HBcAg distribution predicts virologic response to PEG-IFN α therapy in HBeAg-positive CHB with minimally elevated ALT

Lingyun Zhou^{‡,1}, Xinyi Zhang^{‡,2}, Jianwen Cai², Libo Yan¹, Duoduo Lv¹, Xiaoqiong Tang¹, Dafeng Liu¹ & Hong Tang^{*,1}

¹Center of Infectious Diseases, West China Hospital of Sichuan University, Chengdu, PR China

²Clinical Medicine, West China Clinical Medical College, Sichuan University, Chengdu, PR China

*Author for correspondence: htang6198@hotmail.com

‡ Authors contributed equally

Aim: This study evaluated the use of initial Hepatitis B virus (HBV) core antigen (HBcAg) distribution in hepatocytes to predict chronic infection with HBV patients with minimally elevated ALT who can achieve viral response to PEG-IFN α therapy. **Materials & methods:** HBeAg-positive chronic infection with HBV patients with minimally elevated ALT who were treated with PEG-IFN α were enrolled. Predictive values of initial HBcAg distribution in hepatocytes were evaluated. **Results:** HBcAg of no-end-of-treatment viral response group was more likely to be distributed in hepatocyte nuclei ($p = 0.02$) and exhibited a higher degree of HBcAg expression in nuclei ($p < 0.0001$) before treatment. The degree of HBcAg expression in nuclei might be a better predictor of end-of-treatment viral response (area under the curve: 0.88). **Conclusion:** Lower HBcAg expressions in nuclei correspond to higher possibilities of patients benefiting from PEG-IFN α treatment.

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Keywords alanine aminotransferase • chronic hepatitis B • hepatitis B core antigen • hepatitis B e antigen positive • peginterferon α

Hepatitis B virus (HBV) is widely distributed throughout the world and affects over 240 million individuals. It remains a major health problem in China [1,2]. Chronic infection with HBV (CHB) often leads to cirrhosis and liver cancer, creating immense sociological, clinical and economic burdens [3]. The treatment to suppress HBV and retard the progress of liver inflammation are clinically important [4].

In clinical practice, physicians mainly use ALT as a common diagnostic indicator of liver injury, yet some studies indicated that a substantial proportion of CHB patients with a minimally elevated ALT (one to two fold upper limit of normal [ULN]) could not exhibit clinical symptoms but still present inflammation or fibrosis of the liver [5,6]. A program appropriate according to the condition of these patients should be selected for the treatment of chronic hepatitis B [7,8].

Because PEG-IFN α usually acts as an immune mediator [9], high levels of ALT (2 to $5 \times$ ULN) have been found to be an important predictor of antiviral response in patients before treatment in a previous study, with two fold ULN for ALT considered as a rational threshold for initiating therapy in the absence of a liver biopsy [7]. Other published reports show that 20% of patients with low ALT levels have a sustained response to treatment [9,10]. Because a substantial percentage of these patients may have liver damage and a minimally elevated ALT value; this may cause unnecessary delay in starting antiviral therapy [11–13]. Previous studies have not described the means of identifying the category of patients with a minimally elevated ALT level who would benefit from PEG-IFN α therapy.

HBV core antigen (HBcAg) is an antigen that can be detected either in the nucleus or cytoplasm of HBV-infected hepatocytes [14]. There are reports that HBcAg can reflect the relationship between HBV replication and

the host immune response [15–18]. Serum HBV e antigen (HBeAg) and HBV DNA levels correlate significantly with the degree of nuclear or cytoplasmic HBcAg expression. In this regard, the lower the HBcAg staining observed in the hepatocytes, the more active the patients' immune response is. Because PEG-IFN α has antiviral and immunomodulatory effects, the initial distribution of HBcAg in the hepatocytes may provide prognostic values for PEG-IFN α response.

This study seeks to evaluate the use of the initial distribution of HBcAg in hepatocytes to identify patients with a minimally elevated ALT level who could achieve viral response and benefit from PEG-IFN α therapy.

Materials & methods

Patients & study design

Between 2012 and 2014, chronic HBeAg-positive patients who had undergone a liver biopsy and received PEG-IFN α therapy were included in the retrospect study at the Center of Infectious Disease of the West China Hospital, affiliated with Sichuan University and the Infectious Hospital of Shenzhen. The inclusion criteria were as follows: 18–40 years old; Hepatitis B surface antigen (HBsAg)-positive and HBeAg-positive for >6 months; treatment-naïve, minimally elevated ALT levels (one to two fold ULN, with at least three ALT tests done three-times before liver biopsy); Serum HBV DNA level $\geq 20,000$ IU/ml; no treatment before liver biopsy; liver biopsy showing a moderate to severe inflammation or fibrosis; completed treatment of PEG-IFN α therapy (180 mg/weekly) without interruption for 48 weeks. Exclusion criteria were: those who were infected with other hepatitis viruses (including A, C, D and E) or human immunodeficiency virus; an inadequate liver biopsy size (<15 mm) evidence of drug-induced liver injury, alcoholic liver disease, autoimmune liver diseases, diabetes, severe systemic illnesses, hepatocellular carcinoma and other tumors, pregnancy.

Definitions

The end-of-treatment viral response (ETVR) was defined as a serum HBV DNA level that was undetectable by real-timePCR (rt-PCR) at the end of a 48-week treatment period (<500 copies/ml). Sustained viral response was defined as a serum HBV DNA level that was undetectable by rt-PCR at 48 weeks post-treatment (<500 copies/ml).

Determination of laboratory variables

Basal HBeAg, antibody to HBeAg (HBeAb), HBV DNA and serum ALT were regularly detected at every 3 months. The blood parameters and serum biochemical markers were evaluated by a blood analyzer and automatic biochemical analyzer (Olympus AU5400, Olympus Corporation, Tokyo, Japan) following standard procedures. Serum HBV markers were detected by electrochemiluminescence immunoassay (Elecsys; Roche Diagnostics, China). Serum HBV DNA levels were measured by rt-PCR.

Liver immunohistochemical assays

Menghini needle was used to obtain liver biopsies. A 1- to 3-cm biopsy core was obtained from each patient for further analysis. Liver histological results including inflammatory grades and fibrosis stages were analyzed according to the scoring system proposed by Ishak *et al.* [19].

Immunostaining of HBcAg in hepatocytes was detected by the avidin–biotin immunoperoxidase method with paraffin sections of liver specimens as previously described in the literature [15,18]. Briefly, this involves four major steps: deparaffinization of tissue, antigen retrieval, tissue permeabilization and immunostaining. The slides were screened and observed by three pathologists who had no prior access to the clinical reports to avoid bias. Analysis of staining was made by microscopic examination. The degree of viral antigen expression was expressed as a proportion of the immunolabeled cells without consideration for the staining intensity. The degree of HBcAg expression in the nuclei and cytoplasm was semi-quantitated using a score of 0–4, corresponding to positivity in 0, 1–10, 11–25, 26–50 and >50% of hepatocytes examined respectively, as previously reported (Figure 1 & Table 1) [14,15].

Statistical analysis

Quantitative variables were expressed as the mean $\pm$ standard deviation or the median with interquartile ranges, while categorical variables were expressed as absolute and relative frequencies. The t test or the nonparametric Mann–Whitney U-test was performed to calculate differences between quantitative data; and χ^2 test or Fisher's exact test was performed to calculate differences between qualitative data. Serum HBV DNA levels were logarithmically transformed for analysis. A statistically significant result was defined as a p-value <0.05, as calculated by a two-

Figure 1. Images of pathology slides illustrating the classification system for intrahepatic Hepatitis B virus core antigen. (A1) Negative control ($\times 100$). (A2) Negative control ($\times 400$). (B1) HBcAg distributed in the nucleus ($\times 100$). (B2) HBcAg distributed in the nucleus ($\times 400$). (C1) HBcAg distributed in the cytoplasm ($\times 100$). (C2) HBcAg distributed in the cytoplasm ($\times 400$).
HBcAg: Hepatitis B virus core antigen.

Table 1. Pathologic classification and semiquantitative determination of intrahepatic Hepatitis B virus core antigen distribution.

Degrees of expression (HBcAg)	Score
Positive rate	
0	0
1–10%	1
11–25%	2
26–50%	3
>50%	4
HBcAg: Hepatitis B virus core antigen.	

Table 2. Characteristic of the study patients.

Baseline characteristics	n
Patients	62
Age (years)	30.7 ± 7.1
Sex:	
– Male	49 (79)
– Female	13 (21)
HBV DNA (log ₁₀ IU/ml):	
– 4–6	33 (53)
– >6	29 (47)
ALT	59 (49.00–71.75)

Data are expressed as mean ± SD, n (%) or median (interquartile range).
HBV DNA: Hepatitis B virus DNA.

Table 3. Baseline characteristics and Hepatitis B virus core antigen distribution patterns according to end-of-treatment viral response of the study patients.

Baseline characteristics	Virological response		p-value
	ETVR	no-ETVR	
Time	48 weeks		
Sex M/F	13/4 (76.5)	36/9 (80.0)	0.74
Age (years)	31.4 ± 9.6	30.5 ± 6.0	0.73
HBcAg distribution pattern (nucleus/cytoplasm)	2/15 (13.3)	19/26 (42.2)	0.02*

Data are presented as mean ± standard deviation or n (%).
ETVR was defined as the serum HBV DNA level undetectable by real-time PCR at the end of 48-week treatment (<500 copies/ml).
*p-values were determined using χ^2 .
ETVR: End-of-treatment viral response; HBcAg: Hepatitis B virus core antigen.

sided test. All statistical analyses were done with the SPSS Version 18.0 (SPSS, IL, USA). The figures were drawn using GraphPad Prism 6 (GraphPad Software Inc., CA, USA).

Results

Baseline characteristics

The results from 62 HBcAg-positive patients with minimally elevated ALT level were included in our study. The mean age of patients was 30.7 ± 7.1 years old, with a remarkable male predominance (79%). Among them, 33 patients (53%) had a serum HBV DNA level 4–6 log₁₀ IU/ml while 29 patients had a serum HBV DNA level >6 log₁₀ IU/ml (47%). The mean value of serum ALT was 59 (49.00–71.75) U/l. HBcAg was found in all the 62 patients. The detailed baseline characteristics are shown in Table 2.

Predictive value of initial HBcAg distribution

Patients were divided into two groups (ETVR group and no-ETVR group) according to the achievement of ETVR. Of the 62 patients included, 17 (27.4%) achieved an ETVR. Between patients in the ETVR and no-ETVR groups, the basal features showed no differences in terms of sex ($p = 0.74$), age ($p = 0.73$) and initial HBV DNA level ($p = 0.51$). Initial HBcAg distribution seemed to be important for the prediction of therapeutic response: HBcAg of the no-ETVR group was more likely to be distributed in the nuclei of hepatocytes ($p = 0.02$) before treatment (Table 3). Moreover, patients in the no-ETVR group had higher degrees of HBcAg expression in the nuclei ($p < 0.0001$). However, the degree of HBcAg expression in the cytoplasm was not different between the patients in the ETVR and no-ETVR groups ($p = 0.92$; Figure 2). These observations suggest that the degree of HBcAg expression in the hepatic nucleus was a good predictor to viral response in CHB patients with normal or minimally elevated ALT levels. The less patients had of HBcAg expression in the hepatic nuclei, the more likely they benefited from PEG-IFN α treatment.

Receiver operating characteristic curves were generated from the analyses of HBcAg distribution in hepatocytes in order to evaluate the predictive values of HBcAg distribution patterns, degree of HBcAg expression

Figure 2. Degrees of Hepatitis B virus core antigen expression in the nuclei and cytoplasm according to end-of-treatment viral response. (A) Degrees of HBcAg expression in the nucleus. (B) Degrees of HBcAg expression in the cytoplasm.

p-values were determined using Mann–Whitney U-test.

ETVR: End-of-treatment viral response; HBvAg: Hepatitis B virus core antigen.

in the nuclei and cytoplasm for an ETVR in chronic HBeAg-positive patients with minimally elevated ALT levels. The area under the curve (AUC) of HBcAg distributed mainly in the nuclei was only 0.65, with a sensitivity of 88.2%, specificity of 44.2%, positive predictive value of 36.6% and a negative predictive value of 90.5%. When we analyzed these data according to the degree of HBcAg expression, the AUC of the degree HBcAg expression in the nuclei was 0.88, and a cut-off value 0 had a sensitivity of 70.6%, specificity of 95.6%, positive predictive value of 85.7% and negative predictive value of 89.6%, suggesting that it is a better predictor of ETVR than HBcAg distribution patterns. However, the AUC of the degree HBcAg expression in the cytoplasm was 0.507, indicating that it might be not suitable as a predictor for ETVR ($p = 0.93$; Figure 3).

Discussion

Chronic HBeAg-positive patients with minimally elevated ALT levels often require liver biopsy to make treatment decisions. Identifying patients that would most likely benefit from PEG-IFN α therapy may help improve personalized therapy for this special patient population [6].

In this study, 27.42% of chronic HBeAg-positive patients with minimally elevated ALT levels had a viral response to PEG-IFN α therapy. The response rate was consistent with those reported previously and comparable with those achieved with other pegylated interferon therapies, including PEG-IFN α . As mentioned above, it is of great importance to analyze certain characteristics exerting a negative or positive influence on response rates. HBcAg intrahepatic distribution was investigated and revealed that HBcAg distribution degrees in the nuclei were correlated with the ETVR. Patients in the no-ETVR group had higher degrees of HBcAg expression in the nuclei. However, the degree of HBcAg expression in the cytoplasm was not different between the patients in the ETVR and no-ETVR groups. These results indicate that the expression level of HBcAg in the hepatic nucleus was a good predictor to viral response in chronic HBeAg-positive patients with normal or minimally elevated ALT levels. The less the patients had of HBcAg expression in the hepatic nuclei, the more likely they benefited from PEG-IFN α treatment.

Compared with nucleos(t)ide analogs (NUC) treatment, PEG-IFN α is not readily drug-resistant and its limited duration is an attractive therapeutic strategy for patients with CHB [9]. PEG-IFN α treatment showed high rates of off-therapy host immune control over HBV and increased rates of HBeAg/HBsAg loss or seroconversion over time [20]. However, only 30–40% patients benefiting from PEG-IFN α treatment remains an obstacle in CHB management [21]. Recent studies have sought to find new predictors for the outcomes of PEG-IFN α treatment in order to identify patients with a high probability of achieving a satisfactory endpoint or nonresponders as early as possible for optimal clinical management and cost-effectiveness [13,22]. Unlike NUCs, which inhibit HBV

Figure 3. Receiver operating characteristic curves of intra hepatocytes Hepatitis B virus core antigen distribution for predicting the end-of-treatment viral response.

AUC: Area under curve; HBvAg: Hepatitis B virus core antigen.

replication polymerase directly, it is known that the antiviral effect of interferon as an immunomodulator depends on the basic immune function of CHB patients [9,10,20]. Previous studies have shown that responders to interferon therapy demonstrated higher levels of lymphocytic 2'5'oligoadenylate synthesis, an interferon-inducible enzyme that activates intracellular ribonucleases that cleave viral mRNA [23,24].

HBcAg is the main structural protein of the HBV capsid assembly which is important for RNA packaging, reverse transcription and nucleocapsid assembly [14]. It has long been recognized that positive staining of HBcAg in the liver indicates active viral replication. Previous studies have indicated that serum HBV DNA level was significantly correlated with HBcAg expression in nucleus; however, there was no significant correlation between serum HBV DNA and HBcAg expression in cytoplasm HBsAg expression [15]. This finding seems to be in accordance with previous observations, wherein HBcAg is more likely to be expressed in the nucleus, and HBV DNA levels were elevated in the serum during the immune tolerance phase of chronic HBV infection. Hence, HBcAg expression in nucleus can be recognized as a marker of high levels of HBV replication. The expressions of HBcAg have been thought to induce liver necroinflammation through HBV specific and nonspecific T cells [17]. Previous studies have shown that during the immune tolerance phase of chronic HBV infection, HBcAg expressed in nucleus with normal ALT and during the immune clearance phase, HBcAg shifted from the nucleus to the cytoplasm with elevated ALT [15,16,18]. Summed up, these observations suggest that HBcAg distributed in the cytoplasm rather than the nucleus is the target for T cells mediated immunity. As such, interferon therapy could hardly target and cleave the HBcAg in the nuclei.

Because of the lack of efficacy of PEG-IFN monotherapy, many new treatment strategies such as NUCs plus peg-IFN combination therapy or NUC/peg-IFN sequential therapy were proposed to improve the response rate to interferon therapy [25]. These studies suggest that serum HBsAg, hepatitis B core-related antigen (HBcrAg)

and HBV DNA levels are useful for predicting responses to NUC/peg-IFN sequential therapy [22,26]. Our study indicated that the lower the expression of HBcAg in nuclei, the more likely it will be that chronic HBeAg-positive patients with minimally elevated ALT levels will benefit from PEG-IFN α treatment, which suggested that the plasma HBsAg level combined with degree of HBcAg expression in hepatocyte nuclei can better predict outcome of NUC/IFN sequential therapy.

Although liver biopsy is an invasive procedure, it remains an important diagnostic method to make treatment decisions for HBeAg-positive patients with minimally elevated ALT levels. Degrees of HBcAg expression in the nuclei can help physicians make optimal treatment decisions for this specific patient population. Hence, if the patients are willing to take a liver biopsy, anti-HBcAg-staining would be a more direct indicator for predicting viral response to PEG-IFN α therapy. HBcrAg indicates serum levels of HBcAg, HBeAg and empty particle (p22). HBcrAg was a good predictor for viral response of NUC/peg-IFN sequential therapy, and it can be continuously monitored during treatment [27,28]. The correlation between serum HBcrAg level and HBcAg distribution in hepatocytes warrants further research. The correlation between serum HBcrAg level and HBcAg distribution in hepatocytes and the combination of these two predictors require further studies.

Although PEG-IFN therapy in chronic hepatitis B has limited indications, NUCs was the more commonly used treatment for patients with CHB [25]. Currently, the number of studies reporting the correlation between HBcAg distribution in hepatocytes and NUCs antiviral effects are very few. This field is worth relevant research in the future. At the time of the study (2012–2014), both medical centers did not perform high precision detection of HBV DNA and quantitative test of HBsAg routinely; therefore, we were unable to study the quantitative results of HBsAg in the baseline and HBV DNA clearance rate. We will conduct relevant research in the future.

Our research provides a possible strategy for evaluating the use of initial HBcAg distribution in hepatocytes to predict CHB in patients with minimally elevated ALT, and who can achieve viral response to PEG-IFN α therapy. However, there are some limitations associated with it. First, the chronic HBeAg-positive patients with minimally elevated ALT who received PEG-IFN α therapy were selected on the basis of strict criteria, and the sample size of the study was relatively small. Second, the patient selection process may have been affected by referral bias because of the retrospective design. Third, for long-term or life-long antiviral therapy, it would be better to check the sustained viral response at the end of the second and third year.

Conclusion

In conclusion, liver biopsy remains an important diagnostic method to make treatment decisions for HBeAg-positive patients with minimally elevated ALT levels. Lower HBcAg expressions in nuclei correspond to higher possibilities of patients benefiting from PEG-IFN α treatment.

Summary points

- The expression level of Hepatitis B virus (HBV) core antigen in the hepatic nucleus was a good predictor to viral response in chronic HBV e antigen-positive patients with normal or minimally elevated ALT levels.
- Degrees of HBV core antigen expression in the nuclei can help physicians make optimal treatment decisions for this special patient population.

Financial & competing interests disclosure

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Ethical conduct of research

The study was approved by the ethics committee of West China Hospital of Sichuan University. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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