Title: Hepatitis B core-related antigen (HBcrAg): an emerging marker for chronic hepatitis B virus infection

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Structured Summary

Background: Chronic hepatitis B (CHB) cannot be completely eradicated due to the presence of covalently closed circular DNA (cccDNA) in the nuclei of infected hepatocytes. While quantification of intrahepatic cccDNA requires liver biopsies, serological markers can be non-invasive alternatives to reflect intrahepatic viral replicative activity. Recently, hepatitis B core-related antigen (HBcrAg) has been advocated as a novel serum marker for disease monitoring and prognostication of CHB.

Aim: We aimed to examine the virological aspect and clinical application of HBcrAg with respect to the natural history and treatment of CHB.

Methods: We reviewed all papers published in the PubMed journal list and abstracts from major international meetings that included the keyword "HBcrAg" or "hepatitis B core-related antigen" until March 2017. Selected studies were compared and summarized on the basis of existing theories, as well as the authors' experience.

Results: HBcrAg exhibited good correlation with intrahepatic (ih) cccDNA, ih total hepatitis B virus (HBV) DNA, serum HBV DNA and to a lesser extent HBV surface antigen (HBsAg). In situations where serum HBV DNA levels become undetectable or HBsAg loss is achieved, HBcrAg can still be detectable. This marker is helpful in differentiation of HBeAg-negative chronic hepatitis from HBeAg-negative chronic infection, predicting spontaneous or treatment-induced HBeAg-seroconversion, sustained response to nucleos(t)ide analogue (NA), risk of HBV reactivation in occult HBV infection under immunosuppressive therapies,

and risk of hepatocellular carcinoma (HCC) development as well as post-operative HCC recurrence.

Conclusions: HBcrAg is a potential surrogate marker of cccDNA. It may soon become a useful marker for disease monitoring, predicting treatment response and disease outcome of chronic hepatitis B.

Introduction

Chronic hepatitis B (CHB) affects approximately 240 million persons worldwide[1], with an estimated 15-40% progressing to cirrhosis, decompensation and/or hepatocellular carcinoma (HCC).[2] Currently, hepatitis B virus (HBV) cannot be completely eradicated due to the presence of covalently closed circular DNA (cccDNA) in the nuclei of infected hepatocytes.[3-5] Therefore, the present goal of monitoring is to ensure a high degree of virological suppression, ideally with hepatitis B surface antigen (HBsAg) seroclearance, leading to biochemical remission, histological improvement and reduction of the risk of complications.[6,7] Although liver biopsy for the quantification of intrahepatic cccDNA and intra-hepatic HBV DNA remains the most accurate measurement for viral reservoir, it is limited by its invasive nature, the potential for sampling error, and the lack of a standardized assay. Non-invasive serological markers can be used as surrogate markers of intrahepatic viral replicative activity. Established serological markers include serum HBV DNA levels and serum HBsAg titers, both of which have been shown to predict the risk of cirrhosis and HCC.[8-11] However, as cirrhosis and HCC can still occur in many patients despite undetectable HBV DNA[12] and HBsAg seroclearance[13,14], any promising markers with predictive value in these scenarios are very much welcome. Moreover, given that almost all patients treated with antiviral therapy have undetectable HBV DNA, more accurate biomarkers for risk stratification are needed. Recently, linearized HBsAg (HQ-HBsAg) assay achieving an even lower limit of detection for HBsAg[15] has been shown to correlate with serum HBV DNA and HBsAg levels, which makes it a potential marker for stratifying patients with HBsAg seroclearance.[16] Another new marker, the hepatitis B core-related antigen

(HBcrAg), has also been advocated as a serum marker for disease monitoring and prognostication of CHB. In this review, the clinical application of HBcrAg in CHB patients based on its virological features, the distinctive profiles in different disease stages, and the profile under antiviral treatment will be discussed.

HBV replication

After intial viral entry into the infected hepatocyte and formation of stable cccDNA minichromosomes in the nucleus, the first step of HBV replication starts with HBV transcription. Five major messenger RNAs (mRNAs) species, namely the core mRNA/pregenomic RNA (3.5 kb), precore mRNA (3.5 kb), LHBs mRNA (2.4 kb), MHBs and SHBs mRNA (2.1 kb) and X mRNA (0.9 kb), are generated from the minus strand of the genome. These mRNAs encode for various structural and non-structural proteins. The pregenomic RNA acts as a precursor for the synthesis of viral DNA genome by reverse transcription. The nascent viral genome, encapsidated by the hepatitis B core protein (HBcAg), is then packaged by the hepatitis B surface (HBs) proteins in the endoplasmic reticulum. The mature virions are then secreted into the blood stream, giving rise to a large number of progeny virions. The measurement of serum HBV DNA therefore can provide an estimate of the viral replicative activity, and widely used as a marker of antiviral efficacy. However, nucleos(t)ide analogues (NAs) only act on limited steps of the viral replication cycle, and production of viral intermediate proteins may not be affected significantly. Therefore, measurement of viral proteins can be useful in monitoring HBV activities, especially in patients receiving NAs when HBV DNA levels are undetectable. One such candidate is HBsAg, which is found in mature virions as well as HBV DNA-negative empty particles, in either

spherical or filamentous forms. Another candidate is HBcrAg, which consists of three species of related proteins sharing an identical 149 amino acid sequence: HBcAg, hepatitis B e antigen (HBeAg), and a truncated 22kDa precore protein (p22Cr). HBcAg is the structural component of the viral capsids, which can be found in mature virions. HBeAg, a non-structural HBV protein, is the N-terminal processed product of the precore protein. Like HBeAg, p22cr is also a processed product of the precore protein, but with protein processing at both the N- and C-terminals.[17] The viral replication cycle and the origins of HBV DNA, HBsAg, HBeAg and HBcrAg are illustrated in Figure 1.

HBcrAg measurement

The first prototype HBcrAg assay was reported in 2002.[18] Since HBcAg, HBeAg, and p22cr all share an identical 149 amino acid sequence, the same monoclonal antibodies can be used for the detection of all three proteins. The specimen is mixed with anti-HBcrAg-coated particles and allowed to form antigen-antibody immunocomplexes. Alkalaine phosphatase(ALP)-labeled anti-HBcrAg specifically binds to HBcrAg of the immunocomplexes on the particles, and additional immunocomplexes are formed. Luminescence is generated and the luminescent signal reflects the amount of HBcrAg. Quantitation of HBcrAg is made by comparing the chemiluminescence signal generated by known concentration of recombinant ProHBeAg. This assay is currently available in an automated format, using the Lumipulse G1200 CLEIA analyser (Fujirebio, Tokyo, Japan), with a lower limit of detection of 2.0 log U/mL, and a linear range of 3.0 logU/mL – 7.0 logU/mL (1 kU/mL is equal to 3 logU/mL). Samples with HBcrAg above 7 logU/mL can be diluted and retested in order to calculate the quantitative

HBcrAg level. According to the Lumipulse® G HBcrAg Immunoreaction cartridges set product insert, the accuracy of the test is confirmed by assay values (calculated with the assay values in log U/mL) for three in-house controls range within ± 5% of their control values. The coefficient of variation for specimens (calculated with the assay values in log U/mL) are less than 5% when subjected to the assay six replicates. The analytical sensitivity of the reagent is 3.0 logU/mL. Serum-plasma equivalence is reflected by correlation coefficient of 0.999.

Correlation with existing hepatitis B viral markers

Serum and intrahepatic HBV DNA

The serum HBcrAg concentration correlates strongly with the serum HBV DNA concentration in a positive and linear manner, regardless of the HBeAg status (Table 1).[19-22] Three out of 4 studies showed that the correlation coefficient is greater than 0.8. Intrahepatic total HBV DNA also correlates well with serum HBcrAg (correlation coefficient 0.67-0.70) in patients who are either treatment-naïve or treatment-experienced (Table 2).[21, 22]

Intrahepatic cccDNA

It has been shown that serum cccDNA levels correlated only moderately (r=0.481) with intrahepatic cccDNA (N=39) and the assay involves multiple complicated steps[23] rendering it an unfavourable surrogate marker. HBcrAg has a much stronger correlation with intrahepatic cccDNA.[21, 22, 24] Except for a study with small number of patients (N=31)[24], the correlation coefficient is 0.664 - 0.70 (Table 3).[21, 22] In comparison, serum HBV DNA also correlates equally well with

intrahepatic cccDNA (correlation coefficient 0.7, p<0.001).[22] However, patients receiving antiviral therapy often have undetectable serum HBV DNA, while 78% of these patients still have detectable serum HBcrAg.[22] Therefore, in the context of serum DNA undetectability, HBcrAg would be the preferred serum marker to estimate intrahepatic cccDNA quantity.

HBsAg & Linearized HBsAg (HQ-HBsAg)

Serum HBsAg was shown to correlate moderately with intrahepatic cccDNA (r=0.46, p<0.001) (N=82).[25] HBcrAg exhibited linear correlation with serum HBsAg titre (r=0.703, p<0.001) as well as the more sensitive assay of HBsAg, i.e. linearized HBsAg (HQ-HBsAg) (r=0.818, p<0.001) (N=404).[16]

With the above findings, HBcrAg seems to have the advantageous property of good correlation with both viral replicative activities i.e. HBV DNA, and viral protein synthesis i.e. HBsAg. In addition, HBcrAg is preferred over serum HBV DNA as a surrogate marker for intrahepatic cccDNA in the majority of on-therapy patients with undetectable serum HBV DNA.

Profile of HBcrAg in the Natural History of CHB

In the natural history of CHB infection, HBcrAg levels change significantly during the four phases of CHB infection. The profiles were well characterized by two landmark studies performed in treatment-naïve Asian (N=404) and European (N=249) CHB patients across genotypes A-D.[16, 26] In both studies, the HBcrAg levels differed significantly between HBeAg-positive and negative patients. In

general, HBeAg-positive patients have a higher HBcrAg level compared to HBeAg-negative patients (Figure 2). This is related to the diminished production of HBeAg after HBeAg seroconversion. Specifically, in HBeAg-positive patients, the HBcrAg levels were 8.54 and 7.92 log U/mL in HBeAg-positive chronic infection (also known as immunotolerant phase) and HBeAg-positive chronic hepatitis (also known as immune clearance phase), respectively ($p<0.001$).[16] It suggests that HBeAg-positive patients with lower HBcrAg levels are more likely to be under a more intense immune control. Therefore, whether HBcrAg levels can reflect immune clearance activity deserves more future studies to examine.

For HBeAg-negative patients, the HBcrAg levels were significantly lower in HBeAg-negative chronic infection (also known as inactive carrier state) patients compared to HBeAg-negative chronic hepatitis (also known as HBeAg negative active phase) patients (2.60 vs. 4.92 log U/mL, respectively; $p<0.001$) (Figure 2).[16] A higher HBcrAg in HBeAg-negative chronic hepatitis compared to HBeAg-negative chronic infection was associated with more significant necroinflammatory activity and significant fibrosis.[27] Unlike in HBeAg-positive phase, if HBeAg-negative patients (although with lower HBcrAg level compared with HBeAg-positive patients) still have relatively high HBcrAg levels after HBeAg seroconversion, the disease activities are in fact more advanced.

For patients who underwent spontaneous HBsAg seroclearance, most of them (79%) had undetectable HBcrAg levels signifying a more quiescent disease state. Of the 21% still having detectable HBcrAg in the serum, the median HBcrAg was 2.7 log U/mL.[16, 28]

All of the above findings offer a potential role for HBcrAg to further define the phases of CHB infection, although the optimal cut-offs remain to be determined. The latest EASL guideline acknowledged the potential of HBcrAg to help define the phase of chronic HBV infection, especially in HBeAg-negative patients.[7]

In addition, HBcrAg may be useful in predicting important milestones in natural history of CHB infection such as HBeAg seroconversion. A number of host and virological factors are found to be favourable for spontaneous HBeAg seroconversion, including alanine aminotransferase (ALT) > 2 times the upper limit of normal (ULN), low HBV DNA, low HBsAg titre, non-C genotype and low serum interleukin-27 levels.[29-33] More recently, HBcrAg has been shown to be predictive of early spontaneous HBeAg seroconversion at 12 months in a Japanese study (N=234), with an area under receiver-operating-characteristic (ROC) curve (AUROC) of 0.708.[34] In another Chinese study of 113 patients, the HBcrAg levels at week 28 of follow up were significantly lower for patients in the HBeAg-positive chronic hepatitis phase who underwent spontaneous HBeAg seroconversion compared to those who did not (4.32 vs. 5.16 log U/mL, respectively; p=0.004). To predict spontaneous HBeAg seroconversion, baseline HBcrAg level below 4.9 log U/mL or a decline of HBcrAg by ≥ 2 logs at week 28 would confer positive predictive values (PPV) of 73.9% and 76.2% respectively, and negative predictive values (NPV) of 96.7% and 93.8%, respectively.[35] Whether HBcrAg levels can be predictive of spontaneous HBsAg seroclearance remains to be determined.

HBcrAg and Antiviral Therapy

Despite achieving an undetectable serum HBV DNA during antiviral therapy, the majority will still have persistent infection.[4, 23, 36] In a study of 43 patients treated with NA for a median of 126 months, 98% had undetectable serum HBV DNA, while 51% still had detectable intrahepatic cccDNA.[37] Similar findings were reported in 24 patients treated with sequential therapy of pegylated-interferon (PEG-IFN) and adefovir (ADV) followed by ADV monotherapy, with 46% and 66% having undetectable serum HBV DNA and detectable intrahepatic cccDNA, respectively.[38] Therefore, a decline in serum HBV DNA does not correlate well with a reduction in intrahepatic cccDNA for those receiving antiviral therapy. In contrast, HBcrAg levels decline in a similar manner as cccDNA when patients are treated with NA (Table 4).[21] In patients treated with entecavir (ETV), the annual rate of HBcrAg decline was shown to be 0.244 kU/mL/ year after 7 years of treatment.[39] More rapid decline was seen in patients who were HBeAg-positive, had high baseline HBV DNA, and in the first year of ETV therapy compared to subsequent years (Figure 3).[39] More importantly, the decline in HBcrAg demonstrates good correlation with the magnitude of change in intrahepatic cccDNA.[21,22] For patients treated with up to 5 years of ETV, there was a significant correlation, although moderately, between the decline of serum HBcrAg and intrahepatic cccDNA ($r=0.419$, $p=0.005$).[40] In contrast to serum HBV DNA, the decline in HBcrAg was slower during NA exposure[41], with an increment in the ratio of serum HBcrAg: HBV DNA after three months of lamivudine (LAM).[20] The divergence between the two can be explained by the action of NA

on reverse transcription and subsequent prevention of HBV DNA replication, while HBcrAg production remains unaffected. Therefore it is not surprising that in NA-treated patients with undetectable serum HBV DNA, 78% had persistence of HBcrAg.[22] Even in patients with documented HBsAg seroclearance, 21% had detectable serum HBcrAg, in contrast to detectable serum HBV DNA in only 2.1%.[24, 28] For PEG-IFN treatment, two studies exploring the decline of cccDNA and HBcrAg found conflicting results. While one study described a similar and significant decline of HBcrAg levels as cccDNA[42], the other study did not find a significant decline of HBcrAg with PEG-IFN (Table 5).[43] The number of patients in these two studies were too small (N=58 and 8 respectively) to demonstrate any specific pattern of HBcrAg levels during PEG-IFN therapy.

The profile of HBcrAg was further described in 58 CHB patients treated with a combination of NA and and a novel RNA interference compound (ARC-520) targeting cccDNA-derived transcription that initially entered phase 2 clinical trial in year 2015. The baseline HBcrAg was higher in HBeAg-positive patients and the mean reduction of HBcrAg was 1.4 log kU/mL over 85 days after a single dose of ARC-520. Although this trial was discontinued in December 2016 due to deaths in non-human primates receiving doses higher than those used in human subjects, the intial findings suggested that interfering with RNA transcription reduced the antigen production driven by cccDNA in HBeAg-positive patients.[44]

These studies demonstrated that HBcrAg is a responsive viral marker to be measured whenever treatment can suppress viral DNA and protein synthesis.

In particular for a situation where serum HBV DNA becomes undetectable, HBcrAg seems to remain as a measurable serum marker to correlate with the cccDNA.

Baseline and changes in HBcrAg levels while on antiviral therapy may also predict useful milestones in CHB patients. For patients treated with PEG-IFN, a baseline high HBcrAg level >8 log U/mL would confer >94.4 % NPV for achieving HBeAg seroconversion and suppressed HBV DNA at 12 weeks (N=46).[45] In another study of 50 patients receiving sequential therapy of PEG-IFN plus NA for 4 weeks followed by PEG-IFN for 20 weeks, a high HBcrAg level (>4.5 log U/mL) at initiation of therapy predicted non-response and no HBeAg seroconversion at 24 months after completion of treatment.[46] Furthermore, the on-treatment changes in HBcrAg levels may be useful to predict clinical outcomes. In 58 patients treated with PEG-IFN, the HBcrAg at week 12 of therapy was predictive of HBeAg seroconversion at 24 weeks after completion of therapy with AUROC of 0.896.[42] For patients treated with NA therapy (N=39), the HBcrAg levels were shown to be lower in patients with NA-induced HBeAg seroconversion compared to those who remained HBeAg-positive.[21]

Studies have also reported on the use of HBcrAg in predicting HBsAg seroclearance, although the findings remain inconclusive. In a study of 62 HBeAg-negative CHB patients, the NPVs for achieving HBsAg loss were 79% and 89% for HBeAg-negative patients with baseline HBcrAg >3.7 log U/mL receiving PEG-IFN alone or in combination with tenofovir-disoproxil-fumarate (TDF), respectively.[47] The AUROC for HBsAg loss was 0.763 if both mean baseline

HBcrAg plus HBsAg were used to predict HBsAg loss, although this performed no better than using HBsAg titer alone (AUROC 0.771).[48] It seems that HBcrAg may not be more advantageous than HBsAg titre in terms of predicting HBsAg loss in treatment-experienced patients.

Although the majority of patients treated with NA will remain on long-term therapy, a proportion of patients may opt to discontinue therapy. The decision to stop therapy has been traditionally based on viral serological markers, serum HBV DNA, ALT, and more recently, HBsAg levels. The decline in HBcrAg observed under treatment with NA therapy, and the pattern of decline, might provide prognostic information on the risk of post-treatment reactivation of HBV.[43] For patients treated with LAM or ETV, a high end-of-treatment HBcrAg levels (reported range: 3.2 – 3.7 log U/mL) was shown to predict relapse after cessation of therapy despite undetectable HBV DNA for at least 6 months (Table 6).[49-52] Another study showed that an end-of-treatment HBcrAg level >3.7 log IU/mL predicted virological relapse within 1 year of cessation of NA.[52] Therefore, additional markers such as HBcrAg may better risk-stratify patients that are contemplating cessation of NA. The Japanese Society of Hepatology guidelines have recently incorporated the use of HBcrAg levels in identifying patients at low risk of relapse using the cut-offs <1.9 log U/mL or <3.0 log U/mL for HBsAg and HBcrAg, respectively.[53] Validation studies using these criteria for selecting patients to stop NA should be performed in different populations.

HBcrAg and Hepatocellular Carcinoma

Apart from individual risk factors such as age, liver cirrhosis, serum HBV DNA and serum HBsAg level[54, 55] , risk stratification models have been developed to predict risk of HCC in CHB patients, including the REACH-B, GAG-HCC, CU-HCC and LSM-HCC score.[56-59] Although these scores are simple to use and some of them had been externally validated,[60, 61] they are mainly developed to estimate risk of HCC in treatment-naïve patients. Well defined risk factors and models for treatment-experienced patients are lacking. More recently, HBcrAg has been shown to be associated with HCC development.[62-64] For treatment-naïve patients, the time-dependent AUROCs of HBcrAg for predicting HCC incidence were superior to HBV DNA levels for up to 10 years of follow-up.[64] For treatment-experienced patients, NA can only reduce but cannot eliminate the risk of HCC[65], and HBcrAg positivity after NA for at least 2-year duration was an independent risk factor for HCC.[62] In 76 CHB patients treated with NA with undetectable serum HBV DNA, the pre-treatment HBcrAg levels were significantly higher in the HCC group compared to the matched control group (279.0 vs. 35.4 kU/mL, i.e. 5.45 log U/mL vs. 4.55 log U/mL, respectively; p=0.005) and a pre-treatment cut-off of 47.1 kU/mL (i.e. 4.67 log U/mL) independently predicted HCC. Furthermore, a post-treatment HBcrAg >7.8 kU/mL (i.e. 3.89 log U/mL) predicted HCC with an odds ratio of 3.27. When only non-cirrhotic patients were considered, a cut-off of >7.9 kU/mL (i.e. 3.90 log U/mL) predicted HCC with an odds ratio of 5.95.[66] These findings highlight the importance of conducting further studies to examine the predictive capacity of using HBcrAg for the development of HCC in patients who are already on antiviral treatment.

Serological markers have also been used to predict HCC recurrence after resection or radio-frequency ablation. However, post-surgical HCC recurrence rates remained high despite the use of NA, with reported recurrence rates of up to 41.8% in 2 years.[67-70] Several factors have been shown to be associated with higher recurrence, including the pre-operative serum HBsAg titre >1,000 IU/mL, baseline HBeAg positivity, the presence of cirrhosis, tumor size, tumor number, macrovascular invasion and the use of NA other than ETV or TDF.[71, 72] More recently, studies have also demonstrated the predictive value of HBcrAg in HCC recurrence after curative surgery. In a study of 55 patients, HBcrAg level >4.8 log U/mL at the time of HCC diagnosis gave a hazard ratio of 8.96 of subsequent HCC recurrence within 2 years.[73] In another study of 21 HCC patients undergoing liver transplantation (LT), 5 developed HCC recurrence after LT (2 out of 14 HBcrAg-positive and 3 out of 7 HBcrAg-negative), but HBcrAg positivity post-LT did not show significant correlation with risk of HCC recurrence.[74] Pre-operative HBcrAg might therefore be a potential marker to stratify post-surgical surveillance strategies and in identifying those at high risk of recurrence.

HBcrAg and HBV Flare with Immunosuppression

Occult HBV infection refers to patients who have prior exposure to HBV. They are at risk of HBV reactivation if treated with immunosuppressive therapy, especially anti-CD 20 monoclonal antibodies like rituximab[75-78] and hematopoietic stem cell transplantation (HSCT).[79] Although the observed reactivation rate could be up to 41.5% at 2 years, among several factors, only baseline anti-HBs negativity (<10 IU/mL) was shown to be significantly

associated with the risk of reactivation compared to those with positive anti-HBs (68.3% vs. 34.4%).[77] Recently, HBcrAg positivity at baseline was also shown to be a significant risk factor of HBV reactivation in 124 Asian patients receiving rituximab-containing chemotherapy or HSCT. Despite undetectable HBV DNA at baseline in all 124 anti-HBc positive patients, the 2-year cumulative reactivation rate was 40.4%. Baseline HBcrAg-positive patients had a significantly higher 2-year HBV reactivation rate compared to HBcrAg-negative patients (71.8% vs. 31%, respectively, p=0.002).[80] The latest EASL guideline recommends prophylactic NA in high-risk patients with occult hepatitis B receiving rituximab or HSCT (>10% risk of reactivation), while HBV DNA monitoring is the recommended strategy for patients receiving all other types of immunosuppressive therapy, i.e. moderate-risk (1-10% risk of reactivation) or low-risk (<1% risk of reactivation) group.[7] HBcrAg might have a role for risk stratification in the latter 2 groups of patients for intensification of HBV DNA monitoring or even prophylactic antiviral therapy. Although the guideline recommends at least 18 months duration of NA after cessation of rituximab or stopping immunosuppressant following HSCT, there are currently no studies examining the use of HBcrAg levels to identify the optimal time point for stopping therapy in this patient population.

HBcrAg and Liver Transplantation

Although LT is curative for cirrhosis and HCC, it does not completely eradicate HBV due to extra-hepatic reservoirs, and life-long antiviral prophylaxis is required.[81-85] Therefore the goal of antiviral therapy in this setting is to prevent

recurrence of graft hepatitis, rather than re-infection as the patients are already chronically infected.[86-88] The role of HBcrAg in post-LT patients remains to be determined. In a study of 11 patients with pre-LT positive serum HBcrAg, six of them still had detectable serum HBcrAg at 6 months post-operatively, although at lower levels (pre-LT: 5.25 log U/mL vs. post-LT: 2.87 log U/mL). In all the 6 patients, serum HBsAg and HBV DNA remained negative and none of them had elevated ALT. Similar findings were reported by another study of 32 post-LT patients, which showed that 50% remained positive for HBcrAg even though only 18.8% had reappearance of HBV DNA and/or serum HBsAg.[74] There was a correlation between HBcrAg and cccDNA in the liver grafts ($r=0.616$, $p<0.001$) and higher histological stages of liver fibrosis were observed in patients with detectable serum HBcrAg and positive intrahepatic cccDNA.[89]

Figure 4 summarizes the major findings and potential clinical applications of HBcrAg.

Unresolved issues of HBcrAg

There are several unresolved issues in HBcrAg measurement. Firstly, the lower limit of detection for serum HBcrAg is 2 logU/mL, and this is particularly an issue for HBeAg-negative patients. Up to 42% patients with HBeAg-negative chronic infection had undetectable HBcrAg, while on the contrary, 21% patients with loss of HBsAg still had detectable HBcrAg.[16] With the available data so far, no good explanatory mechanisms and implications could be provided for this phenomenon and more investigations are needed. Secondly, the absolute HBcrAg levels are significantly reduced in the presence of precore or basal core promoter

mutants due to eradication of HBeAg expression in HBeAg-negative patients[90], therefore supplementary mutation testing of patients may be required to further characterize this biomarker in the context of HBeAg negativity. Thirdly, although HBcrAg correlated well with intrahepatic cccDNA[21, 22], serum HBcrAg were still detected in a small proportion of patients with undetectable intrahepatic cccDNA.[21] This could be explained by the aforementioned issue of sampling bias leading to falsely low quantification of intrahepatic cccDNA.

Further research for clinical application of HBcrAg

Before widespread clinical utilization of HBcrAg in CHB, the validity of HBcrAg should be further established. From the available data described, HBcrAg correlates best with intrahepatic cccDNA among all viral markers. Therefore, *content validity* i.e. the correlation of HBcrAg with intrahepatic cccDNA[19-22] (even in post-LT patients)[89] is well demonstrated. *Construct validity* is also established, given the linear correlations with the other viral markers including serum HBV DNA and intrahepatic total DNA. Indeed, *criterion validity* is the major area that needs further exploration. In most of the clinical scenarios described in the previous sections, only inter-group differences in the median HBcrAg values were described, while the predictive power i.e. specificity, sensitivity, false positivity, false negativity etc. for a certain cut-off value is not yet evaluated. Among them, ROCs were only analyzed when evaluating for spontaneous or PEG-IFN induced HBeAg-seroconversion. To prove *criterion validity*, similar analyses should be carried out to identify the performance characteristics of HBcrAg for predicting the clinical outcomes of interest as mentioned above, including post-NA cessation HBV flare, development of HCC, and occult HBV reactivation in

immunosuppressed patients. The same token applies if HBcrAg is used for characterization of disease state in HBeAg-positive and HBeAg-negative group. A second concern is *group variability* of HBcrAg among CHB patients of different ethnic groups and genotypes, as shown by the differences in median HBcrAg in two studies (Figure 2)[16,26], although it is not possible to directly compare the median levels of HBcrAg between the two study populations for a statistically significant difference. Future studies should include a mixed population to improve generalizability. *Intra-individual variability* of HBcrAg levels is also observed in different disease states. This is inevitable as long as viral replication and persistence in the hepatocyte is a dynamic process. One should therefore beware of the specific clinical scenario at which HBcrAg is measured, as the cut-off values will differ widely and have various clinical implications.

Conclusive remarks

In summary, HBcrAg is a good surrogate marker of intrahepatic cccDNA. It correlates with HBV DNA in all disease states. HBcrAg is helpful in differentiation of HBeAg-negative chronic hepatitis from inactive carrier state as shown by 2 large scale studies. These two disease states carry entirely different clinical implications and prognosis, and the role of HBcrAg in here should be further elaborated in particular for HBeAg-negative patients with apparently normal ALT. In patients with undetectable serum HBV DNA and HBsAg, i.e. achieving "functional cure", complications would still occur. Certain proportion of patients achieving "functional cure" still have detectable HBcrAg and this warrants prospective studies comparing the long-term outcome between HBcrAg-positive

and HBcrAg-negative patients. HBcrAg, similar to HBsAg titre, is potentially useful in predicting HBsAg loss under NA therapy, although the cumulative rates of HBsAg loss is still disappointing. On the other hand, sustained off-therapy response to NA is a more attractive outcome to investigate, since most NA is continued indefinitely and the role of HBcrAg in selecting low-risk patients for NA cessation should be further explored.

Finally, use of HBcrAg needs further exploration in two special populations. First, as HBcrAg demonstrated good predictive value for risk of HBV reactivation in occult HBV, and more studies should be performed on moderate-risk and low-risk group for risk stratification, as well as for determining the optimal duration of NA after cessation of rituximab or immunosuppressive therapy for HSCT. Second, predicting HCC by HBcrAg for both treatment-naïve and treatment-experienced group is an exciting area.

However, as remarked in the EASL guideline, most studies were performed in Japan and other Asia countries and large correlation studies derived from Caucasian patients are lacking. As such, in spite of being a very promising new viral marker, more extensive clinical validation is needed before routine use in clinical practice.

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Tables

Table 1: Correlation coefficients of HBcrAg and serum HBV DNA

No. of subjects	correlation coefficient	p value	Reference no.
190	0.79 (genotype B)* 0.87 (genotype C)*	<0.001 <0.001	19
82	Overall: 0.807	<0.001	20
	HBeAg-positive: 0.847	<0.001	
	HBeAg-negative: 0.632	<0.001	
93	0.820*	<0.001	21
138	Overall: 0.69	<0.0001	22
	HBeAg-positive: 0.66	<0.0001	
	HBeAg-negative: 0.59	<0.0001	

HBcrAg: hepatitis B core-related antigen, HBeAg: HBV e antigen, no.: number
* The correlation coefficients were not separately reported in subgroups of HBeAg-positive and HBeAg-negative patients.

Table 2. Correlation coefficients of HBcrAg and intrahepatic total DNA

No. of subjects	correlation coefficient	p value	Reference no.
93^	0.70	<0.001	21
305#	0.67	<0.0001	22

HBcrAg: hepatitis B core-related antigen, no.: number
^ 54 patients were treatment-naïve and 39 patients were treated with 1-year duration of entecavir or lamivudine.
# 138 patients were treatment-naïve and 167 patients were treated with 1-year duration of various nucleos(t)ide analogues including entecavir, lamivudine, telbivudine, clevudine, or adefovir.

Table 3. Correlation coefficients of HBcrAg and intrahepatic cccDNA

No. of subjects	correlation coefficient	p value	Reference no.
93	0.664	<0.001	21
138	0.70	<0.0001	22
31	0.482	<0.006	24

cccDNA: covalently-closed circular DNA, HBcrAg: hepatitis B core-related antigen, no.: number

Table 4. Change of HBcrAg and intrahepatic cccDNA levels under NA therapy

No. of subjects	Type of NA	Duration	HBcrAg decline	ih-cccDNA decline	correlation	Ref no.
39	ETV: 20 LAM: 19	48 weeks	1.38 logkU/mL	1.00 log copies/ cell	r=0.378 (p=0.027)	21
222	ETV	>5 years	0.244 logkU/mL/year	2.94 log copies/ cell*	r=0.419 (p=0.005)	37, 39

*43 patients had paired liver biopsies
cccDNA: covalently-closed circular DNA, ETV: entecavir, HBcrAg: hepatitis B core-related antigen, ih: intrahepatic, LAM: lamivudine, NA: nucleos(t)ide analogue, no.: number, Ref: reference

Table 5. Change of HBcrAg levels under PEG-IFN therapy

No. of subjects	Type of PEG-IFN	Duration	HBcrAg (logU/mL)					Ref no.
			baseline	week 12	end of therapy	12 week FU	24 week FU	
58	alfa-2b	48 weeks	8.042	6.575	6.870	5.854	5.301	42
8	alfa	18 months	3.30	no significant variation (p=0.172)				43

FU: follow-up, HBcrAg: hepatitis B core-related antigen, PEG-IFN: pegylated interferon, no.: number, Ref: reference

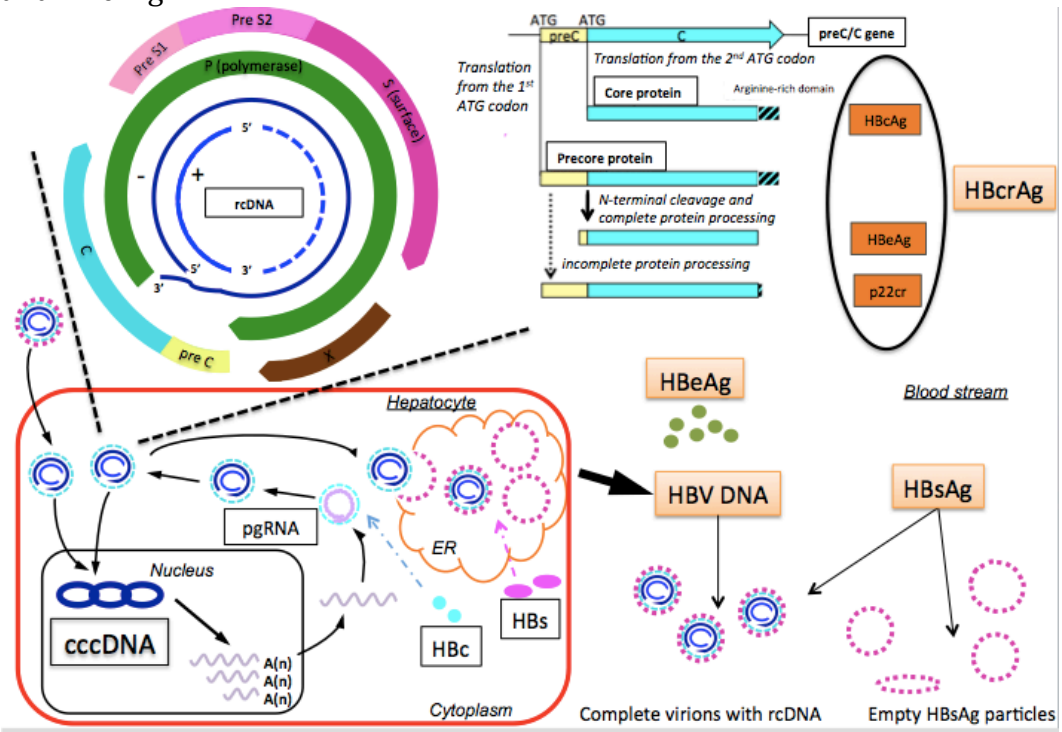
Table 6. End-of-treatment HBcrAg levels and risk of relapse after NA cessation

No. of subjects	Type of NA	Duration of therapy	Relapse rate	End-of-treatment HBcrAg level (logkU/mL)			Ref no.
				Relapse	Non-relapse	p value	
34	LAM	6 months	59%	4.9	3.2	0.009	49
22	LAM	6 months of undetectable DNA	50%	4.5	3.4	0.0145	51
113	LAM: 32 ETV: 81	HBeAg +ve: HBeAg seroconversion and 12 months of UD DNA	57.8%	-	-	NS	52
		HBeAg –ve:18 months of UD DNA	54.4%	>3.7	-	0.010	

ETV: entecavir, HBcrAg: hepatitis B core-related antigen, LAM: lamivudine, NA: nucleos(t)ide analogue, no.: number, NS: not statistically significant Ref: reference, UD: undetectable

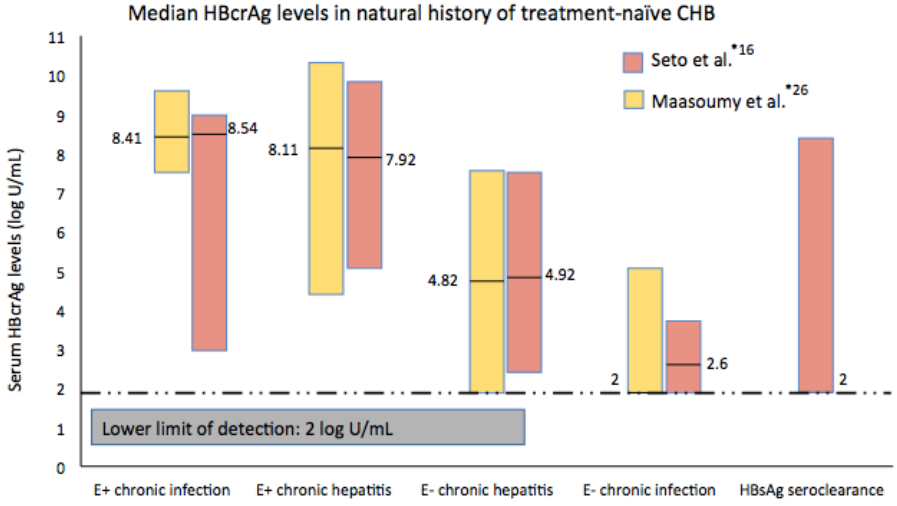
Figure legends

Figure 1. The viral replication cycle and the origins of HBV DNA, HBsAg, HBeAg and HBcrAg.



cccDNA=covalently closed circular DNA, ER=endoplasmic reticulum, HBc=hepatitis B core protein, HBcrAg=hepatitis B core-related antigen, HBeAg=hepatitis B e antigen, HBs=hepatitis B surface protein, HBsAg=hepatitis B virus surface antigen, HBV=hepatitis B virus, p22cr=truncated 22kDa precore protein, pgRNA=pre-genomic RNA, rcDNA=relaxed circular DNA

Figure 2. Median HBcrAg levels in natural history of CHB and correlation with other viral markers.

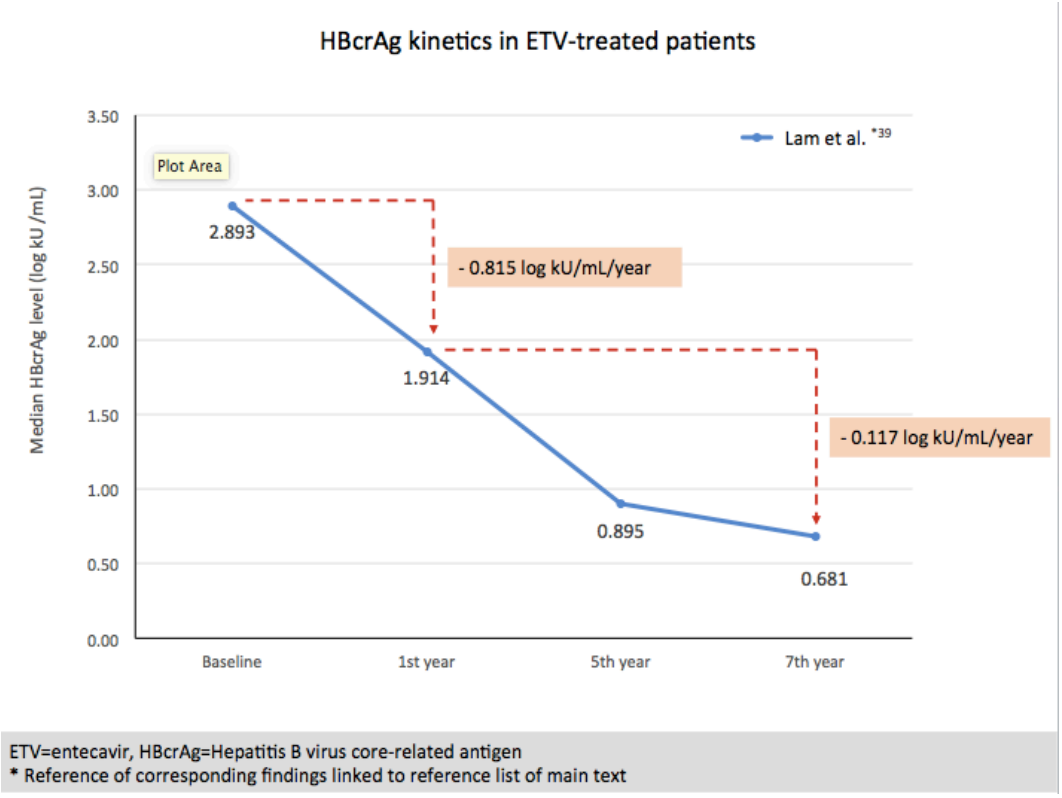


Correlation coefficients of HBV DNA, HBsAg and HBV RNA with HBcrAg

	E+ chronic infection	E+ chronic hepatitis	E- chronic hepatitis	E- chronic infection	Ref.
HBV DNA	r=0.369 (p=0.007)	r=0.484 (p<0.001)	r=0.537 (p<0.001)	r=0.472 (p<0.001)	16
	r=0.45 (p=0.013)	r=0.66 (p<0.0001)	r=0.74 (p<0.0001)	r=0.18 (p=0.054)	26
HBsAg	r=0.286 (p=0.040)	r=0.406 (p=0.017)	r=0.245 (p<0.001)	0.388 (p<0.001)	16
	r=0.47 (p=0.0095)	r=0.53 (p<0.0001)	r=0.40 (p=0.0045)	r=0.47 (p<0.0001)	26

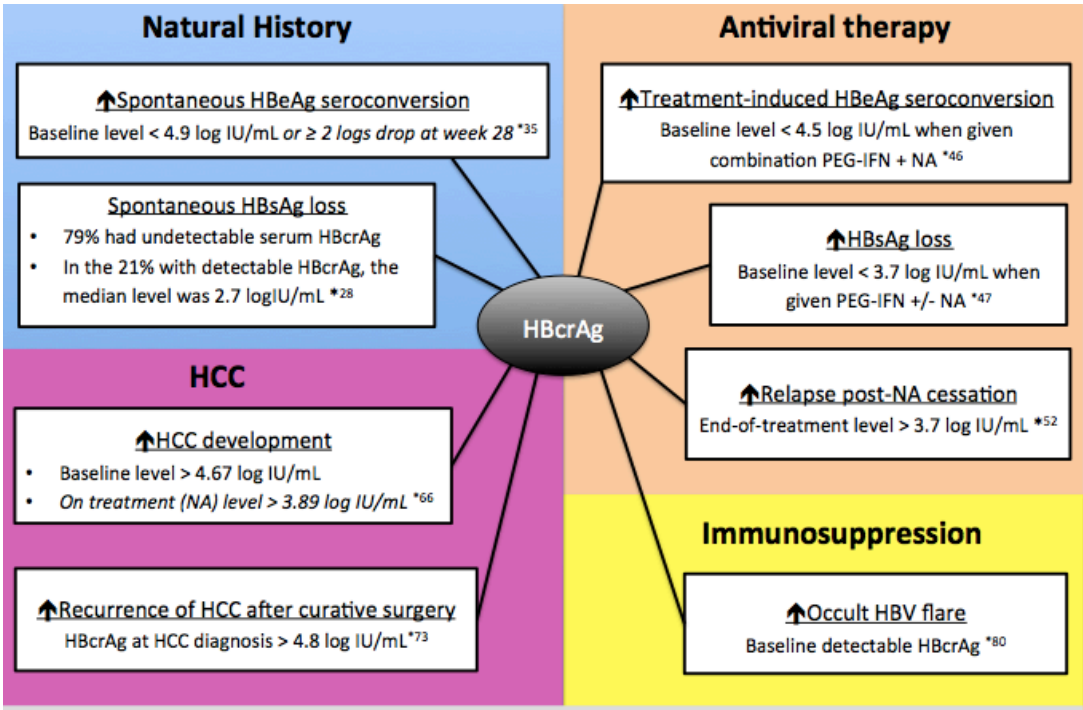
CHB=chronic hepatitis B infection, E+ =Hepatitis B e antigen positive, E-=Hepatitis B e antigen negative, HBcrAg=Hepatitis B virus core-related antigen, HBsAg=Hepatitis B surface antigen * References of corresponding findings linked to reference list of main text. The bars represent the range of values.

Figure 3. Median HBcrAg levels in CHB patients treated with ETV.



ETV=entecavir, HBcrAg=Hepatitis B virus core-related antigen
* Reference of corresponding findings linked to reference list of main text

Figure 4. The major findings and potential clinical applications of HBcrAg.



HBeAg-HBV e antigen, HBV=hepatitis B virus, HBcrAg=Hepatitis B virus core-related antigen, HBsAg=hepatitis B virus surface antigen, HCC=hepatocellular carcinoma, NA=nucleos(t)ide analogue, PEG-IFN=pegylated-interferon
Words shown in Italic represent dynamic change of HBcrAg
References of corresponding findings linked to reference list of main text

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