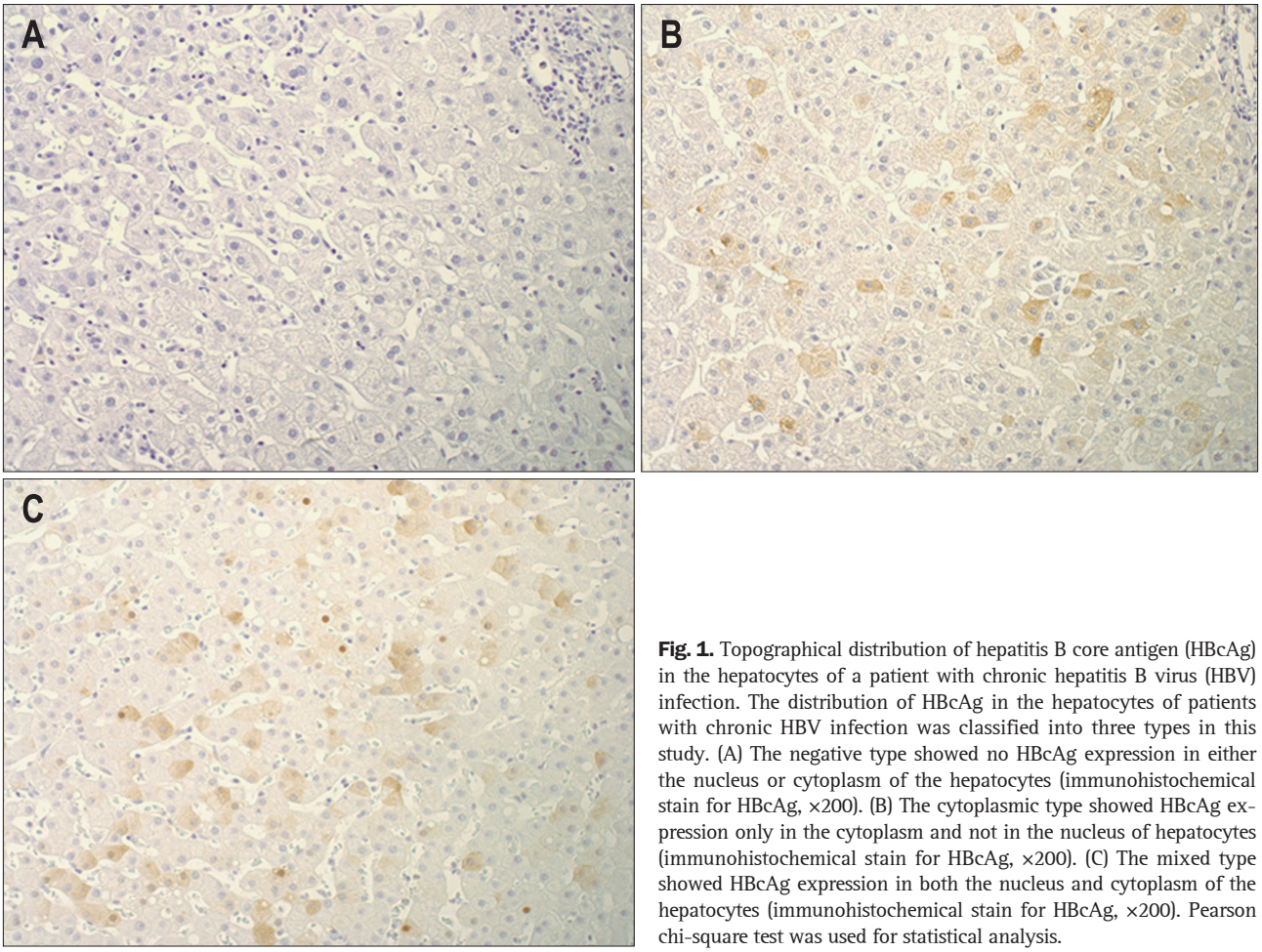

Fig. 1. Topographical distribution of hepatitis B core antigen (HBcAg) in the hepatocytes of a patient with chronic hepatitis B virus (HBV) infection. The distribution of HBcAg in the hepatocytes of patients with chronic HBV infection was classified into three types in this study. (A) The negative type showed no HBcAg expression in either the nucleus or cytoplasm of the hepatocytes (immunohistochemical stain for HBcAg, ×200). (B) The cytoplasmic type showed HBcAg expression only in the cytoplasm and not in the nucleus of hepatocytes (immunohistochemical stain for HBcAg, ×200). (C) The mixed type showed HBcAg expression in both the nucleus and cytoplasm of the hepatocytes (immunohistochemical stain for HBcAg, ×200). Pearson chi-square test was used for statistical analysis.

active host immune T-cell response against a major viral target. Antiviral response by a nucleoside analogue seems to depend not only on inhibiting viral replication but also on modulating immune response. This explains the predictive values of negative expression in viral response to nucleoside analogues. The purpose of this study is to evaluate whether the HBcAg expression pattern in hepatocytes can predict viral response to entecavir in chronic hepatitis B.

MATERIALS AND METHODS

1. Patients

We enrolled 91 patients with chronic hepatitis B, who were admitted to CHA Bundang Medical Center from January 2007, to May 2010; we reviewed their medical charts retrospectively. The Institutional Review Board of the CHA Bundang Medical Center approved this study (IRB no. BD2012-035D).

All patients had elevated alanine aminotransferase (ALT) (>upper limit of normal) and elevated HBV DNA levels by real time polymerase chain reaction (PCR) (>10^5 copies/mL in HBeAg-positive patients and >10^4 copies/mL in HBeAg-negative patients)[8] and tested negative for serological markers of the hepatitis C virus. None of the patients showed evidence of other liver diseases, including autoimmune disease, metabolic liver disease, or drug toxicity; alcohol intake was absent or <20 g/day in all patients. All patients received 0.5 mg of entecavir daily, after liver biopsy, and we checked HBV DNA titer at baseline, 3, 6, and 12 months during antiviral therapy. We defined viral response as a decrease in serum HBV DNA to undetectable levels (<70 copies/mL)[8] by PCR assays (COBAS TaqMan HBV test; Roche Diagnostics, Meylan, France) and viral unresponse as a nondecrease to undetectable levels.

2. Evaluation of liver biopsy specimens

All patients gave their informed consents to the liver biopsy procedure. Liver biopsy was performed with Trucut needles (16G; TSK Laboratory, Tochigi, Japan), using a sono-guided technique. Liver biopsy specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin.

Histologic grade was evaluated based on the modified histology activity index (HAI) which is summation of four individual scores representing periportal or periseptal interface hepatitis, confluent necrosis, cocal (spotty) lytic necrosis, and portal inflammation. The combined score ranges from 0 to 18. The stage and modified staging system were classified as 0 to 4 and 0 to 6 respectively by observing the degree of fibrosis on Gomori's

silver impregnation stain and Masson's trichrome stain.[9,10]

Biopsied tissue sections cut at 4 mm from representative tissue blocks are prepared and placed in a paraffin-oven to remove most of the paraffin. For complete deparaffinization, specimens are then passed through xylene and a series of alcohol Dilutions for 5 minutes, and microwave treatment was used for the antigen retrieval for 10 minutes. After soaking in the methanol solution with 3% H_2O_2 for 5 minutes for blockage of endogenous peroxidase, peroxidase conjugated Envision kit (Envision-PO, Envision System; DAKO, Carpinteria, CA, USA) for rabbit primary antibodies are applied on the specimens for immunohistochemical staining for the HBcAg in the hepatocytes.

3. Statistics

All statistics were analyzed using PASW version 18.0 software (IBM Co., Armonk, NY, USA); Student t-test, Pearson chi-square test, logistic regression, and Cramer's V were used where appropriate, and a $p<0.05$ was considered statistically significant.

Table 1. Clinical and Laboratory Features of the Patients

Feature	All patients (n=91)	HBcAg (+) (n=64)	HBcAg (-) (n=27)	p-value
Age, yr	42.2±9.9	42.0±9.8	42.5±10.3	0.821
Sex				0.298
Male	56 (61.5)	41 (64.1)	15 (44.4)	
Female	35 (38.5)	23 (35.9)	12 (55.6)	
BMI, kg/m^2	24.2±3.4	24.8±.3.3	23.0±3.1	0.210
HBeAg				0.017
Positive	72 (79.1)	55 (85.9)	17 (37.0)	
Negative	19 (20.9)	9 (14.1)	10 (63.0)	
AST, IU/L	177.5±202.8	149.4±174.1	244.5±250.1	0.440
ALT, IU/L	189.2±170.1	156.1±124.5	267.6±231.5	0.040
PT	87.6±16.2	87.7±16.2	87.5±16.7	0.960
Albumin	4.1±0.4	4.15±0.4	4.07±0.4	0.402
Total bilirubin	1.3±2.2	1.1±2.2	1.7±2.3	0.186
Total cholesterol	170.6±39.0	170.0±41.7	172.3±32.4	0.798
$\log_{10}$ HBV DNA*	6.69±1.77	7.03±1.66	5.89±1.80	0.020

Data are presented as mean±SD or number (%). Pearson chi-square test and Student t-test were used for statistical analysis.
HBcAg, hepatitis B core antigen; BMI, body mass index; HBeAg, hepatitis B e antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PT, prothrombin time; HBV, hepatitis B virus.
*$\log_{10}$ HBV DNA: mean $\log_{10}$ HBV DNA level at the baseline, $\log_{10}$ copies/mL±SD.

Table 2. Histologic Features according to Hepatitis B Core Antigen Distribution

Feature	All patients (n=91)	HBcAg-negative (n=27)	HBcAg-positive			p-value*
			Total (n=64)	Cytoplasmic (n=14)	Mixed (n=48)	
Grade						
Lobular inflmmation	2.1±1.0	1.90±1.1	2.2±0.9	2.2±1.3	2.2±0.8	0.116
Portoperiportal activity	3.4±0.8	3.2±1.1	3.5±0.7	3.6±0.5	3.5±0.7	0.049
Modified HAI	8.9±2.0	8.3±2.2	9.2±1.9	9.8±2.1	9.04±1.9	0.056
Stage	2.3±0.8	2.5±0.8	2.3±0.8	2.7±0.7	2.1±0.8	0.173
Modified stage†	3.0±1.2	3.4±1.2	2.8±1.2	3.9±1.0	2.5±1.1	0.051

Data are presented as mean±SD. Student t-test was used for statistical analysis.
HBcAg, hepatitis B core antigen; HAI, histologic activity index.
*A p-value shows significant differences between the HBcAg-negative and HBcAg-positive groups in this table. Only two patients showed nuclear HBcAg, and they were excluded from statistical analysis because of the small sample size; †Stage of modification: no fibrosis=0, fibrosis of some of the portal area=1, fibrosis of most of the portal area=2, mostly portal fibrosis with occasional portal-to-portal bridging=3, portal fibrosis with marked bridging (portal-to-portal as well as portal-to-central)=4, marked bridging with occasional nodules (incomplete cirrhosis)=5, cirrhosis=6.

Table 3. Clinical and Laboratory Features of Responder versus Nonresponder Patients

Feature	Responders (n=59)	Nonresponders (n=32)	p-value
Age, yr	42.4±9.3	41.8±11.0	0.776
Gender			0.208
Male	34 (57.6)	22 (68.8)	
Femal	25 (42.4)	10 (31.2)	
BMI, kg/m^2	24.0±3.6	24.6±3.0	0.435
HBeAg	60	40	0.039
Positive	43 (72.9)	29 (90.6)	
Negative	16 (27.1)	3 (9.4)	
AST, IU/L	204.6±236.4	127.7±104.9	0.089
ALT, IU/L	200.5±176.7	168.3±157.8	0.392
PT, %	85.5±14.9	91.5±18.1	0.101
Albumin	4.1±0.4	4.2±0.4	0.689
Total bilirubin	1.6±2.7	0.7±0.4	0.090
Grade			
Lobular inflmmation	2.1±1.0	2.1±0.9	0.960
Portoperiportal activity	3.4±0.8	3.3±0.8	0.591
Modified HAI	9.0±2.0	8.8±2.2	0.706
Fibrosis (=stage)	2.5±0.8	2.0±0.8	0.006
Modified stage	3.2±1.3	2.5±1.1	0.011
$\log_{10}$ HBV DNA*	6.22±1.6	7.57±1.39	<0.001

Data are presented as mean±SD or number (%). Pearson chi-square test and Student t-test were used for statistical analysis.
BMI, body mass index; HBeAg, hepatitis B e antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PT, prothrombin time; HAI, histologic activity index; HBV, hepatitis B virus.
*$\log_{10}$ HBV DNA: mean $\log_{10}$ HBV DNA level at baseline.

RESULTS

1. Expression and distribution of HBcAg in hepatocytes

Sixty-four cases (70.3%) expressed HBcAg in immunohistochemical staining. The demographic details are listed in Table 1. There were statistical differences in the ratio of HBeAg-positivity, ALT, and mean $\log_{10}$ HBV DNA levels at the baseline, between the HBcAg-positive and HBcAg-negative group.

2. Correlation between histologic activity of hepatitis and intracellular expression of HBcAg

Fig. 1 shows the three types of HBcAg expression in liver tissue. HBcAg-positive group showed higher modified stage and lower modified HAI than HBcAg-negative group, but they were not significantly different (p=0.051 and 0.056, respectively in modified stage and modified HAI). Portoperiportal activity in HBcAg-negative and HBcAg-positive group was 3.15±1.06 and 3.52±0.67 respectively, which was statistically significant (p=0.049) (Table 2). In a subgroup analysis, the modified stage of the cases which expressed cytoplasmic HBcAg was significantly higher than that of the mixed type (p<0.001). Portoperiportal activity was not significantly different between the cases with mixed and cytoplasmic HBcAg expression (p=0.443).

3. HBcAg expression patterns and viral response to entecavir

Throughout the entire treatment period, HBV DNA level in the HBcAg-negative group was significantly lower than in the HBcAg-positive group, but there was no difference between the cases with mixed and cytoplasmic HBcAg expression in a subgroup analysis. After 12 months of entecavir treatment, 59 patients (64.8%) were responders. Responders included a significantly more HBeAg-positive patients, higher fibrosis stage and

Table 4. Hepatitis B Virus DNA Level and Viral Response to Entecavir according to Hepatitis B Core Antigen Distribution

	All patients (n=91)	HBcAg-negative (n=27)	HBcAg-positive			p-value*
			Total (n=64)	Cytoplasmic (n=14)	Mixed (n=48)	
$\log_{10}$ HBV DNA						
Baseline	6.69±1.77	5.89±1.80	7.03±1.66	7.02±1.57	7.02±1.73	0.004
3 mo	4.01±1.48	3.10±1.41	4.39±1.35	4.24±1.88	4.42±1.8	<0.001
6 mo	2.65±1.14	2.06±0.43	2.90±1.26	2.53±0.84	3.00±1.34	<0.001
12 mo	2.35±0.91	1.89±0.14	2.55±1.02	2.36±0.92	2.61±1.07	<0.001
Virologic response						
3 mo	12 (13.2)	9 (33.3)	3 (4.7)	3 (21.4)	0 (0.0)	<0.001
6 mo	41 (45.1)	19 (70.4)	22 (34.4)	6 (42.9)	15 (31.2)	0.002
12 mo	59 (64.8)	24 (88.9)	35 (54.7)	10 (71.4)	24 (50.0)	<0.001

Data are presented as mean±SD or number (%). Pearson chi-square test and Student t-test were used for statistical analysis.
HbcAg, hepatitis B core antigen; HBV, hepatitis B virus.
*A p-value shows differences between HBcAg-negative and HBcAg-positive groups. The number of patients showing nuclear HBcAg was only two, and they were excluded from statistical analysis because of small sample size.

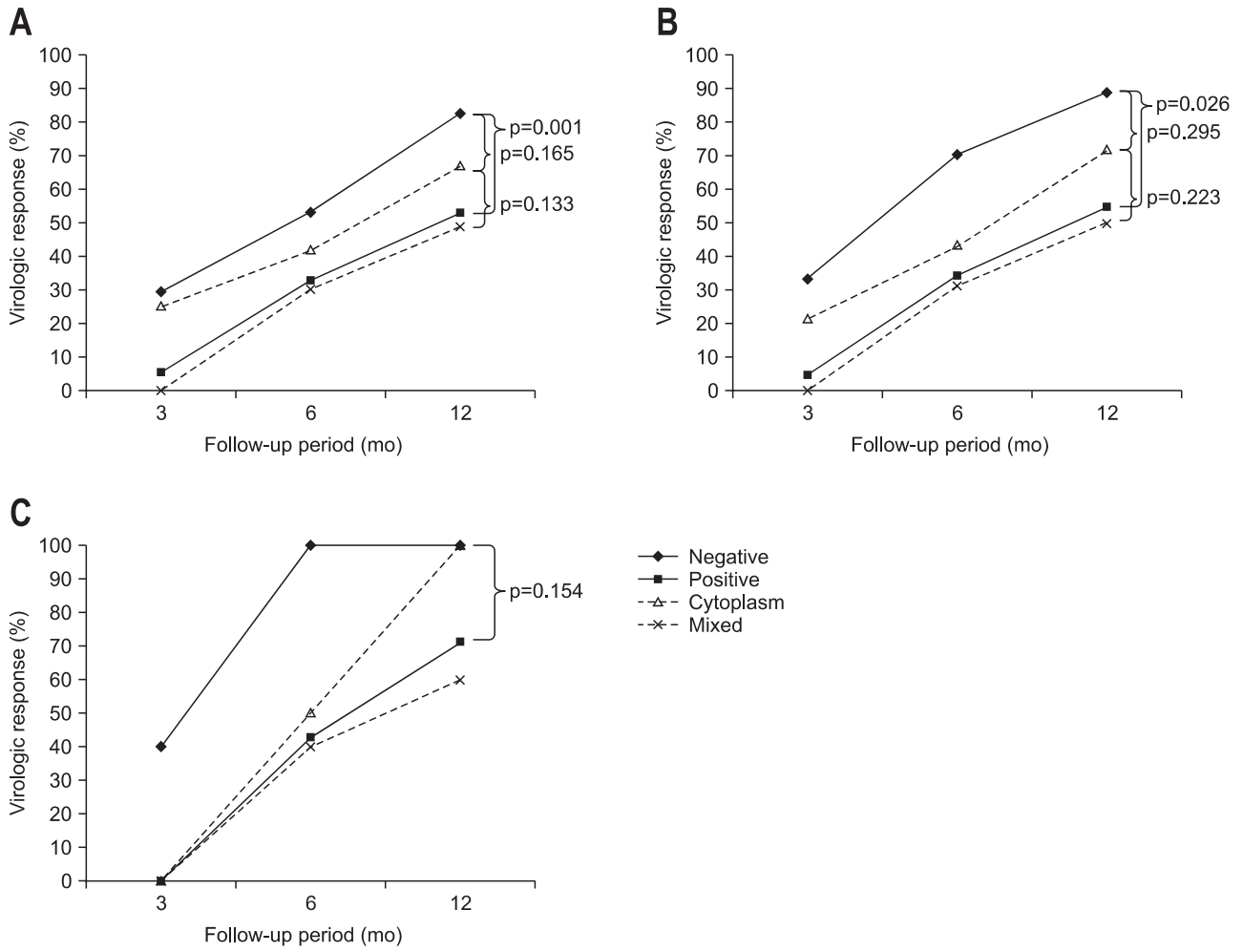

Fig. 2. Virologic response to entecavir therapy according to the pattern of hepatitis B core antigen (HBcAg) expression in all patients, hepatitis B e antigen (HBeAg)-positive patients, and HBeAg-negative patients. (A) The HBcAg-negative group showed a better response to entecavir therapy than the HBcAg-positive group, but no significant differences were shown among subtypes of HBcAg expression. (B) Similarly to the HBeAg-positive group, the HBcAg-negative group shows a higher viral response than the HBcAg-positive group, (C) and in the HBeAg-negative groups, no significant difference was shown between the two groups; this apparent similarity may be the result of low patient numbers; the data revealed a strong correlation.

Table 5. Viral Response to Entecavir according to Hepatitis B Core Antigen and Hepatitis B e Antigen Expression

	All patients	HBcAg-negative	HBcAg-positive			p-value*
			Total	Cytoplasmic	Mixed	
HBeAg (+)	72	17	55	12	43	
3 mo	8 (11.1)	5 (29.4)	3 (5.5)	3 (25.0)	0 (0.0)	0.015
6 mo	27 (37.5)	9 (52.9)	18 (32.7)	5 (41.7)	13 (30.2)	0.112
12 mo	43 (59.7)	14 (82.4)	29 (52.7)	8 (66.7)	21 (48.8)	0.026
HBeAg (-)	17	10	7	2	5	
3 mo	4 (23.5)	4 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.088
6 mo	13 (76.4)	10 (100.0)	3 (42.9)	1 (50.0)	2 (40.0)	0.015
12 mo	15 (88.2)	10 (100.0)	5 (71.4)	2 (100.0)	3 (60.0)	0.154

Data are presented as number (%). Pearson chi-square test was used for statistical analysis.
HbcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen.
*A p-value shows the significances of the difference between the HBcAg-negative and HBcAg-positive groups. Only two patients showed nuclear HBcAg, and they were excluded from the statistical analysis because of the small sample size.

modified stage, and lower HBV DNA titer at baseline compared to nonresponders (Table 3). However, logistic regression shows that periportal activity, degree of fibrosis, and modified stage did not affect significantly on entecavir response. Overall viral response was 13.2%, 45.1%, and 64.8% at 3, 6, and 12 months. A significant difference in viral response was seen between the HBcAg-negative and HBcAg-positive groups at 3, 6, and 12 months of treatment (33.3% vs 4.7%, p=0.001; 70.4% vs 34.4%, p=0.002; 88.9% vs 54.7%, p=0.001; and odds ratio, 5.88 at 12 months) (Table 4). We also found a sequential increase in viral response from mixed cytoplasmic to negative type but could not find significant differences between each subtypes (Fig. 2A). We evaluated a viral response according to HBeAg positivity. In HBeAg-positive group analysis, the HBcAg-negative group shows a significantly higher viral response of entecavir, at 12 months, than the HBcAg-positive group (82.4% vs 52.7%, p=0.026) (Fig. 2B). However, we could not find a significant difference in viral responses between the HBcAg-negative and HBcAg-positive groups, among patients who show HBeAg negativity (100% vs 71.4%, p=0.154) (Fig. 2C, Table 5). In addition, subtypes of HBcAg expression showed no significant differences in viral response among both HBeAg-negative and HBeAg-positive groups (data was not shown).

DISCUSSION

Many investigators suggested that the chronicity of HBV infection is caused by a deficient cellular immune function, but the mechanism has not been defined.[11] HBcAg is one of the main antigens causing the T cell immune reaction and has a stronger antigenicity than other viral proteins. The $CD4^+$ lymphocyte is known to be a very specific immune reaction to HBcAg, and the $CD8^+$ lymphocyte plays an important role in viral clearance.[12,13] HBcAg expression pattern in hepatocytes had been found to be related to the activity of liver disease, hepatocyte proliferation, and HBV DNA level.[14] In previous studies, the cytoplasmic type was seen in patients with active hepatitis and hepatocyte regeneration.[3,4] The nuclear type was observed in patients with minimal liver injury, in the absence of hepatocyte regeneration,[6,15] and the degree of nuclear HBcAg reflects the level of viral replication in chronic HBV infection.[16] Serinoz *et al*.,[6] suggested that expression of HBcAg correlates with the liver pathology and the three phases of chronic HBV infection: 1) the early immune tolerance phase is characterized by nuclear HBcAg, mild disease, and low HBeAg seroconversion rate; 2) the virus replication/elimination phase by cHBcAg or negative HBcAg, frequent active hepatitis, and high HBeAg seroconversion rate; and 3) the inactive virus replication phase by negative HBcAg. Thus, the rate of hepatocyte proliferation and expression of the HBcAg may be important in determining the prognosis and the viral resposnse to treatment in chronic HBV hepatitis patients. Uzun *et al*.[7] investigated the role of HBcAg expression in response to antiviral treatment in patients with chronic hepatitis B, who were treated with lamivudine and interferon combined or with lamivudine alone, and supported the idea that the absence or a low level of HBcAg expression may predict good viral response, especially in the HBeAg-negative group. Other studies showed that viral response to lamivudine differed significantly between the cytoplasmic and mixed type.[17] The nucleoside analogue, entecavir, is known to inhibit viral replication and to restore T lymphocyte subpopulations.[18] Therefore, we thought that entecavir could enhance immune reaction and might have a different level of immune reaction, according to HBcAg distribution. In the present study, the HBcAg-negative group had a stronger immune reaction and a better viral response to entecavir than did the HBcAg-positive group. In the HBeAg subgroup analysis (the HBeAg-negative group), however, we failed to show the significant higher responses of HBcAg-negative patients to entecavir therapy, statistically; this statistical irrelevance might be due to the small number of HBeAg-negative patients, and previous studies supported the correlation in HBeAg-negative patients treated with antiviral therapy.[7] Furthermore, we could find stronger correlations between the HBcAg and entecavir responses in the HBeAg-negative group than in HBeAg-positive group (Cramer's V, 0.471 in HBeAg-negative group and 0.244 in HBeAg-positive group). Only two nuclear-type patients were found in our study as we enrolled patients who had abnormal liver funsctions and needed antiviral therapy, suggesting the immune clearance phase. This is the first study that shows a correlation between distribution patterns of HBcAg and the viral response to entecavir therapy. Our study suggested that the distribution of HBcAg in hepatocytes can help predict viral response to entecavir in the treatment of naïve chronic HBV hepatitis. Interestingly, in the present study, as Kim *et al*.[1] suggested, the HBcAg-negative group displayed far more advanced fibrosis and lower HBV DNA titer than the HBcAg-positive group. These findings could be explained by selective lysis of hepatocytes containing HBcAg during the immune clearance phase. Eventually, fibrosis is more developed, and HBV DNA titer decreased after this clearance phase.

Our study was a retrospective study, and we could not enroll patients in immune tolerance phase. Moreover, HBcAg is not the only viral target for immune control.[19] In the future, a larger-scale, long-term prospective study will be needed in order to validate the value of the HBcAg expression pattern in hepatocytes in predicting response to nucleoside analogues.

In conclusions, HBcAg-negative chronic hepatitis B patients have a better response to entecavir therapy than do HBcAg-positive patients, and evaluation of existence and pattern of HBcAg by liver biopsy might contribute to predict response of entecavir therapy.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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