Association between HBV DNA Level and Intrahepatic HBcAg Protein Expression of Hepatocytes in Patients with Chronic Hepatitis B

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ABSTRACT

Background: *Correlation between serum Hepatitis B viral deoxyribonucleic acid (HBV DNA) and hepatitis B core antigen (HBcAg) protein expression in the liver regarding hepatitis B e-antigen (HBeAg) status have not been well studied. This study was aimed to demonstrate association between serum HBV DNA and HBeAg levels with HBcAg expressions in the liver.*

Method: *A total of 55 naive chronic hepatitis B (CHB) patients were enrolled. All samples were tested for HBeAg serum by serological test enzyme-linked immunosorbent assay (ELISA) and HBV DNA was determined by polymerase chain reaction (PCR) HBsAg and HBcAg were evaluated immunohistochemically in the liver tissue.*

Results: *Of 55 patients, 44 (80%) were HBeAg positive and 11 (20%) were HBeAg negative. There was a positive correlation between serum HBV DNA and HBcAg expression in the nuclei (r = 0.383; p = 0.004). There was association between HBcAg expression in the nucleus and qualitative HBeAg (p = 0.017).*

Conclusion: *In the patient with chronic HBV, there is a very significant positive correlation between the level of viral replication and HBcAg expression in the nucleus of hepatocytes. Moreover, there is association between HBcAg expression in the hepatocytes nucleus and HBeAg serum level. These finding lead to the proposition that nucleus localization of HBcAg protein function to amplify the pool of cccDNA in the replication cycle of HBV.*

Keywords: *HBV DNA, Imunohistochemistry, HBcAg*

INTRODUCTION

Chronic hepatitis caused by viral infection is still becoming a health issue in the world. It is assumed that about 4–20% people worldwide, especially in Asia – Africa regions have been infected by chronic hepatitis. Hepatitis B virus (HBV) has infected approximately 350 million people worldwide and has been reported as a major cause of chronic hepatitis, liver cirrhosis, and even hepatocellular carcinoma (HCC).[1-3]

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Patients with HBV infection have differentiation in their severity of illness, which is related to various factors that play a role in liver damage. A multidisciplinary integration is necessary in determination of chronic hepatitis B prognosis and the decision to provide viral eradication therapy, including physical examination, serum hepatitis B e-antigen (HBeAg) and serum hepatitis B surface antigen (HBsAg) tests, blood chemistry test in order to evaluate liver function as well as the abdominal imaging. Moreover, in determining treatment for chronic hepatitis B patients, utilization of some parameters should be considered such as liver function test and examination with transient elastography. Liver biopsy for determining treatment has only been used in some health centers. It is still being an essential examination and scientifically

accountable for evaluating the severity of liver damage compared to blood chemistry test and viral load.

It has been known that HBV replication cycle is not directly causing cytotoxicity and cytopathic effects in liver cells.[4,5] Currently, it has been noticed that the host immune responses played a role as viral antigen, which can be shown when liver cells have been infected. However, the immune response to HBV and its pathogenesis has not been understood completely.[5]

HBV particles/virions are bound to surface receptors of the liver cells and after internalized to cells membranes, the virus core particles will be delivered to liver nucleus where its genomes converted to the covalently closed circular DNA (cccDNA), a template for viral mRNA. Subsequently, the mRNA is translated in cytoplasm resulting in encapsidation/virus enveloping, viral core, polymerase, and protein X. One of mRNA, the so-called pregenomic mRNA is used for synthesizing the core (nucleocapsid sub-unit) and reverse-transcriptase of viral protein.

In cytoplasm, the nucleocapsid of synthesized virus will be combined with viral RNA genomes to be packaging RNA. The RNA will experience reverse – transcription into the viral DNA. The resulted core particle will then be released to the endoplasmic reticulum where it forms capsule or envelope encapsulating the viral DNA particles. Some of the complete viral particles or mature core particles will be excreted out of cells and others may have the genomes cycle repetition by re-entering the nucleus of liver cells, which consequently converted into cccDNA again.[5,6,7]

The C gene is a HBV's genome component that coded the nucleocapsid protein transformation, i.e. HbcAg, which will stay in hepatocytes and paired to the complete virions; while pre core area is associated to HBeAg polypeptide secretion into blood. Thus, the existence of hepatitis B core antigen (HBcAg) in liver cells is correlated to the presence of HBeaAg as a marker of HBV replication.[8]

Viral load examination is used for monitoring the presence of HBV replication which actually persistent during chronic HBV infection, also to evaluate the reduction of viral load as a result of antiviral and/or interferon treatment. [9,10]

Currently, several methods for evaluating virus replication in tissues have been known, including cccDNA and pre–genomic tests which are more accurate. However, since there was limitation of funding and equipments in our country, our study utilized immunohistochemistry (IHC) methods to detect HBcAg as a viral replication marker in liver tissues as it was technically easy with affordable cost so that it could be conducted in Indonesia. IHC methods and serology test will provide more strength in the chronic hepatitis B diagnostics.

Some investigators have studied about viral expression in liver tissues using HBcAg marker. They found that HBcAg expression and assay intensity in liver biopsy may reveal HBV infection which is correlated to viral replication degree.[11,12,13] Kim et.al. found that HBcAg expression degree in the nucleus of hepatocytes was effected by viral load.[3] The presence of HBcAg in hepatocytes nucleus was correlated to HBeAg, as HBV replication markers. [3,14]

The aim of this study was to find the quantitative correlation between HBcAg expression in hepatocytes nucleus and viral load as well as its qualitative association with HbeAg serum level in patients with chronic hepatitis B infection.

METHOD

The design was analytical cross–sectional study. It was conducted between August 2009 and October 2010 in Department of Anatomical Pathology Cipto Mangunkusumo hospital. The collected samples were microscopic slides and paraffin blocks of patients who had been clinically diagnosed with chronic hepatitis B during 2007 – 2008. Inclusion criteria were (1). Slides and paraffin blocks of patients who had complete clinical data, serum HBeAg level, viral load, and serum HBsAg level. (2). Paraffin blocks with an adequate size of tissue for histological evaluation and IHC test. Exclusion criteria included slide and paraffin blocks of patients who had been diagnosed with concomitant infections, such as hepatitis B and C as well as HIV based on their medical records.

All cases had undergone the serology examination (ELISA) to measure the qualitative serum HBeAg level and PCR testing (Cobas TaqMan 48) to measure the quantitative serum HBV DNA level. IHC assay was performed by using avidin-biotin methods with HBcAg primary antibody (Dakkopatts, Copenhagen, Denmark).

IHC assay with primary antibody HBcAg was first prepared by deparaffinization for 5 minutes, rehydration for 4 minutes, washing with flowing water, endogenous peroxide blocking with hydrogen peroxide 0.3% in tris buffered saline (TBS) for 30 minutes, washing with flowing water for 5 minutes, heating the retrieval antigen using the 750 watt oven for 10 minutes, soaking with PBS pH 7.4 for 30 minutes, sniper background blocking for 15 minutes, primary antibody incubation for 1 night (HBcAg) by 1: 1,500 dilution, washing with PBS pH 7.4 for 15 – 30 minutes, secondary antibody incubation (universal link) for 15 – 30 minutes, washing in PBS pH 7.4, performing standard avidin–biotin peroxide complex technique with Trekavidin–HRP label for 15 – 30 minutes, washing in PBS pH 7.4, enhancing with diaminobenzidine (DAB) for 2 – 5 minutes,

washing with flowing water and counterstaining with hematoxyllin meyer for 2 minutes, washing with flowing water, lithium carbonate saturation, washing with flowing water, dehydration for 5 minutes, clearing process for 5 minutes, mounting and covering with cover glass.

Every assay was accompanied by negative controls for each case and positive controls of patients with chronic HBV infection as assay technique and standard controller. The positive HBcAg expression was shown by brown color in hepatocytes nucleus and then it was count quantitatively on 10 high power fields. Data analysis: all data was being expressed in the main table, calculated for its frequency and distribution, and statistical test was performed by using SPSS 17.0. The Spearman statistical test was used after normality test showed abnormal data to determine the correlation between numerical and numerical variables; Mann-Whitney was performed to determine the correlation between numerical and categorical variables. Limit of significance was determined as $p < 0.05$.

RESULTS

Of 55 cases based on clinical data, there were 18 (32.7%) female and 37 (67.3%) male subjects. The range age of subjects was 20-65 years.

Table. 1. Subjects baseline characteristics (n= 55)

Variables	n (%)
Sex	
Male	37 (67.27)
Female	18 (32.73)
Age (years)	
≤ 40	23 (41.82)
≥ 40	32 (58.18)
HBeAg Serum	
Positive	44 (80.00)
Negative	11 (20.00)

Brown in nucleus by IHC staining was regarded as positive cells of HBcAg (figure 1). Positive expression in hepatocyte nucleus was detected in 36 cases (65.45%) (table 2).

Table. 2. HBcAg Expression in Hepatocytes

Variable	n (%)
HBcAg expression in nucleus	
Positive	36 (65.45)
Negative	19 (42.23)

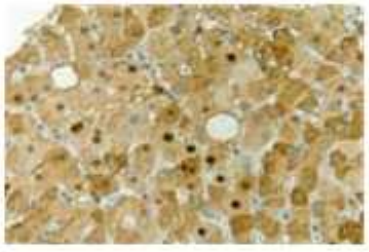

Figure. 1. HBcAg expression in the nucleus of hepatocytes, 400 x

Statistic analysis showed that there was a significant correlation between HBcAg expression in hepatocyte nucleus and viral load concentration of serum HBV DNA ($r = 0.383$; $p = 0.004$) (table 3). Mann-Whitney U test indicated a significant correlation between HBcAg expression in hepatocytes nucleus and qualitative serum HbeAg ($p = 0.017$) (table 3)

Table. 3. Correlation of HBV DNA viral load, HBsAg expression with serum HBeAg and HBcAg in hepatocytes

HBcAg expression in hepatocytes		Viral load HBV	HBeAg serum
HBcAg expression in nucleus	r	0.383	
	p	0.004	0.017

r: correlation

HBcAg expression assessment between two observers showed a moderate strength of agreement, with a kappa coefficient value of 0.643.

DISCUSSION

Some literatures demonstrate that chronic hepatitis B in adults often occur in men at their third and fourth decade of life (table 1). The result of our study indicated that male were more likely to have the disease than female, which is consistent with the existing knowledge in literatures. [1,15]

From this study we found that IHC assay positive was showed by brown coloration in hepatocytes nucleus, which suggested expression of chronic hepatitis B virus in hepatocyte nucleus (intrahepatic) (figure 1). The statistic analysis demonstrated a significant correlation between HBcAg expression in hepatocytes nucleus and qualitative serum HBeAg ($p = 0.017$) (table 3). A similar result had also been found by Hsu et al who have also demonstrated correlation between HBcAg expression and serum HBeAg especially in HBeAg - seropositive patients.[16] Such results are consistent with prior opinions in which HBeAg formation in serum indicated the occurrence of viral replication activities, and this also in keeping with HBcAg formation in nucleus that indicated viral activities in hepatocyte nucleus.[8,17]

The interesting point of our study is the correlation between HBcAg expression in hepatocytes nucleus and increasing titer of serum HBV DNA ($r = 0.383$; $p = 0.004$) (table 3). Similar results had also been demonstrated by Kim et.al and Chu et al. [3,18] By this study, we found that HBcAg location in nucleus and the occurrence of HBcAg found in nucleus indicated that the virus may lay dormant (quiescent); however, it may also indicate that the viral replication is active. HBcAg expression in hepatocytes nucleus is very important in viral replication. [18] However, the significant mechanism about the HBV DNA correlation with HBcAg expression in hepatocytes nucleus has not been completely clear.

HBcAg is translational product of pre–core/core gene of HBV and it is an arginine rich–carboxyl-terminal region that has a nucleus localization signal. After internalized to hepatocytes and converted to uncoated virus, then it transported into nucleus, DNA virus converted to stable DNA (cccDNA) which ready for template of RNA pregenomic transcription. Some of pregenomic RNA are moved into cytosol, where viral nucleocapsid is formed and polymerated into core particles.[3,17,18]

DNA core virus– cytoplasm package may have re-entered to the nucleus for core cccDNA replication, thus leads to the continuous new virions production. Finding of correlation between HBcAg expression in nucleus and DNA HBV viral load in this study supported amplification mechanism of re-entry theory.[3,17,18]

CONCLUSION

There was a significant correlation between HBcAg expression in hepatocytes nucleus and DNA HBV viral load in chronic hepatitis B patients and there was a significant relationship between HBcAg expression in hepatocytes nucleus and qualitative serum HBeAg. These findings may confirm that HBcAg protein was located in nucleus, which also has a responsibility in cccDNA pool multiplication in replication cycle of HBV. IHC assay for liver biopsy preparation is very helpful for quantitative estimation of viral replication. Therefore, we recommend the utilization of IHC in routine assay for determining HBV diagnosis.

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