Int J Clin Exp Pathol 2016;9(3):3563-3570
www.ijcep.com /ISSN:1936-2625/IJCEP0021758

Original Article

Hepatitis B core antigen expression in hepatocytes reflects viral response to Peg-IFNα-2a in HBeAg-positive chronic hepatitis B patients

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Received December 13, 2015; Accepted February 18, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: Hepatitis B core antigen is known to be a major target for virus-specific T cells and also reflects the progression of liver disease and viral replication. This study aims to evaluate whether topographical distribution of hepatitis B core antigen expression can predict viral response to Peginterferon alfa-2a (Peg-IFNα-2a) in patients with chronic hepatitis B. We enrolled 207 patients with chronic hepatitis B. All patients underwent liver biopsy, and the existence and pattern of hepatitis B core antigen evaluated by immunohistochemistry. All patients received 180 μg of Peg-IFNα-2a once weekly for 48 weeks following a liver biopsy. We checked viral response at 48 weeks during antiviral therapy. Of 207 patients, 11 (5.31%) had no hepatitis B core antigen expression (HBcAg-negative), others had hepatitis B core antigen expression. Of the hepatitis B core antigen expression, 83 (42.35%) belong to cytoplasmic expression (cHBcAg), 105 (53.57%) cases was cytoplasmic dominance expression (cdHBcAg), while only 8 (4.08%) belong to cytoplasmic and nuclear mean expression (mHBcAg). HBcAg-negative group has lowest baseline HBV DNA among four groups ($P<0.01$). Liver tissue inflammation in HBcAg-negative group and cHBcAg group reached G3 (respectively 54.55%, 42.17%), and were higher than other groups ($P<0.01$). The viral response was significantly higher in HBcAg-negative group than in hepatitis B core antigen-positive group (88.9% and 54.7%, respectively; $P<0.001$) after 48 weeks of Peg-IFNα therapy. In conclusion, chronic hepatitis B patients who are hepatitis B core antigen-negative have a better response to Peg-IFNα therapy than do hepatitis B core antigen-positive patients.

Keywords: Chronic hepatitis B, core antigen of hepatitis B, expression pattern, histology, interferon alpha

Introduction

Hepatitis B virus (HBV) is a circular, partially double-stranded DNA virus [1]. HBV infection often leads to chronic hepatitis when it occurs in the neonatal period or early childhood. The natural history of chronic hepatitis B (CHB) has been divided into 4 phases: immune tolerance, immune clearance, immune control, and reactivation after HBV e antigen (HBeAg) seroconversion [2]. HBV c antigen (HBcAg) is an intracellular antigen that is expressed in HBV-infected hepatocytes. Immunodetection of HBcAg in hepatocytes in liver tissue thus provides helpful information about the replicative status of HBV and is usually performed as part of a histopathological diagnosis of patients with chronic hepatitis B [3]. According to immunochemical staining patterns, HBcAg can be classified as cytoplasmic expression (cHBcAg), cytoplasmic dominance expression (cdHBcAg), cytoplasmic and nuclear mean 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, cytoplasmic dominance type shows more than 2/3 HBcAg expression in the cytoplasm, and mean type shows half HBcAg expression in the nucleus and half HBcAg expression in cytoplasm of the hepatocytes (**Figure 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 [4]. In the viral replicative stage,

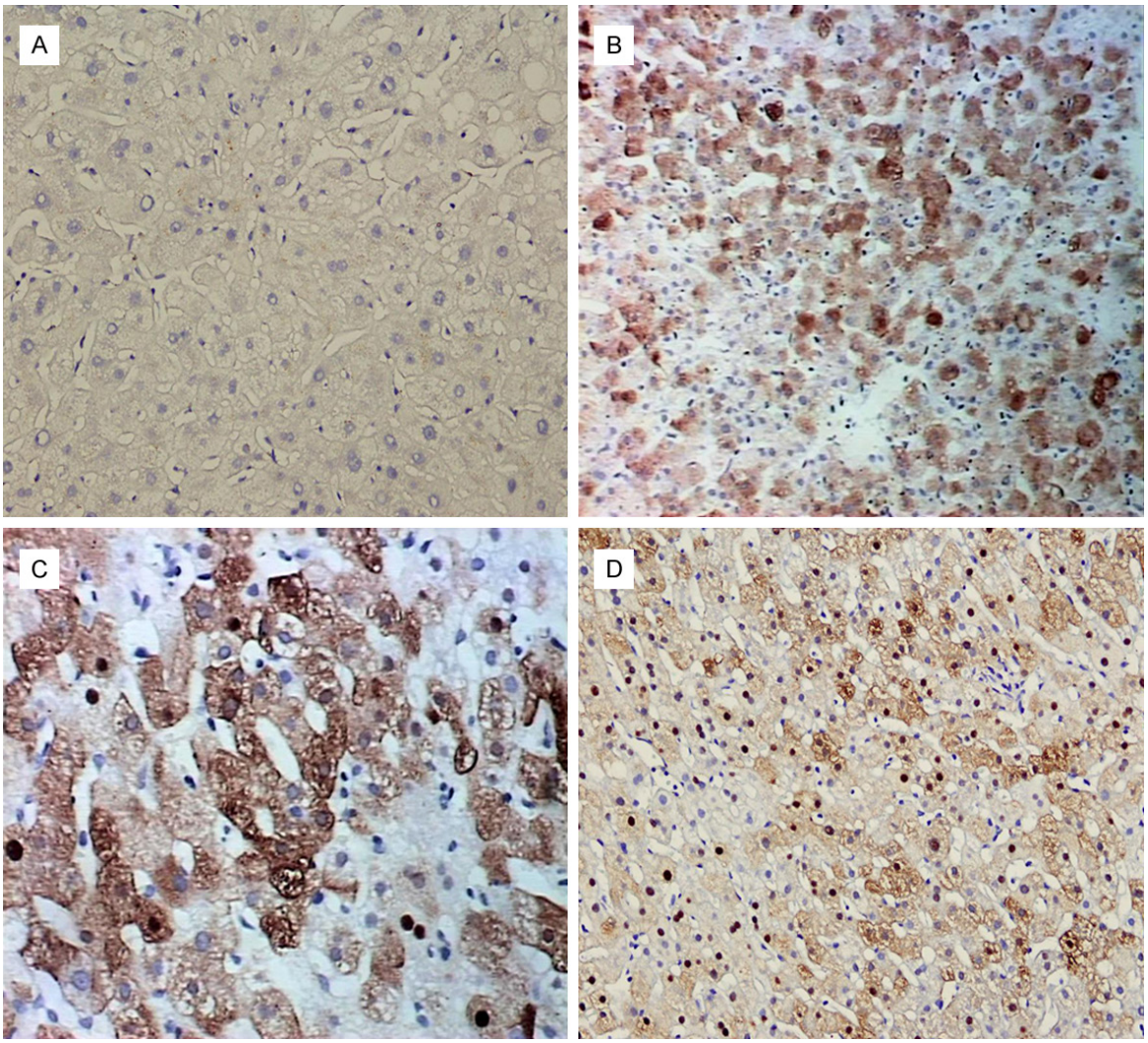

Figure 1. Topographical distribution of hepatitis B core antigen (HBcAg) in the hepatocytes of a patient with chronic hepatitis B virus (HBV) infection. The distribution of HBcAg in the hepatocytes of patients with chronic HBV infection was classified into four types in this study. A. The negative type 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 cytoplasmic dominance type showed more than 2/3 HBcAg expression in the cytoplasm of hepatocytes (immunohistochemical stain for HBcAg, ×200). D. The mean type showed half of HBcAg expression in the nucleus and half of HBcAg expression in the cytoplasm of the hepatocytes (immunohistochemical stain for HBcAg, ×200).

HBcAg is localized primarily in the nucleus and has minimal liver injury and high HBV-DNA load. However, HBcAg is found in the cytoplasm of hepatocytes in the viral clearance phase and suggests severe liver cell injury and low viral load [5]. It was 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. HBcAg-negative staining likely result from a more 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 Peg-IFNα in chronic hepatitis B.

Materials and methods

Patients

We enrolled 207 patients with CHB, who were admitted to Hepatology Unit of Xiamen Hospital of Traditional Chinese Medicine from September 2011 to August 2013; we reviewed their medical charts retrospectively. The study protocol was reviewed and approved by the Human Research Ethics Committee of Xiamen Hospital of Traditional Chinese Medicine. The written informed consent was obtained from each recruited patient before the questionnaire survey.

All patients had been positive for hepatitis B surface antigen (HBsAg) for at least 6 months, were HBeAg-positive, and had a serum HBV DNA level of at least 20000 IU/mL and an ALT level that was 2 to 10 times the upper limit of normal (ULN, 40 U/L). And tested negative for serological markers of the hepatitis A, C or D virus infection. None of the patients showed evidence of other liver diseases, including decompensate liver cirrhosis, HCC on presentation, autoimmune disease, metabolic liver disease, or drug toxicity, alcohol intake was absent or <20 g/day in all patients. All patients received 180 μg of Peg-IFNα-2a once week after liver biopsy, and we checked HBV DNA titer at baseline and during antiviral therapy at 48 weeks (end of treatment). We defined viral response as a decrease in serum HBV DNA to undetectable levels (<500 IU/mL) by PCR assays (COBAS TaqMan HBV test; Roche Diagnostics, Meylan, France) and viral unresponse as a nondecrease to undetectable levels.

Evaluation of liver biopsy specimens

All patients gave their informed consents to the liver biopsy procedure. Liver biopsy was performed with 16 G biopsy needles guided by ultrasonography. A qualified biopsy specimen was either a minimum 1.5 cm long or displayed 6 or more portal tracts. The specimens were fixed, paraffin-embedded, and stained with hematoxylin and eosin (HE staining). Scheuer's scoring system was used to semi-quantify the histological necroinflammation from G0 to G4 and fibrosis stages from S0 to S4 by the same pathologist, who was blinded to the biochemical and virologic results of the patients. Fibrosis was evaluated in all specimens by subjecting them to Masson-Trichrome staining. 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.

Combination response

All clinical trials using antiviral agents address these responses individually or in combination. Different types of responses resulting from Peg-IFNα-2a therapy have been identified, namely, complete response (CR), partial response (partial response, PR), non-response (no response, NR), and HBeAg seroconversion.

CR is defined by ALT less than normal level and HBV DNA less than 500 IU/ml and disappearance of HBeAg or appearance of HBeAb.

PR is defined by decrease of ALT, HBV DNA or disappearance of HBeAg or appearance of HBeAb.

NR is defined by no progression of markers above mentioned.

HBeAg seroconversion means that disappearance of HBeAg and appearance of HBeAb, in the meantime, HBV DNA <500 IU/mL.

Statistics

All statistics analyses were performed using SPSS 13.0 software (IBM Co., Armonk, NY, USA); measurement data were analyzed with mean ± SD, abnormal distribution was analyzed with a median (interquartile spacing). Student t-test, Pearson chi-square test, Spearman rank correlation analysis, independent Wilcoxon rank sum test, Wilcoxon signed rank sum test were used where appropriate, and a $P<0.05$ was considered statistically significant.

Table 1. Clinical and laboratory features of the patients

Feature	HBcAg-negative (n=11)	cHBcAg (n=83)	cdHBcAg (n=105)	mHBcAg (n=8)	*p*-value
Age, yr	31.2±5.1	29.5±6.8	29.0±6.5	29.8±5.0	0.7543
Sex					0.5674
Male	9	55	72	7	
Female	2	28	33	1	
ALT, U/L	157.0 (169.0)	183.0 (186.0)	197.0 (169.0)	222.5 (389.0)	0.4626
AST, U/L	60.0 (36.0)	60.0 (36.0)	101.0 (75.5)	97.5 (128.3)	0.0502
HBV DNA	5.40 (1.48)	7.15 (1.08)	7.37 (0.91)	7.14 (0.54)	0.000

Continuous variables are expressed as mean ± SD or median (interquartile range), and categorical variables are described by count. Pearson chi-square test and Student t-test were used for statistical analysis. Abbreviations: HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HBV, hepatitis B virus.

Table 2. Histologic necroinflammation of hepatitis and intracellular expression of HBcAg

Feature	HBcAg-Negative (n=11)	cHBcAg (n=83)	cdHBcAg (n=105)	mHBcAg (n=8)	Spearman rank correlation	Coefficient of contingency
G1	1	1	2	0	0.0332	-0.1499
G2	4	47	73	7		
G3	5	30	28	0		
G4	1	5	2	1		

Categorical variables are described by count and proportions. Pearson chi-square test, Spearman rank correlation analysis and coefficient of contingency were used for statistical analysis.

Results

Expression and distribution of HBcAg in hepatocytes

The baseline characteristics of our cohort are listed in **Table 1**. Of the 207 patients, 11 (5.31%) had no hepatitis B core antigen expression (HBcAg-negative), others had hepatitis B core antigen expression. Of the hepatitis B core antigen expression, 83 (42.35%) belong to cytoplasmic expression (cHBcAg), 105 (53.57%) cases was cytoplasmic dominance expression (cdHBcAg), while only 8 (4.08%) belong to cytoplasmic and nuclear mean expression (mHBcAg). HBcAg-negative group has the lowest baseline HBV DNA among the four groups (P<0.01), while other features such as age, sex, ALT, and AST has no differences.

Correlation between histologic activity of hepatitis and intracellular expression of HBcAg

From **Table 2**, there were statistical differences in the histologic necroinflammation among four groups (P<0.05) by the Spearman rank correlation analysis and coefficient of contingency. In a subgroup analysis, the histologic necroinflammation in the HBcAg-negative group and cHBcAg group reached G3/G4 (respectively 54.55%, 42.17%), while most of cdHBcAg group and mHBcAg group maintain G1/G2. **Figure 1** shows the four types of HBcAg expression in liver tissue. Histologic necroinflammation which expressed in HBcAg-negative or cHBcAg group were significantly higher than that of the cdHBcAg or mHBcAg group.

HBcAg expression patterns and ALT, AST and HBV DNA levels to Peg-IFNα

After 48 weeks of Peg-IFNα treatment, the ALT, AST and HBV DNA levels in the four groups were significantly decreased compared to pre treatment in **Figure 2**, while and the AST and HBV DNA levels in the mHBcAg was no significantly decreased.

HBcAg expression patterns and HBeAg serum conversion rate *to Peg-IFNα*

After Peg-IFNα treatment, the rate of HBeAg seroconversion was difference in four groups, and Fisher's exact test is 0.0028. HBeAg seroconversion in the HBcAg-negative group was the highest compared to other groups (P<0.01). There were no significant difference was found among groups of cdHBcAg, cdHBcAg and mHBcAg (P>0.05) in **Figure 3**. There was no HBeAg seroconversion in mHBcAg group.

HBcAg expression patterns and combination response to Peg-IFNα

After 48 weeks of Peg-IFNα treatment, a significant difference in viral response was seen among the four groups (P=0.0227). We also found a sequential increase in combination response from mean type to negative type. The HBcAg-negative group shows the highest rate

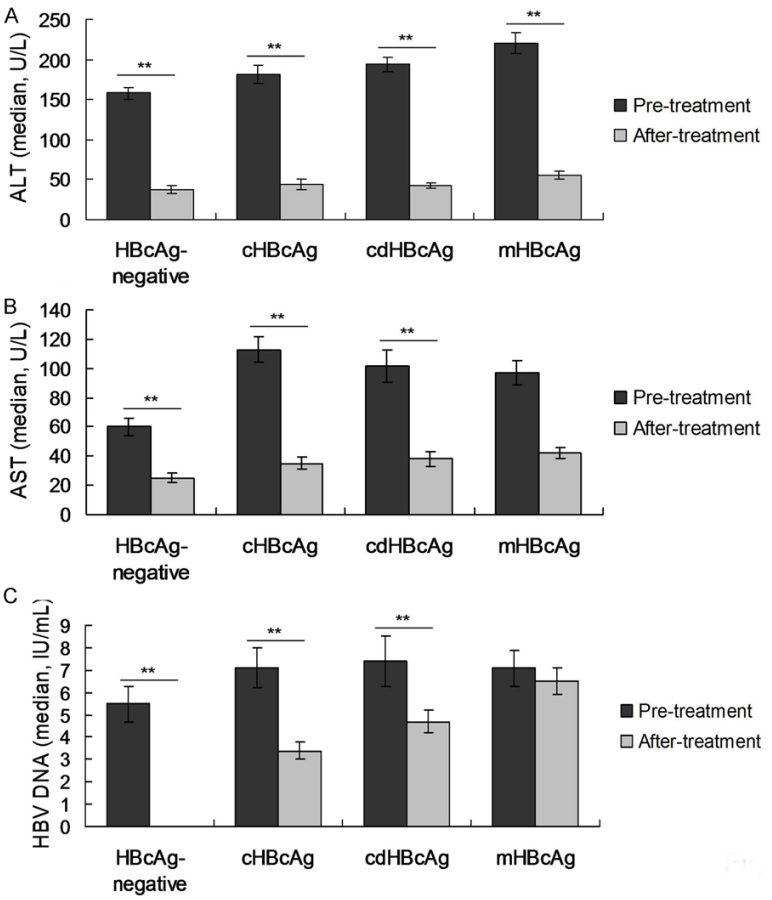

Figure 2. HBcAg expression patterns and ALT, AST and HBV DNA levels to Peg-IFNα. A. ALT levels significantly decreased in four groups after treatment (P<0.01). B. AST levels significantly decreased in HBcAg-negative, cHBcAg and cdHBcAgr groups after treatment (P<0.01), while mHBcAg group has no significance. C. HBV DNA significantly decreased in HBcAg-negative, cHBcAg and cdHBcAgr groups after treatment (P<0.01), while mHBcAg group has no significance. Continuous variables are expressed as median (interquartile range). Pearson chi-square test and Student t-test were used for statistical analysis.

of complete response to Peg-IFNα, reached to 72.7% (**Table 3**).

Histologic activity of hepatitis pre-treatment and combination response to Peg-IFNα

According to histologic activity of hepatitis, of the 207 patients, most of cases (131) were G2, 63 cases were G3, and only 4 and 9 patients were G1 and G4 respectively. After 48 weeks of Peg-IFNα treatment, a significant difference in combination response was seen according to histologic activity of hepatitis (P=0.0404) (**Table 4**). It was found that higher histologic activity of hepatitis, higher combination response.

Discussion

Chronic hepatitis B (CHB) caused by persistent infection with hepatitis B virus (HBV), is a common chronic liver disease in mainland China. According to the latest national hepatitis B seroepidemiological survey [6], the estimated current HBV carriers in mainland China run up to 93 million, including 20-30 million patients with CHB [7]. Current international guidelines recommend first-line treatment of CHB infection with pegylated interferon α, entecavir, or tenofovir, but the optimal treatment for an individual patient is controversial.

Peg-IFNα was recommended by 2012 APASL of CHB treatment guidelines for its higher combined response rate and higher HBeAg seroconversion rate [8]. Researchers have shown that the clinical effect of Peg-IFNα is affected by several factors, such as baseline HBV DNA level, baseline ALT level, gender, disease duration, whether the mother-to-fetus transmission, baseline liver fibrosis, adherence to treatment and if combined with other viruses infection [9, 10]. Predictors of response are useful to provide the most appropriate antiviral therapy to the most suitable patients, in order to achieve the best response and improve the clinical outcome of chronic hepatitis B patients. If we can find more predictors of response to guide the Peg-IFNα therapy, we can acquire the better effects and reduce the waste of financial resources.

It is believed that cytoplasmic HBcAg is the main target antigen of CD8 T-lymphocytes (CTL) [11], which plays an important role in the immune injury of CHB. In the immune tolerant phase, HBcAg mainly in hepatocyte nucleus, and less in the hepatocyte cytoplasm. In the

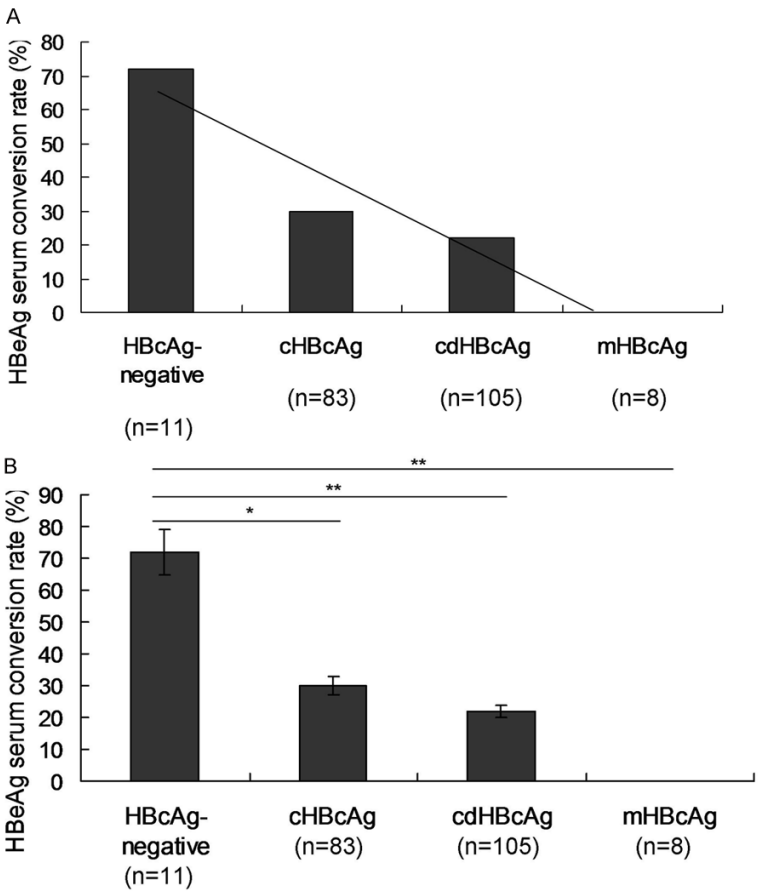

Figure 3. HBcAg expression patterns and HBeAg serumconversion rate to Peg-IFNα. A. After Peg-IFNα treatment, the rate of HBeAg seroconversion was difference in four groups. Data are presented as number (%) and Fisher's exact test was used for statistical analysis. B. Comparison of HBeAg serumconversion rate to Peg-IFNα among HBcAg expression patterns, HBcAg-negative group was the highest compared to other groups ($P<0.01$). There were no significant difference was found among groups of cdHBcAg, cdHBcAg and mHBcAg ($P>0.05$). Pearson chi-square test and Student t-test were used for statistical analysis.

Table 3. HBcAg expression patterns and combination response to Peg-IFNα

Feature	HBcAg-negative (n=11)	cHBcAg (n=83)	cdHBcAg (n=105)	mHBcAg (n=8)	Spearman rank correlation	coefficient of contingency
CR	8	19	23	0	0.0227	0.1583
PR	2	42	51	4		
NR	1	22	31	4		

Data are presented as number. Pearson chi-square test, Spearman rank correlation analysis and coefficient of contingency were used for statistical analysis.

subsequent immune clearance phase, Cytoplasmic HBcAg increases and nuclear HBcAg reduces. The expression of cytoplasmic HBcAg was positively correlated with the degree of liver inflammation. Patients with nuclear HBcAg or dominant nuclear HBcAg staining had lower HAI and a lower PCNA score while cytoplasmic HBcAg or dominant cytoplasmic HBcAg staining had higher HAI and a higher PCNA score [12-14]. The relationship between intrahepatic expression of HBcAg and the liver inflammatory activity was investigated in this study. In CHB patients, the histologic necroinflammation in the HBcAg-negative group and cHBcAg group reached G3/G4 (respectively 54.55%, 42.17%), while most of cdHBcAg group and mHBcAg group maintain G1/G2. Histologic necroinflammation which expressed in HBcAg-negative or cHBcAg group were significantly higher than that of the cdHBcAg or mHBcAg group. HBcAg negative group, cHBcAg group showed more piecemeal necrosis and focal necrosis by using microscope. This study suggests that cytoplasmic HBcAg is the main target antigen in CHB. In the process of immune clearance, HBcAg migrate from hepatocyte nucleus to cytoplasm to stimulate immune response and induce histologic necroinflammation. When HBcAg completely migrated to the cytoplasm, the immune function was fully motivated response to cytoplasm HBcAg and HBVDNA, and some cytoplasm HBcAg and HBVDNA were removed and the Hepatitis B virus (HBV) was reduced. It can be proved that lower HBVDNA lever in HBcAg negative group. However the immune function is not enough to remove HBV completely, then the immune function declined slowly and the inflammation was alleviated and HBV repro-

Table 4. HBcAg expression patterns and viral response to Peg-IFNα

Feature	G1 (n=4)	G2 (n=131)	G3 (n=63)	G4 (n=9)	Spearman rank correlation	Coefficient of contingency
CR	1	25	22	2	0.0404	-0.1426
PR	2	65	26	6		
NR	1	40	15	1		

Data are presented as number. Pearson chi-square test, Spearman rank correlation analysis and coefficient of contingency were used for statistical analysis.

duced again. We can use Peg-IFNα-2a to increase the immune function and anti HBV when the expression pattern of HBcAg was the HBcAg-negative group or cHBcAg group, which can further promote viral clearance and increase treatment efficiency.

This study also showed that HBeAg seroconversion rate and complete response rate was the highest in HBcAg negative group after Peg-IFNα-2a treatment, followed by cHBcAg. mHBcAg has the worst HBeAg seroconversion rate and complete response rate, which seroconversion rate and complete response rate was only 0. This study suggests that the expression pattern of HBcAg and Peg-IFNα-2a antiviral effect is closely related and whether to choose IFNα antiretroviral therapy according to HBcAg expression patterns before antiviral therapy.

At the same time, the results showed that the activity of liver histologic necroinflammation was negatively correlated with the combination response to Peg-IFNα-2a. The higher the activity of liver histologic necroinflammation has before treatment, the better the antiviral effect has. The complete response rate of patients with G3 and G4 was 33.33% while the complete response rate of G2 and G1 was 19.26%.

As a result, we can draw the following conclusions that in the immune tolerance, HBcAg is mainly distributed in hepatocyte nucleus, and the expression of the cytoplasm is less, and the activity of liver histologic necroinflammation is mild. In the early stage of immune clearance phase, the expression of cytoplasm HBcAg was increased, and the expression of hepatocyte nucleus decreased gradually. In the middle stage of immune clearance phase, HBcAg was dominant expressed in cytoplasm. In the late stage of immune clearance phase, HBcAg was completely transferred to the cytoplasm, and the immune response was fully activated, the hepatocytes where HBVDNA replicate and cytoplasmic HBcAg express were dissolved. The expression of HBcAg is HBcAg-negative and HBVDNA quantitative decline. However, such immune activation is not sufficient to eliminate the virus, only a temporary alleviate inflammation and immune control. With the gradual cease of immunity, liver HBVDNA replication gradually increased, a new circle of immune activation occurs, and the repeated immune activation and necroinflammation is bound to cause liver fibrosis, cirrhosis and even liver cancer. Therefore, in the middle and later immune clearance stage, when the immune activated, HBVDNA and HBcAg decreased, the use of Peg-IFNα-2a can enhance the immunity and had a better anti HBV effect. It is a good treat timing.

This study is a prospective study which has clearly diagnostic criteria and unified treatment, accuracy of the testing method, the integrity of the data. It has a certain clinical value. However, the source of this information is from Xiamen Hospital of Traditional Chinese Medical and the object is HBeAg-positive chronic hepatitis B which is lacking of inactive carriers and HBeAg-negative chronic hepatitis B. Therefore, it is lacking of hepatocyte nuclear HBcAg group and hepatocyte nuclear dominant HBcAg group which make the results could not accurately represent the overall population of patients with chronic HBV infection. It is bound to cause the selection bias and the results have a certain impact. Be undertaken multicenter, large randomized controlled study can better control the bias and get more realistic results.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81274155, 81503529), the Natural Science Fund of Fujian Province (No. 2014J01374), the Science and Technology Plan Projects of Fujian Province (No. 2016D012), the Traditional Chinese Medicine Scientific research Projects of Fujian Province (No. WZPW201308) and the Higher Education Support Program for New Century Excellent Talents of Fujian Province, the Science and Technology PlanProjects of Xiamen City (No. 3502Z20134020).

Disclosure of conflict of interest

None.

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