Viral Biomarkers for Hepatitis B Virus-Related Hepatocellular Carcinoma Occurrence and Recurrence

Yuanyuan Liu1,2, Vaishnavi Veeraraghavan2,3, Monica Pinkerton2,3, Jianjun Fu1, Mark W. Douglas2,4,5, Jacob George2 and Thomas Tu2,4*

1 Department of Infectious Diseases, The Affiliated Xi'an Central Hospital of Xi'an Jiaotong University, Xi'an, China, 2 Storr Liver Centre, The Westmead Institute for Medical Research, The University of Sydney and Westmead Hospital, Sydney, NSW, Australia, 3 School of Medical Science, The University of Sydney, Camperdown, NSW, Australia, 4 Marie Bashir Institute for Infectious Diseases and Biotechnology, University of Sydney, Sydney, NSW, Australia, 5 Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, NSW, Australia

Viral Biomarkers for Hepatitis B Virus-Related Hepatocellular Carcinoma Occurrence and Recurrence

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the fourth leading cause of cancer-related death. The most common risk factor for developing HCC is chronic infection with hepatitis B virus (HBV). Early stages of HBV-related HCC (HBV-HCC) are generally asymptomatic. Moreover, while serum alpha-fetoprotein (AFP) and abdominal ultrasound are widely used to screen for HCC, they have poor sensitivity. Thus, HBV-HCC is frequently diagnosed at an advanced stage, in which there are limited treatment options and high mortality rates. Serum biomarkers with high sensitivity and specificity are crucial for earlier diagnosis of HCC and improving survival rates. As viral–host interactions are key determinants of pathogenesis, viral biomarkers may add greater diagnostic power for HCC than host biomarkers alone. In this review, we summarize recent research on using virus-derived biomarkers for predicting HCC occurrence and recurrence; including circulating viral DNA, RNA transcripts, and viral proteins. Combining these viral biomarkers with AFP and abdominal ultrasound could improve sensitivity and specificity of early diagnosis, increasing the survival of patients with HBV-HCC. In the future, as the mechanisms that drive HBV-HCC to become clearer, new biomarkers may be identified which can further improve early diagnosis of HBV-HCC.

Keywords: hepatitis B, hepatocellular carcinoma, biomarkers, HBV surface antigen (HBsAg), HBV DNA integration, HBV RNA, HBcr antigen

INTRODUCTION

Chronic infection with the Hepatitis B virus (HBV) is the predominant risk factor for primary liver cancer, specifically hepatocellular carcinoma (HCC; Bosch et al., 2004; Kew, 2010; Ozakyol, 2017). Overall, the lifetime incidence of HCC in HBV has been reported to be approximately 10–25% (McGlynn et al., 2015). Moreover, most cases of HBV-associated HCC occur in cirrhotic liver...
disease, present in 70–90% of cases (Yang et al., 2011). Liver cancer is the fourth most deadly cancer (Bray et al., 2018), with a median survival time as short as 11 months (Greten et al., 2005; Yang and Roberts, 2010). There is also a broad range of indirect health impacts driven by chronic HBV, including anxiety about disease progression, stigma and discrimination, and health care costs associated with treatment (Tu et al., 2020a).

Chronic HBV infection leads to a repeated cycle of liver damage and regeneration, which promotes tumorigenesis (Wang et al., 2006). Treatment of the underlying HBV infection can reduce, but not eliminate HCC risk (Papatheodoridis et al., 2015). Currently, oral nucleo(s)tide analogs (NAs) are used as first-line therapy for HBV infection. NA therapy targets the reverse transcriptase of HBV and suppresses HBV DNA replication, reduces progression to end-stage liver disease and improves long-term patient survival (Bitton Alaluf and Shlomai, 2016). NAs suppress viral replication (Ghany and Liang, 2007) but do not target HBV covalently closed circular DNA (cccDNA; Revill et al., 2020; Tu et al., 2020b). cccDNA is the template for HBV replication and expression of viral proteins, so its persistence plays a crucial role in chronic infection, inflammation, and cancer formation.

**CURRENT CLINICAL DETECTION OF HCC**

Early screening of HBV-infected patients for HCC reduces mortality (Zhang B.H. et al., 2004). Current AASLD guidelines advise abdominal ultrasound surveillance for HBV-infected patients with advanced fibrosis or cirrhosis at 6-month intervals (Marrero et al., 2018), as marked liver fibrosis is a strong risk factor for HCC. However, ultrasound can miss HCC at early stages [sensitivity 63% (Singal et al., 2009)] and is strongly affected by operator- and patient-dependent factors (Singal et al., 2009; Pocha et al., 2013). Moreover, HCC can occur at any stage of liver fibrosis; hence AASLD guidelines recommend HCC surveillance of people with HBV who are ≥40 (for males) or ≥50 (for females) years old, regardless of fibrosis levels (particularly in those of Asian descent) (Xu et al., 2017). A cohort of studies suggested that liver stiffness measurement using FibroScan can predict HCC development in HBV patients with cirrhosis (Jung et al., 2011; Pesce et al., 2012; Adler et al., 2016) but fail to predict HCC in non-cirrhotic chronic hepatitis B (CHB) patients with liver stiffness measurement <8.0 kPa as well as patients with body mass index >28 kg/m² and waist circumference ≥102 cm (Foucher et al., 2006; Jung et al., 2011; Cassinotto et al., 2016).

Alpha-fetoprotein (AFP) is the most widely used serum biomarker for the diagnosis of HCC (Marrero et al., 2018). However, elevated serum AFP is only found in 60–70% of HCC patients (Luo et al., 2019). Lectin-reactive AFP (AFP-L3) and des-gamma-carboxy prothrombin (DCP) have also been proven to be useful biomarkers for HCC (Li et al., 2001; Volk et al., 2007) and increase the sensitivity compared to using AFP alone (Marrero et al., 2009; Wang et al., 2020). Unfortunately, considering the low sensitivity (55%) of AFP-L3, HCC detection (particularly in early stages) is still suboptimal (Choi et al., 2019). DCP has a poorer diagnostic power for small HCC compared to AFP, but is better at detecting intermediate and advanced HCC (Nakamura et al., 2006).

Therefore, more sensitive, non-invasive biomarkers for better HCC diagnosis are needed. Here, we review the current knowledge on circulating viral biomarkers to screen for HCC, which may improve detection rates in combination with existing host-derived markers.

**HBV Structure, Natural History, and Replication Cycle**

Hepatitis B virus is the prototypic member of the Hepadnaviridae family. The HBV virion contains a ∼3.2 kbp double-stranded DNA genome contained in a nucleocapsid composed of hepatitis B core antigen (HBcAg) subunits. The majority (∼90%) of virions contain a relaxed-circular DNA (rcDNA) genome, while a minority contain a double-stranded linear (dsDNA) form of the viral genome (Venkatakrishnan and Zlotnick, 2016). This nucleocapsid is enveloped in a host-derived lipid bilayer studded with hepatitis B surface antigens (HBsAg).

Infection with HBV itself is not cytopathic and the initial infection is usually asymptomatic, despite the production of high levels of virus antigen and viral particles by the liver. After decades of infection, HBV can trigger the immune response, though this is generally insufficient to clear all HBV-infected liver cells and subsequently causes chronic inflammation and liver damage progression. These two phases can be broadly divided serologically by the presence of circulating HBV e antigen (HBeAg, marking a status prior to extensive immune recognition) or antibodies against HBeAg (anti-HBe, present after antiviral clearance of the majority of infected cells). According to EASL 2017 Clinical Practice Guidelines (European Association for the Study of the Liver, 2017), chronic HBV infection can be separated into five clinical phases (Table 1): HBeAg-positive chronic HBV infection, previously termed “immune tolerant”; HBeAg-positive CHB with serum HBeAg positive, high HBV DNA and elevated ALT, termed “immune clearance phase”; HBeAg-negative chronic HBV infection, formerly known as the “inactive carrier” state; HBeAg-negative CHB with positive anti-HBe, persistent or fluctuating levels of HBV DNA and elevated ALT; HBsAg-negative phase, termed “occult HBV infection.”

On a cellular level, the infection of hepatocytes begins with attachment of the virion to the sodium taurocholate co-transporting polypeptide (NTCP), the entry receptor of HBV (Yan et al., 2012, 2014; Ni et al., 2014; Figure 1). After binding and receptor-mediated endocytosis, viral nucleocapsids are transported through the cytoplasm (Yan et al., 2014) to the nuclear membrane, where uncoating and entry of the HBV DNA genome into the nucleus occurs.

Nuclear HBV rcDNA is converted into the HBV cccDNA form using host cell DNA repair enzymes (Königer et al., 2014; Qi et al., 2016). HBV cccDNA is an episomal “mini-chromosome”
and acts as a stable template for the 5 viral mRNAs. Each of these transcripts has different 5′ transcription start sites but a common 3′ polyadenylation signal. These mRNAs include the 3.5-kb pregenomic RNA (pgRNA), the 3.5-kb precore RNA, the 2.4-kb/2.1-kb surface mRNAs, and the 0.7-kb X mRNA (Blondot et al., 2016). Moreover, post-transcriptional modification of 3.5-kb species can produce spliced HBV RNA variants (Candotti and Allain, 2017).

The functions of pgRNA are both as the template for reverse transcription and the translation of viral polymerase and core protein. The newly translated viral polymerase binds to the 5′-epsilon region of pgRNA, and is packaged together as sub-viral core particles (Jones and Hu, 2013). Reverse transcription occurs within the HBV nucleocapsid through a series of complicated mechanisms, resulting in the synthesis of rcDNA (major pathway) or dslDNA. These mature nucleocapsids are then enveloped by HBsAg and secreted into the blood at multi-vesicular bodies (Blondot et al., 2016).

Nuclear dslDNA genomes follow separate pathways: these can form replication-defective cccDNA (Yang and Summers, 1998) or integrate into the host cell genome (Yang and Summers, 1999; Tu et al., 2017). While the integrated HBV genome is replication-deficient, but still acts as a template viral antigen expression (e.g. HBsAg and HBs) (Wooddell et al., 2017).

A broad range of components generated by virus-infected cells have been investigated as potential biomarkers for predicting HCC occurrence (summarized in Table 2) and recurrence (summarized in Table 3). For each major serum viral marker that has been investigated, we provide in the next section a description, the mode of quantification, their molecular association with HCC, and their predictive power for HCC occurrence and recurrence.

### TABLE 1 | Natural history of patients with chronic HBV infection.

| Phases | New name | Old name | HBs | Anti-HBs | HBe | Anti-HBe | HBV DNA titers | ALT levels | Cirrhosis rate | HCC risk (incidence)a |
|--------|----------|----------|-----|----------|-----|----------|----------------|------------|-----------------|---------------------|
| Phase 1 | HBeAg-positive chronic infection | Immune tolerance | +   | –        | +   | –        | Very High       | Normal     | Very Low        | 0.04–0.5 (Fattovich et al., 2008) |
| Phase 2 | HBeAg-positive chronic hepatitis | Immune active | +   | –        | +   | –        | High            | Elevated   | Low             | 0.5–3 (Chu et al., 2004; Lin S.M. et al., 2007; Fattovich et al., 2008) |
| Phase 3 | HBeAg-negative chronic infection | Inactive carrier phase | +   | –        | –   | +        | Low to Undetectable | Normal     | Low/Mid         | 0.02–0.2 (De Franchis et al., 1993; Hsu et al., 2002; Manno et al., 2004; Raffetti et al., 2018) |
| Phase 4 | HBeAg-negative chronic hepatitis | Immune re-activation | +   | –        | –   | +        | Moderate to High | Elevated   | Mid/High        | No cirrhosis 0.3–0.6 (Fattovich et al., 2008) |
| Phase 5 | HBsAg-negative phase | Clearance or occult HBV infection | – | ±        | –   | +        | Undetectable to low | Normal     | Low             | No cirrhosis 0.3 (Kim et al., 2015) |

aIncidence per 100 person years.

### SERUM VIRAL BIOMARKERS FOR HBV RELATED HCC

#### HBV DNA

**Description**

Hepatitis B virus DNA, the genomic nucleic acid of the virus, reflects active viral replication and secretion. There are two forms of HBV genome: rcDNA and dslDNA (as mentioned in the HBV replication cycle) (Lee et al., 2004; Blondot et al., 2016). Quantitative PCR for serum HBV DNA detects both forms of the virus genome and is used as a clinical marker to measure the efficacy of antiviral therapy in people with CHB.

**Quantification**

Using real-time PCR quantification assays for HBV DNA detection is strongly recommended by EASL (European Association for the Study of the Liver, 2017) and is generally expressed as a WHO-standardized IU/mL (5.26 copies/mL = 1 IU/mL) (Saldanha et al., 2001). At present, with their high sensitivity, specificity, accuracy and broad dynamic range, these techniques are the most widely used assays in clinical practice. The assays include Cobas AmpliPrep/Cobas TaqMan HBV version 2.0 (CAP/CTM HBV 2.0) (Roche Molecular Systems, Pleasanton, CA, United States), with a dynamic range between 105 copies/mL to 9 × 108 copies/mL, and Abbott RealTime HBV assay (Abbott Molecular, Des Plaines, IL, United States), with a dynamic range 50 copies/mL to 5 × 109 copies/mL (Chevaliez et al., 2008, 2010; Yeh et al., 2014). More sensitive pre-clinical tests have also been developed: digital droplet PCR can quantify HBV DNA down to 8 copies/mL (Liu et al., 2017).

The amount of the dslDNA form of HBV (as opposed to rcDNA form) can be measured using quantitative real-time PCR
| Biomarkers       | Antiviral treatment | Patient population | Findings                                                                 | References                        |
|------------------|---------------------|--------------------|--------------------------------------------------------------------------|-----------------------------------|
| **HBV DNA**      | Naïve               | HBeAg (+)          | HBV DNA was not different between HCC and non-HCC                       | Fung et al., 2007                 |
|                  |                     | HBeAg (−)          | HBV DNA is higher in HCC group (AUROC = 0.62)                            | Fung et al., 2007                 |
|                  | Treated             | CHB patients       | HBV DNA was not different between HCC and non-HCC                       | Kim et al., 2017; Lee et al., 2020|
|                  |                     | Cirrhosis patients | Risk of HCC is significantly higher in low-level viremia (<2,000 IU/mL) compared to undetected | Kim et al., 2017                 |
| **HBV integration** | Naïve               |                      |                                                                          |                                   |
|                  | Treated             |                      |                                                                          |                                   |
| **HBV variants** | Splice variants     |                      |                                                                          |                                   |
|                  | Treated             |                      |                                                                          |                                   |
| **Pre-S mutants** | Naïve               | HBeAg-negative patients without liver cirrhosis | HBV DNA with pre-S deletions predicted HCC (HR, 11.26; 95% CI, 2.18–58.1; \(P = 0.004\), median time 84 months | Chen et al., 2007                 |
|                  | Treated             | CHB patients with Genotypes C and B | HBV DNA with pre-S deletions predicted HCC (OR = 3.28). HBV DNA with Pre-S1 or Pre-S2 mutations predicted HCC (OR = 2.42, 3.36) | Wungu et al., 2021               |
| **Total HBV RNA** | Naïve               |                      |                                                                          |                                   |
|                  | Treated             |                      |                                                                          |                                   |
| **Truncated HBV RNA** | Naïve             |                      |                                                                          |                                   |
|                  | Treated             |                      |                                                                          |                                   |
| **HBsAg**        | Naïve               | HBeAg (−), HBV DNA > 2000 IU/mL | HBsAg poorly predicted HCC (AUROC: 0.58)                                  | Tseng et al., 2012               |
|                  |                     | HBeAg (−), HBV DNA ≤2000 IU/mL | HBsAg ≥ 1,000 IU/mL is an independent risk factor for HCC (HR 13.7)       | Tseng et al., 2012               |
|                  | Treated             |                      |                                                                          |                                   |
| **HBcrAg**       | Naïve               | HBeAg (−), HBV DNA 2000–19,999 IU/mL | HBcrAg > 10,000 U/mL could independently define a high HCC risk group (HR 6.29) | Tseng et al., 2019               |
|                  |                     | HBeAg(−), HBV DNA≤10⁴ copies/mL, no cirrhosis | HBcrAg > 5012 U/mL was associated with HCC occurrence (HR 6.13) | Tada et al., 2016               |
|                  |                     | Any HBeAg status, HBV DNA > 10⁴ copies/mL, FIB-4 < 3.8 | HBcrAg > 5012 U/mL was associated with HCC occurrence (HR 5.69) | Tada et al., 2016               |
|                  |                     | Independent of HBV DNA levels, HBeAg | HBcrAg > 794 U/mL was independently associated with HCC occurrence (HR 5.05) | Tada et al., 2016               |
|                  | Treated             | HBeAg (+)          | HBcrAg > 4.9log U/mL predicted HCC (Sensitivity: 90.3%, specificity: 21.7%) | Hosaka et al., 2019             |
|                  |                     | HBeAg (−)          | HBcrAg > 4.4log U/mL predicted HCC. (Sensitivity: 51.9%, specificity: 78.7%) | Hosaka et al., 2019             |
|                  |                     | HBV DNA (−) post-treatment | HBcrAg > 7.8 kU/mL predicted HCC. (AUROC: 0.61, Sensitivity: 57.9%, specificity: 70.4%) | Cheung et al., 2017             |
|                  |                     | Non-cirrhotic      | HBcrAg > 7.8 kU/mL predicted HCC. (AUROC: 0.7, Sensitivity: 62.5%, specificity: 78.1%) | Cheung et al., 2017             |
FIGURE 1 | The HBV replication cycle and its secreted products. The HBV virion enters the hepatocyte by NTCP receptor binding, and uncoats prior to entry into the cytoplasm. The viral nucleocapsid is then transported to the nucleus, where it deposits its DNA genome. HBV relaxed-circular DNA (rcDNA) genomes can be repaired and ligated to form cccDNA, the template for all viral RNAs. HBV core antigen (HBcAg) is translated and forms capsids, some of which form around the pregenomic RNA (pgRNA) and viral polymerase. The pgRNA is reverse-transcribed to form either double stranded linear DNA (dslDNA) or rcDNA forms of the virus genome. The mature nucleocapsid is then enveloped by host membranes studded with HBV surface antigen (HBsAg) and secreted at multi-vesicular bodies. Cytoplasmic HBV capsids are recycled at a poor efficiency to the nucleus and do not appear to significantly add to the cccDNA pool (Tu and Urban, 2018; Revill et al., 2020; Tu et al., 2021). In a secondary pathway, HBV dslDNA can integrate into the host genome at host DNA breaks or form defective cccDNA (not shown). Some of these viral components are released in the serum (bottom) by as yet unclear mechanisms (dashed arrows) including within apoptotic bodies of dying hepatocytes, secretion through alternate pathways, or within exosomes. Even the form in which some of these biomarkers exist in the serum is still unknown and controversial (question marks). Figure was generated using Biorender (https://biorender.com/).

(qPCR) coupled with peptide nucleic acid-mediated clamping (Zhao X.L. et al., 2016). However, this assay is not a standard laboratory test.

Molecular Association With HCC
Serum HBV DNA load in people with CHB has been shown to be closely related to disease activity and progression (Iloeje et al., 2006). Moreover, elevated HBV DNA is considered as a predictive biomarker for HCC, independent of HBeAg and liver cirrhosis (Chen et al., 2006, 2011). HBV DNA is associated with both indirect and direct mechanisms of carcinogenesis. The indirect mechanisms include inducing new HBV infection of hepatocytes, which triggers ongoing liver immune attack, inflammation, and liver injury (Bolukbas et al., 2005; Duygu et al., 2012; Chen and Tian, 2019). Possible mechanisms of direct carcinogenesis include HBV dslDNA integration into the host genome, which reportedly leads to genomic instability, insertional mutagenesis and expression of pro-oncogenic viral proteins (Sze et al., 2013; Zhao I.H. et al., 2016; Gao et al., 2019). Indeed, one study reported that the levels of dslDNA increased to 14% of total serum HBV DNA in people with liver cirrhosis and 20% in those with HCC, compared to 7% in people with CHB alone (Zhao X.L. et al., 2016). However, the utility of dslDNA proportion as a biomarker for HCC has not been examined in clinical trials.

Performance as a Predictor of HCC

HCC occurrence
In NA-naïve patients, two studies in Taiwan have inferred that elevated serum HBV DNA level can be a useful biomarker for monitoring HCC independent of HBeAg and liver cirrhosis (Chen et al., 2006, 2011). A study by Chen et al. (2011) showed that in a cohort of patients with genotype B/C HBV infection aged >30 years, the risk of HCC increased with higher levels of circulating HBV DNA (after excluding patients in immune
tolerance phase with HBV DNA $>10^7$ copies/mL, as people in this phase have low risk of HCC). In a case-control study of HBeAg-negative CHB patients, levels of HBV DNA were found to be higher in people with HCC than those without (Area Under the Receiver Operating Characteristic curve, AUROC = 0.62) (Fung et al., 2007). Tseng et al. (2012) reported that in a cohort of 2688 treatment-naïve people with CHB, HBV DNA predicts the risk of HCC regardless of HBeAg status [AUROC = 0.7(95% confidence interval (CI): 0.65–0.75)]. Together, this suggests HBV DNA has good predictive strength for HCC risk.

However, HBV DNA titers cannot be used for all patients. NA therapy can reduce levels of serum HBV DNA to an undetectable level, preventing its use as a biomarker in this population that is still susceptible to HCC (Vlachogiannakos and Papatheodoridis, 2013; Varbobitis and Papatheodoridis, 2016). Further, in a Korean cohort of 1,246 patients with CHB who received entecavir, baseline HBV DNA did not predict HCC in non-cirrhotic patients under NA treatment ($>$5.7 vs. $<$5.7log IU/mL; $P = 0.166$) (Kang et al., 2017).

Nevertheless, HBV DNA levels can be used to detect poor response to NAs, which is linked to HCC. In a cohort of 875 patients with CHB treated with entecavir, greater HBV DNA levels were linked to increased HCC risk in patients with cirrhosis (adjusted hazard ratio = 2.20, compared to those with persistently undetectable HBV DNA) (Kim et al., 2017). But, HBV DNA did not predict HCC risk in patients without cirrhosis. HCC incidence was not significantly different between people with persistently detectable HBV DNA and those with undetectable levels (13.3% vs. 8.3%, $P = 0.821$) (Lee et al., 2020).

### TABLE 3 | Serum viral biomarkers for the prediction of HCC recurrence.

| Biomarkers   | Antiviral treatment | Patient population | Findings                                                                 | References          |
|--------------|---------------------|--------------------|--------------------------------------------------------------------------|---------------------|
| HBV DNA      | Naive               | Early recurrence (within 2 years) | HBV DNA levels $>20,000$ IU/mL predicted microvascular invasion (HR 2.77; $P < 0.001$) | Sohn et al., 2014   |
|              |                     | Late recurrence (after 2 years)   | HBV DNA level $>10^6$ copies/mL was associated with recurrence (HR 2.548; CI 1.040–6.240) | Wu et al., 2009     |
|              | Treated             | 1040 patients with a high baseline HBV DNA level ($>2,000$ IU/mL) | Undetectable HBV DNA at week 24 post-resection predicted lower late HCC recurrence ($P < 0.001$, HR 0.408, 95% CI 0.269–0.618), but was not associated with early HCC recurrence | Huang et al., 2013  |
| HBV integration | Pre-resection: 21 (42.0%) | 50 HBV-related HCC with 36 genotype B (72.0%) | Detection of tumor-associated HBV DNA integrations in serum predicted HCC recurrence in $>$90% of cases | Li et al., 2020     |
|              | Post-resection: 35 (70.0%) |  |  |  |
| HBV variants | Splice Variants      | Naïve at HCC diagnosis: 35 (46%) | Median HBV DNA: $2.1 \times 10^4$ IU/mL | Unreported          |
|              |                     | Median HBV DNA: $2.1 \times 10^4$ IU/mL | The AUROC of the pre-S2 plus pre-S1 + pre-S2 deletion percentage is 0.6827, followed by the combined pre-S deletion (AUROC,0.6789) | Teng et al., 2020b  |
|              | Pre-S mutants       | Naïve at HCC diagnosis: 35 (46%) | Median HBV DNA: $2.1 \times 10^4$ IU/mL | HBV DNA with Pre-S2 deletions nt 1–54 in serum was associated with HCC recurrence ($P = 0.008$, AUROC = 0.6321) | Teng et al., 2020a  |
| HBV RNA      | Truncated HBV RNA   | Naïve at HCC diagnosis: 35 (46%) | Late HCC recurrence (after 2 years) | Unreported          |
| HBsAg        |                     | Hepatic resection HBeAg$\leq$ | HBsAg ≥4,000 IU/mL is the risk factor for HCC recurrence after 2 years (HR 2.80; $P = 0.023$) | Sohn et al., 2014   |
|              |                     | HBV DNA < 2000 IU/mL | HBsAg ≥1,000 IU/mL is associated with HCC recurrence | Huang et al., 2014  |
| HBcAg        | Treated at HCC      | 55 HCC patients, either curative resection or percutaneous ablation | HBcAg levels ≥ 4.8log U/ml at the time of HCC diagnosis was independent factor for HCC recurrence (HR 8.96, 95% CI 2.47–11.25; $P = 0.005$) | Hosaka et al., 2010  |
|              | diagnosis of HCC    | 119 HCC patients, HBeAg$\leq$ | HBeAg level ≥ 5.1log U/ml was associated with increased tumor recurrence rate ($P = 0.01$) | Beudeker et al., 2021 |
|              |                     | HBeAg$\leq$ 68% |  |  |
|              | Treated at HCC      | 169 HCC patients with liver transplantation, HBeAg$\leq$47 (27.8%) | HBcAg ≥ 5.0 log U/mL predicted HCC recurrence after 5 years (HR 5.27, 95% CI 2.47–11.25; $P = 0.001$) | Yu et al., 2019  |
NAs, HBV DNA titer is useful in predicting HCC only in cirrhotic patients.

**HCC recurrence**

In NA-naive HCC patients, high serum HBV DNA levels were an independent risk factor for HCC recurrence after curative resection or liver transplantation, or percutaneous radiofrequency ablation (Huang et al., 2008; Chuma et al., 2009; Goto et al., 2011; Li et al., 2011). In a study of 248 Korean patients who underwent curative resection for early stage HBV-related HCC, HBV DNA level $\geq 20,000$ IU/mL [hazard ratio (HR) 2.77; $P < 0.001$] was a risk factor for microvascular invasion and early recurrence (within 2 years) (Sohn et al., 2014). However, Wu et al. (2009) found that HBV DNA level $>10^6$ copies/mL (HR 2.548, CI 1.040–6.240) in Taiwan patients with HBV-related HCC was associated with late recurrence (after 2 years). Therefore, the utility of high HBV DNA in predicting HCC recurrence needs further research.

After NA treatment at diagnosis of HCC or follow-up, sustained HBV DNA expression could increase the risk of HCC recurrence (Kim et al., 2008). Moreover, Huang et al. (2013) found that in the 865 HCC patients receiving NAs therapy with a high baseline HBV DNA level (subpopulation of a 1,040 patient cohort), an undetectable HBV DNA before postoperative week 24 ($P < 0.001$, HR 0.408, 95% CI 0.269–0.618) was a protective factor for late HCC recurrence, but not for early tumor recurrence ($P = 0.541$, HR 0.946, 95% CI 0.793–1.130). Therefore, detectable HBV DNA level could predict HCC recurrence in patients receiving NA treatment.

**HBV Integration**

**Description**

Integration of the dslDNA form of HBV DNA can occur throughout the host genome at double-strand DNA breaks (Bill and Summers, 2004), likely without the help of specific viral proteins (Tu et al., 2019) (instead probably using host DNA repair enzymes). The sites of HBV DNA integration during CHB are randomly distributed across the host genome without strong preference for any specific structural genome features (Budzinska et al., 2018a).

**Quantification**

Hepatitis B virus integrations can be detected in the serum and tissue of HBV-infected patients as virus-host chimera DNA. Current detection methods for virus-host chimera DNA include whole-exome sequencing, whole-genome sequencing, Alu PCR and inverse-nested PCR (Budzinska et al., 2018b). These have shown less-than-genome length fragments of HBV dslDNA integrate (with terminal truncations of 100s to 1,000s of base pairs being common). Of these detection assays, the only method enabling absolute quantification of HBV integrations is inverse-nested PCR (Mason et al., 2009), though this method is very time-consuming and technically challenging, limiting its clinical utility.

**Molecular Association With HCC**

While HBV integration sites are randomly distributed across the genome in non-tumor tissue, HBV DNA integrations in HCCs have been reported to be enriched in genes involved in carcinogenesis pathways (i.e., CTNN2, EGFR, and TERT) and have been found to be preferentially located within CpG islands and close to telomeres (Sung et al., 2012; Zhao L.H. et al., 2016; Li et al., 2019). Even when the HBV infection is cleared (marked by HBsAg seroconversion), HCCs risk remains and 70% of HCCs contain HBV integrations (Wong et al., 2020).

The mechanism behind the association of HCC with HBV integration is currently unknown. Many studies indicate that HBV integration causes genetic damage and chromosomal instability, which has the potential to promote carcinogenic transformation (Scotto et al., 1983; Zhao L.H. et al., 2016; Chen et al., 2019; Jang et al., 2020), or drive downstream host protein expression. HBV DNA can integrate into fragile sites, CpG islands and near telomerase reverse transcriptase, lysine methyltransferase 2B, as well as cyclin A2 (Wong et al., 2020), potentially inducing cancer-initiating genomic instability (Zhao L.H. et al., 2016; Furuta et al., 2018; Wong et al., 2020). However, genomic instability is not evident in many cases of HBV-HCC (Sung et al., 2012). The integrated HBV DNA can also disrupt cellular genes by insertional mutagenesis or drive expression of nearby with viral promoters. Insertion in TERT promoter, CCNE1 (cyclin E1), CCNA2, ML4 (Myeloid/lymphoid or mixed-lineage leukemia 4), TP53, and CTNNB1 have been repeatedly detected in HCC (Paterlini-Brechot et al., 2003; Sung et al., 2012; Kawai-Kitahata et al., 2016), but these are not present in all tumors.

In addition, mutant HBsAg produced from integrated HBV DNA could contribute to HBV-related HCC by causing endoplasmic reticulum (ER) stress and immune evasion (Hsieh et al., 2004; Wang et al., 2006).

**Performance as a Predictor of HCC**

**HCC occurrence**

Specific HBV-host fusion genes created by HBV integrations have been suggested as biomarkers for predicting HCC in people with CHB. In NA-treated patients, a prospective study using liver tissue from people with CHB reported that human ESPL1-HBV S fusion gene was detected in 8 of 12 (66.7%) people with HCC, compared to 0 of 11 (0%) CHB patients without HCC (Hu et al., 2020). Moreover, HBV has been reported to integrate into long interspersed nuclear elements (LINEs), leading to fusion HBx-LINE1 transcripts. HBx-LINE1 can activate β-catenin signaling, reduce E-cadherin and enhance cell migration, which has been suggested to promote HCC progression (Liang et al., 2016). These studies suggest that specific fusion genes could be used as a biomarker for the early detection of HCC in people with CHB, but these have not been able to be repeated independently in other cohorts [for example, in a cohort of Vietnamese patients with HBV-associated HCC (Trung et al., 2019)]. Indeed, the majority of integration sites in tumor samples are randomly distributed across the host genome (Zhao L.H. et al., 2016).
biomarker for monitoring HCC recurrence (Wang et al., 2019; Li et al., 2020). A study of 20 people with HBV-HCC found circulating vh-DNA representing 87 different HBV integration sites, which were enriched in genes involved in cancer-related pathways, suggesting they could act as a biomarker for HCC diagnosis (Li et al., 2019). Moreover, Li et al. (2020) detected vh-DNA in 97.7% of people with HBV-related HCC. Two months following HCC resection, the same vh-DNA sequence could be detected in 10 cases (23.3%), nine of whom (90%) experienced HCC recurrence within a year. Thus, vh-DNA of HBV integration could also be a useful circulating biomarker for monitoring HCC recurrence.

HBV Splice Variants

Description
Hepatitis B virus pgRNA has multiple splice donor and acceptor sites and can be spliced by cellular machinery as a post-transcriptional modification. Sixteen spliced pgRNA variants have been identified both in vitro and in tissues of CHB patients (Candotti and Allain, 2017). These splice variants can be encapsidated, reverse-transcribed and secreted into serum as replication-deficient viral particles (Terre et al., 1991).

Spliced viral RNAs can also be translated into HBV spliced proteins. For example, the 2.2-kb singly spliced variant lacking intron 2447/489, can encode hepatitis B spliced protein (HBSP) in the livers of patients with chronic HBV infection (Soussan et al., 2000). The 2447-2901 HBV RNA splice variant can act as the template for a 43 kDa polymerase-surface fusion glycoprotein (P-S FP), which localizes to the ER and is posited to be an HBV structural protein (Huang et al., 2000; Park et al., 2008).

Furthermore, hepatitis B doubly spliced protein and HBSP are respectively encoded by the 2.2-kb doubly spliced pgRNA and the single spliced product 1(SPI) variant (Terre et al., 1991; Huang et al., 2000; Soussan et al., 2000; Lee et al., 2008). However, the specific function of any of these splice variant-derived proteins is currently unclear.

Quantification
Hepatitis B virus splice variants can be quantified by reverse-transcription PCR (RT-PCR). At present, using different combinations of 5′ splice site and 3′ splice site can generate HBV RNA splicing variants, including 16 identified HBV splice variants of pgRNA and 4 splice variants of preS2/S mRNA (Su et al., 1989; Terre et al., 1991; Hass et al., 2005). The 2.2-kb singly spliced variant with a lack of intron 2447/489 which is the most common spliced variant can encode the HBSP, which can be detected by Western blot (Soussan et al., 2003).

Molecular Association With HCC

Some studies have reported increased HBV RNA splicing being associated with HCC (Krensdorf et al., 2006; Bayliss et al., 2013). The 2.2 kb HBV spliced variant has been reported to be more highly expressed in tumor tissues than in the adjacent-tumor tissues (Lin et al., 2002). Moreover, when full-length (3.2 kb) HBV DNA and 2.2 kb spliced variant are co-transfected into HepG2 cells, the replication signal of the 3.2 kb HBV genome was increased 3–7 times (Lin et al., 2002). This suggests the HBV spliced variant plays a role in increasing HBV, which is a strong risk factor for HCC.

Circulating splice variant DNA is most frequently detected as defective HBV particles (dHBV) derived from reverse transcription of the 2.2-kb singly spliced mRNA, the most common spliced variant ( Günther et al., 1997). In NA-naïve patients, the ratio of serum dHBV to wild-type HBV was lower in patients with moderate fibrosis and moderate or no liver necrosis compared to those with severe fibrosis and severe liver necrosis (Soussan et al., 2008). However, the direct clinical relationship between HBV splice variants and HCC remains uncharacterized.

Performance as a Predictor of HCC

HCC occurrence
Many studies have suggested that Pre-S deletion mutants play an important role in HBV-related HCC ( Chen et al., 2007; Xie et al., 2010; An et al., 2018; Chen, 2018). In NA-naïve patients, a study enrolled 141 HBeAg-negative patients with CHB, 7 of whom developed HCC with a median time of 84 months. Univariate analysis showed that the presence of pre-S deletions was a significant factor for prediction of HCC (HR 11.26, 95% CI, 2.18-58.1; P = 0.004) (Chen et al., 2007). A recent meta-analysis revealed that pre-S deletions were related to HCC occurrence (OR 3.28, 95% CI 2.32–4.65; P < 0.00001; random-effects model). Both pre-S1 and pre-S2 were risk factors for HCC development, with OR 2.42 (95% CI 1.25–4.68, P = 0.008) and 3.36 (95% CI 2.04–5.55; P < 0.00001), respectively (Wungu et al., 2021).

In a cohort of 165 people with CHB under NA treatment in Australia (58 of whom were diagnosed with HCC), the median level of serum spliced HBV was higher in HCC patients than in non-HCC patients (P < 0.001) (Bayliss et al., 2013). Using a real-time PCR cut-off value of 7% for serum spliced HBV, the AUROC analysis of spliced HBV is 0.77, with a sensitivity of 45% and a specificity of 96%. Multiple regression analysis found that the serum spliced HBV level increased by about 0.1% per year before the diagnosis of HCC, independent of liver fibrosis (Bayliss et al., 2013).

HCC recurrence
Studies have revealed that HBV-related HCC patients with pre-S mutants are at higher risk of HCC recurrence after curative surgery, even when receiving post-surgical NA therapy (Su et al., 2013; Yen et al., 2018; Teng et al., 2020b). Su et al. (2013) analyzed 73 HCC patients without NAs therapy but with pre-S deletion mutants. They found that pre-S deletion mutants were related to a higher rate of HCC recurrence and higher serum HBV DNA levels ( P = 0.055) (Su et al., 2013). Moreover, a recent study reported that using next-generation sequencing-based quantitative detection of pre-S mutants in serum can be useful for predicting HCC recurrence (AUROC of either pre-S2/pre-S1 or pre-S2 deletion = 0.683) (Teng et al., 2020b). Teng et al. (2020a) reported that only the presence of pre-S2 deletions (nt 1 to 54) in serum was associated with HCC recurrence (P value = 0.0080) with higher AUROC (0.632, 95% CI 0.556–0.708), compared with the pre-S1 deletion or the pre-S1 + pre-S2 deletion (nt 2,855–2,872, 1–54). In summary, pre-S2 deletion mutants may be a useful biomarker for HCC recurrence.
### Circulating HBV RNA

**Description**

Multiple studies have shown that HBV RNA can be detected both in culture supernatants and in the serum of people with CHB (Hatakeyama et al., 2007; Huang et al., 2015; Wang et al., 2016). Given that HBV RNA exists as pgRNA in virus-like particles [produced by defective or partial reverse transcription (Wang et al., 2016; Prakash et al., 2018)], theoretically, serum HBV RNA is derived only from cccDNA in infected hepatocytes. However, the mechanism of the release of HBV pgRNA viral particles from infected hepatocytes into the circulation is unclear (Lam et al., 2017; Butler et al., 2018; Wang et al., 2018).

**Quantification**

Serum HBV RNA can be measured by quantitative RT-PCR, and digital droplet PCR (Wang et al., 2016; van Campenhout et al., 2018; Carey et al., 2019). Butler et al. (2018) used quantitative RT-PCR on the m2000 system (Abbott Molecular) to quantify serum HBV RNA detection with a lower limit of quantitation of 45 U/ml (Carey et al., 2020). There is limited standardization between these approaches to HBV RNA quantification, so further work needs to be done to harmonize these assays if they are to be used for routine diagnosis.

### Molecular Association With HCC

Serum HBV RNA is closely related to the activity of HBV replication, especially in people with CHB during NA treatment (Giersch et al., 2017; Lu et al., 2017; Wang et al., 2017; Huang et al., 2018). However, there are few data on its predictive power for HCC risk. Halgand et al. reported that HBV pgRNA levels in tumor tissues were correlated with a particular HCC subtype (well-differentiated, non-invasive, and associated with better survival) (Halgand et al., 2018). However, serum levels may not be correlated with this. It is possible that high levels of HBV RNA could be a predictor of HCC in people with CHB under NA treatment (given it is a surrogate of cccDNA activity), but there is no clear clinical evidence for this yet.

### HBV Truncated RNA

**Description**

Hepatitis B virus integration can act as a template for truncated HBV RNA (trRNA) transcripts. Hilger et al. (1991) identified HBV trRNA transcripts that terminated at a non-canonical CATAAA polyadenylation signal within the 3′ end region of the HBx open reading frame in tissue samples from two HBV-HCC patients. This signal can be used when the canonical polyadenylation signal is absent (e.g., when truncated as in the integrated HBV DNA form) (Breitkreutz et al., 2001). Later studies suggested that truncated HBx transcripts with a C-terminal deletion could be transcribed from integrated HBV DNA (Wang et al., 2004).

**Quantification**

Using specific primers containing a sequence corresponding to the polyadenylated 3′-end of full-length polyadenylated HBV RNA (fRNA) or trRNA, RACE-PCR targets the 3′-ends of the X gene for quantification of all polyadenylated HBV RNA species (Zhang W. et al., 2004; Ou et al., 2020). The assay’s lower limit of detection for HBV RNA was 794 copies/mL with a quantitative range of 800–10^6 copies/mL.

### Molecular Association With HCC

Studies have shown that HBV trRNA, which can be transcribed from integrated HBV DNA, can encode a C-terminal truncated HBx protein (Hilger et al., 1991; Sze et al., 2013; van Bömmel et al., 2015). C-terminal-truncated HBx has been reported to enhance HCC invasion and reduce apoptotic response (Tu et al., 2001; Ma et al., 2008). In vitro studies suggest that C-terminal-truncated HBx promotes HCC through upregulating caveolin-1 to enhance β-catenin-mediated transcription of FRMD5 (FERM domain containing 5) (Ng et al., 2016; Mao et al., 2019). Sze et al. (2013) analyzed clinical data from 50 HBV-HCC patients and found that C-truncated HBx correlated with venous invasion. Also in vitro experiments reported that C-truncated HBx activates matrix metalloproteinase 10 by increasing C-Jun transcriptional activity, resulting in enhanced cell invasion and metastasis (Sze et al., 2013). Moreover, C-termianlly truncated middle surface protein MHBst initiates c-Raf-1/Erk-2 signaling, resulting in an increased hepatocyte proliferation rate and dysplastic changes in hepatocytes (Hildt et al., 2002; Wang et al., 2006). Although these suggest possible roles for truncated HBx protein in tumor progression, whether it also plays a role in tumor formation is still unclear.

### Performance as a Predictor of HCC

Although serum HBV trRNA has been detected and used as a predictor for virological outcomes (van Bömmel et al., 2015), its association with HCC has only been shown in tumor tissues and not serum. A study with 50 people with HCC revealed that C-terminal truncated HBx was detected in 23 of 50 (46%) tumor tissues, and these had more venous invasion compared to tumors expressing only full-length HBx (P = 0.005) (Sze et al., 2013). This is consistent with another study where C-terminal truncated HBx was detected in 88 of 111 (79.3%) HCC tissues, compared with full-length HBx in all 111 non-tumor tissues and 23 of 111 (20.7%) HCC tissues (Ma et al., 2008). However, these studies only detected truncated HBx in confirmed HCC tissues. Whether serum HBV trRNA can predict HCC occurrence or recurrence is still unclear.

### Hepatitis B Surface Antigen

**Description**

Hepatitis B virus sub-genomic mRNA transcripts (2.4- and 2.1-kb surface mRNAs) encode the large, middle, and small variants of the HBV surface antigen, which can assemble at the ER as sub-viral particles (SVP) and be secreted via the Golgi apparatus (Ganem and Schneider, 2001). The majority of circulating HBsAg exists as non-infectious filamentous and spherical SVP, in 1,000- to 100,000-fold excess compared to virions (Wei et al., 2010). HBsAg may be translated from both HBV cccDNA and integrated DNA; the latter especially in HBeAg-negative chronic HBV-infected patients (Hu et al., 2018).

Secreted HBsAg in SVP could play an immunomodulatory role during HBV infection. SVP capture neutralizing anti-HBsAg
antibodies and divert host immune recognition away from infectious HBV virions (Carman et al., 1996). The host antiviral immune response is subsequently minimized, allowing HBV persistence (Rydell et al., 2017). HBsAg is also the target of HBV functional cure; if serum HBsAg is eliminated then HBV infection is considered to be cleared (Chen et al., 2016; Al Awaidy and Ezzikouri, 2020).

Quantification

Currently, there are three quantitative assays for HBsAg measurement: Architect HBsAg QT (Abbott Diagnostics), Elecsys HBsAg II Quant (Roche Diagnostics) and DiaSorin Liaison XL. All assays provide measurements that correlate well with each other (Burdino et al., 2014; Liao et al., 2015). The Architect assay is capable of quantifying HBsAg concentrations ranging from 0.4 to 250 IU/mL (Deguchi et al., 2004). The range of the Elecsys II and DiaSorin Liaison XL assays are respectively from 0.05 to 130 IU/mL (sensitivity from 0.017 to 0.022 IU/mL) and 0.03 to 150 IU/mL (sensitivity of 0.03 IU/mL) (Burdino et al., 2014; Cornberg et al., 2017). All three assays have automatic dilution (1:400) to increase the upper limit of detection to over 50,000 IU/mL.

Unfortunately, these assays do not distinguish between the three forms of HBsAg (small, medium, and large). In preclinical trials, the ratios and composition of the three HBsAg forms have been reported to predict HBsAg clearance during treatment in patients with HBeAg-positive CHB (Pfefferkorn et al., 2021). Therefore, the quantification of HBsAg variants and monitoring the HBsAg composition throughout treatment could be important to predict the clearance of secreted HBsAg and the associated reduction in HCC risk.

Molecular Association With HCC

Several clinical studies recently reported that high levels of serum HBsAg are associated with an increased risk of HCC (Tseng et al., 2012; Kawanaka et al., 2014). Similarly, HBsAg loss is associated with very low HCC risk (Yip et al., 2017, 2019). While the underlying mechanism is not clear, this may be due to the association of HBsAg with replication levels or the amount of integrated HBV DNA, which are both risk factors for HCC (Xiangji et al., 2011; Yan et al., 2015; Tseng et al., 2019).

Chronic inflammation driven by anti-HBs responses could promote oncogenesis. A chimeric HBV-HCC mouse model was studied by extracting HBsAg-expressing hepatocytes from HBsAg transgenic mice (C57BL/6j) and transferring them into immuno-competent Fah−/− recipient mice (which allow implantation of hepatocytes) (Hao et al., 2021). Persistent HBsAg expression triggered HBsAg-specific CD8+ T cell activation, followed by hepatocyte apoptosis and turnover, progressive chronic inflammation, clonal expansion, and ultimately HCC (Nakamoto et al., 1998; Hao et al., 2021). In in vitro models, HBsAg has been reported to promote HCC invasion through the TLR2/MyD88/NF-kB signaling pathway (Cheng et al., 2017).

Hepatitis B surface antigens with mutations in the Pre-S1 or Pre-S2 regions could be directly oncogenic: these mutated proteins can alter host cell lipid metabolism, lead to ER stress, induce oxidative DNA damage and genomic instability, all of which increase the risk of HCC development (Hsieh et al., 2004; Wang et al., 2006; Yang et al., 2008). HBsAg Pre-S1 and Pre-S2 mutants accumulate intracellularly, forming the characteristic cytopathic effect of ground-glass structures (Roingeard and Sureau, 1998). Ground-glass hepatocytes (GGH) occur as either Type I or Type II GGH containing LHBsAg with mutations in the Pre-S1 or Pre-S2 regions, respectively (Wang et al., 2003). Type I GGH occurs as single hepatocytes during early stages of HBV infection with active HBV replication, while Type II GGH occurs as clusters (suggestive of clonal expansion) during latter stages of reduced HBV replication (Fan et al., 2001; Wang et al., 2003). Type II GGH is associated with cirrhosis and HCC development (Fan et al., 2001). In HBV patients, biopsies of cirrhotic nodules with Type II GGH contained HBV genomes which were integrated and clonally expanded, suggesting that Type II GGH are pre-neoplastic lesions (Fan et al., 2000). Similarly, the presence of Pre-S mutants in the serum of patients with CHB is associated with an increased risk of HCC, with Pre-S1 and Pre-S2 mutants present significantly higher in HCC patients compared to non-HCC carriers (19/64, 29.7% vs 25/202, 12.4%, P = 0.002) (Fan et al., 2001; Lin C.L. et al., 2007). HBsAg mutant may activate ER stress-dependent and -independent pathways to promote genomic instability and cell proliferation needed for HCC tumorigenesis.

Performance as a Predictor of HCC

HCC occurrence

Generally, the predictive value of HBsAg levels for HCC is poorer than HBV DNA or ALT in treatment-naive patients with CHB (Tseng et al., 2012). In HBeAg-negative patients with HBV DNA levels ≥2,000 IU/mL, HBsAg levels (<1,000 IU/mL or >1,000 IU/mL) were not related to the risk of HCC (AUROC 0.58; P = 0.247) (Tseng et al., 2012). However, multivariate analysis showed that HBsAg ≥1,000 IU/mL was an independent risk factor for HCC development in HBeAg-negative patients with HBV DNA level <2,000 IU/mL (HR 13.7, 95% CI: 4.8–39.3) (Tseng et al., 2012).

The true sensitivity and specificity of HBsAg in predicting HCC in these patients on NA therapy remains unknown and requires larger cohorts. Furthermore, HCC can still occur in patients with CHB who have undergone HBsAg loss (Chen et al., 2016). In one retrospective study, the annual incidence of HCC after HBsAg seroconversion was 2.85 and 0.29% in cirrhotic and non-cirrhotic patients, respectively (Kim et al., 2015). The risk factors associated with HCC development post-HBsAg seroconversion are: age above 50 (HR: 12.14; 95% CI: 1.61–91.68), male gender (HR: 8.96; 95% CI: 1.17–68.80), and infection with HBV genotype C (Kim et al., 2015). Given that HCC can occur in patients with CHB following HBsAg seroconversion, quantitative HBsAg is unlikely to be a suitable standalone biomarker for HCC risk (Kim et al., 2015; Chen et al., 2016).

HCC recurrence

HBsAg can also be used to predict HCC recurrence following curative hepatic resection (HR 1.23, 95% CI: 1.04–1.44, P = 0.01) (Huang et al., 2014; Zhou et al., 2015). Moreover, HBsAg level ≥4,000 IU/mL (HR 2.80; P = 0.023) is a risk factor for late...
Hepatitis B Core Related Antigen

Description
The 3.5-kb precore RNA derived from the HBV Pre-C/C gene can act as the template for three viral proteins: HBcAg, HBeAg and a truncated 22 kDa precore protein (p22Cr) (Mak et al., 2018). The so-called hepatitis B core related antigen (HBcrAg) consists of these three proteins which share an identical 149 long amino acid sequence. HBcAg forms the viral capsid subunits. HBeAg (164-amino acid protein) is synthesized by removing the C-terminal region of p22 and is secreted from infected cells (Messageot et al., 2003). p22Cr is the pre-core protein with additional post-translational processing at both the N- and C-termini (Kimura et al., 2005).

Quantification
Hepatitis B core related antigen was first measured by a sensitive enzyme immunoassay that denatures antibodies prior to analysis and therefore can detect HBcAg and HBeAg in anti-HBc or anti-HBe antibody-positive patients (Kimura et al., 2002). Currently, a newly chemiluminescence enzyme immunoassay with monoclonal antibodies to HBcAg and HBeAg was developed for the detection of HBcrAg. This assay showed the HBcrAg concentration correlates strongly with the HBV DNA concentration (P < 0.001) over a 5-log range. Moreover, the correlation between HBV load and circulating HBcrAg was not affected in HBeAg-negative patients nor those with precore mutations (Rokuhara et al., 2003). Particularly for patients under NA treatment, the HBcrAg assay could be a sensitive and clinically useful surrogate marker of intrahepatic HBV cccDNA levels (Rokuhara et al., 2003).

Molecular Association With HCC
Transcriptional activity of intrahepatic cccDNA is recognized as a risk for HBV-induced HCC under NA therapy (Leverero and Zucman-Rossi, 2016; Chen et al., 2017; Mak et al., 2018; Suzuki et al., 2019; Testoni et al., 2019). Several studies have shown that serum HBcrAg is highly correlated with intrahepatic cccDNA activity (Wong et al., 2017; Mak et al., 2018) as it can only be expressed from cccDNA (unlike HBsAg, which can also be expressed from integrated HBV DNA). Importantly, NA therapy only inhibits reverse transcription of HBV RNA, but does not inhibit protein synthesis from cccDNA (Tong and Revill, 2016). Therefore, HBcrAg is a non-invasive biomarker of active viral replication, which in turn may predict HCC.

Performance as a Predictor of HCC
HCC occurrence
A number of studies have suggested that serum HBcrAg can be a useful viral biomarker for HCC risk (Chen et al., 2018; Hosaka et al., 2019; Suzuki et al., 2019; Baudì et al., 2020). A study of 1,031 NA-naive patients with CHB (78 of whom were diagnosed with HCC during the follow-up period, median duration 10.7 years) revealed that serum HBcrAg was significantly related to the risk of developing HCC. HBcrAg > 794 U/mL (HR, 5.05; 95% CI, 2.40–10.63) was associated with the risk of developing HCC, independent of HBV DNA titers. In the subgroup of HBeAg-negative, non-cirrhotic patients with HBV DNA levels ≤ 10,000 copies/mL, HBcrAg > 5,012 U/mL was significantly related to the risk of HCC (HR 6.13, 95% CI 1.71–22.06). However, in the subgroup of CHB patients with HBV DNA levels > 10,000 copies/mL, any HBeAg status, and FIB-4 index ≤ 3.6 (an index of fibrosis), HBcrAg > 794 U/mL was associated with the incidence of HCC (HR 5.69, 95% CI 1.37–23.72) (Tada et al., 2016). Another study of 2,666 patients with CHB (of whom 209 developed HCC) reported that baseline HBcrAg levels of > 10 kU/mL in HBeAg-negative patients with HBV DNA levels from 2,000 to 19,999 IU/mL are at increased risk of HCC (Tseng et al., 2019). Conversely lower HBcrAg levels (< 10 kU/mL) were linked to a low risk of HCC.

Hepatitis B core related antigen has been reported to be superior to HBV DNA or HBsAg in predicting HCC in NA-naive patients with CHB. Tada et al. (2016) reported that HBcrAg could predict HCC in 2, 5, and 10 years with AUROC curves 0.80, 0.68, 0.70 (compared to HBV DNA at 0.75, 0.63, 0.65, respectively). Moreover, Tseng et al. (2019) found that AUROC of HBcrAg, HBV DNA, HBsAg was 0.73, 0.72, 0.57, respectively with 10 years follow-up, or 0.70, 0.69, 0.56 with 15 years follow-up. These studies show a high correlation between HBV DNA and HBcrAg levels, with HBcrAg being more sensitive than HBsAg in predicting HCC in untreated patients.

In patients with CHB under NA treatment, persistently high HBcrAg levels were associated with HCC development (Kumada et al., 2013; Hosaka et al., 2019). Hosaka et al. (2019) reported that in a study of 1,268 patients treated with NAs for more than 1 year, among the 60 of 667 HBeAg-positive patients, high HBcrAg levels (≥ 4.9 log U/mL) after 1-year on-treatment was associated with increased HCC incidence within 15 years (HR, 6.15, 95% CI: 1.89–20.0, P = 0.003). Using a HBcrAg cut-off value of 4.9 log U/mL gave positive and negative predictive values of 0.95 and 0.19, with sensitivities and specificities of 0.903 and 0.217, respectively. Moreover, in 601 HBeAg-negative patients, the risk of HCC was higher in those with HBcrAg values > 4.4 log U/mL (HR, 2.54, 95% CI: 1.40–4.60; P = 0.002). In this cohort positive and negative predictive values were 0.51 and 0.79, sensitivity and specificity were 0.519 and 0.787 (Hosaka et al., 2019). This result is similar to another study enrolling 76 NA-treated patients with CHB with undetectable HBV DNA diagnosed with HCC.
and 152 matched controls who did not develop HCC (Cheung et al., 2017). The AUROC of HBcrAg in the HCC group was 0.61 (95% CI: 0.54–0.69) for predicting HCC. Using a cut-off value of ≥7.8 kU/mL, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 57.9, 70.4, 49.4, and 77.0%, respectively, with an odds ratio (OR) of 3.27 (95% CI: 1.84–5.80) for HCC development. HBcrAg was more predictive of HCC in non-cirrhotic patients: AUROC was 0.70 (95% CI: 0.58–0.81) using a HBcrAg cut-off value of ≥7.9 kU/mL, with a sensitivity, specificity, PPV and NPV of 62.5, 78.1, 58.8, 80.6%, respectively, and with an OR of 5.95 (95% CI: 2.35–15.07) for HCC development (Cheung et al., 2017).

**HCC recurrence**

Hepatocellular carcinoma recurrence after HCC resection is still high, with a rate of ~50% within two years (Wu et al., 2009). High serum HBcrAg has been reported to predict HCC recurrence: Chen et al. (2018) reported that in 56 of 89 HCC patients with both positive cccDNA and HBcrAg who had been followed up for 5 years, recurrence rates of HCC in patients with high HBcrAg (>5.2 log U/mL) were higher than those with low HBcrAg (≤5.2 log U/mL; P = 0.003).

During NA therapy, higher HBcrAg levels at HCC diagnosis can predict post-treatment recurrence of HCC (Hosaka et al., 2010). In a study of 55 HCC patients with NA treatment at diagnosis of HCC receiving curative surgery, serum HBcrAg levels ≥4.8 log U/mL at the time of HCC diagnosis was an independent risk factor for HCC recurrence with HR of 8.96 (95% CI: 1.94–41.4) (Hosaka et al., 2010). A long-term follow-up study in Netherlands revealed that higher HBcrAg level (≥5.1 log U/mL) was associated with an increased tumor recurrence rate in 53 of 119 HCC patients who were identified with early stage HCC receiving NAs at the time of HCC diagnosis (Beudeker et al., 2021). Moreover, in a cohort of 357 CHB-related HCC patients who underwent liver transplantation followed by NA treatment, HBcrAg ≥5.0 log U/mL was an independent risk factor for HCC recurrence, with a higher 5-year cumulative recurrence rate, compared with an HBcrAg <5.0 log U/mL (37.6 vs 6%, P < 0.001) (HR:5.27, 95% CI 2.47–11.25, P < 0.001) (Yu et al., 2019). In conclusion, HBcrAg may be a useful biomarker for HCC recurrence, however the sensitivity and specificity of HBcrAg in predicting HCC recurrence needs further research.

**FUTURE WORK AND CONCLUSION**

This review has assessed the value of serum viral biomarkers in HBV-related HCC. Of all the potential biomarkers that have been studied, growing evidence supports the use of serum HBcrAg and preS mutations as biomarkers for predicting HCC occurrence in people with CHB, both in NA-naive patients and in patients receiving NA treatment. In combination with AFP and abdominal ultrasound serum biomarkers might improve HCC screening and increase early diagnosis, although further validation studies are required to confirm their clinical performance in predicting and/or detecting HCC. Moreover, several biomarkers remain to be tested in a clinical setting (e.g., HBV integrations and HBV RNA, both full length and truncated forms), laying the groundwork for future exploratory studies.

Challenges remain in this field of research. Firstly, some of these markers (e.g., HBV RNA and HBcrAg) have no standardized quantification assay. To accurately and robustly compare HCC risk between different studies, equivalent cut-off values need to be used and this can only be done with appropriate reference samples and standardized assays. Moreover, we lack the appropriate laboratory models to investigate new and existing HCC markers in HBV infection. Even if there were a practical animal system that supported HBV infection, no known models recapitulate the decades long-process of HBV-initiated HCC. This makes discovery, characterization, and confirmation of new and existing viral biomarkers difficult.

While this field awaits further developments to enable more in-depth analysis, our review has shown signs of promise in viral biomarkers and their ability to predict HBV-associated HCC occurrence and recurrence. We expect that (in combination with existing markers) viral biomarkers will increasingly become incorporated into HCC risk algorithms, improving health outcomes for the ~300 million people worldwide living with CHB.

**AUTHOR CONTRIBUTIONS**

YL initiated the writing, led the organization, and did the majority of the research for the review. VV and MP produced the figure and contributed to large sections of the review. JG, JF, and MD were involved in editing the manuscript and provided crucial input into several sections of the review. TT was responsible for the conceptualization, coordination, structure, and editing of the review. All authors contributed to the article and approved the submitted version.

**FUNDING**

This study was supported by the Robert W. Storr Bequest to the Sydney Medical Foundation, University of Sydney. TT and MD are supported by grant funding from the Australian Centre for HIV and Hepatitis Virology Research and NHMRC Ideas grant GNT2002565.

Al Awaidy, S. T., and Ezzikouri, S. (2020). Moving towards hepatitis B elimination in Gulf Health Council states: from commitment to action. J. Infect. Public Health 13, 221–227. doi: 10.1016/j.jiph.2019.08.004

An, P., Xu, J., Yu, Y., and Winkler, C. A. (2018). Host and viral genetic variation in HBV-related hepatocellular carcinoma. Front. Genet. 9:261. doi: 10.3389/fgene.
Baudi, I., Inoue, T., and Tanaka, Y. (2020). Novel biomarkers of hepatitis B and hepatocellular carcinoma: clinical significance of HBeAg and M2BPQgI. Int. J. Mol. Sci. 21:949. doi: 10.3390/ijms21030949
Bayliss, J., Lim, L., Thompson, A. J., Desmond, P., Angus, P., Locarnini, S., et al. (2013). Hepatitis B virus splicing is enhanced prior to development of hepatocellular carcinoma. J. Hepatol. 59, 1022–1028. doi: 10.1016/j.jhep.2013.06.018
Beudeker, B. J., Groothuismink, Z. M., de Man, R. A., van Der Eijk, A. A., Boonstra, A., et al. (2021). Hepatitis B core-related antigen levels predict recurrence-free survival in patients with HBV-associated early-stage hepatocellular carcinoma: results from a Dutch long-term follow-up study. J. Viral Hepat. 28, 205–208. doi: 10.1111/jvh.13394
Bill, C. A., and Summers, J. (2004). Genomic DNA double-strand breaks are targets for hepadnaviral DNA integration. Proc. Natl. Acad. Sci. U.S.A. 101, 11135–11140. doi: 10.1073/pnas.0403925101
Bitton Alaluf, M., and Shlomai, A. (2016). New therapies for chronic hepatitis B. Liver Int. 36, 775–782. doi: 10.1111/liv.13086
Blondot, M.-L., Bruss, V., and Kann, M. (2016). Intracellular transport and egress of hepatitis B virus. J. Hepatol. 64, S49–S59.
Boluksbas, C., Bolukbas, F. F., Horoz, M., Aslan, M., Celik, H., and Erel, O. (2005). Increased oxidative stress associated with the severity of the liver disease in various forms of hepatitis B virus infection. BMC Infect. Dis. 5:95. doi: 10.1186/1471-2334-5-95
Bosh, P. X., Ribes, J., Díaz, M., and Cléries, R. (2018b). Sequence analysis targets for hepadnaviral DNA integration. Proc. Natl. Acad. Sci. U.S.A. 115, 7779–7784. doi: 10.1073/pnas.1715371115
Budzinska, M. A., Shackel, N. A., Urban, S., and Tu, T. (2018b). Hepatitis B virus nucleic acids circulating in the blood: distinct patterns in HBs antigen carriers with hepatocellular carcinoma. Int. J. Cancer. 143, 2106–2117. doi: 10.1002/ijc.31602
Chan, H.-C., Yang, H.-L., Su, J., Jen, C.-L., You, S.-L., Lu, S.-N., et al. (2006). Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 295, 65–73. doi: 10.1001/jama.295.1.65
Chen, E.-Q., Feng, S., Wang, M.-L., Liang, L.-B., Zhou, L.-Y., Du, L.-Y., et al. (2017). Serum hepatitis B core-related antigen is a satisfactory surrogate marker of intrahepatic covalently closed circular DNA in chronic hepatitis B. Sci. Rep. 7:173.
Chen, S., Gao, Y., Li, H., Fang, M., Feng, H., Guan, W., et al. (2018). Clinical evaluation of hepatitis B core-related antigen in chronic hepatitis B and hepatocellular carcinoma patients. Clin. Chim. Acta 486, 237–244. doi: 10.1016/j.cjca.2018.07.027
Chen, X.-P., Long, X., Jia, W.-L., Wu, H.-J., Zhao, J., Liang, H.-F., et al. (2019). Viral integration drives multifocal HCC during the occult HBV infection. J. Exp. Clin. Cancer Res. 38:261.
Chen, Y.-G., Feng, W., Chien, R., Chu, C., and Liaw, Y. (2016). Clinical outcomes after spontaneous and nucleus (t) ide analogue-treated HB sAg seroclearance in chronic HBV infection. Aliment. Pharmacol. Ther. 43, 1311–1318. doi: 10.1111/apt.13630
Chen, Y., and Tian, Z. (2019). HBV-induced immune imbalance in the development of HCC. Front. Immunol. 10:2048. doi: 10.3389/fimmu.2019.02048
Chevaliez, S., Bouvier-Alias, M., Lapercie, S., Hézode, C., and Pawlotsky, J.-M. (2010). Performance of version 2.0 of the Cobas AmpliPrep/Cobas TaqMan real-time PCR assay for hepatitis B virus DNA quantification. J. Clin. Microbiol. 48, 3641–3647. doi: 10.1128/jcm.01306-10
Chevaliez, S., Bouvier-Alias, M., Lapercie, S., and Pawlotsky, J.-M. (2008). Performance of the Cobas AmpliPrep/Cobas TaqMan real-time PCR assay for hepatitis B virus DNA quantification. J. Clin. Microbiol. 46, 1716–1723. doi: 10.1128/jcm.01248-07
Choi, J., Kim, G. A., Han, S., Lee, W., Chun, S., and Lim, Y. S. (2019). Longitudinal assessment of three serum biomarkers to detect very early-stage hepatocellular carcinoma. Hepatology 69, 1983–1994. doi: 10.1002/hep.30233
Chu, C.-M., Hung, S.-J., Lin, J., Tai, D.-L., and Liaw, Y.-F. (2004). Natural history of hepatitis B antigen to antibody seroconversion in patients with normal serum aminotransferase levels. Ann. Intern. Med. 136, 829–834. doi: 10.1001/anninternmed.2003.12.040
Chuma, M., Hige, S., Kamiyama, T., Meguro, T., Nagasaka, A., Nakashiki, K., et al. (2009). The influence of hepatitis B DNA level and antiviral therapy on recurrence after initial curative treatment in patients with hepatocellular carcinoma. J. Gastroenterol. 44, 991–999. doi: 10.1007/s00535-009-0993-z
Comberg, M., Wong, V. W.-S., Locarnini, S., Brunetto, M., Janssen, H. L., and Chan, H. L. (2017). The role of quantitative hepatitis B surface antigen revisited. J. Hepatol. 66, 398–411. doi: 10.1016/j.jhep.2016.08.009
De Franchis, R., Meucci, G., Vecchi, M., Tatarrella, M., Colombo, M., Del Ninno, E., et al. (1993). The natural history of asymptomatic hepatitis B surface antigen carriers. Ann. Intern. Med. 118, 191–194. doi: 10.1001/anninternmed.118.3.199302010-0006
Deguchi, Y., Yamashita, N., Kagita, M., Asari, S., Iwatsuki, Y., Tsuchida, T., et al. (2004). Quantitation of hepatitis B surface antigen by an automated
Hatakeyama, T., Noguchi, C., Hiraga, N., Mori, N., Tsuge, M., Imamura, M., et al. (2000). Identification of a pre-S2 mutant in hepatocytes expressing a novel marginal pattern of surface antigen in advanced diseases of chronic hepatitis B virus infection. J. Hepatol. 33, 370–398. doi: 10.1016/S0168-8278(00)002187.x

Fan, Y. F., Lu, C. C., Chang, Y. C., Chang, T. T., Lin, P. W., Lei, H. Y., et al. (2000). Identification of a pre-S2 mutant in hepatocytes expressing a novel marginal pattern of surface antigen in advanced diseases of chronic hepatitis B virus infection. J. Gastroenterol. Hepatol. 15, 519–528. doi: 10.1046/j.1440-1744.2000.02187.x

Huang, Y., Wang, H.-C., Chang, W.-W., Lei, H.-Y., Lai, M.-D., et al. (2004). Pre-S mutant surface antigens in chronic hepatitis B virus infection induce oxidative stress and DNA damage. Carcinogenesis 25, 2023–2032. doi: 10.1093/carcin/bgh207

Hu, B., Huang, W., Wang, R., Zang, W., Su, M., Li, H., et al. (2020). High rate of detection of human ESPL1–HBV S Fusion Gene in Patients With HBV-related Liver Cancer: a Chinese Case–Control Study. Anticancer Res. 40, 245–252. doi: 10.21873/anticanres.13946

Huang, H.-L., Jeng, K.-S., Hu, C.-P., Tsai, C.-H., Lo, S. J., and Chang, C. (2000). Prevalence of S gene deletions in acute and chronic hepatitis B virus infection. Hepatol. Int. 49, 457–471. doi: 10.1111/apt.15108

Huang, G., Yang, Y., Shen, F., Pan, Z.-Y., Fu, S.-Y., Lau, W. Y., et al. (2013). Pre-S2 activator MHBst of hepatitis B virus induces c-raf-1/Erk2 signaling in transgenic mice. EMBO J. 21, 525–535. doi: 10.1038/embo/j.21.4.525

Huang, Y.-W., Takahashi, S., Tsuge, M., Chen, C.-L., Wang, T.-C., Abe, H., et al. (2008). Role of hepatitis B virus pgRNA as a clinical marker for cccDNA activity. Hepatology 47, 519–527. doi: 10.1002/hep.21581
development using liver stiffness measurement (FibroScan). *Hepatology* 53, 885–894. doi: 10.1002/hep.24211

Kang, N. K., Chung, J. W., Jang, H. Y., Park, D. W., Jang, E. S., and Kim, J.-W. (2017). PO-021: role of HBsAg Titer and HBV DNA Level in Predicting Hepatocellular Carcinoma in Chronic Hepatitis B Patients Receiving Entecavir Therapy. *Liver Cancer* 6, 97–98. doi: 10.1159/000438587

Kew, M. C. (2010). Epidemiology of chronic hepatitis B virus infection, *Frontiers in Microbiology* | www.frontiersin.org 15

Kimura, T., Rokuhara, A., Sakamoto, Y., Yagii, S., Tanaka, E., Kiyosawa, K., et al. (2004). Three novel cis-acting elements required for efficient plus-strand DNA synthesis of the hepatitis B virus genome. *J. Virol.* 78, 7455–7464. doi: 10.1128/jvi.2008.05.007

Lee, H. W., Lee, J. I., Kim, S., Kim, S., Chang, H. Y., and Lee, K. S. (2020). Cumulative incidence of hepatocellular carcinoma and hepatitis B virus infection in patients with hepatitis B virus surface antigen Seroclearance after Nucleos (t) ide-analogue therapy. *Front. Med.* 6, 45–52. doi: 10.1007/s11684-017-0590-z

Luo, C.-L., Rong, Y., Chen, H., Zhang, W.-W., Wu, L., Yang, J., et al. (2018). Effect of nucleos (t) ide analogues on hepatitis B virus integration in hepatocellular carcinoma patients. *Gastroenterology* 154, 134–141. doi: 10.1053/j.gastro.2016.11.005

Manno, M., Cammà, C., Schepis, F., Bassi, F., Gelmini, R., Giannini, F., et al. (2017). Development of a digital droplet PCR assay to measure HBV DNA in patients receiving long-term TDF treatment. *J. Virol. Methods* 249, 189–193. doi: 10.1016/j.viromet.2017.09.015

Ma, N.-F., Lau, S. H., Hu, L., Xie, D., Wu, J., Yang, J., et al. (2008). COOH-terminal truncated HBV X protein plays key role in hepatocarcinogenesis. *Cancer Res.* 68, 723–750. doi: 10.1159/000291646

Manno, M., Cammà, C., Schepis, F., Bassi, F., Gelmini, R., Giannini, F., et al. (2004). Natural history of chronic HBV carriers in northern Italy: morbidity and mortality after 30 years. *Gastroenterology* 123, 756–763. doi: 10.1053/j.gastro.2004.06.021

Mason, W. S., Low, H. C., Xu, C., Aldrich, C. E., Scougall, C. A., Grosse, A., et al. (2009). Detection of clonally expanded hepatocytes in chimpanzees with chronic hepatitis B virus infection. *Methods Mol. Biol.* 83, 8396–8408. doi: 10.1186/1478-661x-2017-9.15.118.31230

Li, D., Mallory, T., and Satomura, S. (2001). AFP-L3: a new generation of tumor marker for hepatocellular carcinoma. *Clin. Chim. Acta* 313, 15–19. doi: 10.1016/s0009-8981(00)00644-1

Li, M.-R., Chen, G.-H., Cai, C.-J., Wang, G.-Y., and Zhao, H. (2011). High hepatitis B virus DNA level in serum before liver transplantation increases the risk of hepatocellular carcinoma recurrence. *Digestion* 84, 134–141. doi: 10.1159/000324197

Li, W., Cui, X., Hoo, Q., Qi, Y., Sun, Y., Tan, M., et al. (2019). Profile of HBV integration in the plasma DNA of hepatocellular carcinoma patients. *Carr. Genomics* 20, 61–68. doi: 10.2174/183982921966618100214336

Liang, H.-W., Wang, N., Wang, Y., Fang, F., Xu, Z., Yan, X., et al. (2016). Hepatitis B virus-human chimeric transcript HBx-LINE1 promotes hepatic injury via sequestering cellular microRNA-122. *J. Hepatol.* 64, 278–291. doi: 10.1016/j.jhep.2015.09.013

Liu et al. Viral Biomarkers for HBV-HCC

Lee, J., Shin, M.-K., Lee, H.-J., Yoon, G., and Ryu, W.-S. J. (2004). Three novel cis-acting elements required for efficient plus-strand DNA synthesis of the hepatitis B virus genome. *J. Virol.* 78, 7455–7464. doi: 10.1128/jvi.2008.05.007

Lee, J. H., Lee, J. I., Kim, S., Kim, S., Chang, H. Y., and Lee, K. S. (2020). Cumulative incidence of hepatocellular carcinoma and hepatitis B virus surface antigen Seroclearance after Nucleos (t) ide analogue-induced hepatitis B e antigen Seroclearance. *BMC Gastroenterol.* 20:113. doi: 10.1186/s12876-020-01236-9

Lee, J., Shin, M.-K., Lee, J.-H., Yoon, G., and Ryu, W.-S. J. (2004). Three novel cis-acting elements required for efficient plus-strand DNA synthesis of the hepatitis B virus genome. *J. Virol.* 78, 7455–7464. doi: 10.1128/jvi.2008.05.007

Levrero, M., and Zucman-Rossi, J. (2016). Mechanisms of HBV-induced hepatocarcinoma. *J. Hepatol.* 64, S84–S101.
McGlynn, K. A., Petrick, J. L., and London, W. T. (2015). Global epidemiology of hepatocellular carcinoma: an emphasis on demographic and regional variability. Clin. Liver Dis. 19, 223–238.

Messageto, F., Salhi, S., Eon, P., and Rossignol, J.-M. (2003). Proteolytic processing of the hepatitis B virus e antigen precursor: cleavage at two furin consensus sequences. J. Biol. Chem. 278, 891–895. doi: 10.1074/jbc.m207634200

Nakamoto, Y., Guidotti, L. G., Kuhlen, C. V., Fowler, P., and Chisari, F. V. (1998). Immune pathogenesis of hepatocellular carcinoma. J. Exp. Med. 188, 341–350.

Nakamura, S., Nouchi, K., Sakaguchi, K., Ito, Y. M., Ohashi, Y., Kobayashi, Y., et al. (2006). Sensitivity and specificity of des-gamma-carboxy prothrombin for diagnosis of patients with hepatocellular carcinomas varies according to tumor size. Am. J. Gastroenterol. 101, 2038–2043. doi: 10.1111/j.1572-0241.2006.00681.x

Ng, K.-Y., Chai, S., Tong, M., Guan, X.-Y., Lin, C.-H., Ching, Y.-P., et al. (2016). C-terminal truncated hepatitis B virus X protein promotes hepatocellular carcinogenesis through induction of cancer and stem cell-like properties. Oncotarget 7, 24005–24017. doi: 10.18632/oncotarget.8209

Ni, Y., Lempf, F. A., Mahrle, S., Nkongolo, S., Kaufman, C., Falth, M., et al. (2014). Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. Gastroenterology 146, 1070–1083. doi: 10.1053/j.gastro.2013.12.024

Ou, Q., Guo, J., Zeng, Y., and Chen, H. (2020). Insights for clinical diagnostic indicators of virus and host in chronic hepatitis B infection. J. Viral Hepat. 27, 224–232. doi: 10.1111/vrh.13260

Ozakyol, A. (2017). Global epidemiology of hepatocellular carcinoma (HCC) epidemiology. J. Gastrointest. Cancer 48, 238–240. doi: 10.1007/s10432-019-09959-0

Papatheodoridis, G. V., Chan, H. L.-Y., Hansen, B. E., Janssen, H. L., and Papatheodoridis, G. V. (2006). Sensitivity and specificity of des-gamma-carboxy prothrombin for diagnosis of patients with hepatocellular carcinomas varies according to tumor size. Am. J. Gastroenterol. 101, 2038–2043. doi: 10.1111/j.1572-0241.2006.00681.x

Qi, Y., Gao, Z., Xu, G., Peng, B., Liu, C., Yan, H., et al. (2016). DNA polymerase κ have a role in decision making in HCC carcinogenesis through induction of cancer and stem cell-like properties. J. Hepatol. 65, 48–56.

Sangal, A., Vokk, M., Waljee, A., Salgia, R., Higgins, P., Rogers, M., et al. (2009). Meta-analysis: surveillance with ultrasound for early-stage hepatocellular carcinoma in patients with cirrhosis. Aliment. Pharmacol. Ther. 30, 37–47. doi: 10.1111/j.1365-2036.2009.04014.x

Soi, W., Paik, Y.-H., Kim, J. M., Kwon, C. H., Joo, J. W., Cho, J. Y., et al. (2014). HBV DNA and HBsAg levels as risk predictors of early and late recurrence after curative resection of HBV-related hepatocellular carcinoma. Ann. Surg. Oncol. 21, 2429–2435. doi: 10.1111/j.1445-2494.2014.06318.x

Soussan, P., Garreau, F., Zylberberg, H., Ferray, C., Brechot, C., and Kremsdorf, D. (2000). In vivo expression of a new hepatitis B virus protein encoded by a spliced RNA. J. Clin. Invest. 105, 55–60. doi: 10.1172/jci8098

Soussan, P., Pol, J., Garreau, F., Schneider, V., Le Pendeven, C., Nalpas, B., et al. (2008). Expression of defective hepatitis B virus particles derived from singly spliced RNA is related to liver disease. J. Infect. Dis. 198, 218–225. doi: 10.1086/598623

Su, C.-W., Chou, Y.-W., Tsai, Y.-H., Teng, R.-D., Chau, G.-Y., Lei, H.-J., et al. (2013). The influence of hepatitis B virus load and pre-S deletion mutations on post-operative recurrence of hepatocellular carcinoma and the tertiary preventive effects by anti-viral therapy. PLoS One 8:e66457. doi: 10.1371/journal.pone.0066457

Sung, W.-K., Zheng, H., Li, S., Chen, R., Liu, X., Li, Y., et al. (2012). Genome-wide survey of recurrent HBV integration in hepatocarcinoma cells. Nat. Genet. 44, 765–769.

Suzuki, Y., Maekawa, S., Komatsu, N., Sato, M., Tatsumi, A., Miura, M., et al. (2005). NTCP and OATP1B1 mediate active uptake of anti-HBV drug lamivudine treatment. J. Virol. 69, 4011–4018. doi: 10.1128/jvi.69.6.4011-4018.1999

Sung, W.-K., Feng, H., Li, S., Chen, R., Liu, X., Li, Y., et al. (2012). Genome-wide survey of recurrent HBV integration in hepatocarcinoma cells. Nat. Genet. 44, 765–769.

Tada, T., Kumada, T., Toyoda, H., Kiiyama, S., Tanikawa, M., Hisanaga, Y., et al. (2003). Hepatitis B virus pre-S mutants in plasma predicts hepatocellular carcinoma recurrence. Gut Pathog. 5, 24. doi: 10.1186/1750-937x-5-24

Teng, C.-F., Li, T.-C., Huang, H.-Y., Chen, W.-S., Shyu, W.-C., et al. (2008). Non-interferon therapy for treatment of hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. J. Viral Hepat. 15, 86. doi: 10.1111/j.1365-2893.2003.00437.x

Teng, C.-F., Li, T.-C., Huang, H.-Y., Lin, J.-H., Chen, W.-S., Shyu, W.-C., et al. (2016). Next-generation sequencing-based quantitative detection of hepatitis B virus pre-S1 mutants in plasma predicts hepatocellular carcinoma recurrence. Viruses 8:796. doi: 10.3390/v8030796
Vlachogiannakos, J., and Papatheodoridis, G. (2013). Hepatocellular carcinoma in patients with low HBV load. Hepatol. Res. 157, 1518–1529.e3.

Tseng, T.-C., Liu, C.-J., Hsu, C.-Y., Hong, C.-M., Su, T.-H., Wang, Y.-T., et al. (2016). Cell-Free Junctional DNA Fragment from Hepatitis B Virus Integration in HCC for Monitoring Postresection Recurrence and Clonality. (Alexandria, VA: American Society of Clinical Oncology), 4090–4090.

Wei, Y., Neveuct, C., Tsiollais, P., and Buendia, M.-A. (2010). Molecular biology of the hepatitis B virus and role of the X gene. Pathol. Biol. 58, 267–272. doi: 10.1016/j.pathbio.2010.03.005

Wong, D., Seto, W. K., Cheung, K. S., Chong, C. K., Huang, F. Y., Fung, J., et al. (2017). Hepatitis B virus core-related antigen as a surrogate marker for covalently closed circular DNA. Liver Int. 37, 995–1001. doi: 10.1111/liv.13346

Wong, D. K., Cheng, S. Y., Mak, L. L., To, E. W., Lo, R. C., Cheung, T.-T., et al. (2020). Among patients with undetectable hepatitis B surface antigen and hepatocellular carcinoma, a high proportion has integration of HBV DNA into hepatocyte DNA and no cirrhosis. Clin. Gastroenterol. Hepatol. 18, 449–456. doi: 10.1016/j.cgh.2019.06.029

Wooddel, C. I., Yuen, M.-F., Chan, H. L.-Y., Gish, R. G., Locarnini, S. A., Chavez, D., et al. (2017). RNAi-based treatment of chronically infected patients and chimpanzees reveals that integrated hepatitis B virus DNA is a source of HBsAg. Sci. Transl. Med. 9, eaan2041. doi: 10.1126/scitranslmed.aan2041

Wu, J.-C., Huang, Y.-H., Chau, G.-Y., Su, C.-W., Lai, C.-R., Lee, P.-C., et al. (2009). Risk factors for early and late recurrence in hepatitis B-related hepatocellular carcinoma. J. Hepatol. 51, 890–897. doi: 10.1016/j.jhep.2009.07.009

Wungu, C. D. K., Ariyanto, F. C., Prabowo, G. I., Soetjipto, S., and Handajani, R. (2021). Meta-analysis: association between hepatitis B virus pre-S mutation and hepatocellular carcinoma risk. J. Viral Hepat. 28, 61–71. doi: 10.1111/jvh.13402

Xia, J.-X., Zhao, J., Yin, J.-H., Zhang, Q., Pu, R., Lu, W.-Y., et al. (2010). Association of novel mutations and hepatitis B virus genotypes with hepatocellular carcinoma. Front. Med. China 4, 419–429. doi: 10.1007/s11684-010-0160-0

Xu, W., Wu, Y., and Wong, V. W.-S. (2017). Mechanism and prediction of HCC development in HBV infection. Best Pract. Res. Clin. Gastroenterol. 31, 291–298. doi: 10.1016/j.bpg.2017.04.011

Yan, H., Peng, B., Liu, Y., Xu, G., He, W., Ren, B., et al. (2014). Viral entry of hepatitis B and D viruses and bile salts transportation share common molecular determinants on sodium taurocholate cotransporting polypeptide: J. Virol. 88, 13737–13743.

Yan, H., Yang, Y., Zhang, L., Tang, G., Wang, Y., Xue, G., et al. (2015). Characterization of the genotype and integration patterns of hepatocellular carcinoma. Hepatology 61, 1821–1831. doi: 10.1002/hep.27222

Wang, H.-C., Wu, H.-C., Chen, C.-F., Fausto, N., Lei, H.-Y., and Su, I.-J. (2003). Different types of ground glass hepatocytes in chronic Hepatitis B virus infection contain specific Pre-S Mutants that May induce endoplasmic reticulum stress. Am. J. Pathol. 163, 2441–2449. doi: 10.1016/s0002-9440(10)61237-7

Wang, J., Du, M., Huang, H., Chen, R., Niu, J., Jiang, J., et al. (2017). Reply to: “Serum HBV pgRNA as a clinical marker for cccDNA activity”: consistent loss of serum HBV RNA might predict the “para-functional cure” of chronic hepatitis B J. Hepatol. 66, 462–463. doi: 10.1016/j.jhep.2016.10.034

Wang, J., Shen, T., Huang, X., Kumar, G. R., Chen, X., Zeng, Z., et al. (2016). Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound. J. Hepatol. 65, 700–710. doi: 10.1016/j.jhep.2016.05.029

Wang, J., Yu, Y., Li, G., Shen, C., Meng, Z., Zheng, J., et al. (2018). Relationship between serum HBV-RNA levels and intrahepatic viral as well as histologic activity markers in entecavir-treated patients. J. Hepatol. 68, 16–24. doi: 10.1016/j.jhep.2017.08.021

Wang, X., Zhang, Y., Yang, N., He, H., Tao, X., Kou, C., et al. (2020). Evaluation of the Combined Application of AFP, AFP-L3%, and DCP for Hepatocellular Carcinoma Diagnosis: A Meta-analysis. Biomed. Res. Int. 2020:5087643.

Wang, Y., Lai, S. H., Sham, J. S., Wu, M.-C., Wang, T., and Guan, X.-Y. (2004). Characterization of HBV integrants in 14 hepatocellular carcinomas: association of truncated X gene and hepatocellular carcinoma. Oncogene 23, 142–148. doi: 10.1038/sj.onc.1206889

Wang, Y.-C., Li, C.-L., Lin, Y.-Y., Tseng, S.-T., Chen, Y.-J., et al. (2019). Cell-Free Functional DNA Fragment from Hepatitis B Virus Integration in HCC for Monitoring Postresection Recurrence and Clonality. (Alexandria, VA: American Society of Clinical Oncology), 4090–4090.

Wei, Y., Neveuct, C., Tsiollais, P., and Buendia, M.-A. (2010). Molecular biology of the hepatitis B virus and role of the X gene. Pathol. Biol. 58, 267–272. doi: 10.1016/j.pathbio.2010.03.005

Wong, D., Seto, W. K., Cheung, K. S., Chong, C. K., Huang, F. Y., Fung, J., et al. (2017). Hepatitis B virus core-related antigen as a surrogate marker for covalently closed circular DNA. Liver Int. 37, 995–1001. doi: 10.1111/liv.13346

Wong, D. K., Cheng, S. Y., Mak, L. L., To, E. W., Lo, R. C., Cheung, T.-T., et al. (2020). Among patients with undetectable hepatitis B surface antigen and hepatocellular carcinoma, a high proportion has integration of HBV DNA into hepatocyte DNA and no cirrhosis. Clin. Gastroenterol. Hepatol. 18, 449–456. doi: 10.1016/j.cgh.2019.06.029

Wooddel, C. I., Yuen, M.-F., Chan, H. L.-Y., Gish, R. G., Locarnini, S. A., Chavez, D., et al. (2017). RNAi-based treatment of chronically infected patients and chimpanzees reveals that integrated hepatitis B virus DNA is a source of HBsAg. Sci. Transl. Med. 9, eaan2041. doi: 10.1126/scitranslmed.aan2041

Wu, J.-C., Huang, Y.-H., Chau, G.-Y., Su, C.-W., Lai, C.-R., Lee, P.-C., et al. (2009). Risk factors for early and late recurrence in hepatitis B-related hepatocellular carcinoma. J. Hepatol. 51, 890–897. doi: 10.1016/j.jhep.2009.07.009

Wungu, C. D. K., Ariyanto, F. C., Prabowo, G. I., Soetjipto, S., and Handajani, R. (2021). Meta-analysis: association between hepatitis B virus pre-S mutation and hepatocellular carcinoma risk. J. Viral Hepat. 28, 61–71. doi: 10.1111/jvh.13402

Xia, J.-X., Zhao, J., Yin, J.-H., Zhang, Q., Pu, R., Lu, W.-Y., et al. (2010). Association of novel mutations and hepatitis B virus genotypes with hepatocellular carcinoma. Front. Med. China 4, 419–429. doi: 10.1007/s11684-010-0160-0

Xu, W., Wu, Y., and Wong, V. W.-S. (2017). Mechanism and prediction of HCC development in HBV infection. Best Pract. Res. Clin. Gastroenterol. 31, 291–298. doi: 10.1016/j.bpg.2017.04.011

Yan, H., Peng, B., Liu, Y., Xu, G., He, W., Ren, B., et al. (2014). Viral entry of hepatitis B and D viruses and bile salts transportation share common molecular determinants on sodium taurocholate cotransporting polypeptide: J. Virol. 88, 13737–13743.

Yan, H., Yang, Y., Zhang, L., Tang, G., Wang, Y., Xue, G., et al. (2015). Characterization of the genotype and integration patterns of hepatocellular carcinoma. Hepatology 61, 1821–1831. doi: 10.1002/hep.27222
Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., et al. (2012). Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *eLife* 1:e00049.

Yang, F., Yan, S., He, Y., Wang, F., Song, S., Guo, Y., et al. (2008). Expression of hepatitis B virus proteins in transgenic mice alters lipid metabolism and induces oxidative stress in the liver. *J. Hepatol.* 48, 12–19. doi: 10.1016/j.jhep.2007.06.021

Yang, J. D., Kim, W. R., Coelho, R., Benson, J. T., Sanderson, S. O., et al. (2011). Cirrhosis is present in most patients with hepatitis B and hepatocellular carcinoma. *Clin. Gastroenterol. Hepatol.* 9, 64–70. doi: 10.1016/j.cgh.2010.08.019

Yang, J. D., and Roberts, L. R. (2010). Hepatocellular carcinoma: a global view. *Nat. Rev. Gastroenterol. Hepatol.* 7, 448–458. doi: 10.1038/nrgastro.2010.100

Yang, W., and Summers, J. J. (1998). Infection of ducklings with virus particles containing linear double-stranded duck hepatitis B virus DNA: illegitimate replication and reversion. *J. Virol.* 72, 8710–8717. doi: 10.1128/jvi.73.12.9710-9717.1999

Zhang, B.-H., Yang, B.-H., and Tang, Z.-Y. (2004). Randomized controlled trial of screening for hepatocellular carcinoma. *J. Cancer Res. Clin. Oncol.* 130, 417–422

Zhang, L. Xie, X.-Y., Chen, Y., Ge, N.-L., Chen, R.-X., Gan, Y.-H., et al. (2017). Hepatitis B surface antigen predicts recurrence after radiofrequency ablation in patients with low hepatitis B virus loads. *Medicine* 96:e9377. doi: 10.1097/md.0000000000009377

Yu, J., Ye, Y., Liu, J., Xu, Y., Lou, B., Zhu, J., et al. (2019). The role of hepatitis B core-related antigen in predicting hepatitis B virus recurrence after liver transplantation. *Aliment. Pharmacol. Ther.* 50, 1025–1036. doi: 10.1111/apt.15429

Copyright © 2021 Liu, Veeraraghavan, Pinkerton, Fu, Douglas, George and Tu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.