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Persistent Hepatitis B Virus in Hepatic and Extrahepatic Reservoirs in Hepatitis B Virus Related Oncogenesis

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Persistent Hepatitis B Virus in Hepatic and Extrahepatic Reservoirs in Hepatitis B Virus Related Oncogenesis

by

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A THESIS

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ABSTRACT (344 / 350 words)

Chronic infection by Hepatitis B virus (HBV) may result in hepatocellular carcinoma (HCC). Numerous viral factors are associated with oncogenesis and development of HCC, including integration, genetic variations in the X/basal core promoter/precore (X/BCP/PC) region, and occult infection. Although primarily hepatotropic, HBV is frequently found in circulating peripheral blood mononuclear cells (PBMCs) of the lymphoid system. Few studies have characterized this extrahepatic reservoir particularly in HBV-related HCC individuals. In this thesis, we hypothesized that pro-oncogenic HBV is present in both liver and extrahepatic reservoirs in chronic HBV (CHB) carriers with and without malignant disease.

A highly sensitive molecular tool for accurate detection of low-level HBV and determination of genotype is described in this thesis. Subsequently, this technique was applied to plasma and PBMCs from post-liver transplant CHB carriers in combination with next generation sequencing to identify HCC-associated viral genotypes and genetic variants. The lymphoid reservoir in 32 CHB carriers with HCC and a representative patient with an extrahepatic lymphoid malignancy (i.e., dendritic cell sarcoma [DCS]), was evaluated for pro-oncogenic HBV. Interestingly, integrated virus was identified in genes implicated in cancer development in liver, PBMCs and DCS tumor. HCC-associated X/BCP/PC variants and genotypes were also present within lymphoid cells. Functional characterization of the most commonly detected variants (Guanine-1896-Adenine and Adenine-1762-Thymine/Guanine-1764-Adenine) were evaluated *in vitro*. Compared to wild-type, these mutants showed reduced viral HBeAg, modulated cytokine/chemokine expression, decreased APOBEC3G protein expression. The A1762T/G1764A mutant had more robust HBV replication than the G1896A variant.

Overall, the findings in this thesis contributes to the literature on the epidemiological association of occult HBV, specific HBV SNPs, and integrated virus in hepatic and extrahepatic reservoirs. Persistent carcinogenic HBV poses continual risks of HCC recurrence and viral reactivation thus advocating for long-term HBV prophylaxis use post-transplant. Pro-oncogenic HBV within lymphoid cells may contribute towards oncogenesis of extrahepatic malignancies such as DCS. HCC-associated variants show functional differences in viral replicative fitness and host immune responses. Taken together, this thesis is significant for advancing the currently limited understanding of HBV molecular virology and pathogenesis of HBV-related carcinogenesis within the hepatic and extrahepatic reservoirs.

PREFACE

The work included in this PhD thesis was performed, designed, and written by Keith Chi Kei Lau at the University of Calgary, Calgary, Alberta, Canada under the supervision of Dr. Carla Coffin and guidance of supervisory committee members: Drs. Kelly Burak, Douglas Mahoney, and Guido van Marle. The collaboration with Dr. Carla Osiowy and her staff at the National Microbiology Lab, Winnipeg, Manitoba, Canada was instrumental in collection and analysis of data collected in this thesis. Chapter 1 provides essential background information to hepatitis B virus and hepatocellular carcinoma relevant to this thesis. In addition, this chapter references our manuscript reporting on HBV epidemiology in Alberta:

CHAPTER 1:

Lau KCK, Shaheen AA, Aspinall AA, Ricento T, Qureshi K, Congly SE, Borman MA, Jayakumar S, Eksteen B, Lee SS, Stinton L, Swain MG, Burak KW, Coffin CS. Hepatitis B virus testing and linkage to care in a Canadian urban tertiary referral centre: a retrospective cohort study. CMAJ Open. 2017 Jun;5(2):E431-E436. doi: 10.9778/cmajo.20170002.
Author contributions: AA Shaheen, KW Burak and CS Coffin were responsible for the study conception and design. KCK Lau and AA Shaheen were responsible for statistical analysis. KCK Lau, AA Shaheen and CS Coffin analyzed and interpreted the data, and drafted and revised the manuscript. All authors acquired the data, approved the final version to be published, and agreed to act as guarantors of the work.

The body of this PhD thesis is based upon the following manuscripts, both published and under consideration for publication:

CHAPTER 2:

Lau KCK, Osiowy C, Coffin CS. Hepatitis B virus (HBV) genome detection and genotyping in virally suppressed patients using nested polymerase chain reaction-based Sanger sequencing. Diagn Microbiol Infect Dis. 2019 Apr;93(4):318–24. doi: 10.1016/j.diagmicrobio.2018.10.015.

Author contributions: KCK Lau acquired the data. KCK Lau and CS Coffin drafted and revised the manuscript. All authors were responsible for the study conception and design, analysis and interpretation of the data, and approved the final version to be published and agreed to act as guarantors of the work.

CHAPTER 3:

Lau KCK, Osiowy C, Giles E, Lusina B, van Marle G, Burak KW, Coffin CS. Deep sequencing shows low-level oncogenic hepatitis B virus variants persists post–liver transplant despite potent anti-HBV prophylaxis. J Viral Hepat. 2018 Jan;25(6):724–32. doi: 10.1111/jvh.12860.

Author contributions: KCK Lau, C Osiowy, E Giles, B Lusina, KW Burak, and CS Coffin contributed to data acquisition. KCK Lau, G van Marle, and CS Coffin analyzed and interpreted the data. CS Coffin was responsible for study conception and design. KCK Lau and CS Coffin drafted and revised the manuscript. All authors approved the final version to be published and agreed to act as guarantors of the work.

CHAPTER 4:

Lau KCK, Joshi SS, Gao S, Giles E, Swidinsky K, van Marle G, Bathe OF, Urbanski SJ, Terrault NA, Burak KW, Osiowy C, Coffin CS. Oncogenic HBV variants and integration are present in hepatic and lymphoid cells derived from chronic HBV patients. Cancer Lett. 2020 Jun 28;480:39-47. doi: 10.1016/j.canlet.2020.03.022. Epub 2020 Mar 27.

Author contributions: KCK Lau, SS Joshi, S Gao, E Giles, K Swidinsky, OF Bathe, SJ Urbanski, NA Terrault, KW Burak, C Osiowy, CS Coffin contributed to data acquisition and curation. KCK Lau, SS Joshi, G van Marle, C Osiowy, and CS Coffin performed formal analysis and interpretation of the data. KCK Lau and CS Coffin were responsible for study conception and design as well as writing of the original manuscript. All authors approved the final version to be published and agreed to act as guarantors of the work.

CHAPTER 5:

Lau KCK, Joshi SS, Mahoney DJ, Mason AL, van Marle G, Osiowy C, Coffin CS. Differences in HBV replication, APOBEC3 family expression, and inflammatory cytokine levels between wild-type HBV and pre-core (G1896A) or basal core promoter (A1762T/G1764A) mutants. Manuscript in preparation for submission to Frontiers in Microbiology.

Author contributions: KCK Lau, AL Mason, DJ Mahoney, G van Marle, and CS Coffin contributed to study conception and design. KCK Lau and C Osiowy acquired the data. KCK Lau, SS Joshi, and CS Coffin were responsible for analysis and interpretation of the data. KCK Lau and CS Coffin drafted and revised the manuscript. All authors approved the final version for journal submission.

CHAPTER 6 includes a thorough discussion and description of the significance of the findings in this thesis. Additional avenues of research and future experimentation are discussed that may address outstanding questions that remain or have arisen from this PhD thesis.

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Many individuals have contributed in both direct and indirect ways to the completion of my graduate studies whom I will be forever indebted to. Of course, this PhD thesis would not have been possible without the support, guidance, and mentorship of Dr. Carla Coffin. Her passion, pragmatic vision, and dedication to her students and their research was fundamental to reaching the finish line of this academic marathon. Beyond the research work, Carla was a constant mentor by encouraging and supporting my future career aspirations and she serves as an exceptional role model as a clinician scientist. She was supportive of my participation of both national and international conferences, providing professional networking and travel opportunities which I never anticipated but will endlessly cherish. I would also like to thank the University of Calgary Cumming School of Medicine, Canadian Institutes for Health Research, and the Killam Trusts for the financial support and funding throughout my graduate studies.

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The members of the Carla Coffin lab (Dr. Shan Gao, Dr. Shivali Joshi, Dr. Vanessa Meier-Stephenson, Aaron Lucko, Nishi Patel, Simmone D'souza, Gurmeet Bindra) has been central players to the success of this degree. In particular, I want to thank Dr. Shivali Joshi for her consistent friendship, guidance, and research support especially with regards to the immunology-related aspects of this thesis. Further, I want to recognize the fellow students of the University of Calgary Virology Research group for their academic support, communal spirit, and friendship. Members from this group, including Chimoné Dalton, William Bremner, Jane Fletcher, Morgan

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Lastly, I would like to express my gratitude to my family and partner for their unconditional love and unwavering support. The completion of this part of my educational journey is only possible with the bravery and courage of my parents, Jacky Lau and Joyce Chan. Their many years of continual hard work facing the never-ending challenges of immigration permitted me to have the opportunity to pursue my academic aspirations. I am forever indebted to the sacrifices they have made to provide me with a better quality of life and opportunity for higher education. I must also recognize the support of my sisters, Jennica and Furla Lau. They are important role models in my life with success in their chosen career fields through strong work ethics and dedication. Finally, I would like to acknowledge my life partner Calista Yim. She has been my closest friend and most avid supporter throughout the peaks and valleys of this academic trek. She is the first to celebrate each achievements or milestone and typically with more eagerness than myself. She has consistently provided everlasting love, laughter, and a shoulder to cry on during the struggles, challenges, and frustrations. Thank you.

DEDICATION

This thesis is dedicated to my family: Jacky Lau, Joyce Chan, Jennica Lau, and Furla Lau as well as my significant other: Calista Yim. It takes a village to raise a child (or a PhD in this case).

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LIST OF ABBREVIATIONS

Symbol	Definition
ALT	Alanine aminotransferase
Anti-HBc	Antibodies targeting HBcAg
Anti-HBe	Antibodies targeting HBeAg
Anti-HBs	Antibodies targeting HBsAg
APOBEC	Apolipoprotein B mRNA editing enzyme
BCP	Basal core promoter
cDNA	Complementary DNA
cccDNA	covalently closed circular DNA
CHB	Chronic Hepatitis B
DCS	Dendritic cell sarcoma
ddPCR	droplet digital PCR
DLBCL	Diffuse large B cell lymphoma
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
dslDNA	double stranded linear DNA
ELISA	Enzyme linked immunosorbent assay

ER	Endoplasmic reticulum
FFPE	Formalin-fixed paraffin-embedded
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBIg	Hepatitis B immune globulin
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
hTERT	human telomerase reverse transcriptase
IL	Interleukin
IQR	Interquartile range
IU	International units
LT	Liver transplant
MIBI	Multiplexed ion beam imaging
MMEJ	Microhomology mediated end joining
mRNA	messenger RNA
NA	Nucleos/tide analogues

NAH	Nucleic acid hybridization
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NHL	Non-Hodgkin’s lymphoma
NTCP	Sodium Taurocholerate Co-transporting Polypeptide
OBI	Occult hepatitis B infection
P	Polymerase
PBMCs	Peripheral blood mononuclear cells
PC	Pre-core
PCR	Polymerase chain reaction
PEG-IFN	Pegylated Interferon
pgRNA	pregenomic RNA
PHA	Phytohemagglutinin
PWM	Pokeweed mitogen
qPCR	quantitative PCR
qRT-PCR	quantitative real-time PCR
RACE	Rapid amplification of cDNA ends
rcDNA	relaxed circular DNA

RNA	Ribonucleic acid
RT	Reverse transcriptase
S	Surface
ssDNA	single stranded DNA
SNP	Single nucleotide polymorphism
SVP	Subviral particle
vge	viral genome equivalents
WHO	World Health Organization
WHV	Woodchuck hepatitis virus
3D-PCR	Differential DNA denaturation PCR

學而不思則罔，思而不學則殆。

- 孔夫子，論語

Learning without thought is labour lost, thought without learning is perilous.

- Confucius, The Analects

CHAPTER 1: INTRODUCTION

1.1 Hepatitis B Virus (HBV) epidemiology

The Hepatitis B Virus (HBV) is a significant global viral pathogen. An estimated two billion individuals worldwide have been exposed to the virus with approximately 257 million living with a chronic HBV infection (1). The introduction of the HBV vaccine has effectively reduced the spread of HBV, particularly in young children (1). However, HBV remains endemic in many areas of the Western Pacific region and Africa which have an estimated prevalence of 6.2% and 6.1%, respectively (1,2). Within Canada, there are an estimated 260,000 chronically infected individuals. The majority of these chronic hepatitis B (CHB) carriers are primarily new Canadians and migrants from endemic areas (2,3). HBV is responsible for over 800,000 deaths a year primarily due to the induction of hepatocellular carcinoma, cirrhosis, and acute hepatitis (4). In the Calgary Health Zone in Alberta, our prior research study reported a total of 1214 individuals that tested positive for HBV surface antigen (HBsAg) within a single calendar year (2014) (3). Vast majority of these cases are likely newly diagnosed CHB carriers, many of which lacked appropriate monitoring and management of their disease. CHB represents a significant disease burden in both Canada and worldwide, one which will require substantial investment in prevention, research, and patient care to achieve the World Health Organization (WHO) goals for elimination of viral hepatitis by 2030.

1.2 Horizontal and vertical transmission of the HBV

As a bloodborne pathogen, the HBV is capable of both horizontal and vertical transmission into new human hosts. Horizontal transmission may occur through exposure to materials such as blood or semen containing infectious HBV particles. However, horizontal transmission into

immunocompetent hosts most frequently develops into a self-limiting acute infection as the immune system during adulthood is more effective against the virus. The vast majority of CHB carriers are initially infected via transmission of the virus by mother-to-child transmission or close contact with other family members, typically either during childbirth or early childhood (i.e. <5 years of age) (5).

1.3 Structure of HBV virions and subviral particles

HBV is the prototypic member of the *hepadnaviridae* which is unique for their incomplete double stranded DNA genome (relaxed circular [rc]DNA) and the use of reverse transcription within their viral life cycle (6). The infectious viral particle (Figure 1.1) consists of the rcDNA covalently bound to the HBV polymerase which is encapsulated within an icosahedral (T = 3 or 4) capsid composed from the HBV core proteins (i.e. HBcAg) (7). In turn, the viral capsid is enveloped with a lipid membrane originating from host cells embedded with the HBV large, middle, and small surface/envelope proteins (i.e. HBV surface antigen [HBsAg]) (Figure 1.1A). This entire infectious virion with an approximately 42nm diameter is termed the Dane particle (6,8). However, in addition to complete infectious virions, host cells infected by HBV also abundantly produce and release subviral particles (SVP) (Figure 1.1B). Typically, HBV SVPs are secreted as 25nm filamentous and 22nm spherical comprised primarily of small surface antigen with trace amounts of large and middle surface particles (7). The formation of the filamentous SVPs generally arises from the presence of a higher ratio of large surface proteins (6,7). Both the filamentous and spherical SVPs lack HBV capsid and nucleic acids, but they are nonetheless important contributors towards host immune evasion, particularly by sequestration of HBsAg targeting antibodies and potentially inducing T-cell exhaustion (6,8–10). Further, it has been recently recognized that immature or incomplete virions may also be released from

HBV infected cells (Figure 1.1B) (8). Indeed, the detection of enveloped empty capsids or capsids containing pregenomic (pg)RNA or single stranded (ss)DNA instead of rcDNA have been reported and may serve as future clinical markers of disease or treatment response (8,11–13).

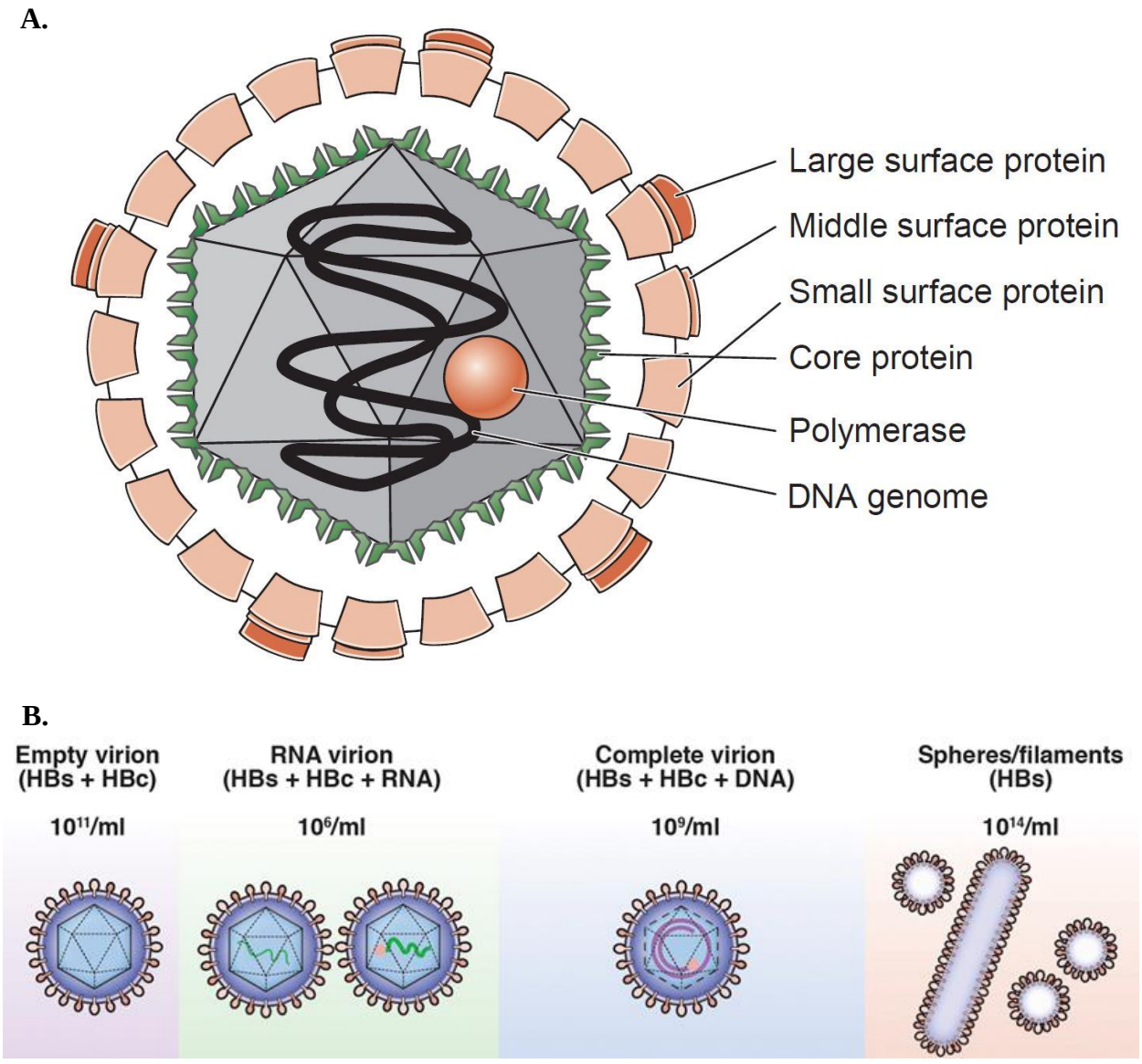


Figure 1.1: A. Structure of the 42nm infectious HBV virion, also known as the Dane particle, adapted from Acheson (6). **B.** SVPs and viral particles with their relative abundances adapted from Hu *et al*. (14).

1.4 General life cycle of the HBV (Figure 1.2)

Infection of hepatocytes by the HBV is initiated by viral attachment to surface heparan sulfate proteoglycan molecules mediated by the antigenic loop found within the HBV surface proteins (i.e. HBsAg) (15,16). Following this attachment, entry of the HBV occurs through direct binding of the preS1 region of the large HBV surface protein with the cellular sodium taurocholate co-transporting polypeptide (NTCP) (17–19). The virus is then internalized into the cell by endocytosis followed by endosomal escape and nuclear entry via the nuclear pore complexes; essential steps in the viral life cycle that has yet to be fully characterized (20,21). Once the viral genome in the form of rcDNA has entered the nucleus, it is converted into covalently closed circular (cccDNA) likely though the exploitation of host DNA repair machinery (Figure 1.2) (22).

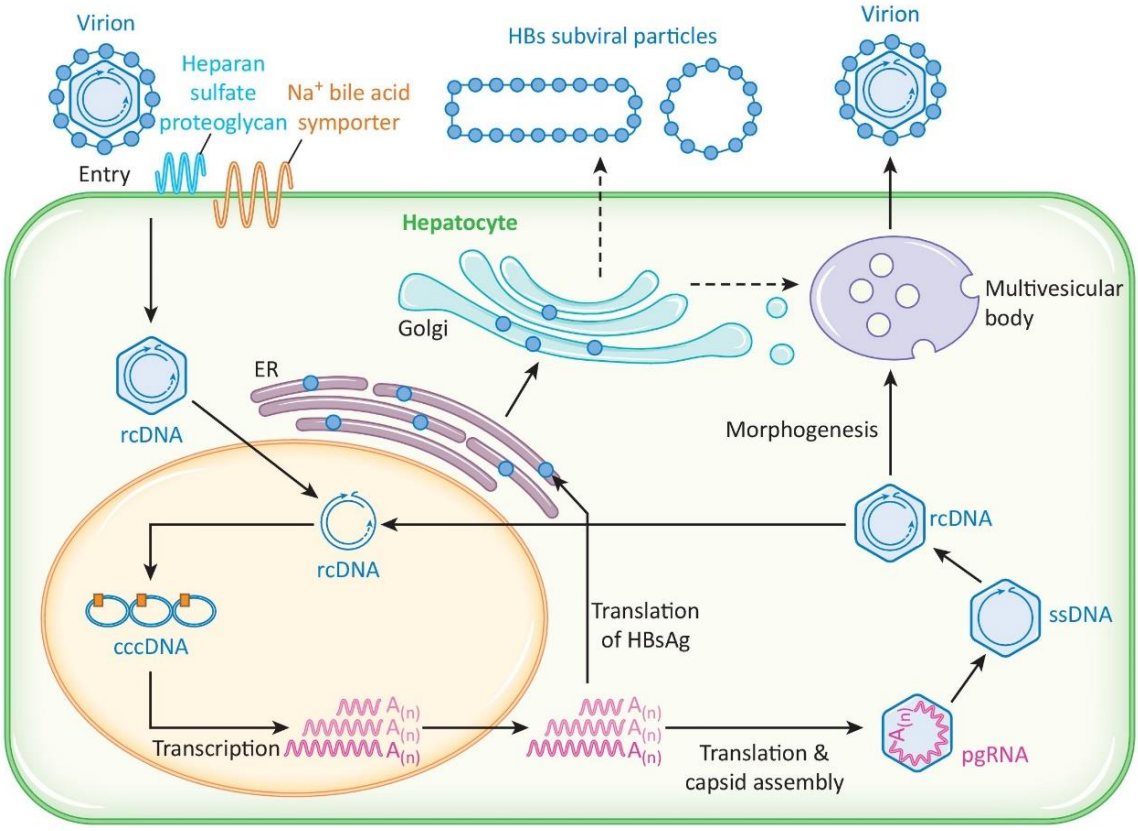


Figure 1.2: General life cycle of the HBV adapted from Shih *et al* (23).

The highly persistent episomal cccDNA is an important replicative intermediate which serves as a template to produce five viral RNA transcripts: 3.5kb preC RNA, 3.5kb pgRNA, 2.4kb and 2.1kb preS/S RNAs, and the 0.7kb HBx RNA (Figure 1.2) (24). These viral RNAs facilitate the production of essential proteins required for viral replication (Table 1.1). The HBV pgRNA is packaged alongside the polymerase (P) protein into the viral core (ie. capsid) proteins. The P protein terminal domain recognizes the viral pgRNA via the 5' epsilon stem loop which serves to initiate reverse transcription mediated by the P protein reverse transcriptase domain (24,25). Within the encapsulated shell, the P protein utilizes the pgRNA as a template to create the minus strand DNA followed by digestion of the pgRNA with the RNase H domain located at the C-terminal of the P protein (6,25). Several RNA nucleotides are left undigested which subsequently translocate to the DR2 present at the 5' of the minus strand DNA thus functioning to prime plus strand DNA synthesis (6,25). The incomplete synthesis of the plus strand results in the partially double stranded rcDNA. Newly formed rcDNA buds into the endoplasmic reticulum (ER) induced by association of the capsid core proteins with HBV envelope proteins present in the ER membrane. Assembled virions are released through exocytosis to produce infectious viral progeny capable of invading new host cells. In addition, the newly formed rcDNA can also recycle back into the nucleus to replenish the cccDNA reservoir thereby facilitating continual viral persistence.

Table 1.1: Viral proteins encoded by HBV RNA transcripts.

HBV RNA TRANSCRIPTS	PROTEINS ENCODED
3.5kb preC	HBV precore protein (which is eventually processed into secreted Hepatitis B e antigen [HBeAg])
3.5kb pregenomic	Core (ie. capsid) and P proteins
2.4kb preS/S	L envelope protein
2.1kb preS/S	M and S envelope proteins
0.7kb HBx	HBV X protein

1.5 Organization of HBV genome and non-structural proteins

HBV is a small and compact virus with an approximately 3.2kb DNA genome consisting of four overlapping reading frames encoding for five viral RNA transcripts and seven proteins (Table 1.1, Figure 1.3) (24,26). Four promotors (core, SPI, SPII, and X) are responsible for transcription of the HBV RNA which share a common poly-adenylation site (24). In addition, the direct repeat (DR) regions, DR1 and DR2, are important features of the HBV genome by contributing towards template switching and primer translocation during reverse transcription of pgRNA (Figure 1.3) (6,27). Located between the DR1 and DR2 region is the basal core promoter (BCP), a component of the core promoter that influences expression of precore and pregenomic RNAs (24). Single nucleotide polymorphisms (SNPs) within the BCP have been described with an association to liver cancer development or affecting anti-viral treatment response (Section 1.11). Due to the overlapping nature of the viral genes and transcripts, the development of a mutation within the HBV genome may have pleiotropic effects. For example, the development of anti-viral treatment resistance mutations (e.g. rtA181T) within the P gene results in a corresponding S gene mutation (i.e., W172*) that leads to production of truncated surface proteins and an increased risk of HCC (24,28).

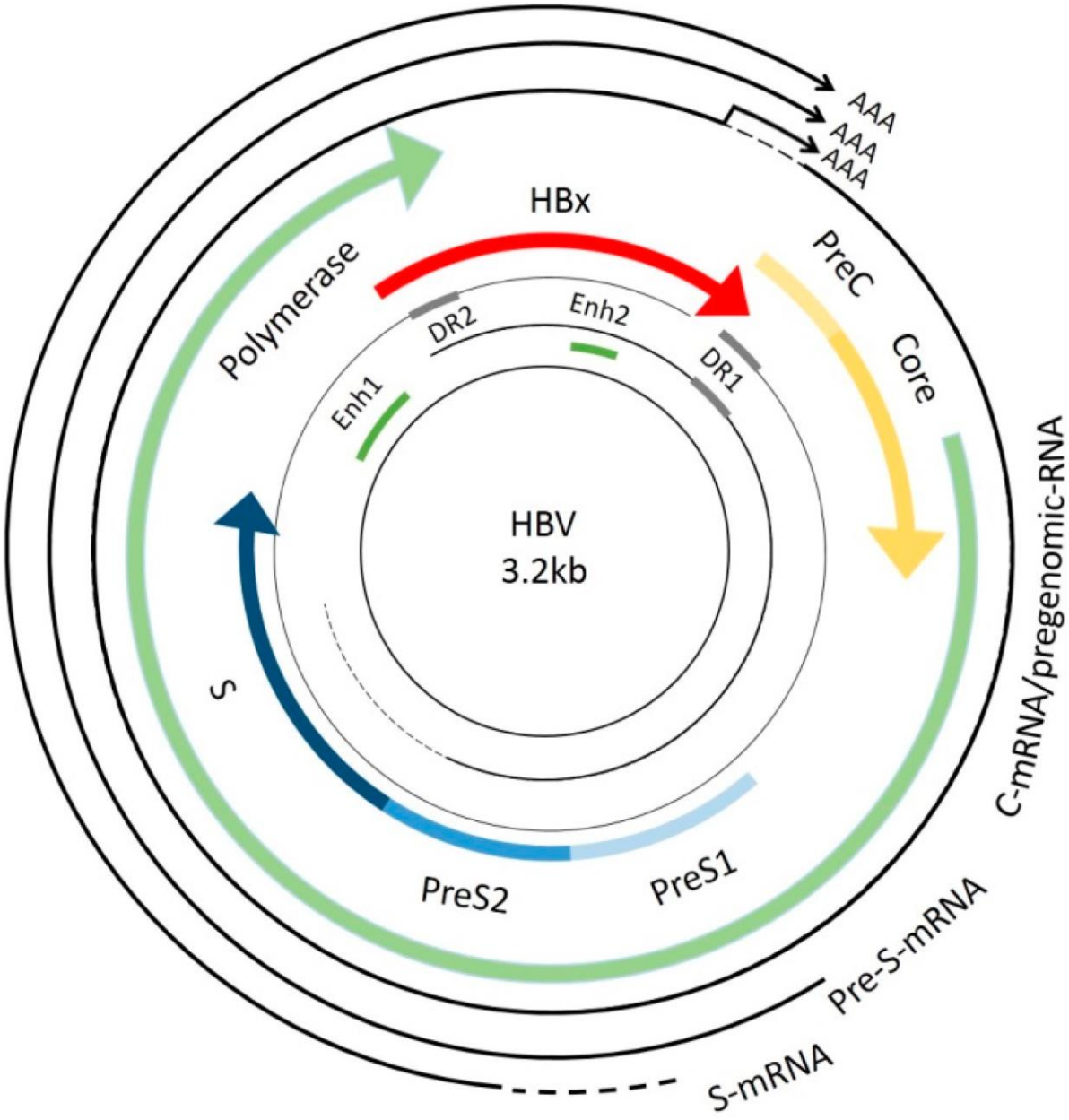


Figure 1.3: HBV genome organization and features adapted from Liu *et al* (29).

Of the seven proteins encoded by the HBV genome, precore (i.e., HBeAg) and HBx are two non-structural proteins that nonetheless function to enhance viral infection and persistence. HBeAg is a non-essential protein with regards to the viral replicative life cycle but serves to induce chronic infection and is frequently utilized as a clinical disease marker (30). Indeed,

HBeAg is an immunomodulator that has been shown to inhibit both innate and adaptive immunity through a variety of mechanisms such as interfering with interferon expression and induction of T-cell exhaustion (30,31). HBx has a wide variety of pleiotropic effects, many of which impact oncogenesis through epigenetic modulation and alteration of oncogene/tumor-suppressor expression (29). With regards to HBV viral infection, HBx has now been characterized to significantly enhance HBV cccDNA transcription. This recently established mechanism involves the association of HBx with the E3 ubiquitin ligase DDB1-Cul4 in order to direct ubiquination of the Smc5/6 complex for proteasomal degradation (32–34). The uninhibited Smc5/6 complex functions to suppress HBV cccDNA transcription thus preventing viral pgRNA and protein production (33). In addition, HBx contributions to immune suppression via disruption of host pathogen recognition pathways and subsequent induction of an innate antiviral response (29,35,36).

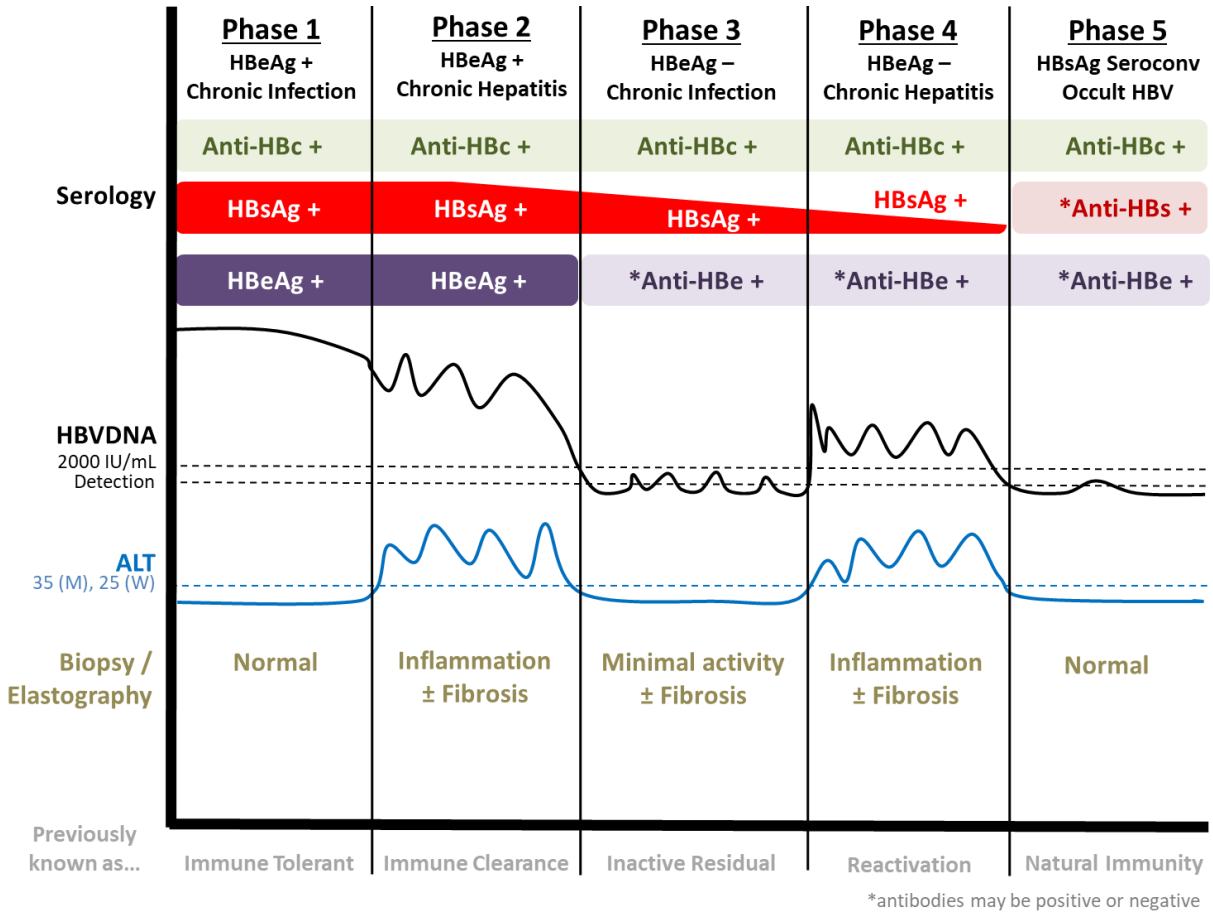
1.6 Natural history of chronic HBV infection

CHB infection is defined by positive HBsAg in serum for greater than 6 months duration. Persistent infection with HBV most frequently arises in individuals exposed to HBV at a young age (<5 years of age) as majority of these cases eventually develop into CHB. In contrast, only 5% or less of adolescents and adults who are exposed to HBV eventually progress to CHB infection (37). Individuals persistently infected with HBV are at risk of development of severe liver complications including cirrhosis and primary liver cancer (ie., hepatocellular carcinoma [HCC]).

The natural history of CHB infection have been divided into multiple distinct phases (30,38,39). The first phase is defined as a high replicative and low inflammatory state (Figure 1.4). Typically, CHB carriers in this phase are characterized with extremely high levels of viral

load and positivity for HBeAg (39). Despite, the high levels of viral protein and DNA, liver inflammation and damage are minimal. CHB carriers may progress into the second stage, the HBeAg positive hepatitis phase, characterized with occasional hepatic flares in which liver inflammation and damage occurs (30,39). Liver damage occurs within this phase which could be observed through elevated levels of alanine aminotransferase (ALT).

Transition to the third stage occurs with the loss of viral HBeAg and the presence of antibodies targeting HBeAg (anti-HBe) (39). Within this phase, CHB carriers typically experience HBeAg-negative infection with low levels of HBV viremia and ALT due to minimal liver inflammation. However, some patients may develop HBeAg-negative chronic hepatitis in which viral replication occurs at higher levels resulting in hepatic flares (30,39). These generally occur with the presence of HBV precore or BCP mutations that disrupt non-structural HBeAg production, but maintains or enhances viral replication (24,38,39). BCP mutations present within the core promoter alter viral cccDNA transcription by reducing precore RNA production to favor increased pgRNA production (Section 1.11) (24). The final phase is the HBsAg negative phase which may occur either spontaneously or with treatment and is associated with improved prognostic outcomes including reduced risks of cirrhosis and HCC (39,40). However, only a small proportion of CHB carriers eventually progress to this last stage and rarely occurs despite many years of oral antiviral therapy.



ALT = alanine aminotransferase; IU = international units; M = male; W = woman

Figure 1.4: Natural course of chronic HBV infection courtesy of KW Burak.

1.7 Occult hepatitis B infection (OBI)

Individuals infected with HBV may present as occult hepatitis B infection (OBI), a condition defined primarily with HBsAg-negative serology despite the presence of replication competent HBV (41). OBI cases may or may not have detectable HBV viremia due to frequently suppressed viral expression and replication. This suppression may arise primarily due to host immune system control of HBV (42,43). Typically, individuals with OBI are identified with positive results from at least two HBV genomic regions using highly sensitive molecular techniques, such as nested polymerase chain reaction (PCR) (41). Nonetheless, detection and accurate genotyping

of the HBV can be particularly challenging in OBI individuals with very low-level HBV viremia (i.e., < 10 IU/mL or 50 virus copies/mL).

Despite the HBsAg-negativity and low-level viremia, OBI remains clinically significant as HBV may reactivate with immunosuppression and individuals with OBI have increased risks of malignant disease relative to HBV-negative individuals (44). Indeed, HBV replicative intermediates can be frequently detected and found in HCC patients without overt CHB disease (45–48). In fact, many cases of cryptogenic HCC (those with no known cause) have been linked to OBI (48–50). Studies have demonstrated that oncogenic viral integration is detectable and likely poses a continual risk for malignant transformation of hepatocytes (50–52). In addition to HCC and liver disease, reports have suggested that the presence of occult low-level HBV are also associated with extrahepatic and hematological malignancies including leukemia and lymphoma (53–55). HBV reactivation in OBI may also be particularly problematic within patients who undergo chemotherapeutics or immunosuppressive therapies that impact host adaptive immunity. The most well-characterized cases occur in patients receiving anti-CD20 therapy (e.g. rituximab) (56–58). As a consequence of the disruption of host anti-HBV immunity, the virus can reactivate potentially leading to acute liver failure and fatal outcomes. These situations emphasize the appropriate identification of OBI to prevent and monitor potential HBV reactivation.

1.8 Treatment of chronic HBV infection

CHB management is limited by a lack of effective curative therapy, but persistent HBV infection can be treated with the use of pegylated-interferon α (PEG-IFN) or nucleos/tide analogues (NAs) such as tenofovir and entecavir. HBV is not only a weak inducer of innate immunity (i.e. Type I IFNs), but also actively suppresses immune detection and activity (9).

Thus, the mechanisms underlying PEG-IFN targeting of HBV primarily involve harnessing the host innate immune system to target viral replication and replicative intermediates through multi-faceted approaches (59). Indeed, studies have demonstrated the capability of IFN in inducing expression and activation of innate anti-viral interferon stimulated genes/proteins which serves to effectively inhibit HBV replication (60–65). Activation of ISGs including but not limited to the apolipoprotein B mRNA editing enzyme (APOBEC)3 family, MX2, ISG20, and the TRIM family have been described with innate anti-HBV effects (60–65). Further, studies have reported the capability of IFNα therapy to inhibit viral replication and suppress HBV gene expression through epigenetic modulation of the HBV cccDNA (66,67). PEG-IFN treatment can be associated with higher rates of HBsAg clearance/loss (i.e. clinical HBsAg negative serology) and therefore achievement of a functional cure. However, these rates seem to be genotype specific with improved treatment responses in HBV genotype A (38,68,69).

NAs inhibit the viral RT enzyme, an essential component in the viral life cycle, thereby attenuating production of new HBV particles. However, NA's do not directly target the HBV cccDNA and only a small minority of patients on therapy eventually develop HBsAg clearance (ie., HBsAg negative/loss) which serves as a marker of viral suppression or functional cure (30,70). Therefore, NAs are typically life-long treatments for CHB patients. Despite these disadvantages, NAs induces substantial reduction of HBV viral load with minimum adverse side-effects which effectively prevents and delays the progression of CHB to cirrhosis or HCC in a wide variety of patients (71,72).

1.9 HBV-related hepatocellular carcinoma (HCC)

Primary liver cancer accounts for approximately 840 000 new cases each year and is the fourth leading cause of cancer deaths worldwide accounting for an estimate 780 000 deaths per

year (73). The majority of primary liver cancer presents as HCC, which primarily arises due to viral hepatitis (HBV and Hepatitis C Virus) (1,74). HBV-related HCC accounts for approximately 56% of all HCC cases worldwide (74). The development of HBV-related HCC is complex and arises from a contribution of host, environmental, and viral factors (Figure 1.5). Major host factors include age, sex, and comorbidities. Similar to the majority of neoplastic diseases, increasing age is a risk factor generally through the collection of mutations and genetic abnormalities over time. Males are more prone towards HCC development which has been suggested to arise from hormonal differences particularly between the effects of androgens and estrogens towards HBV replication and viral gene transcription (75,76). Host comorbidities or co-infection primarily affecting the liver such as non-alcoholic fatty liver disease (77,78) or hepatitis C virus infection (79) also increases the likelihood of developing HCC. In addition, environmental factors such as aflatoxin, a fungal dietary contaminant prevalent in Africa, is linked to HCC development (80).

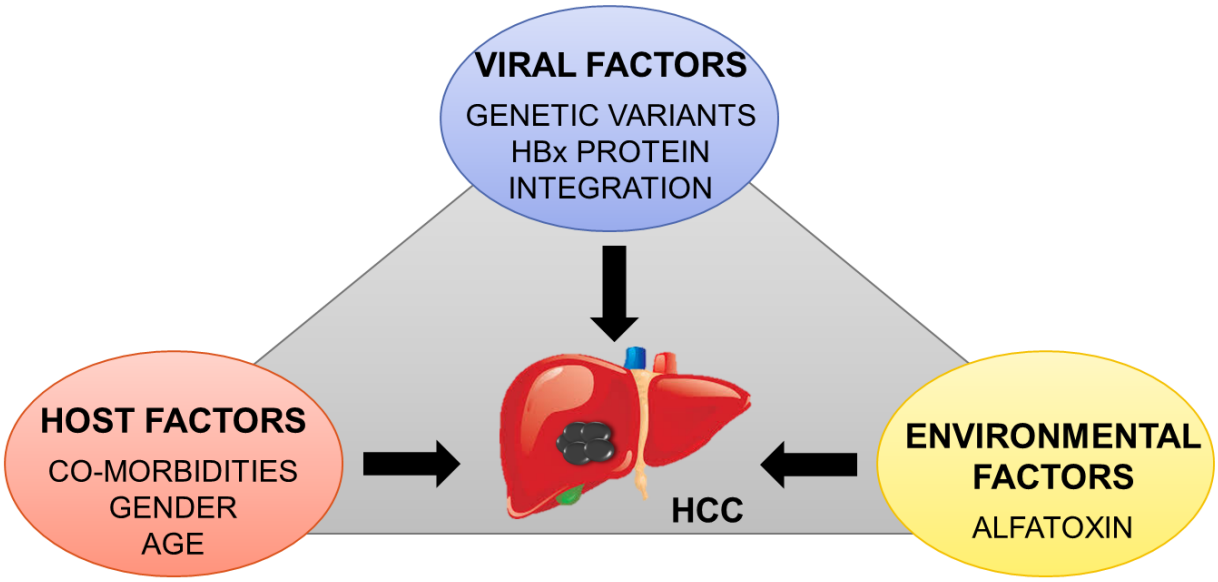


Figure 1.5. Development of HBV-related HCC are influenced by a variety of host, viral, and environmental factors.

Individuals that develop HCC typically progresses from liver fibrosis to cirrhosis before the presentation of HCC. However, HBV-related HCC is unique as presentation of liver cancer may occur even in the absence of a cirrhotic liver, albeit at lower rates (48,81–83). In fact, certain viral features and characteristics are well recognized to directly induce oncogenesis including viremia (HBV DNA levels), HBeAg, HBx protein, and HBV integration. The positive correlation between HBV viral load and risk of cirrhosis and HCC has been well documented in the REVEAL-HBV study and amongst other large Asian epidemiological reports (84–87). Similarly, HBeAg positivity has been associated with an increased risk of hepatocarcinogenesis and cirrhosis (88,89). Positive HBeAg stages of CHB is also characterized by additional hepatocarcinogenic features including high viral load levels and liver inflammation.

The importance of HBx towards viral transcription has only recently been described (32), but its role towards oncogenesis and malignant cell transformation is an area of extensive research. Although HBx does not have DNA binding domains, this promiscuous viral protein utilizes a variety of protein-binding domains to interact with many different cellular factors impacting multiple processes implicated in carcinogenesis including apoptosis, cell cycle progression, DNA damage repair, and cell adhesion (90). HBx can induce epigenetic changes by regulating the activity of DNA methyltransferases. Through this mechanism, HBx is observed to dysregulate cell cycle by reducing the appropriate expression of checkpoint signaling pathways or inhibitors, such as retinoic acid (91). Further, HBx is known to alter many signaling pathways including but not limited to NF-kB, extracellular signal-regulated kinases, and JAK/STAT via binding to protein kinases and ubiquitin ligases (90,92).

1.10 Oncogenic implications of HBV integration

HBV integration into the host genome arises with the incorporation of double stranded linear (dsl) variants of HBV. An estimated 10% of HBV reverse transcription results in the synthesis of a dslDNA form of the HBV genome due to a lack of translocation of the viral RNA primer to the 5' DR2 (Figure 1.6) (27). Similar to rcDNA, the dslDNA can be either exocytosed from the cell as new infectious viral progeny or recycled back into the nucleus (27). Once the dslDNA localizes into the nucleus either through *de novo* secondary infection or intracellular recycling, the dslDNA is capable of inserting into the host genome, a process primarily mediated by non-homologous end joining (NHEJ) or microhomology mediated end joining (MMEJ) that utilizes host machinery (27,93–95). These mechanisms of handling double strand DNA breaks are non-specific and frequently causes deletions and insertions which are reflected within the integrated HBV (27,96). HBV integration is not necessary for a productive HBV life cycle and frequently results in a dead-end infection as the integrated virus is incapable of producing viral progeny (27). Estimates of HBV integration frequency have theorized a rate of 1 integration in 1000 hepatocytes within an infected liver (97). Integration of HBV likely occurs shortly if not immediately after infection as suggested by studies in both the clinical and *in vitro* settings. HBV integration has been detected from liver tissues from early stages of CHB (i.e. immune tolerant, now recognized as the HBeAg+ chronic infection phase) (98,99). In addition, *in vitro* studies have shown that HBV integration in immortalized hepatocyte cell lines can occur even within hours after entry into a new host cell (100,101).

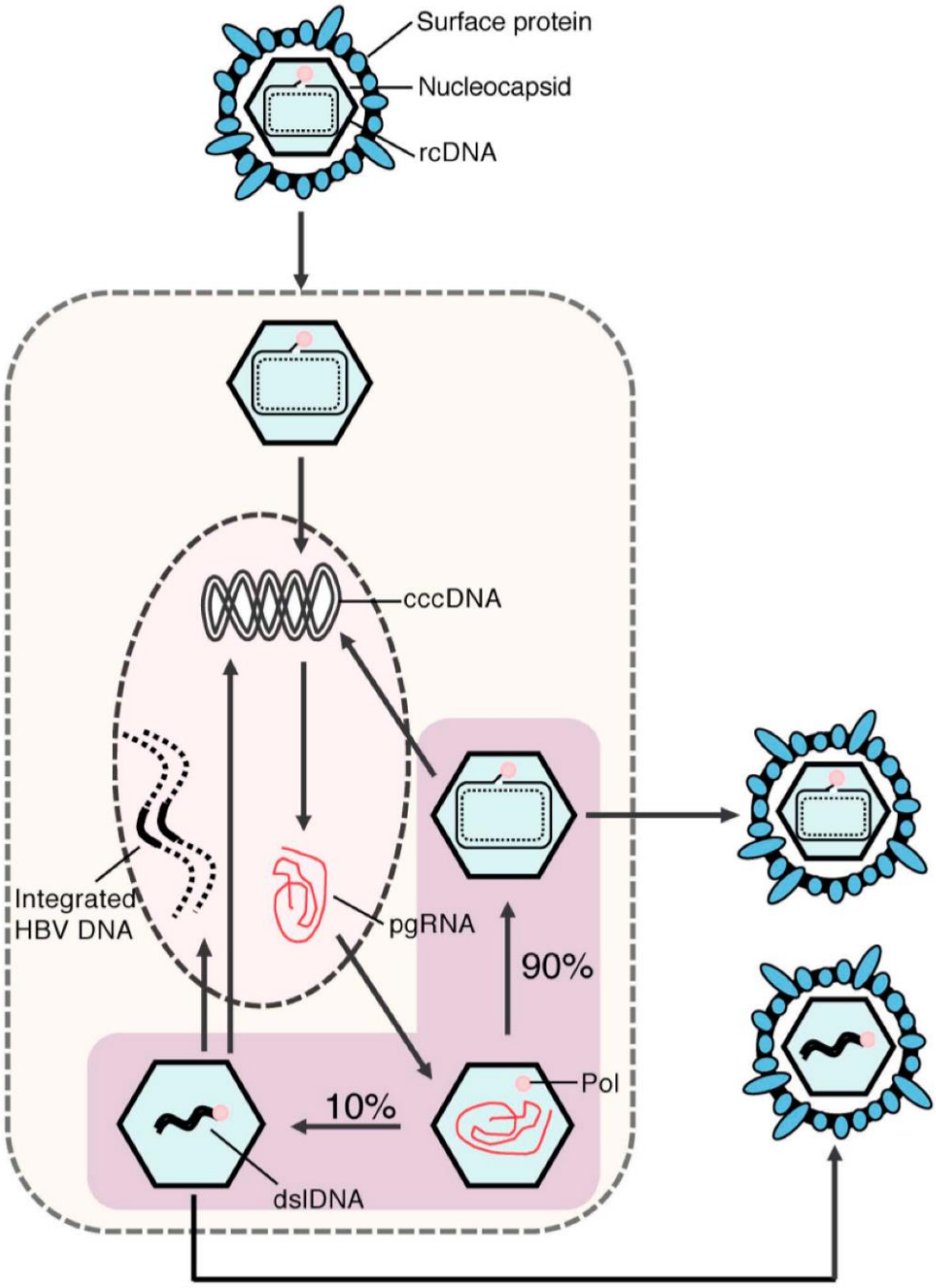


Figure 1.6. Translation error of the RNA primer to the 5' DR2 site during reverse transcription of HBV results in creation of a dslDNA form of the HBV genome which serves as fundamental unit for integration of the virus into the genome of the infected host (via recycling of the nucleocapsid) or integration in newly infected cells (via release of dslDNA infectious virions). Figure adapted from Tu *et al*. (27)

Integration of HBV is traditionally theorized to generally occur at random sites and has been detected in a wide variety of genes. However, preferential sites for HBV integration include host genomic regions prone to dsDNA breaks or fragile sites largely due to the use of NHEJ and MMEJ mechanism (95). Further, recent studies have observed increased detection of HBV integration within coding regions, open chromatin areas, and regions with higher gene expression (102,103). Current theories hypothesize that HBV integration is insufficient by itself to induce cancer, but instead facilitates hepatocarcinogenesis (104,105). Integrations associated with oncogenic implications would likely provide survival advantages which are selected for during clonal expansion of HCC progenitor cells (Figure 1.7). Thus, recurrent integration sites have been characterized from HCC tissues, many of which occur within genes that play a role in cellular processes implicated in oncogenesis. For example, one of the more frequently reported integration site is the human telomerase reverse transcriptase (hTERT) gene (103,106–111). The hTERT is a well known oncogene found to be one of the most frequent genetic alterations present in early developing HCC suggesting a role in development and pathogenesis of HCC (112,113). This gene permits cellular immortalization by activating telomerase activity and is upregulated in cells integrated with HBV (103,107,108,110). In addition to the hTERT, HBV recurrent integration events has been found within other genes implicated in oncogenesis and malignant transformation, such as the MLL4 and SERCA1 genes (103,108,111,114,115). HBV integration may also influence the expression or production of non-coding RNA which are increasingly reported to impact carcinogenesis of many cancers including HCC (116,117). Indeed, Lau *et al.* has elegantly characterized a specific HBV integration site which generates a novel long non-coding RNA (coined HBx-LINE1) with oncogenic implications (118).

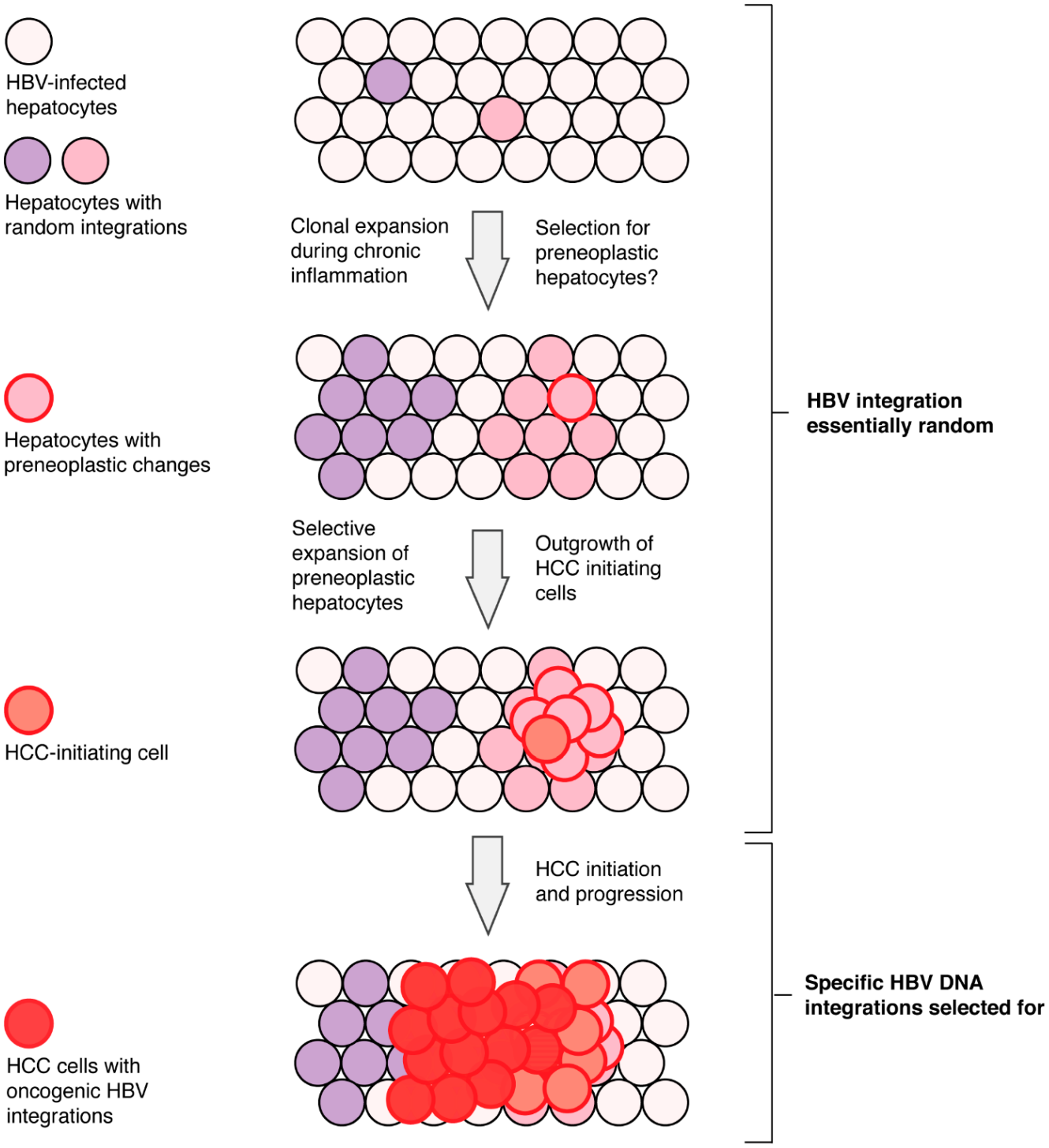


Figure 1.7. Current model for the role of HBV integration in HCC oncogenesis adapted from Budzinska *et al.* (104).

1.11 HBV genotypes and genetic variants

It is particularly interesting to note that not all HBV infections have identical risks towards HCC development, as HBV genotype and genetic variants within the HBV preS1 or X/basal core promotor/pre-core (X/BCP/PC) regions are associated with different risks of HCC. HBV is currently classified into 10 different genotypes, labeled A to J, which are defined as >7.5% genetic divergence within the full genome sequence of the virus (119,120). HBV genotypes are typically geographically distributed (Figure 1.8A). For example, genotype B and C are endemic in the South-East Asia including China and Japan. Countries that experience larger influxes of immigration from a variety of regions worldwide, such as Canada, demonstrate a more diverse array of genotypes (Figure 1.8B) (121,122). Different genotypes have been observed to impact HCC risk, age of onset, and prognosis. Indeed, individuals infected predominantly with HBV genotype C or genotype D have a higher risk of developing cirrhosis and HCC in comparison to genotype B or A, respectively (84,119,123). Further, multiple studies have demonstrated that early onset HCC is more common in HBV genotype B patients whereas genotype C is associated with later onset HCC (110,123,124).

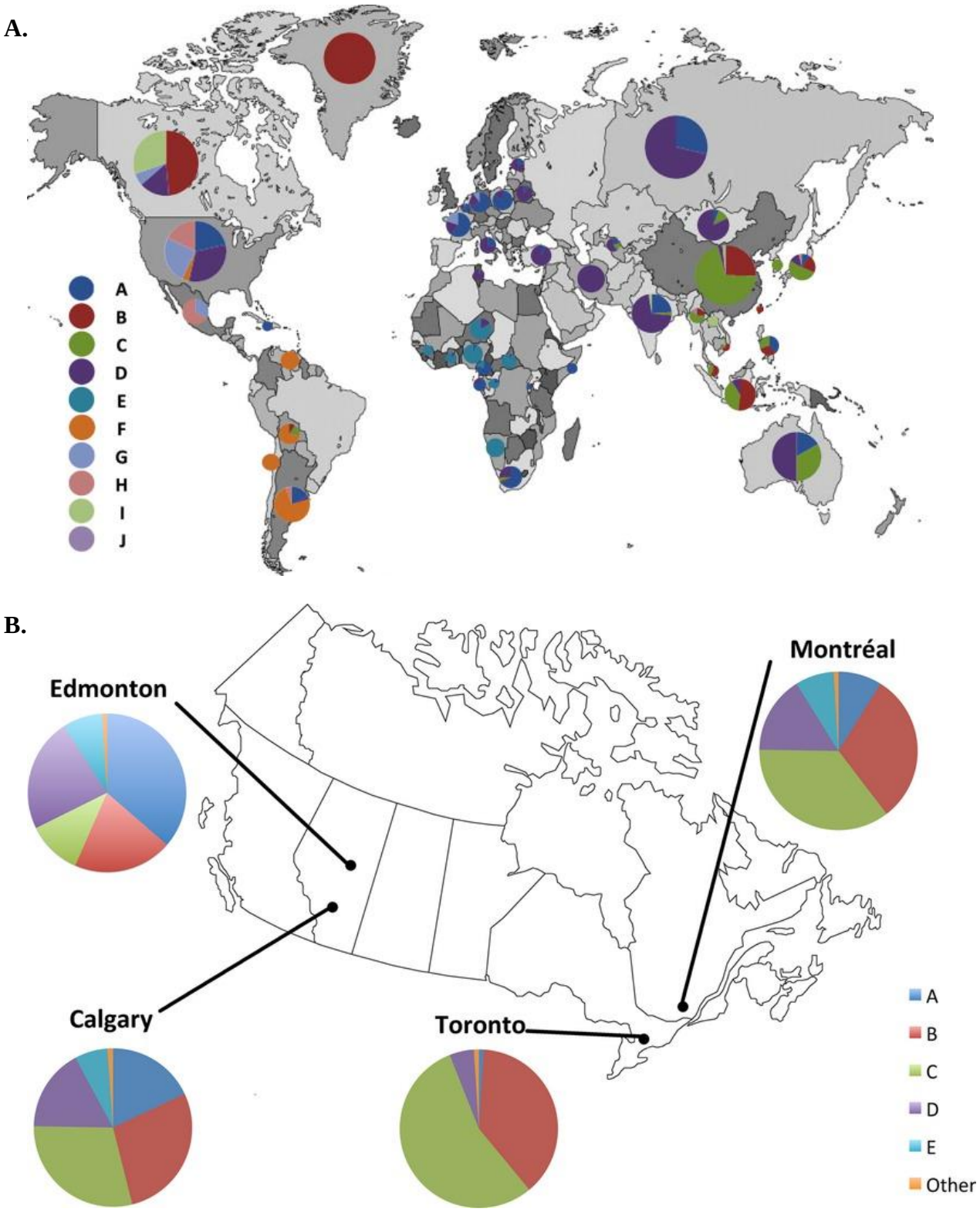


Figure 1.8. Geographic distribution of HBV genotypes **A.** globally as adapted from Shi *et al.* (125) and **B.** within Canada as adapted from Congly *et al.* (121)

Due to the error-prone method of viral replication via the HBV polymerase, the HBV population within a host exists as quasi-species. Indeed, studies evaluating lymphoid cells (i.e. peripheral blood mononuclear cells [PBMCs]) in CHB carriers have reported different genotypes exist within the plasma and PBMCs (126,127). These genotype discrepancies likely arise due to different evolutionary and environmental pressures amongst the reservoirs including antiviral treatment exposure, targeting by the immune system, or genetic drift (126–128). Further, mutations within the preS1 and preS2 regions of the virus have not only been reported, but also demonstrated to induce hepatocarcinogenesis (129–131). Mutations of these regions result in impaired secretion of HBV virions and HBs subviral particles leading to an accumulation of unfolded viral proteins within the cellular ER (132,133). The preS mutations are responsible for the histological phenotype of type II ground-glass hepatocytes (133,134). Consequently, hepatocytes containing HBV preS mutants exhibit ER stress response, oxidative DNA damage, increased inflammation and undergo cellular apoptosis (132,133,135). These cellular processes facilitate the neoplastic transformation of hepatocytes and the induction of HCC.

In addition to the preS mutations, genetic variants of HBV within the X/BCP/PC, have also been associated with an increased - and in some cases decreased - risk of developing cirrhosis and HCC (Table 1.2). Some of the oncogenic variants in this region have been observed at higher frequencies in certain genotypes, which likely is reflected in the difference in HCC risks amongst HBV genotypes. For example, the double mutations A1762T and G1764A are more frequently observed in genotype C. Many variants in this region have been identified from large epidemiological studies generally from Asian populations (136). Most notably, the A1762T and G1764A double mutations are consistently associated with HBV induced cirrhosis and HCC (129,136–141). In addition, T1674C/G, A1752G, T1753V, T1768A, C1773T, A1846T, G1896A,

and G1899A also influence HCC risks (129,136,138–141). Amongst these variants, only the presence of A1752G is correlated with a decreased cirrhosis and HCC risk (139).

Table 1.2: HBV SNPs within the X/BCP/PC region associated with cirrhosis and HCC.

GENETIC VARIANT*	ASSOCIATED HCC RISK
T1674C/G	Increased
A1752G	Decreased
T1753V	Increased
A1762T	Increased
G1764A	Increased
T1768A	Increased
C1773T	Increased
A1846T	Increased
G1896A	Increased
G1899A	Increased

*A = Adenine; C = Cytosine; G = Guanine; T = Thymine; V = Adenine, Cytosine, or Guanine

Current theory with regards to the HCC-associated mutations within the X/BCP/PC region focuses on the alterations towards the HBx protein. Due to the overlapping nature of the HBV genome, the mutations directly affect the genetic coding sequence of HBx. Indeed, a research study by Yan and colleagues exploring the quadruple mutant of A1762T, G1764A, T1753A, and T1768A showed an overall downregulation of p53 in comparison to wild-type HBV X (142). Aside from the HBV X protein, these mutations are also capable of influencing the non-structural protein, HBeAg. The variant G1896A is well recognized for the ability of introducing a premature stop codon within the HBeAg transcript resulting in a defective protein and HBeAg-negative serology (143,144). Other mutations in this region, including the A1762T/G1764A double mutations, also result in reduced HBeAg production and expression by disrupting the

BCP, a component of the core promoter that encodes for precore (i.e. HBeAg) RNA (144). Interestingly, these BCP genetic variants are associated with both a decrease in HBeAg and a corresponding increase in viral replication through enhanced pgRNA expression. Due to this phenotype, a combination of these mutations is theorized to be related to the hepatic flares or high viral load levels in HBeAg-negative CHB cases (30,145,146). However, many of the X/BCP/PC mutants that have been linked to HCC risk and development by epidemiological studies are still poorly understood particularly with regards to the molecular biology. Their impacts on either the viral life cycle or cellular processes have yet to be characterized.

1.12 Liver transplantation and occult HBV persistence post-transplant

Liver transplantation is now an effective curative therapy for HBV-related end-stage liver diseases such as liver failure or HCC. Historically, CHB was a contraindication for liver transplant due to severe HBV recurrence, which arises from the persistent virus present within transplantation patients (147). However, the advent of post-transplant combination prophylaxis with hepatitis B immune globulin (HBIg) and potent oral NAs effectively prevented overt HBV recurrence (148,149). Recent reports suggest that gradual minimization of prophylaxis with HBIg discontinuation and even complete withdrawal of both HBIg and NA is possible in liver transplant recipients with minimal risk of HBV recurrence (150–153). The argument for this atypical practice of complete prophylaxis withdrawal is based upon the fact that many liver transplant recipients receive more potent NA pre-transplant and have continually suppressed HBV DNA at the time of transplant. However, the potential impact of extrahepatic (ie., peripheral blood mononuclear cells [PBMCs]) HBV on relapse risk following withdrawal of long-term prophylaxis is not clearly understood (42). Studies have explored the use of HBV vaccination post-transplant for prophylactic use, but was restricted by limited effectiveness in

preventing HBV recurrence (154–156). However, more recent studies have suggested that vaccination may provide continual viral suppression without HBIg or NA use, particularly in appropriately selected of vaccine responders (157–159). Nonetheless, this method still requires sufficient characterization and validation for regular use as a HBV prophylaxis in post-liver transplant recipients (157–159). Further, it is unknown whether circulating HBV mutants associated with HCC and/or cirrhosis can persist long-term after liver transplant which may have unclear clinical implications with regards to end-stage liver disease or HCC recurrence.

1.13 Extrahepatic infection and lymphotrophism of HBV

HBV is well recognized to infect and establish reservoirs in hepatocytes and the majority of HBV research focuses on the hepatocyte derived virus. However, additional work by a variety of research groups have suggested the lymphoid system also serves as a HBV reservoir. Important HBV replicative intermediates and infection markers have been identified in extrahepatic lymphoid tissues including the spleen, lymphoid nodes, and lymphoid cells (126,128,148,160–165). In fact, Lee *et al.*, Chemin *et al.*, and Trippler *et al.* have independently demonstrated that the virus is universally present in multiple immune cell subsets derived from HBV-infected individuals (166–168). Studies by Yan *et al.* and Huang *et al.* reported that naïve primary PBMCs and hematopoietic stem cells, respectively, can be infected *in vitro* with clinically derived HBV (169,170). Limited studies of lymphoid-derived HBV have demonstrated the infectious potential of this reservoir. Indeed, prior *in vitro* studies performed by Gao *et al.* also suggests that PBMC-derived HBV can establish an infection in differentiated HepaRG cells (171). An important clinical implication of this extrahepatic reservoir was reported by Brind *et al.* in which PBMC-derived HBV was the infectious source responsible for re-infection of the liver post-transplantation (161). The findings from these studies are particularly noteworthy as it

suggests that the lymphoid cell reservoir is not only susceptible for HBV infection, but are also capable of harboring infectious HBV virions.

As a carcinogen, the presence of HBV within the lymphoid cells may contribute towards the development of hematological neoplasms. Indeed, increasing numbers of epidemiological studies have reported associations of CHB with higher risks of non-Hodgkin lymphoma, namely diffuse large B-cell lymphoma (DLBCL) (172–179). Although, the mechanisms underlying this clinical association have yet to be fully elucidated, the direct oncogenic effects of HBV within lymphoid cells may induce malignant transformation. In support of this idea, a recent study by Wang *et al.* not only demonstrates that B-lymphocyte cell lines are infectable by HBV, but also that DLBCL tissues contain HBV infection markers such as HBV DNA and viral HBsAg, HBcAg, as well as HBx proteins (180). HBV integration have previously been detected within the PBMCs and hematopoietic stem cells (160,181). This viral feature is well-recognized to contribute towards hepatocarcinogenesis and may also be implicated in oncogenesis of HBV infected lymphoid cells. In-depth HBV integration studies of PBMCs and of lymphoid malignancies from CHB carriers could clarify this hypothesis. Further, Sinha *et al.* have identified genetic variants of HBV which are associated with increased HCC risks in DLBCL tissues (182). The results of these recent studies highlight the potential oncological importance of HBV lymphotrophism and warrant additional research to clarify the associations of HBV and hematological malignancies.

1.14 Rationale and summary of study

CHB is not only a global health concern, but it also constitutes a significant local disease burden. In our prior study evaluating the epidemiology of CHB in the Calgary health region, we identified 1214 individuals who tested positive for HBsAg within a single calendar year (ie., 2014) (3). As HBsAg testing is infrequent in those who are already known CHB carriers, most of

these individuals are likely newly identified as CHB carriers. Further, we identified that the majority these individuals had yet to receive appropriate consultation with a specialist regarding their disease (3). Therefore, the disease burden of CHB within Calgary and the potential burden on the health care system is substantial, particularly as these individuals have significant risks of progressing to severe liver diseases such as cirrhosis and HCC.

The hepatocarcinogenic capabilities of HBV are well established, but very few studies have explored the lymphoid reservoir in HBV-related HCC patients. This particular reservoir of HBV could be clinically important by serving diagnostic role as markers of CHB disease progression and HCC surveillance. It is also important to consider how this reservoir may influence the recurrence of malignant disease after use of potentially curative therapy in HBV-related HCC patients. Furthermore, studies are demonstrating clinical connections between the CHB carriers and lymphoid-proliferative malignancies, yet few have assessed for the oncogenic potential of HBV within the lymphoid reservoir.

Therefore, our research objective is to enhance our current understanding of the pathogenic mechanisms of persistent HBV reservoirs towards oncogenesis. We hypothesize that HBV persists in hepatic and extrahepatic reservoirs (i.e. lymphoid cells such as PBMCs) in individuals with CHB, even after potentially curative HCC therapy such as liver transplantation. The persistent HBV species present in these reservoirs are expected to contain oncogenic viral features including viral integration, oncogenic HBV genotypes, and SNPs associated with carcinogenesis. In addition, we theorize that HCC associated HBV genetic features will impact cellular processes that increases the likelihood of oncogenesis and HCC development through dysregulation of host innate antiviral restriction factors.

In this thesis, we primarily utilize plasma, serum, PBMC, liver, and/or tumor tissue obtained by CHB patients with or without a history of malignancy. The work presented in this thesis identifies pro-oncogenic HBV within both hepatic and extrahepatic reservoirs. In chapter 2, a highly sensitive molecular tool (nested polymerase chain reaction [PCR]) is described, which can effectively detect HBV and genotype samples from CHB carriers with very low-level viremia. Chapter 3 utilizes the aforementioned molecular techniques in combination with next generation sequencing (NGS) to characterize pro-oncogenic HBV persistence post-liver transplantation. Chapter 4 includes a thorough molecular virological analysis, including novel HBV biomarkers analysis of the tissues from CHB carriers with or without HCC, as well as representative samples from a CHB carrier with a lymphoproliferative disease (i.e. dendritic cell sarcoma [DCS]). Important viral replicative intermediates, genotype, HCC-associated genetic variants, and host-viral integration sites were identified within available samples including plasma, PBMCs, (liver or DCS) tumor, and non-tumorous tissues. In chapter 5, two specific HBV variants most commonly found in association with HCC (ie., G1896A and A1762T/G1764A) were analysed using *in vitro* hepatocyte models of HBV infection. Both viral replication and host cell responses were explored with regards to these mutants associated with liver cirrhosis and cancer. Lastly, chapter 6 contains an in-depth discussion of the findings from this thesis, their significance, and their limitations. Outstanding questions are highlighted with future research direction and experimentation suggested to tackle these areas of ambiguity that may further advance our current understanding of molecular and clinical HBV and HBV-related malignancies.

CHAPTER 2: HEPATITIS B VIRUS (HBV) GENOME DETECTION AND GENOTYPING IN VIRALLY SUPPRESSED PATIENTS USING NESTED POLYMERASE CHAIN REACTION-BASED SANGER SEQUENCING

2.1 Abstract

Hepatitis B virus (HBV) genotypes have important clinical implications. Current genotyping methods are less sensitive in patients with ultra-low HBV viral load. We report a highly sensitive and specific nested polymerase chain reaction (PCR) assay for genotyping patient HBV. Total DNA derived from plasma of 14 (HBsAg+ and/or HBsAg-) HBcAb+ patients was used for HBV-specific nested PCRs targeting the preC/C, X/BCP/preC, and surface regions. All patients were treated with long-term nucleos(t)ide analogues (NAs), and 12/14 have undetectable viremia (clinical PCR: sensitivity >10 IU/mL). Surface amplicons were sequenced, aligned with reference genomes, and used in phylogenetic tree construction to determine genotype. HBV DNA was detected in 14/14, including 3 occult (HBsAg-/HBcAb+) cases. Genotypes identified were 6/14 B, 6/14 C, and 2/14 D. This assay in virologically suppressed patients may be useful for future studies requiring genotype prior to assessment of immunomodulatory and/or direct acting anti-viral therapeutics in patients on potent NAs.

2.2 Introduction

There are an estimated 240 million hepatitis B virus (HBV) surface antigen (HBsAg) positive chronic hepatitis B (CHB) carriers worldwide who are at risk of severe liver complications including cirrhosis, liver failure, or primary liver cancer (i.e. hepatocellular carcinoma, HCC) (2). Current first-line anti-viral therapies (i.e. nucleos(t)ide analogues) target the error-prone reverse transcriptase polymerase of HBV. Although these therapies lack the ability to directly

target the covalently closed circular DNA (cccDNA) of HBV, they are effective in significantly reducing viral load or DNA to low levels.

Occult HBV infection is characterized by HBsAg negativity, but with detectable HBV DNA by using highly sensitive assays in serum, peripheral blood mononuclear cells or in liver (41). Both HBsAg positive individuals with low-level (ie, HBV DNA <2000 IU/mL) as well as those with occult HBV remain at continual risk of liver disease. Numerous studies have suggested that risk of primary liver cancer (HCC) in low-level HBV CHB carriers is not negligible and remains higher than those without HBV infection, particularly when additional liver co-morbidities, such as cirrhosis, are present (183–186). Occult HBV is correlated with liver damage, cirrhosis, HCC severity, and characterized viral oncogenic risk factors (preS mutations) suggesting contributing roles towards liver cancer development (47,187,188). These clinical implications are further supported by reports that individuals with occult HBV are also subject to viral integration, which has inherent oncogenic risk factors and can affect oncogenes and tumor suppressor genes (51,160). In addition, we have previously reported that single nucleotide polymorphisms (SNPs) in the HBV basal core promotor/X gene region, which are associated with liver cancer and end-stage liver disease, persist in low-level and occult HBV patients even many years post-liver transplantation (189).

Due to error-prone replication, the HBV exists as a quasi-species population. There are 10 HBV genotypes, labeled A to J, that have been identified based upon a divergence of >7.5% in the full HBV genome sequence (24). The distribution of HBV genotypes is geographically distinct with certain genotypes endemic in specific regions. For example, genotypes B and C are predominant in East Asia whereas genotype D is prevalent in central Asia and the Mediterranean. However, regions with larger influxes of immigrants, such as Canada, are prone

to a large diversity of HBV genotypes reflective of the origin of the migrants (121,190). HBV genotypes may be associated with distinct clinical outcomes and response to therapy. For example, patients chronically infected with genotype A have improved responses to IFN-based anti-HBV therapies, those with genotype B are linked to earlier seroconversion to HBeAg negativity, and genotype C is highly associated with hepatocellular carcinoma development (38,119). These clinical differences emphasize the value of accurate genotype determination allowing a tailored approach to management of hepatitis B (i.e. precision medicine), particularly in genotypically and ethnically diverse regions such as Canada.

A variety of different techniques are available for genotyping of HBV. The gold standard for genotype determination involves sequence and phylogenetic analysis of the full length HBV genome (191). This approach lacks the large-scale cost-effectiveness and efficiency for regular use in clinical disease management. Further, full genome amplification of HBV is challenging particularly in low viremia CHB carriers. The INNO-LiPa HBV Genotyping kit (Fujirebio US, Inc., Malvern, USA) is commonly used for genotype determination of HBV, and may also be used for determination of drug resistant mutations. This commercial kit utilizes biotin labeled amplicons followed by hybridization with genotype specific probes. However, the conventional INNO-LiPa kit lacks the sensitivity for consistently determining the genotype of low-level or clinically undetectable HBV (i.e. <10 IU/mL) (192). In addition, the cost of the INNO-LiPa kit may restrict usage on a large-scale clinical setting, particularly in developing endemic countries.

Generally, the detection and genotype determination of low-level and occult HBV remains a challenge, particularly in CHB carriers that are successfully treated with potent anti-viral therapies. Thus, in our current study we report the robust use of a nested PCR based technique for accurate detection of HBV DNA in clinically undetectable and/or occult CHB carriers. We

adapted the direct and nested PCR of the INNO-LiPa in combination with sequencing and phylogenetic tree analysis for genotyping. We tested a representative cohort of patients with undetectable HBV DNA according to clinical PCR assay, in which HBV DNA was detected in the plasma by in-house nested PCR, and different HBV genotypes were determined.

2.3 Methods

2.3.1 Summary of chronic HBV carriers and samples collected

This study was approved by the University of Calgary conjoint health research ethics board, CHREB (Ethics ID 16636). All subjects were recruited from the Calgary Liver Clinic, University of Calgary and provided informed written consent to participate according to the guidelines of the 1975 Declaration of Helsinki. Inclusion criteria selected for CHB carriers receiving potent anti-HBV therapy with undetectable HBV DNA in plasma according to clinical PCR assay. Approximately 50 mL of whole blood was collected in EDTA vacutainers from patients for isolation of plasma in addition to relevant clinical and treatment data obtained at the time of sample collection. Viral load measurements closest to date of sample collection, (minimum of 3 months before or after) were collected. HBV DNA viral load was quantified according to clinical PCR assay: Abbott m2000 RealTime HBV Viral Load (sensitivity 10 IU/mL or ~50 virus genome copies/mL).

2.3.2 Isolation and detection of HBV DNA from plasma

Total DNA was isolated from 500 μL of plasma using standard phenol chloroform extraction with ethanol precipitation and resuspended into 50 μL of water. 10 μL of extracted DNA was used for detection of HBV by nested PCR using primers specific for the HBV preC/C, X/BCP/preC, and surface (S) genomic regions (Table S2.1). 5 μL and 2 μL of the direct round

PCR products were used as template for the nested round to account for potential inhibitory effects of excessive template in the second reaction. The X/BCP/preC and surface genomic region PCR protocols were adapted from Takahashi *et al*. and the INNO-LiPA HBV genotyping assay, respectively (193). The preC/C and X/BCP/preC nested PCR techniques have been previously reported (126,128,189). The DNA templates were added to a PCR mastermix consisting of 5 μL of 10X PCR Buffer (Takara Bio USA Inc., Mountain View, USA), 4 μL of 10mM dNTPs (Takara Bio USA Inc.), 1 μL of each primer at 10 μM, and 0.25 μL of TaKaRa Taq DNA polymerase HotStart (Takara Bio USA Inc.) in a final 50 μL reaction volume followed by amplification with a standard thermocycler (T100 Thermal cycler, Bio-Rad Laboratories [Canada] Ltd., Mississauga, CAN) (Table S2.2). PCR products were analyzed with 1% agarose gel electrophoresis. Assay sensitivities for HBV DNA detection with preC/C and X/BCP/preC nested PCRs were <10 virus genome equivalents (vge)/mL of plasma (128,189). Surface region nested PCR sensitivity was assessed as per methods described below.

DNA extraction and PCR set-up were performed in separate locations to minimize carryover of HBV sequences. Negative controls included “mock” DNA isolations with water and plasma from healthy HBV negative individuals under strict conditions to exclude possible environmental risk of contamination. Ultra-low PCR positive control included plasmid DNA containing one full genome copy of the HBV genome constructed in-house.

2.3.3 Evaluation of the sensitivity of the HBV surface genomic region nested PCR

Plasmid DNA containing a single full genome copy of the HBV genome was constructed in-house. Briefly, HBV full genome was amplified from phenol-chloroform DNA isolated from a high viral load clinical sample (>10^8 IU/mL) with high-fidelity Phusion DNA polymerase (New England Biolabs, Whitby, Ontario, Canada) as per manufacturer's protocols. HBV FG P1 and P2

primers for amplification (Tables S2.1, S2.2) were derived from Günther *et al*. (194). PCR amplicons were purified from a 1% agarose gel followed by digestion with HindIII (New England Biolabs) and ligated with T4 DNA ligase into the cloning vector pUC19 which was similarly prepared. Ligations were transformed into TOP10 *E. coli* chemically competent cells (Invitrogen, Carlsbad, USA) and successful clones were identified and confirmed via PCR and sequencing respectively with standard M13 primers (Table S2.1).

Following plasmid isolation with the GenElute plasmid miniprep kit (Millipore Sigma, Oakville, Ontario, Canada), cloned HBV copy number was determined via an established in-house quantitative PCR targeting the HBV surface genomic region (127). 2 sets of 10-fold serial dilutions of the plasmid from 10^3 to 10^{-2} and 2×10^3 to 2×10^{-2} copies/μL were prepared. 1 μL of each dilution was used as template in HBV surface genomic region nested PCR as described above in 3 separate experimental replicates. In addition, to simulate our described extraction and PCR technique, 2 sets of 10-fold serial dilutions of the plasmid from 10^3 to 10^{-2} and 5×10^3 to 5×10^{-2} IU/μL were prepared. Copies/μL calculated in-house were converted to IU/μL using a conversion factor of 5 copies = IU. 1 μL of each dilution was used to inoculate 500 μL of healthy (ie., HBV negative) plasma followed by total DNA extraction and HBV surface genomic region nested PCR as described above in 3 separate experimental replicates. Healthy plasma used in plasmid inoculation was also subjected to DNA extraction and nested PCR as a negative control.

PCR mastermix preparations were performed in a dedicated location to minimize carryover of HBV sequences and environmental contamination. Negative control of water was used to exclude possible environmental risk of contamination. PCR positive control consisted of 10^7 copies of the house-made plasmid DNA.

2.3.4 Determination of HBV genotype and phylogenetic tree analysis

HBV surface genomic region PCR amplicons from clinical samples were agarose gel purified (QIAquick gel extraction kit, Qiagen, Hilden, DEU) followed by bidirectional in-house Sanger sequencing (University of Calgary Core DNA services, Calgary, Alberta, Canada) with the nested PCR primers (HBPr75 and HBPr94). Sequencing results were aligned with reference HBV genomes representative of the major genotypes (Table S2.3) using MAFFT version 7 (https://mafft.cbrc.jp/alignment/server/). Phylogenetic analysis was performed using the MEGA software version 7 (195) to construct a maximum likelihood tree with the Kimura-2-parameter substitution method and 1000 bootstrapping replicates. Ambiguous results from phylogenetic analysis were confirmed with the NCBI genotype sequencing tool for HBV (https://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). Sequence alignments were also used for identification of SNPs implicated in HBV immune escape and drug resistance.

2.4 Results

2.4.1 Summary of clinical and virological data (Table 2.1)

In total, 14 CHB carriers (4 females; median age 55.5 [IQR: 44–65.75], 1 African, 13 Asians) were enrolled in this study (Table 2.1). All subjects recruited received potent first-line nucleos(t)ide analogues for anti-HBV therapy (3 entecavir, 11 tenofovir disporoil fumurate). Two cases (21–2 and 33–2) with detectable levels of HBV (>10 IU/mL) and clinical genotyping results via the INNO-LiPa kit were included for validation of our genotyping methodology. Our cohort includes 12 representative cases with clinically undetectable HBV on nucleos(t)ide analog therapy classified into 3 groups according to liver disease sequelae: (1) 4/12 (33.3%) were liver transplant recipients for HBV-related liver failure. 1/4 transplant recipients subsequently had viral breakthrough (HBsAg positive recurrence) with the remaining 3/4 continually identified as occult HBV infection with negative HBsAg). (2) 5/12 (41.7%) CHB carriers were cirrhotic of

which 3 have progressed to HCC. (3) The remaining 3 of 12 (25%) clinically undetectable cases were treated based on guidelines for active hepatitis B but have no known HBV-related liver co-morbidities.

Table 2.1. Clinical and virological features of 14 CHB carriers with potent nucleos/tide analog therapy included in our cohort. 12 cases are representative of CHB carriers at various stages of disease with clinically undetectable viremia.

CASE ID#	AGE / SEX	ETHNICITY	VIRAL LOAD (IU/ML)*	ANTI-VIRAL THERAPY	ADDITIONAL NOTES
21-2	50 / M	AS	340	TDF	GENOTYPE B^{+}
33-2	57 / M	AS	17000	TDF	GENOTYPE B^{+}
14	48 / M	AS	U	TDF	HCC CIRRHOSIS
64	65 / M	AS	U	TDF HBIG	POST-LT
75	54 / M	AS	U	TDF HBIG	POST-LT OCCULT HBV
87	32 / F	AF	U	TDF HBIG	POST-LT OCCULT HBV
94-3	44 / M	AS	U	TDF	
113-2	62 / M	AS	U	TDF	CIRRHOSIS
114-2	40 / M	AS	U	ETV	
191	68 / M	AS	U	TDF	HCC CIRRHOSIS
200	28 / F	AS	U	TDF HBIG	POST-LT OCCULT HBV
247	69 / M	AS	U	ETV	
266	73 / F	AS	U	ETV	HCC CIRRHOSIS
311	58 / F	AS	U	TDF	CIRRHOSIS
TOTAL	Median age = 55.5 4 / 14 F	1 African (AF) 13 Asian (AS)	12 Undetectable (U)	3 Entecavir (ETV) 11 Tenofovir (TDF) 4 Hepatitis B immune globulin (HBIG)	

*Determined by clinical PCR assay, Abbott m2000 RealTime HBV Viral Load (sensitivity >10 IU/mL; 1 IU/mL = ~5.26 copies/mL)

$^{+}$Genotype determined in clinic using INNO-LiPa Assay

2.4.2 Low level HBV DNA is detectable by the surface genomic nested PCR

To evaluate the sensitivity of our surface genomic nested PCR, serial dilutions of a house-made plasmid containing a single copy of the HBV full genome (pUC19-HBV) was amplified. 10^{3} to 10^{-2} copies and 2×10^{3} to 2×10^{-2} copies of the plasmid HBV (equivalent to approximately 2×10^{-1} to 2×10^{2} IU and 4×10^{-1} to 4×10^{3} IU, respectively) in log10 serial dilutions were tested. Although, the direct round PCR was insufficient for detection of the HBV DNA in both dilution series, all samples ≥1 copy/reaction were detectable in the second (nested) round of PCR (Figure S2.1) thereby demonstrating the high sensitivity of our assay. We further demonstrate the effectiveness of the nested PCR by inoculating healthy plasma with HBV plasmids in serial dilutions (1×10^{-2} to 1×10^{3} IU and 5×10^{-2} to 5×10^{3} IU). HBV DNA in plasma was reliably detected in 500 μL of plasma with addition of 5 IU of plasmid HBV (approximately 25 copies), although faint bands were consistently detected at inoculation of 1 IU (approximately 5 copies) of HBV genomes (Figure 2.1). Similarly, our PCR assay was evaluated in plasma collected from 11 additional CHB carriers with detectable HBV DNA via clinical assays (Abbott m2000 RealTime HBV viral load), but unquantifiable viral load (i.e. <10 IU/mL). 10 of the 11 (90.9%) of these samples were detectable by the HBV surface genomic region nested PCR (Table 2.2).

Table 2.2. Plasma from 11 CHB carriers with detectable HBV viremia, but unquantifiable viral load as determined by clinical PCR assay (Abbott m2000 RealTime HBV viral load: sensitivity >10 IU/mL) was evaluated by nested PCR targeting HBV preC/C, X/BCP/preC, and surface genomic regions.

CASE ID#	PREC/C NESTED PCR	X/BCP/PREC NESTED PCR	SURFACE GENOMIC NESTED PCR
2-2	+	+	+
6-2	+	+	+
13-2		+	
24-2		+	+
83		+	+
261	+	+	+
265	+	+	+
302	+	+	+
313	+	+	+
349	+	+	+
352	+	+	+
TOTAL	8	11	10

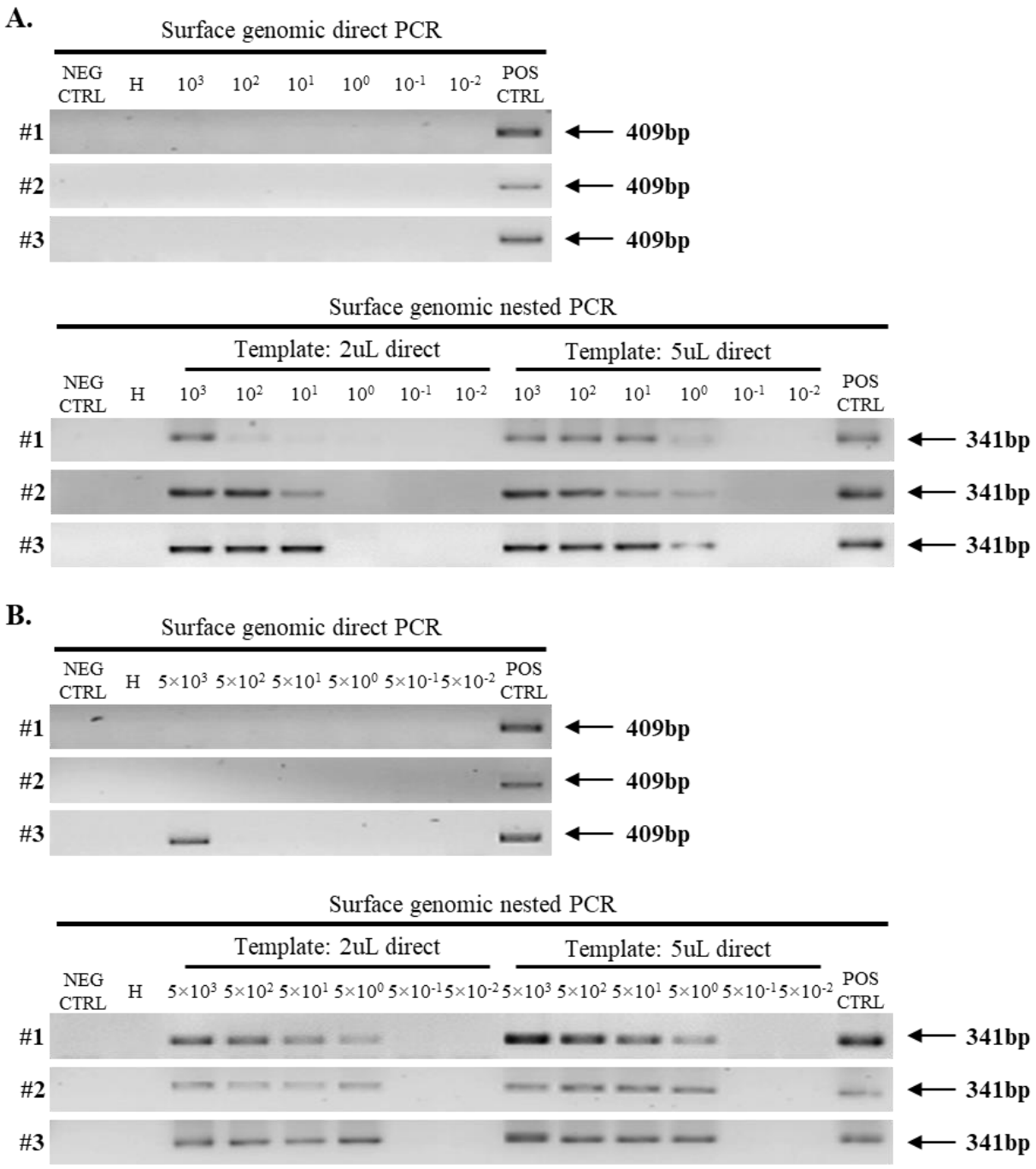


Figure 2.1. Surface genomic region nested PCR was evaluated by inoculating healthy HBV negative patient plasma with 1× HBV genome containing plasmid in 3 different sets of experimental replicates. Total DNA extracted via phenol-chloroform was subjected to direct and nested PCR for HBV surface genomic region. PCR products

from the **A.** plasmid serial dilution 1 (10^3 to 10^{-2} IU/extraction) and the **B.** plasmid serial dilution 2 (5×10^3 to 5×10^{-2} IU/extraction) were analyzed on a 1% agarose gel. 2uL and 5uL of the direct round PCR was used as template in the nested round PCR. Negative included a no template control (ie., water) and healthy (H) HBV negative plasma. 10^7 copies of the HBV full genome plasmid were utilized as a PCR positive.

2.4.3 HBV DNA is consistently detectable in the plasma of clinically undetectable and low-level CHB carriers

We evaluated the surface genomic nested PCR alongside previously established methods of detection used in our laboratory including the preC/C and X/BCP/preC nested PCR (126–128,189,196). HBV DNA was detected in the plasma of all 14 cases with primers specific for the preC/C, X/BCP/preC, and/or surface genomic regions (Table 2.3), despite undetectable viral load by clinical PCR assay in 12/14 (85.7%). Nested PCR consensus was observed in 14/14 (100%) cases with amplification by two or more HBV-specific primer sets and in 11/14 (78.5%) cases with amplification by all 3 primer sets which includes 3 HBsAg negative, putative occult hepatitis B cases (case ID# 75, 87, 200). Detection rate was similar in the X/BCP/preC and surface genomic nested PCRs with HBV DNA amplified in all cases.

Table 2.3. Detection of HBV DNA in the plasma of 14 CHB carriers of which 12 have clinically undetectable viremia with nested PCR targeting HBV preC/C, X/BCP/preC, and surface genomic regions. Surface genomic region amplicons were bidirectionally sequenced for phylogenetic analysis to determine genotype.

CASE ID#	VIRAL LOAD (IU/ML)*	PREC/C NESTED PCR	X/BCP/PREC NESTED PCR	SURFACE GENOMIC NESTED PCR	GENOTYPE
21-2	340	+	+	+	B
33-2	217	+	+	+	B
14	U	+	+	+	C
64	U		+	+	C
75	U	+	+	+	C
87	U	+	+	+	D
94-3	U	+	+	+	B
113-2	U	+	+	+	D
114-2	U	+	+	+	B
191	U	+	+	+	C
200	U	+	+	+	B
247	U		+	+	C
266	U	+	+	+	C
311	U		+	+	B
TOTAL	12 U	11	14	14	6 B / 6 C / 2 D

*Determined by clinical PCR assay, Abbott m2000 RealTime HBV Viral Load (sensitivity >10 IU/mL; 1 IU/mL = ~5.26 copies/mL)

2.4.4 Determination of HBV genotypes and simultaneous identification of clinically important SNPs present in the plasma and PBMC

HBV genotype of the amplified surface genomic region was determined by analysis of phylogenetic clusters, thereby revealing 6 genotype B, 6 genotype C, 2 genotype D (Table 2.3, Figure 2.2). The primary genotypes detected, B and C, correlates directly with our cohort demographics which is pre-dominantly (92.9%) individuals of Asian ethnicity. Genotypes determined of the two clinically detectable cases (ID# 21–2 and 33–2) directly matched the clinical results. As our sequencing region overlaps with the HBV surface and polymerase genes, we simultaneously evaluated the presence of SNPs with clinical importance including the immune escape (sG145R) and nucleos/tide analogue resistant variants (rtI169T, V173 L, L180 M, A181V/T, T184S/A/I/L/G/C/M, A194T, T184G/S, S202I/G, M204I/V). No SNPs were identified in the analyzed samples.

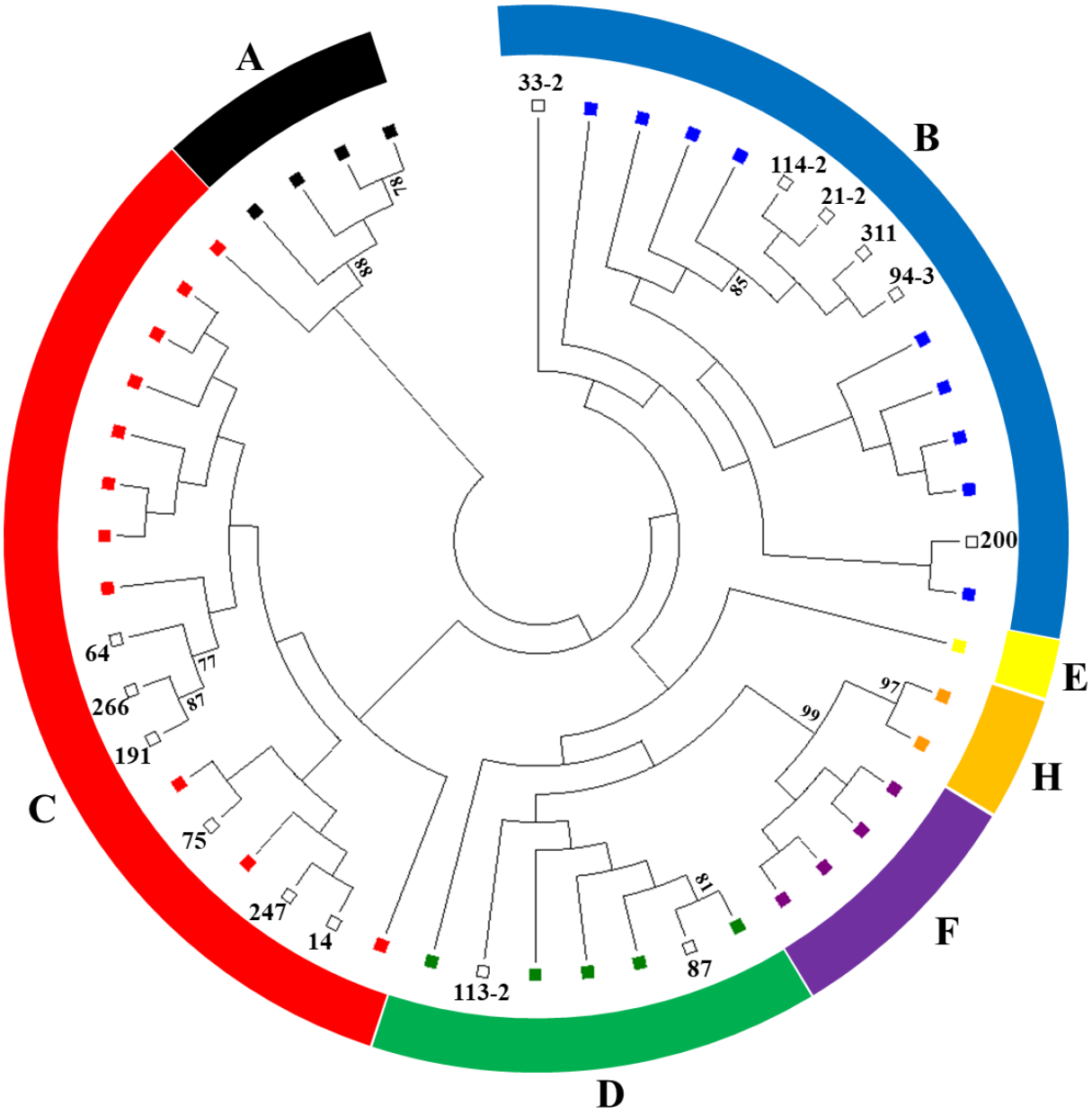


Figure 2.2. HBV genotype determination by maximal likelihood constructed phylogenetic analysis of HBV reference sequences (filled square labels) with HBV surface genomic nested PCR amplicons derived from cohort plasma samples (unfilled square labels). Sequences were aligned with MAFFT and trimmed to remove nested PCR primer sequences to a final length of 297bp. 1000 bootstrap replicates were conducted under the Kimura-2-parameter with MEGA7. Branch nodes with bootstrap

values >70 are included next to the corresponding node. Viral genotypes B to D were identified in 12 plasma samples derived from chronic hepatitis B carriers with clinically undetectable viremia. 2 cases (21-2 and 33-2) previously analysed with INNO-LiPa was similarly identified as genotype B.

2.5 Discussion

The HBV genotype may be clinically important in determining disease management and prognosis (38). Common genotyping methodologies of full genome sequencing and the INNO-LiPa kit are subject to certain limitations, particularly with detection and genotype determination in patients with undetectable (<10 IU/mL) HBV viral load (191,192). In the current study, we evaluated plasma samples from 14 patients, 3 of whom are classified as occult HBV with HBsAg negative serology. All cases included in this study received potent nucleos(t)ide analogue anti-HBV therapy and 12/14 showed undetectable viral load according to clinical PCR assay. Despite the clinical viral suppression, we amplified HBV DNA in all patient samples. All 14 cases were consistently detected with at least two of the three HBV-specific nested PCRs. We have previously utilized the preC/C and X/BCP/preC nested PCRs for detection of persistent and low level HBV in diverse settings including HIV/HBV co-infected, CHB carriers with anti-viral therapies, and post-liver transplant individuals (127,166,189).

The clinical implications of chronic low-level HBV replication have yet to be fully elucidated. Studies have suggested that this phenomenon is correlated with a variety of hepatic and extrahepatic complications including cirrhosis, hepatocellular carcinoma, lymphoma, and leukemia (53,54,183–186). Current clinical methods of HBV viral load quantification and detection are limited by a lower detection limit of 6 IU/mL (~30 vge/mL) via the COBAS TaqMan assay. We demonstrate a HBV surface genomic nested PCR technique capable of

detecting ultra-low levels of HBV DNA within a sample. Our study was limited by the inability to accurately quantify HBV DNA at very low levels of plasmid (Figure 2.1), thereby hindering identification of a lower limit of detection for our assay. Furthermore, to convert between copies of plasmid calculated in-house and IU/mL, we used the approximate factor of 5 thereby introducing potential error into our analysis of the surface genomic region nested PCR (197). Nonetheless, in combination with direct Sanger sequencing, the PCR strategy utilized in this study was effectively and efficiently used for all clinical samples tested. However, a major caveat of this highly sensitive method of HBV detection is the concern of contamination. In our current study, we used strict precautions to exclude environmental contamination and HBV DNA carryover including dedicated working areas, equipment, and reagents. Subsequent application of this technique in a clinical or reference laboratory setting should take these concerns into careful consideration to ensure accurate and reliable results.

Our study cohort was mostly Asian infected by either vertical or early childhood (horizontal) infection, but we were able to successfully determine genotype determination in patients with diverse ethnic backgrounds with HBV genotypes (from B to D). This diversity of HBV genotypes is expected and in line with previous reports of genotype distribution in immigrant-rich nations, including Canada (121,190). Additionally, we demonstrate the effectiveness of this genotyping technique in a wide variety of CHB disease stages including non-cirrhotic, cirrhotic, liver cancer, and post-liver transplant in clinically undetectable cases. We have also previously applied this genotyping methodology to characterize HBV persistence in a larger post-liver transplant cohort (189). In 2 cases (ID# 21–2 and 33–2), the HBV genotype was known pre-treatment (determined by clinical INNO-LiPa), and our reported in-house genotyping analysis produced identical results.

Appropriate detection and genotype analysis of HBV is of increasing importance for precision medicine, particularly with management of CHB and novel developments of a variety of anti-HBV immunomodulatory and direct acting therapeutics. Differences in HBV genotype may impact the progression of CHB or influence the development of liver comorbidities. For example, the increased risk of HCC associated with HBV genotype C is well documented in the REVEAL-HBV study (38,84,198). However, aside from HCC risks, additional effects of viral genotype in disease are poorly understood. With further indications of the role of HBV genotype towards disease progression, patients could be stratified for improved CHB management such as better surveillance of co-morbidities in those with higher risks and specific selection of anti-viral therapies. Previously, it has been demonstrated that HBV genotype correlates with treatment responses to pegylated-interferon (69,199). Similarly, clinical success of novel immunomodulatory anti-viral drugs such as the toll-like receptor (TLR) agonists could be affected by HBV genotype. Indeed, Visvanathan and colleagues (200) have demonstrated that TLR signaling pathways and expression varies with HBV genotype, thus likely influencing responses to TLR agonist therapies.

The simultaneous identification of common drug resistant mutations is possible albeit none were detected in our cohort. Our strategy for HBV genotype could be adapted in clinical or reference laboratory use, with a standardized protocol and methodology, but requires strict precautions and training of laboratory personnel to avoid contamination. The costs associated with this technique are restrained by the lack of plasmid cloning or full HBV genome amplification as required by current gold standard genotyping. Standard PCR reagents and equipment are sufficient for reliable amplification of the target HBV regions thus limiting requirement for specialized equipment or kits. Free-to-use software similar to those applied in

this study are readily available for data analysis of sequence alignments and phylogenetic analysis. The target region amplified coincides with important sites of HBV mutation such as immune escape and drug resistant SNPs which can be simultaneously evaluated thus eliminating multiple work-flows for detection, genotyping, and mutational analysis. In conclusion, we report a sensitive and effective strategy for detecting and genotyping HBV virologically suppressed patients with hepatitis B virus infection.

2.6 Acknowledgements

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2.7 Disclosures and Conflict of Interest

CSC has served as an advisory board member and/or has received research support from Bristol Myers Squibb, Glaxo Smith Kline, Gilead Sciences, Janssen and Merck.

The other authors have no relevant disclosures.

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2.9 Ethics ID

The study received approval from the local ethics review board committee at the University of Calgary (i.e., Conjoint Ethics Review Board, Ethics REB-16636).

CHAPTER 3: DEEP SEQUENCING SHOWS LOW-LEVEL ONCOGENIC HEPATITIS B VIRUS VARIANTS PERSISTS POST–LIVER TRANSPLANT DESPITE POTENT ANTI-HBV PROPHYLAXIS

3.1 Abstract

Recent studies suggest that withdrawal of hepatitis B immune globulin (HBIG) and nucleos(t)ide analogues (NA) prophylaxis may be considered in HBV surface antigen (HBsAg)-negative liver transplant (LT) recipients with a low risk of disease recurrence. However, the frequency of occult HBV infection (OBI) and HBV variants after LT in the current era of potent NA therapy is unknown. Twelve LT recipients on prophylaxis were tested in matched plasma and peripheral blood mononuclear cells (PBMCs) for HBV quasispecies by in-house nested PCR and next-generation sequencing of amplicons. HBV covalently closed circular DNA (cccDNA) was detected in Hirt DNA isolated from PBMCs with cccDNA-specific primers and confirmed by nucleic acid hybridization and Sanger sequencing. HBV mRNA in PBMC was detected with reverse-transcriptase nested PCR. In LT recipients on immunosuppressive therapy (10/12 male; median age 57.5 [IQR: 39.8-66.5]; median follow-up post-LT 60 months; 6 pre-LT hepatocellular carcinoma [HCC]), 9 were HBsAg-. HBV DNA was detected in all plasma and PBMC tested; cccDNA and/or mRNA was detected in the PBMC of 10/12 patients. Significant HBV quasispecies diversity (ie 143-2212 nonredundant HBV species) was noted in both sites, and single nucleotide polymorphisms associated with cirrhosis and HCC were detected at varying frequencies. In conclusion, OBI and HBV variants associated with severe liver disease persist in LT recipients on prophylaxis. Although HBV control and cccDNA transcriptional

silencing may occur despite immunosuppression, complete virological eradication does not occur in LT recipients with a history of HBV-related end-stage liver disease.

3.2 Introduction

There are an estimated 240 million hepatitis B virus (HBV) surface antigen (HBsAg)-positive chronic hepatitis B (CHB) carriers worldwide who are at risk of cirrhosis and hepatocellular carcinoma (HCC) (2). A virological cure for CHB is not possible with current therapies due to the presence of a stable intranuclear episomal form of the HBV genome called covalently closed circular or cccDNA that persists for the life of the hepatocyte (201). Consequently, even after HBsAg clearance, traces of replication-competent HBV DNA are present in the nuclei of infected hepatocytes (202). Such individuals are diagnosed with occult HBV infection (OBI) with low-level HBV DNA detected in serum and liver with or without antibodies to HBV core antigen (anti-HBc) and HBsAg (anti-HBs) (202). Individuals with OBI who lose immune control due to intense immunosuppression such as bone marrow transplantation or receiving B-cell-depleting therapies (ie anti-CD20 monoclonal antibodies) are at risk of HBV reactivation and reappearance of circulating HBsAg (203).

Liver transplantation (LT) is an effective therapy for HBV-related end-stage liver disease. Historically, CHB was a contraindication to LT due to severe HBV recurrence, likely originating from extrahepatic reservoirs such as peripheral blood mononuclear cells (PBMCs) (147,161,204,205). Prophylaxis with hepatitis B immune globulin (HBIG) and potent oral nucleos/tide analogues (NAs) can now prevent HBsAg reappearance and overt HBV recurrence (149) yet OBI can persist after LT (148). Recent reports suggest that gradual minimization of prophylaxis with discontinuation of HBIG and then NA may be possible in LT recipients with low risk of disease recurrence (150–153). The argument for complete withdrawal is based on the

fact that in the recent transplant era, many LT recipients receive more potent NA prior to LT and have continually suppressed serum HBV DNA at the time of transplant. However, in the current era of potent NA, the potential impact of extrahepatic OBI on HBV relapse risk following prophylaxis withdrawal is unclear (42).

In CHB, HCC development has been associated with HBV genotypes and variants (ie genotype C and mutations in the HBV precore/basal core promoter and X gene) (198). Most notably, the A1762T and G1764A double mutations are consistently associated with cirrhosis and HCC (136,138,139,141). In addition, specific variants T1674C/G, A1752G, T1753V, T1768A, C1773T, A1846T, G1896A and G1899A are frequently reported to increase HCC risk (136,138,139,141). LT is curative for HBV-related end-stage liver disease, and post-LT HCC recurrence is rare in carefully selected patients (206–208). However, it is unknown whether circulating HBV mutants associated with HCC and/or cirrhosis can persist long term after transplant, despite removal of the main viral reservoir, explant liver.

In the current study, we evaluated 12 LT recipients for the presence of low-level HBV genomes, including HBV cccDNA in PBMC for up to 60 months post-LT. We found that low-level HBV DNA including HBV genotypes and variants associated with oncogenesis persist long term in both HBsAg+ and HBsAg- post-LT recipients despite HBIG and potent oral NAs therapy.

3.3 Methods

3.3.1 Summary of liver transplant recipients and samples collected

This study was approved by the University of Calgary Conjoint Health Research Ethics Board, CHREB (Ethics ID 16636). All subjects were recruited from the Southern Alberta Liver

Transplant Clinic, University of Calgary, and provided informed written consent to participate according to the guidelines of the 1975 Declaration of Helsinki. Whole blood was collected from patients for isolation of plasma and PBMCs using a Ficoll gradient. All patients were followed as per standard of care post-transplant. Prior to 2008, most LT recipients with HBV-related end-stage liver disease received first-generation oral NAs (ie lamivudine and/or adefovir) given pre-LT and continued post-LT, as well as high-dose i.v. HBIG in the anhepatic period followed by i.v. HBIG weekly, then 2-3 monthly to maintain anti-HBs titres >100 IU/L indefinitely. In the recent era, most LT patients received more potent tenofovir (or entecavir pre- and post-LT as well as HBIG for the first 2 years after LT) (209). HBV DNA monitoring (by PCR assay: Roche TaqMan sensitivity >55 IU/mL or Abbott Architect sensitivity >10 IU/mL), testing for HBsAg and anti-HBs (Abbott Architect) and ultrasound screening for HCC (in those with history of HCC) were carried out as per transplant protocols according to treating hepatologist discretion (usually every 3-6 months post-LT). All patients received standard immunosuppression (usually Tacrolimus based), and stable patients were typically followed quarterly in the first year after transplant and then biannually/annually thereafter.

3.3.2 Detection of HBV DNA and messenger RNA (mRNA) in plasma and PBMC post–liver transplant

Total DNA was isolated from 500 μL of plasma and ~$2x10^7$ PBMCs using standard phenol chloroform extraction. PBMCs were pretreated with DNase/trypsin before extraction to remove extracellular HBV as previously described (126). The isolated DNA was tested by nested PCR using primers specific for the HBV preC/C region (direct: preCC DF and DR; nested: preCC NF and NR, Table S3.1) under the following conditions: denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1.5 minutes; final extension at

72°C for 10 minutes. Amplification of the HBV surface genomic region (direct: HBPr134 and 135; nested: HBPr75 and 94, Table S3.1) used the above conditions with an annealing temperature of 45°C and 30 second 72°C extension. The X/BCP/preC region was amplified with primers (Table S3.1) and reaction conditions as described by Takahashi *et al*. (193). Assay sensitivities for HBV DNA detection were <10 virus genome equivalents (vge)/mL of plasma and <10 vge/μg of total PBMC DNA (148).

Total RNA was isolated from ~$2x10^7$ PBMCs, pretreated with DNase/trypsin, using TRIzol™ reagent (Invitrogen, Carlsbad, USA). Potential DNA carry-over was digested with PerfeCTa® DNase I (Quanta Biosciences, Beverly, USA) followed with cDNA synthesis (qScript™, Quanta Biosciences). Total cDNA was tested by nested PCR using HBV preC/C region-specific primers and reaction conditions as described above. Human β-globulin housekeeping gene was also amplified using specific primers (βglo F and R, Table S3.1) with the generic conditions with an annealing temperature of 58°C and 72°C extension for 60 seconds.

Negative controls included "mock" DNA/RNA isolations with water or PBMC from healthy HBV-negative volunteers, cDNA synthesis without reverse transcriptase and PCR water, under strict conditions to exclude possible environmental risk of contamination. PCR-positive control included DNA isolated from serum or liver of an HBsAg+ individual.

3.3.3 Detection of HBV covalently closed circular DNA (cccDNA) by nested PCR/nucleic acid hybridization (NAH) assay

Protein-free DNA was isolated from ~$2x10^7$ PBMCs using Hirt DNA extraction as described (210). HBV relaxed circular DNA (rcDNA) was removed with T5 exonuclease (New England Biolabs, Ipswich, USA) digestion which was inactivated with addition of EDTA. HBV cccDNA

products were cleaned by column purification (E.Z.N.A. Cycle-Pure Kit, Omega Bio-tek, Norcross, USA). These products were PCR-amplified using primers specific for the nicked region of HBV (direct: cccDNA F1 and R1, Table S3.1) under the following conditions: 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 1.5 minutes; final extension at 72°C for 10 minutes. The direct PCR template was amplified by nested PCR (nested: cccDNA F2 and R2, Table S3.1) with the above conditions with annealing at 55°C, and extension at 72°C for 60 seconds. HBV cccDNA presence was confirmed with nucleic acid hybridization with an HBV nicked region-specific digoxigenin (DIG)-labelled probe using DIG Luminescent Detection Kit for Nucleic Acids (Roche Applied Science, Germany) according to manufacturer's instructions. Assay sensitivities for cccDNA were <100 copies/µg of total PBMC DNA (148). PCRs were performed in parallel with positive controls from liver of an HBsAg+ CHB carrier and negative controls as described above.

3.3.4 Analysis of HBV genotype and quasispecies by population and deep sequencing

Hepatitis B virus surface genomic region and cccDNA PCR amplicons were agarose gel purified (QIAquick Gel Extraction Kit, Qiagen, Hilden, DEU) followed by in-house Sanger sequencing (University of Calgary Core DNA Services). HBV cccDNA sequencing results were aligned with reference HBV genomes of genotypes B (accession# AB602818) and C (accession# AB014381) using MEGA version 6 (211). HBV surface gene sequencing results were used for genotype determination by comparison with NCBI BLAST and HBV genotyping tool. For NGS, adaptor sequences were added to HBV X/BCP/preC gel-extracted PCR products in a high-fidelity Phusion polymerase (New England Biolabs)-based reaction (NGS-X/BCP/preC F and R, Table S3.1) under the reaction conditions: 95°C for 3 minutes; 25 cycles of 98°C for 20 seconds, 55°C for 15 seconds, 72°C for 15 seconds. PCR amplicons were column purified (Omega Bio-

tek) and sequenced with the Illumina MiSeq platform (San Diego, USA). Reads were merged, filtered and cleaned with the Galaxy software to generate clusters of homologous sequences, grouped using a threshold of 99% similarity. These clusters are representative of genetically diverse HBV sequences/species and defined as quasispecies. These quasispecies were aligned using MAFFT version 7 (www.mafft.cbrc.jp) followed by nucleotide frequency determination at each position within the HBV quasispecies in the plasma and PBMC. NGS error rate was calculated using a cloned PCR-amplified HBV fragment as an internal control. Nonparametric statistical tests, Wilcoxon signed rank test and Mann-Whitney test, were used to compare median single nucleotide polymorphism (SNP) frequencies between compartments and between subgroups, respectively.

3.4 Results

3.4.1 Summary of clinical and virological Data (Table 3.1)

In total, 12 LT recipients (10 males; median age 57.5 [IQR: 39.8-66.5], 7 Asian, 3 Caucasian, 1 African, 1 Middle Eastern) were enrolled with a median follow-up of 60 months post-transplant (IQR: 11.3-114.3) (Table 3.1). All subjects recruited were serum HBsAg+ pre-LT and received anti-HBc-negative grafts. The indications for LT were fulminant hepatitis (n = 2), decompensated cirrhosis (n = 4) and HCC (n = 6). All 12 cases received HBV prophylaxis of HBIG and NA (Table 3.1). HBIG was stopped at 12-24 months after transplant with continued NA monotherapy. All patients received standard post-LT immunosuppression as per treating hepatologist discretion. Three patients initially treated with lamivudine post-LT had recurrent HBV infection (defined by serum HBsAg positivity with detectable HBV DNA by PCR [>10 IU/mL]) and were switched to tenofovir with suppressed HBV DNA thereafter with low-level viremia (<10, 13, and 217 IU/mL) at last follow-up. One patient died 152 months post-LT from a

non-HBV-related cause (colon cancer). The remaining 11 are stable with no reports of HCC recurrence, HBV-related disease or graft complications at last follow-up (Table 3.1).

Table 3.1. Clinical and virological features of post-LT cases with potent HBV prophylaxis.

Case ID: (Age / Sex / Ethnicity[1])	Follow-up post-LT (months)	LT indication[2]	Viral load, pre-LT (IU/mL)[3]	Viral load, sample collection (IU/mL)[3]	Post-LT HBV prophylaxis[4]	Time of post-LT HBV recurrence (months)[5]	Current status
53: (54 / M / As)	82	C	Unk	217	HBIG, TDF	36	Deceased Non-HBV
57: (67 / M / As)	48	HCC	U	U	HBIG, ETV	NR	Stable
64: (65 / M / As)	119	HCC	Unk	U	HBIG, TDF	41	Stable
68*: (45 / M / C)	5	HCC	U	U	HBIG, ETV	NR	Stable
72: (73 / M / As)	143	FH	Unk	U	HBIG. LMV	NR	Stable
75: (54 / M / As)	11	HCC	U	U	HBIG, TDF	NR	Stable
87: (32 / F / Af)	50	C	$6.6x10^6$	U	HBIG, TDF	NR	Stable
124: (38 / M / As)	6	C	$5.2x10^5$	U	HBIG, TDF	NR	Stable
126: (77 / F / C)	153	HCC	Unk	13	HBIG, TDF	52	Stable
178: (62 / M / Me)	70	HCC	Unk	U	HBIG, ETV	NR	Stable
200: (28 / F / As)	100	FH	Unk	U	HBIg, TDF	NR	Stable
263: (61/ M / C)	16	C	$3.3x10^3$	U	HBIg, ETV	NR	Stable

*Second sample (Case ID# 68-2) at 19 months post-LT is available.

[1]Af = African; As = Asian; C = Caucasian; Me = Middle Eastern

[2]C = Cirrhosis; FH = Fulminant hepatitis; HCC = Hepatocellular carcinoma

[3] U = undetectable; Unk = Unknown

[4]TDF = Tenofovir; ETV = Entecavir; LMV = Lamivudine

[5] NR = No HBV recurrence

3.4.2 Detection of low-level HBV DNA in matched plasma and PBMCs and cccDNA and mRNA in PBMC (Table 3.2)

HBV DNA was detected in the plasma in all 12 cases with primers specific for the preC/Core, X/BCP/preC and/or surface genomic regions (Table 3.2), even despite undetectable HBV DNA by PCR in 10/12 patients. Similarly, HBV DNA was detected in 12 cases (including a second PBMC sample from one case: ID# 68) by nested PCR. HBV cccDNA was detected in 8/10 available PBMC samples (80%) (Table 3.2), including one that was HBV mRNA-positive (Table 3.2).

3.4.3 HBV genotypes and variants associated with cirrhosis and HCC are present in the plasma and PBMC of LT recipients years after transplant

Hepatitis B virus genotyping was not routinely available pre-LT in all cases. However, HBV genotype determination in plasma after LT revealed that 7/12 (58.3%) cases carried genotype C, 2 genotype D, 2 genotype B and 1 genotype A (Table 3.2). In contrast, the dominant genotypes in the PBMC were C (n = 5) and B (n = 7) (Table 3.2). Thus, in 8/12 cases, the dominant genotype in the plasma differs from that of the PBMC. NGS analysis allowed for characterization of the quasispecies, as defined as genetically diverse clusters of HBV sequences, present in the plasma and PBMC. A large number of quasispecies were present in both the plasma (median = 1179; IQR: 594-1303) and PBMC (median = 578; IQR: 385-933) (Table 3.2). Although statistically insignificant (P = .15, Wilcoxon matched-pairs signed rank test), the number of HBV quasispecies in the plasma was noted to be generally higher than in the PBMC compartment. The mean error rate of the NGS of amplified X/BCP/preC region (nt1653-1959) was <1%.

Table 3.2. HBV genotype, quasi-species number, and detection of HBV DNA, mRNA, and cccDNA in plasma and PBMC from post-LT patients with a history of HBV-related end-stage liver diseases*.

CASE ID	PLASMA			PBMC				
	DNA	GENOTYPE	# REP QS	DNA	GENOTYPE	mRNA	cccDNA	# REP QS
53	+	B	N/A	+	B	-	N/A	1592
57	+	C	1263	+	B	-	+	916
64	+	C	766	+	C	-	+	1730
68	+	C	1272	+	C	-	+	534
68-2+	N/A	N/A	N/A	+	N/A	-	N/A	950
72	+	A	2213	+	C	-	+	351
75	+	C	677	+	C	-	+	459
87	+	D	1179	+	B	-	+	418
124	+	C	594	+	B	-	+	691
126	+	D	1793	+	B	-	-	234
178	+	C	143	+	B	+	-	715
200	+	B	1303	+	C	-	+	211
263	+	C	423	+	B	-	+	578

+Second sample (Case ID# 68-2) is available.

*HBV DNA was detected in both plasma and PBMC compartments from all post-LT cases. HBV genotype analysis of the surface genomic region revealed a predominance of genotype C in plasma (n=7) and PBMC (n=5). HBV cccDNA and mRNA was also detected from the PBMC of 8 and 1 case, respectively. A large number of representative quasi-species (#rep qs), defined as 1% difference in nucleotide sequence, were identified by NGS analysis of X/BCP/preC nested PCR amplicons in all amplified samples.

We analysed the HBV X/BCP/preC region in plasma and PBMC by NGS for specific SNPs previously reported to be associated with cirrhosis and HCC development (ie T1674C/G, A1752G, T1753V, A1762T, G1764A, T1768A, C1773T, A1846T, G1896A and G1899A) (136,138,139,141). Of these SNPs, A1752G is considered as a protective mutant and has been found more often in patients who do not develop HCC compared to those with a history of liver cancer (139). In our cohort of post-LT cases (Figure 3.1A), we observed a low median A1752G frequency of 1.57% (IQR: 0.84%-10.15%) of plasma HBV quasispecies and a median of 0.77% (IQR: 0.41%-1.30%) of PBMC HBV quasispecies. Interestingly, whilst some SNPs reported to be associated with increased cirrhosis and HCC risk (139,141) were observed at low frequencies (median 0%-1.33%: T1674C/G, T1768A, C1773T, Figure 3.1A), other SNPs were detected in both sites at much higher frequencies (median 6.01%-92.22%: T1753V, A1762T, G1764A, A1846T, G1896A and G1899A, Figure 3.1B). The median SNP frequencies of G1899A in plasma and G1764A in PBMC were dominant (>50%) within the HBV quasispecies of each respective compartment. Only one variant (C1773T) had a significantly higher frequency ($P < .001$) in the plasma compared to the PBMC compartment.

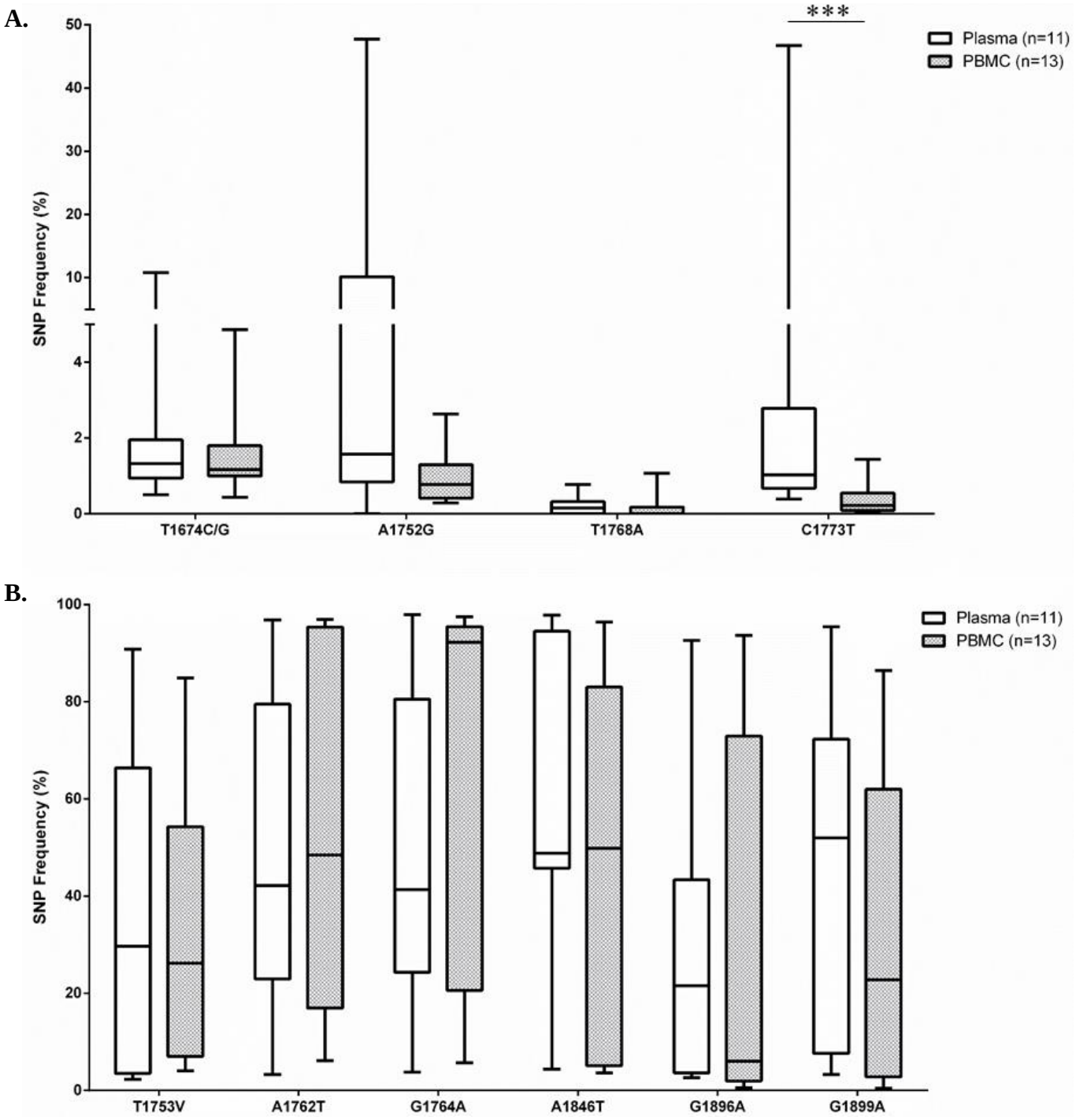


Figure 3.1. Frequency of SNPs in the HBV quasispecies of plasma and PBMC from post-LT cases. Nucleotide frequency analysis of HBV quasispecies revealed SNPs present at **A**, low median frequency mutations, and **B**, high median frequency mutations. With the exception of A1752G (observed at median SNP frequency of 1.57%), all

variants were associated with increased risk of HCC development. Only C1773T was observed to be higher in plasma than PBMC. ***P-value < .001 (Wilcoxon signed rank test)

3.4.4 The frequency of HBV genetic variants associated with HCC and cirrhosis differs by age of LT recipients

Current guidelines recommend that HCC surveillance starts at age 40 in Asian male chronic HBV carriers. The frequency of A1762T and G1764A mutations was significantly higher in plasma (P = .049) in post-LT cases <40 years of age (Figure 3.2). In the PBMC, only G1899A was significantly higher (P = .049) in the <40 age group. It is also interesting to note that all 3 of the post-LT cases <40 years of age (case# 87, 124 and 200) were non-HCC LT cases. In addition, no differences were observed in SNPs in either the plasma or PBMC compartments when cases were separated by LT indications (HCC vs non-HCC) or by overt HBV recurrence post-LT (HBsAg+ vs HBsAg-).

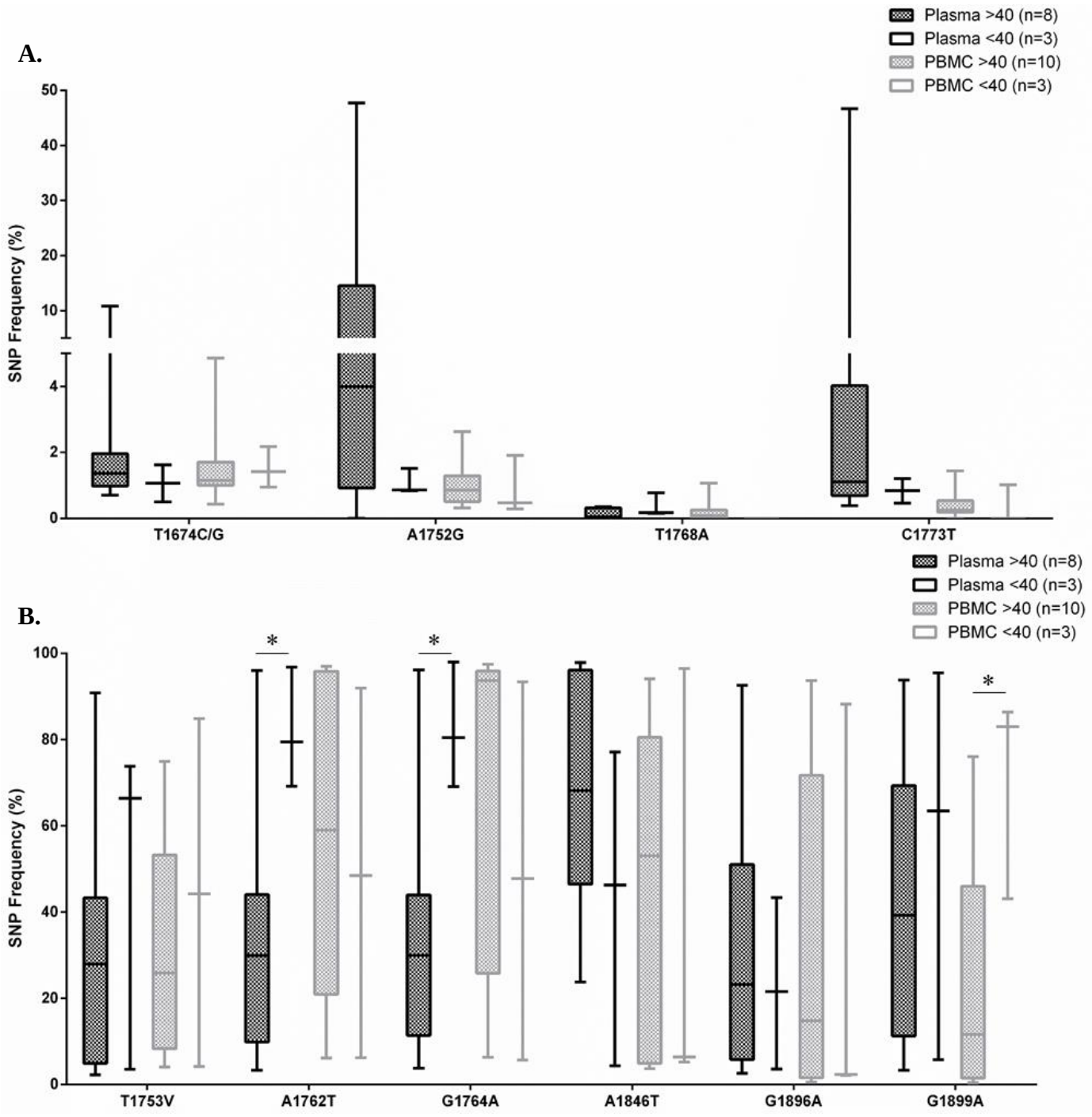


Figure 3.2. Frequency of SNPs in the HBV quasispecies of plasma and PBMC from post-LT cases separated by age groups. SNP frequencies of **A**, low median frequency mutations, and **B**, high median frequency mutations, were separated by the cases >40 years of age and <40 years of age at the time of sample collection. Higher

frequencies of A1762T, G1764A and G1899A which are well recognized HCC-associated HBV variants were observed in plasma and/or PBMC of post-LT cases <40 years of age. *P-value < .05 (Mann-Whitney test).

3.5 Discussion

Most LT recipients for HBV-related liver disease on prophylaxis have excellent post-transplant outcomes with low risk of HBV recurrence and graft failure. Over the last decade, the use of second-generation NA has allowed effective long-term viral suppression both pre- and post-LT; thus, some transplant programmes are questioning the need for ongoing expensive prophylaxis. In the current study, we found that HBV persisted in 12 post-LT patients despite continual use of potent HBV prophylaxis followed up to 153 months post-LT. In the majority of cases (9/12), low-level virus persists even in HBsAg- infection (ie OBI). In the non-LT setting, observational studies in large Asian cohorts report an association of OBI to development of cancer (ie. HCC) (47,187), as well as extrahepatic malignancies including lymphoma and leukaemia (53–55). All LT recipients received continuous albeit low-dose, immunosuppressive therapy, which potentially increases their risk of possible HBV reactivation or extrahepatic malignancy (ie post-transplant lymphoproliferative disorder). However, many immunosuppressive or antirejection therapies used post-LT such as corticosteroids and tacrolimus have a relatively low reported HBV reactivation risk of up to 10% (212).

In the current study, we found that HBV genomes (DNA and mRNA or cccDNA) persist in plasma and/or PBMC of all post-LT patients. Many studies by our group (126,148) and others (161,165,213,214) have shown that HBV may be detected in extrahepatic tissues. HBV uniquely evolves (126,161,165,204,213,215) and persists despite potent antiviral therapy (127,128). In the transplant setting, following removal of the HBV-infected graft, accumulated evidence lends

support towards the idea that the lymphoid system can serve as a HBV reservoir for continual persistence and source of re-infection post-LT (161,213). By sequencing the HBV before and after LT, Brind *et al*. (161) demonstrated that surface and core variants unique in patient PBMC pre-LT are the dominant species in the serum and liver post-LT. Ciesek *et al*. (213) have also shown that OBI can persist for many years in PBMCs of post-LT patients with a history of HBV infection (ie anti-HBc-positive) despite negative HBsAg at time of transplant. It is also speculated that PBMC-derived virus contributes towards HBV reactivation in LT patients who are anti-HBc-positive and HBsAg- at time of transplant.

In all cases, despite clinical HBV DNA suppression, we were able to determine the dominant HBV genotype by sensitive nested PCR/NGS of PCR amplicons. It is also noteworthy that the dominant HBV genotype differed in 8/12 cases between the plasma and PBMC in our post-LT cohort. This phenomenon has been reported by others (214) and by our group (126) in treatment-naïve CHB carriers in which the HBV genotype profiles varied between the plasma and PBMC compartments, as well as in the transplant setting as noted above (161). Although pre-LT HBV genotype was unavailable, the determined genotypes post-LT were unexpected, in some cases, with respect to the case ethnicity. Certain horizontal HBV transmission methods may account for some of these discrepancies and viral genotype-ethnicity expectations. For example, case ID# 68 contracted HBV via a blood transfusion in Canada that was contaminated with HBV from an ambiguous source (ie unknown donor ethnicity and HBV genotype). Switching of HBV genotype post-LT has been previously reported (216) and might be more prevalent than previously expected. Indeed, changes in the HBV genome pre- and post-LT are well recognized, particularly within the polymerase and surface genomic regions (217).

Hepatitis B virus genomic differences between the compartments are also reflected in the NGS determination of SNP frequency (ie C1773T, in the plasma and PBMC). This intercompartmental HBV diversity is comparable to previously reported findings in which certain anti-HBV treatment-resistant variants were present in different frequencies between the plasma, liver and PBMCs (128). Interestingly, some variants associated with HCC and cirrhosis (ie T1753V, A1762T and G1764A) were present at high frequencies in both the plasma and PBMC of post-LT cases. Recent work by Kim *et al*. (185) showed an increased risk of HCC in patients with low-level persistence of HBV despite NA therapy. Thus, in the current study context, the detection of persistent HBV with oncogenic variants suggests that virological risk factors implicated in severe and/or end-stage liver disease remain post-LT despite use of HBV prophylaxis.

An interesting observation from our study is the detection of HBV cccDNA but not viral mRNA in the PBMC compartment despite the presence of cellular mRNA (ie β-globin). A possible explanation for this finding could be that the HBV cccDNA exists at a low level of transcription and viral gene expression in PBMCs. The cccDNA inactivity is potentially related to episomal regulation of viral gene transcription and production such as through histone modification (218,219) or epigenetic effects of HBV proteins (ie HBV X) (220). We speculate that episomal modulation of cccDNA by these mechanisms differs in HBV-infected PBMC, in post-LT patients, or both, a concept that requires further experimental investigation. In our current study, we used strict precautions to exclude environmental contamination as well as rigorous enzymatic treatment protocols to remove extracellular or adhered virus, to assess HBV cccDNA using protein-free DNA templates.

Our study is limited by a lack of pre-LT blood and tissue samples as well as post-transplant liver biopsy tissue allowing HBV genotype and quasispecies comparison to the plasma and PBMC compartments. It is difficult to obtain post-LT liver biopsy in stable graft recipients, especially with the noninvasive use of transient elastography to monitor liver status. A few cases within our cohort were recruited years after transplantation (> 6 years), some of which occurred in other health care regions, accounting for a lack of pre-LT serology and HBV testing. Despite this limitation, our findings emphasize the long-term persistence of HBV in these patients. Additional follow-up for collection of serial samples from this post-LT cohort is ongoing and will be important to demonstrate continual HBV persistence. Finally, although recent studies in small cohorts suggest that the risk of clinical disease and overt/aggressive HBV recurrence after withdrawal of prophylaxis is low, a large multisite study using more sensitive and standardized assays for assessing HBV genomes in plasma, PBMC and liver is necessary before changing our current standard post-LT protocol.

In conclusion, our current study demonstrates that virological eradication of HBV is not possible and low level or occult HBV persists in post-LT patients in both the plasma and PBMC compartments despite the use of potent HBV prophylaxis. HBV cccDNA was found in the PBMC compartment in the majority of cases. HBV variants and genotypes associated with cirrhosis and HCC were present, and in some cases, were the dominant population of HBV quasispecies. This study raises the possibility that if complete withdrawal of potent post-LT HBV prophylaxis is attempted, individuals may still be at risk of losing HBV immune control and leading to overt HBV recurrence.

3.6 Acknowledgements

The authors would like to thank all patients and staff of the Southern Alberta Liver Transplant clinic.

3.7 Conflict of Interest

CSC has served as an advisory board member and/or has received research support from Bristol Myers Squibb, Glaxo Smith Kline, Gilead Sciences, Janssen and Merck. KWB has served as a speaker, advisory board member and/or has received research support from Bayer, Verlyx, Lupin, Astellas, Gilead, Amgen and Merck. The other authors have no relevant disclosures.

3.8 Ethics ID

The study received approval from the local ethics review board committee at the University of Calgary (ie Conjoint Ethics Review Board, Ethics REB-16636).

CHAPTER 4: ONCOGENIC HBV VARIANTS AND INTEGRATION ARE PRESENT IN HEPATIC AND LYMPHOID CELLS DERIVED FROM CHRONIC HBV PATIENTS

4.1 Abstract

The hepatitis B virus (HBV) is a major cause of hepatocellular carcinoma (HCC), partly driven by viral integration and specific oncogenic HBV variants. However, the biological significance of HBV genomes within lymphoid cells (i.e., peripheral blood mononuclear cells, PBMCs) is unclear. Here, we collected available plasma, PBMC, liver, and tumor from 52 chronic HBV (CHB) carriers: 32 with HCC, 19 without HCC, and one with dendritic cell sarcoma, DCS. Using highly sensitive sequencing techniques, next generation sequencing, and AluPCR, we demonstrate that viral genomes (i.e., HBV DNA, RNA, and cccDNA), oncogenic variants, and HBV-host integration are often found in all sample types collected from 52 patients (including lymphoid cells and a DCS tumor). Viral integration was recurrently identified (n=90 such hits) in genes associated with oncogenic consequences in lymphoid and liver cells. Further, HBV genomes increased in PBMCs derived from 7 additional (treated or untreated) CHB carriers after extracellular mitogen stimulation. Our study shows novel HBV molecular data and replication not only liver, but also within 63.8% of lymphoid cells analyzed (including a representative lymphoid cell malignancy), that was enhanced in *ex vivo* stimulated PBMC.

4.2 Introduction

The hepatitis B virus (HBV) is a significant global pathogen with an estimated 257 million chronic HBV (CHB) carriers worldwide (1). CHB can lead to cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (221). HBV-related liver oncogenesis is complex and is influenced by viral characteristics including viral load (i.e., serum HBV DNA levels), HBV

genotype, genetic variants within the HBV X/basal core promotor/pre-core (X/BCP/PC) region, and viral integration into the host genome. Many of these associations have been identified in Asian cohort studies (84,87), such as the REVEAL-HBV study which demonstrated that serum HBV DNA levels were significantly associated with HCC across a biological gradient (84). Moreover, due to the error-prone viral replication, HBV exists as quasi-species within a host. Genetic features including genotype C and X/BCP/PC mutants were also predictors of HCC risk (84,198), especially the A1762T/G1764A mutations (136,138,141). HBV genotype differences have been associated with prognosis and early onset HCC (110,124). The oncogenic HBV potential is augmented by viral integration in the host genome arising from the incorporation of double stranded linear HBV DNA (27). Recent studies have increased our understanding of HBV integration with regards to the mechanism and timing (100,101) with integration found in liver tissues from multiple CHB disease stages including occult infection (51,98,99). It is suggested that HBV insertion into the host genome may create fusion proteins, alter non-coding RNA patterns, or induce expression of oncogenes leading to neoplastic transformation of infected cells (103,118,222).

Although the pathogenesis is poorly understood, CHB is associated with extrahepatic disease, including B-cell non-Hodgkin lymphoma (NHL), polyarteritis nodosa and membranous glomerulonephritis (175–177,223,224). The strongest associations are observed with lymphoma risk, particularly NHL (175–177). HBV is considered a hepatotropic virus, but evidence supports its lymphotropic nature (225). HBV nucleic acids such as genomic DNA, HBV covalently closed circular (ccc)DNA, messenger (m)RNA and integrated virus has been identified in extrahepatic lymphoid tissues including spleen, lymph nodes, and lymphoid cells (126,128,148,160,163–165). Other studies found unique HBV variants in PBMC compared to plasma and liver of CHB

carriers (161,214). Our studies showed that the HBV evolves in a compartment specific fashion in PBMC when compared to matching plasma (126,127), and low-level oncogenic variants persists in PBMC of CHB liver transplant recipients despite potent prophylaxis (189). We reported that HBV DNA was detectable in all PBMC subsets of CHB patients, especially B-cells (166). HBV persistence in lymphoid cells is hypothesized to play a role in HBV recurrence in the graft of patients undergoing liver transplant for HBV-related end-stage liver disease (148,161). Although, multiple investigators have reported the detection of HBV genomes within the lymphatic system, the biological and clinical significance of extrahepatic persistence remains controversial. HBV integration within PBMCs may contribute towards extrahepatic carcinogenesis of hematological malignancies.

In the current study, we evaluated HBV+/-HCC patients for the presence of HBV replicative intermediates including DNA, RNA, and cccDNA. We detected single nucleotide polymorphisms (SNPs) associated with HCC development and evidence of HBV genomic integration within PBMC of CHB carriers with or without HCC. We show that mitogen-stimulation of PBMCs increases viral replicative markers including DNA, cccDNA, and RNA. Interestingly, in one CHB carrier with dendritic cell sarcoma (DCS) (i.e., an extrahepatic lymphoproliferative malignancy), we demonstrate the presence of viral DNA, cccDNA, and antigens including HBV surface (HBsAg) and core (HBcAg). The study provides compelling evidence that HBV can replicate and integrate in both liver and extrahepatic (lymphoid) cells, with potential oncogenic sequelae in these sites.

4.3 Materials and Methods

4.3.1 Patient population and sample collection

52 CHB carriers were recruited (n=19 with CHB, 32 with HCC, 1 with extrahepatic malignancy [DCS]) from the University of Calgary Liver Unit, University of California San Francisco, including subjects enrolled in our previously published study (128) or Beijing YouAn Hospital, Capital Medical University (Figure 4.1). This study was approved by the UofC conjoint health research ethics board, CHREB (Ethics ID#16636). All subjects of this study were above the age of majority and provided informed written consent to participate according to the guidelines of the 1975 Declaration of Helsinki. Whole blood was collected for isolation of serum, plasma, and/or PBMCs using Ficoll density gradients. Clinical data and laboratory assays such as serum HBV DNA (according to clinical PCR) was collected (Table 4.1). Additional HBV serology (i.e., HBsAg, HBeAg, anti-HBeAg) was determined clinically with commercial chemiluminescent microparticle immunoassays (Abbott Architect; quantitative anti-HBc II and anti-HBs).

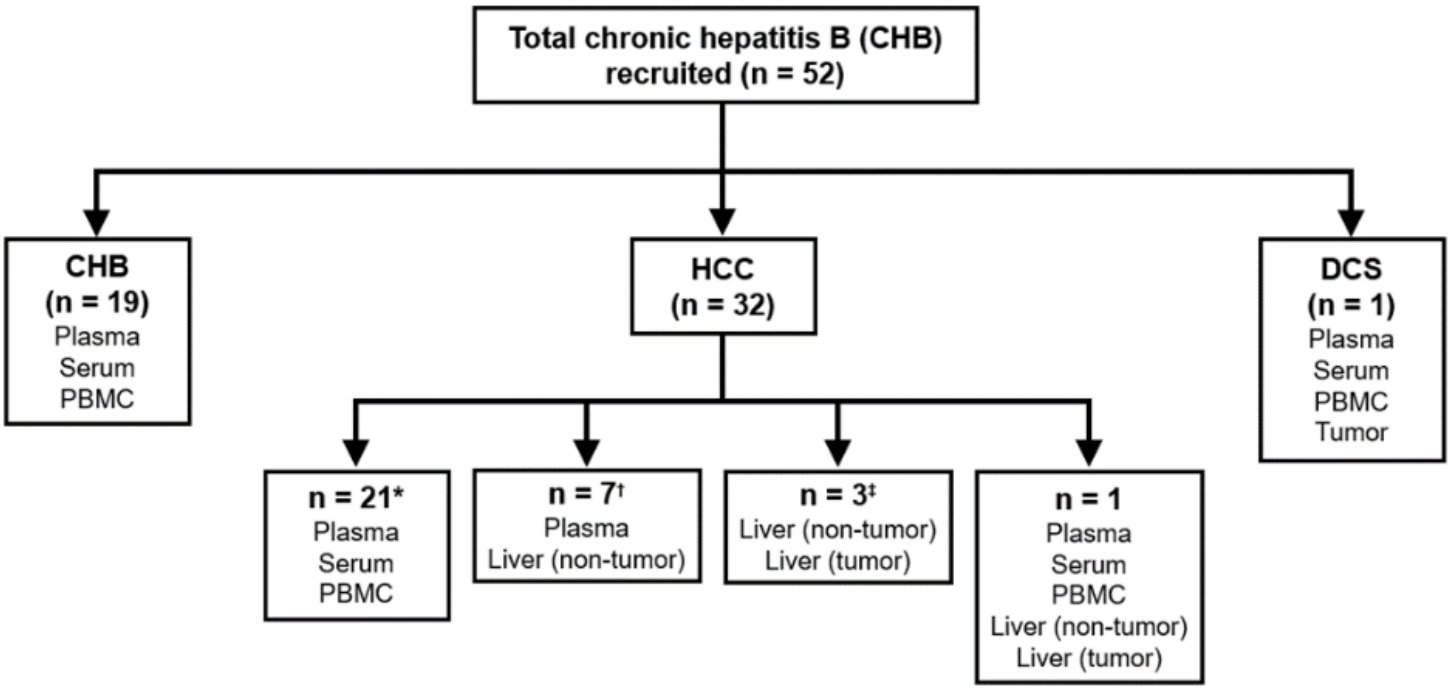


Figure 4.1. Summary of chronic hepatitis B (CHB) carriers recruited and biological samples collected for the study. A total of 52 CHB carriers were retrospectively recruited; 19 with no malignancy, 32 with diagnosis of hepatocellular carcinoma (HCC) and 1 with dendritic cell sarcoma (DCS) (i.e., extrahepatic lymphoproliferative malignancy). Biological samples collected from each group are indicated. *****3/21 cases have 1 follow-up sample; 1/21 has 2 follow-up samples. †7 cases collected from University of California San Francisco, 4/7 have matching plasma. ‡3 cases from Beijing YouAn hospital (total DNA isolated from liver tissue).

Table 4.1. Demographic and clinical information of CHB carriers with and without a history of HCC[1].

VARIABLE	CHB ONLY (n = 19)	CHB + HCC (n = 32)
SEX, F / M	6 / 13	8 / 24
MEAN AGE, YEARS (RANGE)	47.9 (26 – 69)	57.4 (19 – 83)
ETHNICITY*, AF / AS / CA	4 / 13 / 2	4 / 27 / 1
HBV VIRAL LOAD†, Q / D / U MEDIAN, IU/mL (RANGE)	8 / 11 / 0 $2.7x10^3$ ($280 - 8.5x10^4$)	11 / 7 / 14 310 ($14 - 1x10^8$)
ANTIVIRAL THERAPY	15	29
CIRRHOSIS, N	3	29
MALIGNANCY	NO	HCC

***AF** = African; **AS** = Asian; **CA** = Caucasian
†Determined by clinical PCR assay (Roche TaqMan or Abbott Architect, LLOD:55 or 10 IU/mL, respectively).
Q = quantifiable; **D** = detectable but not quantifiable; **U** = Undetectable

[1]Extrahepatic tumour tissue and blood was collected from one 46 years old African male with a dendritic cell sarcoma (lymphoid cell malignancy), with non-cirrhotic CHB, HBV DNA of 390 IU/mL, and on NA therapy.

4.3.2 Isolation and detection of HBV DNA, RNA, and cccDNA

Total DNA was isolated from samples using phenol-chloroform extraction. PBMCs were pre-treated with DNase/trypsin/DNase before extraction to remove extracellular HBV as previously described (126). Total RNA was isolated from ~1mg of liver or tumor tissue with TRIzol™ (Invitrogen) and eluted into RNase-free water. Contaminant DNA was removed followed by cDNA synthesis (PerfeCTa DNase I and qScript cDNA synthesis kits, Quanta Biosciences). HBV DNA and RNA was detected using previously published (226) nested PCR

methodology targeting the HBV X/BCP/PC, PC/C, and surface (S) genomic regions (Table S4.1 for primers). HBV cccDNA was selectively isolated by Hirt DNA extraction followed by T5 exonuclease digestion and detected using published methods (189). Nested PCR targeting the nicked region of HBV with subsequent nucleic acid hybridization (NAH) and Sanger sequencing was employed to identify cccDNA (Table S4.1).

4.3.3 Identification of HBV-host integration junctions with AluPCR

Viral-host integration sites in PBMCs, liver, or tumor tissue were detected using AluPCR (51,160). 100ng of total DNA derived from phenol-chloroform extraction was subjected to nested PCR amplification with Alu (i.e., host) and HBV specific primers. Sense and anti-sense Alu primers as well as primers specific for 3 regions of HBV (X, C, and S) (Table S4.2) were used in separate nested reactions to increase detection of integrated viral sequences. AluPCR reactions with detectable fragments, visualized by agarose gel, were purified (QIAquick PCR purification kit, Qiagen) followed by ligation into the pGEM-T Easy vector (Promega Corporation) and subsequent transformation into TOP10 *E. coli* cells (Invitrogen). 10-15 clones were selected for inserted viral-host junctions which were identified via PCR with M13 primers and Sanger sequencing. Viral-host junctions were analysed with NCBI BLAST databases. Subsequent Circoplots were created using ShinyCircos (227).

4.3.4 Controls for nucleic acid extractions and PCR analyses

Negative controls for nested PCRs and AluPCRs included mock extractions, plasma/PBMC from healthy HBV-negative volunteers, and no template (water) controls. Appropriate reverse transcriptase controls were included for RNA analysis. Strict conditions were undertaken to exclude possible environmental risk of contamination including dedicated workspace and equipment for extraction, PCR preparation, and template addition. PCR positive controls

included HBV-dimer (in-house) and HBV-trimer plasmids (kindly provided by Dr. TI Michalak, Memorial University, Canada). Detailed methods are found in the supplementary materials.

4.4 Results

4.4.1 Summary of clinical and virological data (Table 4.1)

A total of 52 CHB carriers were enrolled in this study including 19 with CHB-only, 32 CHB+HCC, and 1 CHB+DCS cases (Figure 4.1). Whole blood (separated into plasma, serum, or PBMC), non-tumor liver, liver tumor, and DCS tissue was collected from individuals when available. The majority of patients were male (n=35/52) and Asian ethnicity (n=40/52), the CHB+HCC patients were older compared to those with CHB-only (mean age 57.4 vs. 47.9, t-test p-value = 0.0157). An intraabdominal extralymphatic tumor and matching blood sample was collected from one 46 year old male African CHB patient with an extrahepatic malignancy (i.e., CHB+DCS). This individual also presented with autoimmune complications including bullous pemphigoid and paraneoplastic autoimmune multiorgan syndrome. Table S4.5 summarizes clinical data on CHB+HCC cases recruited from the Calgary Liver Unit, the majority (n=29/32) of which have underlying cirrhosis. At last follow-up, 3 CHB+HCC cases are deceased due to disease progression, with the remaining in remission (n=14/32), with recurrent/residual disease (n=4/32) or lost to follow-up (n=1). The majority of patients from UofC were treated for HCC with liver resection (n=10/32), ablation (n=10/32), and/or trans-arterial chemo-embolization (n=9/32) with 2 cases currently awaiting liver transplant (Table S4.5).

4.4.2 Detection of HBV DNA, cccDNA, and serum RNA in all liver and/or extrahepatic samples from individuals with CHB

HBV DNA was detectable in all cohorts and samples (serum/plasma, PBMC, liver and tumour) using nested PCRs targeting three regions of HBV (PC/C, X/BCP/PC, and S) (Table 4.2). The HBV S amplicons were utilized for genotype determination which showed the predominance of genotype C with the exception of the plasma from CHB-only carriers (n=19) (predominantly genotype B). HBV cccDNA was detected within all liver samples from Hirt+T5 extracted DNA with subsequent nested PCR targeting the HBV nicked region (Table 4.2). Detection rates of cccDNA within the PBMC compartment was lower than that of the liver. Interestingly, a higher rate of HBV cccDNA detection was noted in the CHB+HCC cohort (16/23 tested) in comparison to the CHB-only group (6/12 tested). We also found a non-significant trend of higher serum RNA within the CHB+HCC cohort in comparison to the CHB-only group in both the truncated (p-value = 0.16) and full-length (p-value = 0.21) forms of HBV RNA (Table 4.2).

Table 4.2. Comparison of HBV molecular markers in CHB patients with or without HCC or extrahepatic malignancy.[1]

COHORT	SAMPLE TYPE	# OF SAMPLES DETECTED BY NESTED PCR			GENOTYPE	DETECTION OF cccDNA	MEAN LOG SERUM RNA (SEM)		TOTAL # INTEGRATIONS DETECTED	# INTEGRATIONS IN CODING GENES
		X/BCP/ PC*	PC/C	Surface			Truncated	Full Length		
CHB ONLY (n = 19)	Plasma (n = 19)	19	15	18	1 A / 9 B / 3 C / 2 D / 3 E	N/A	2.17 (0.20)	1.90 (0.26)	N/A	N/A
	PBMC (n = 14)	10	8	13	9 C / 5 D	6 of 12 tested			95	47 (38 unique genes)
CHB + HCC+ (n = 33)	Plasma (n = 32)	29	27	28	1 A / 1 B / 21 C / 5 D	N/A	2.62 (0.21)	2.38 (0.25)	N/A	N/A
	PBMC (n = 26)	18	9	26	1 A / 2 B / 19 C / 4 D	16 of 23 tested			156	67 (58 unique genes)
	Liver non-tumor (n = 11)	10	9	9	1 A / 8 C	8 of 8 tested			117	52 (48 unique genes)
	Liver tumor (n = 4)	3	4	3	3 C	1 of 1 tested			48	16 (15 unique genes)
CHB + DCS (n = 1)	Plasma	1	1	1	1 C	N/A	1.68	0	N/A	N/A
	PBMC	1	0	1	1 C	1 of 1 tested			20	9 (9 unique genes)
	Tumor	1	1	1	1 D	1 of 1 tested			18	10 (10 unique genes)

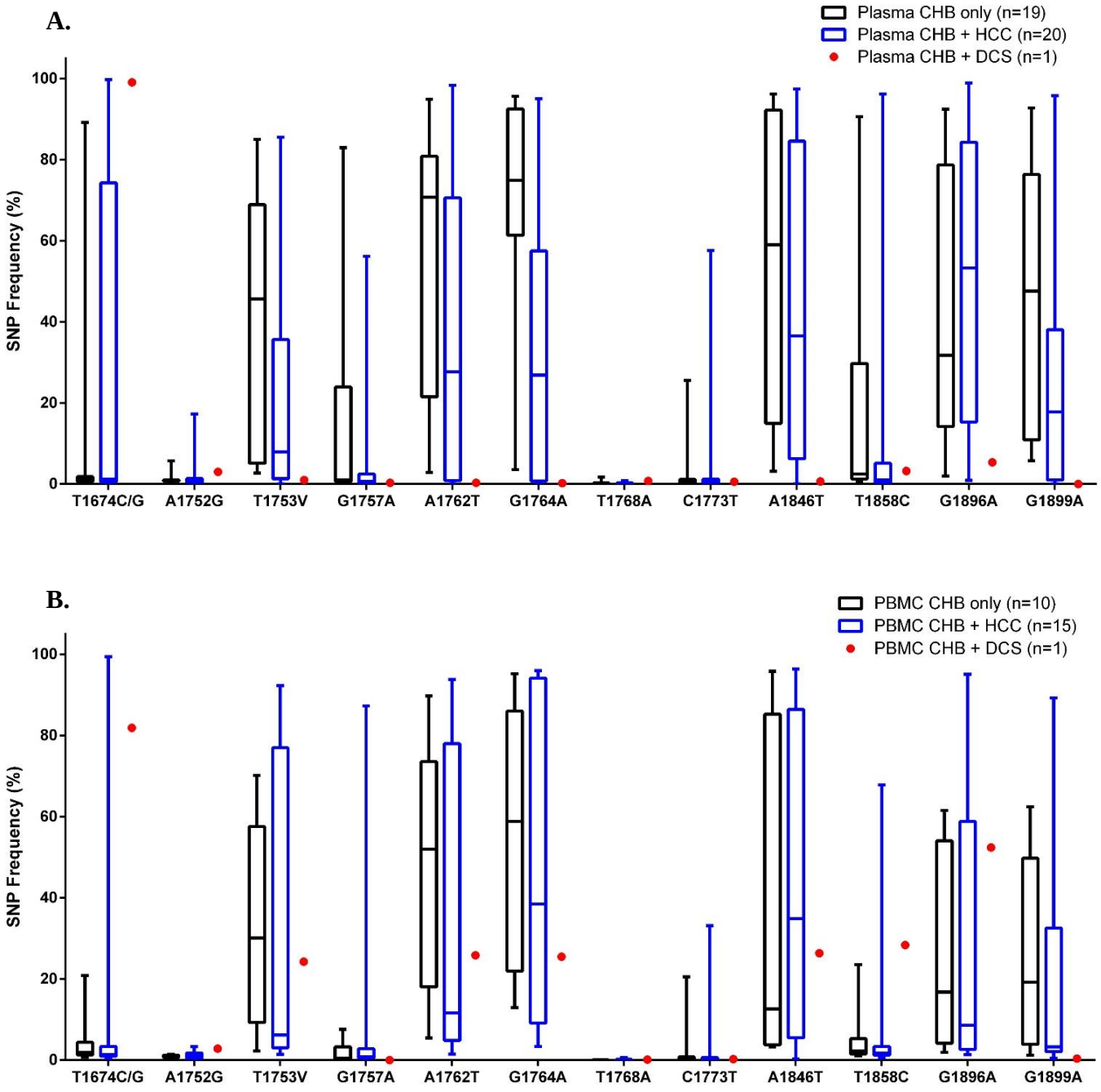
*X/BCP/PC amplicons were subsequently adapted for Illumina MiSeq NGS analysis.

+1 case (#405) has matching PBMC, liver and liver tumor samples; 3 cases (Beijing YouAn, Capital Medical University) have matching liver and liver tumor samples

[1]Number of samples from each cohort detected by nested PCR of the X/BCP/PC, PC/C, and S regions. HBV genotype was determined following Sanger sequencing of the S PCR amplicons. HBV cccDNA was detected following Hirt+T5 followed by nested PCR, NAH, and sequencing confirmation. Serum HBV RNA was quantified by qPCR. Integrations in host genomes were detected by AluPCR.

4.4.3 HBV genetic variants associated with cirrhosis and HCC are present in all liver and/or extrahepatic samples from individuals with CHB

The PCR amplicons for X/BCP/PC region were subjected to NGS for quasi-species diversity analysis and determination of specific SNPs previously reported to be associated with cirrhosis and HCC (136,138,141). Although a wide range of frequencies was observed, most of the SNPs analysed were found in all samples regardless of a history of malignancy (Figure 4.2A-C). However, the A1752G, T1768A, and C1773T mutations were present at low frequencies in all samples (Figure 4.2A-C). A comparison of the HBV variants found within the CHB+DCS case showed more HCC-associated SNPs within the PBMC and tumor (Figure 4.2D). Of particular note, the A1762T/G1764A double mutation, as well as T1858C, was observed at higher frequencies in the tumor than either the PBMC or plasma from this individual. Interestingly, a lower number of haplotypes were observed amongst the malignant liver (mean=330) and DCS tumor (103) tissues potentially due to clonal selection and proliferation of cancerous cells (Table S4.6). However, no differences were noted amongst the cohorts/samples with our quasi-species diversity analysis with the exception of higher diversity observed in the PBMC of the individual with CHB+DCS.


A.
Plasma CHB only (n=19)
Plasma CHB + HCC (n=20)
Plasma CHB + DCS (n=1)
SNP Frequency (%)
0
20
40
60
80
100
T1674C/G
A1752G
T1753V
G1757A
A1762T
G1764A
T1768A
C1773T
A1846T
T1858C
G1896A
G1899A
B.
PBMC CHB only (n=10)
PBMC CHB + HCC (n=15)
PBMC CHB + DCS (n=1)
SNP Frequency (%)
0
20
40
60
80
100
T1674C/G
A1752G
T1753V
G1757A
A1762T
G1764A
T1768A
C1773T
A1846T
T1858C
G1896A
G1899A

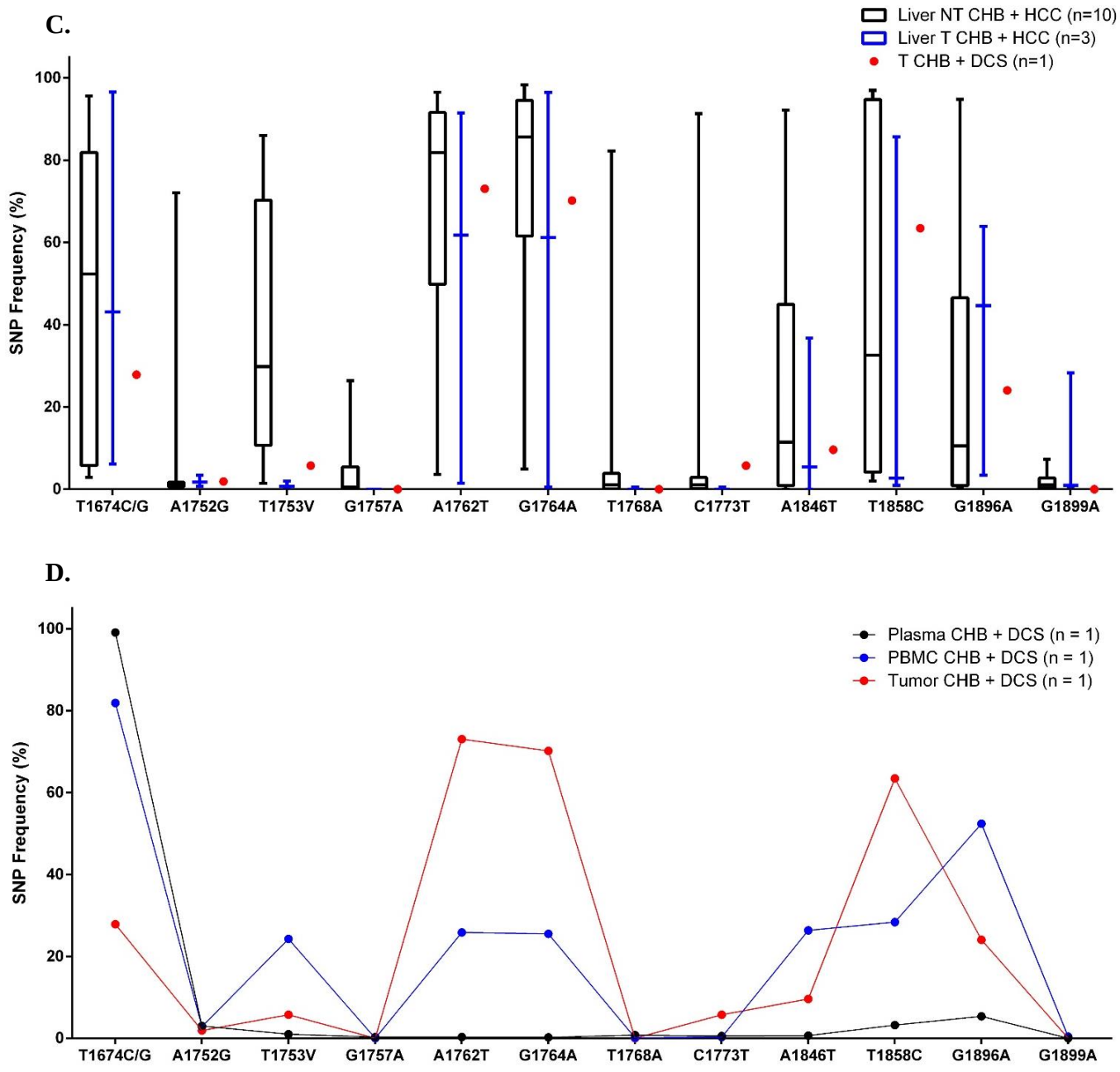


Figure 4.2. Frequency determination of single nucleotide polymorphisms (SNPs) within the HBV X/BCP/PC region associated with HCC. NGS analysis of the HBV X/BCP/PC region was performed with the Illumina MiSeq. HCC-associated SNPs determined from sample quasi-species were plotted in **(A)** plasma of all patients (n=40); **(B)** PBMC from all patients (n=26); and **(C)** Liver (non-tumor [NT] or tumor

[T]) from CHB+HCC carriers (n=10) and DCS tumor from 1 CHB case; **(D)** CHB+DCS plasma, PBMC, and tumor. Box and whisker plots (**a-c**) shows median SNP frequency enclosed by the interquartile range with whiskers representing full range of SNPs.

4.4.4 Detection of viral-host junctions in coding and non-coding regions of HBV DNA from PBMC of CHB individuals

HBV integration in both host non-coding and coding genomic regions was detectable within the PBMCs, liver, and available tumors from each cohort (Table 4.2, Figure 4.3). A total of 271 integration events were found in PBMCs, of which 123 hits were located within coding genes. Many of such coding genes (n=58 hits) identified within the lymphoid cells may impact oncogenesis and hence represent a pre-malignant population of cells with HBV integration (Table S4.7). No significant differences were observed in the number of integrations detected based on age, sex, or HBV genotype. Two CHB cases with HCC (#405) or DCS (#420) had matching PBMCs and tumor tissue. A comparable number of integrations were detected in the different samples from each patient (Table S4.8, Figure S4.2). Shared sites of integration were also observed within the HCC patient such as GOLGA5 in both PBMC and liver tumor; ABCB4 in both non-tumor and tumor liver tissues. Further, HBV integration was not only observed in the DCS tissue, but also within coding genes implicated in cancer development or progression including PTPRT and ZFR2 (Table S4.7).

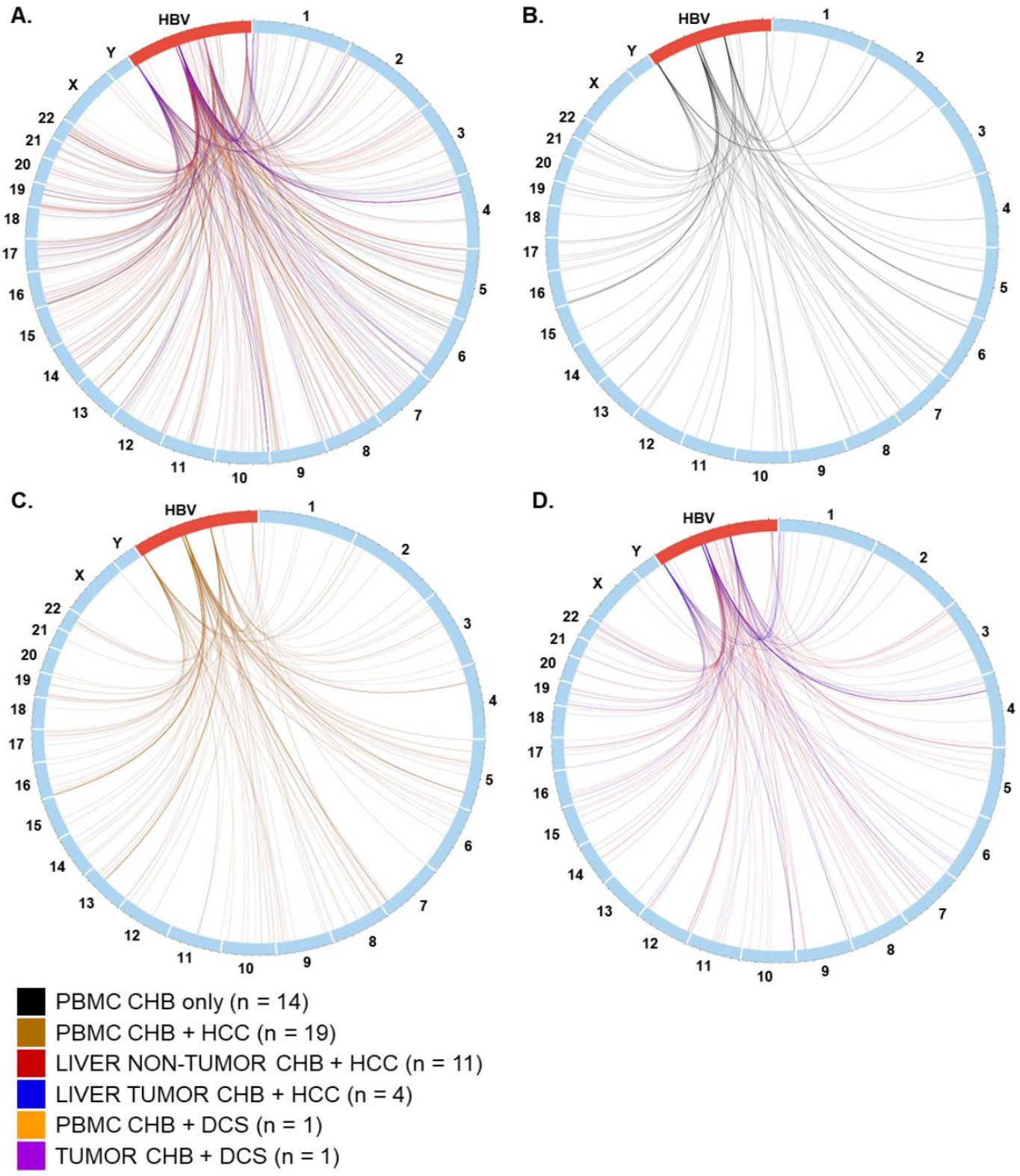


Figure 4.3. HBV-host integration sites identified with AluPCR followed by clonal sequencing. The outside bands indicate the human chromosome (light blue) and the

HBV chromosome (red, not to scale). Lines in the Circoplots connects the location of each integration junction in the viral and host genomes in **(A)** all patients and tissues; **(B)** PBMC of CHB only carriers (n=14); **(C)** PMBC of CHB+HCC cases (n=19); and **(D)** Non-tumor liver (n=11) and liver tumor (n=4).

4.4.5 HBV genomes, RNA and viral proteins are detected in extrahepatic tumor tissue of a patient with CHB and extrahepatic lymphoproliferative malignancy (i.e., DCS)

Plasma, PBMC, and tumor tissue from an individual with CHB+DCS (#420) was collected and utilized for analysis of HBV genomes alongside a CHB+HCC case (#405) with matching samples. Utilizing our in-house PCR assays, we detected HBV DNA within all samples in both cases (Figure 4.4A), as well as HBV RNA within all liver and tumor tissues tested from our CHB+HCC cases (CC06 and #405) or DCS case (#420) (Figure 4.4B). We also detected HBV cccDNA via nested PCR targeting the HBV nicked region from Hirt+T5 extracted samples (Figure 4.4C). Linearized cccDNA samples was used for a direct NAH with no prior PCR amplification. HBV cccDNA was not detected by NAH in all cases tested including liver explant or liver tumor tissue from NA-suppressed CHB+HCC carriers (CC06 and #405), likely due to low levels of cccDNA present and overall lower sensitivity of direct NAH (lower limit of detection for HBV DNA is ~10^5 virus genome copies/mL) (Figure 4.4D). Interestingly, both HBsAg and HBcAg was identified from protein extracts from the DCS tissue and liver or liver tumor tissue from CHB+HCC carriers (CC06 and #405) (Figure 4.4E). Overall, our results suggest the presence of HBV DNA, RNA, cccDNA, and viral proteins within the extrahepatic tissue.

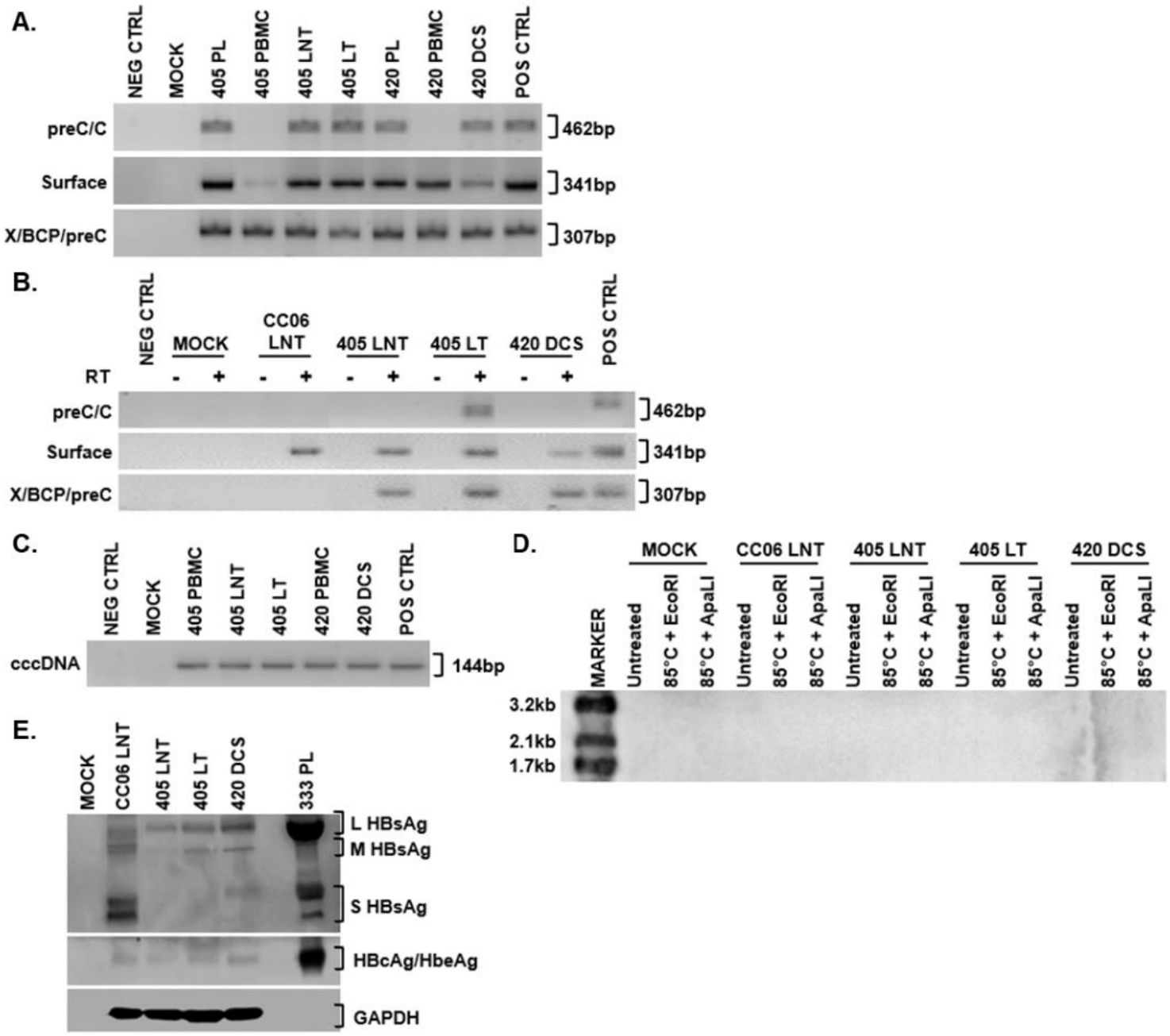


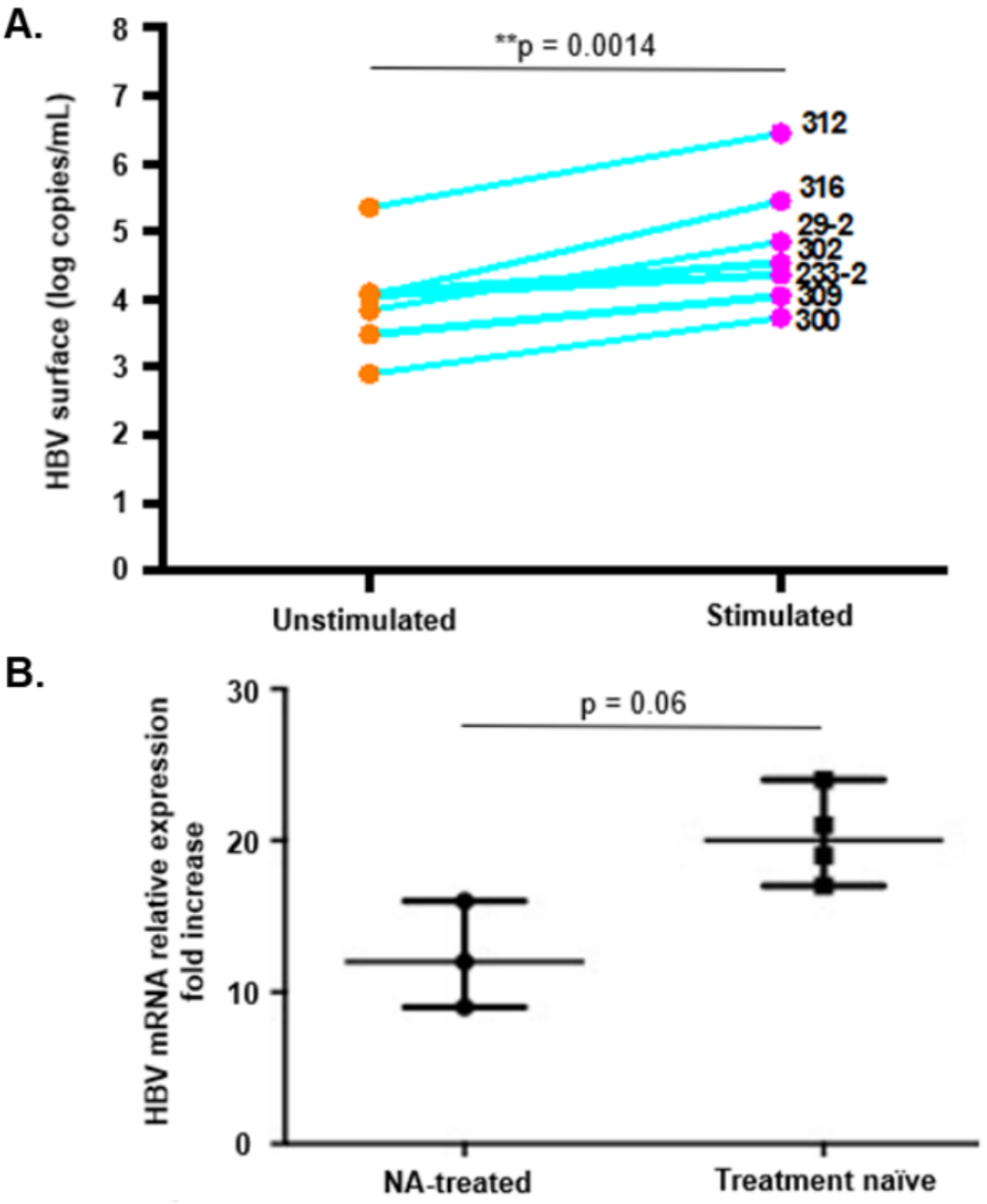
Figure 4.4. Plasma, PBMC, and tumor tissue of CHB+DCS case (ID#420) contains detectable HBV genomes and proteins. Samples of non-tumor liver (LNT) from CHB+HCC carriers treated with NA therapy (ID#s CC06 and 405) and liver cancer tumor from ID#405 (LT) was used. **(A)** HBV-specific nested PCR demonstrated the presence of viral DNAs within plasma, PBMC, and DCS tissue. **(B)** Nested RT-PCR

was used to detect HBV RNAs within liver and tumor tissues. **(C)** HBV cccDNA was isolated by Hirt+T5 extraction followed by detection using nested PCR targeting the nicked region. **(D)** Hirt extracted DNA was subjected to heat (85°C for 5 minutes) and enzymatic treatment (EcoRI or ApaLI) to linearize and identify cccDNA under a direct NAH (no prior PCR amplification). **(E)** Total protein was collected from patient samples and HBsAg, HBcAg/HBeAg, and GAPDH was detected. Diluted plasma (PL) from a HBeAg+ CHB+HCC case (ID#333) was used as a positive control.

4.4.6 HBV replication is upregulated by mitogen-stimulation in patient-derived ex vivo PBMC

Cells undergoing malignant transformation are prone to stimulation via a variety of non-specific mitogen-induced pathways and may affect HBV replication within the PBMCs. PBMC was isolated from untreated CHB carriers with high viremia or NA-treated cases with undetectable viral load (cases 29-2, 233-2, and 302) (Table S4.3) and exposed *ex vivo* to a cocktail of B-cell and T-cell stimulating mitogens including PHA and PWM for 72 hours. Compared to unstimulated PBMC, in both untreated and NA-treated CHB carriers, we found a subtle but significant increase (p-value = 0.0014) in HBV DNA levels in the PBMC supernatant after mitogen-stimulation (Figure 4.5A). Interestingly, similar levels of HBV DNA were found in cultured PBMC supernatants from cases with high viremia (#312, #316) compared to NA-treated cases with undetectable plasma viral load (cases 29-2, 233-2, 302). The amount of HBV secreted by unstimulated or stimulated PBMC appears to be unrelated to plasma-viral load, and also suggesting that there is minimal effect of antiviral therapy on HBV replication upon *ex vivo* PBMC stimulation. In addition, there was a median 12- and 20-fold increase of HBV mRNA relative expression found in stimulated PBMC vs. unstimulated PBMC in NA-treated group and

treatment naïve group, respectively (p-value = 0.06; Figure 4.5B). Overall, the HBV RNA levels were upregulated within the stimulated PBMC (cases 312, 316, 29-2) in comparison to unstimulated cells (Figure 4.5B), as well as an increase of HBV cccDNA in 2 analyzed cases (#233-2, #312) (Figure 4.5C). Thus, our results suggest an increase in HBV replication occurs following *ex vivo* mitogen-stimulation of whole PBMC.



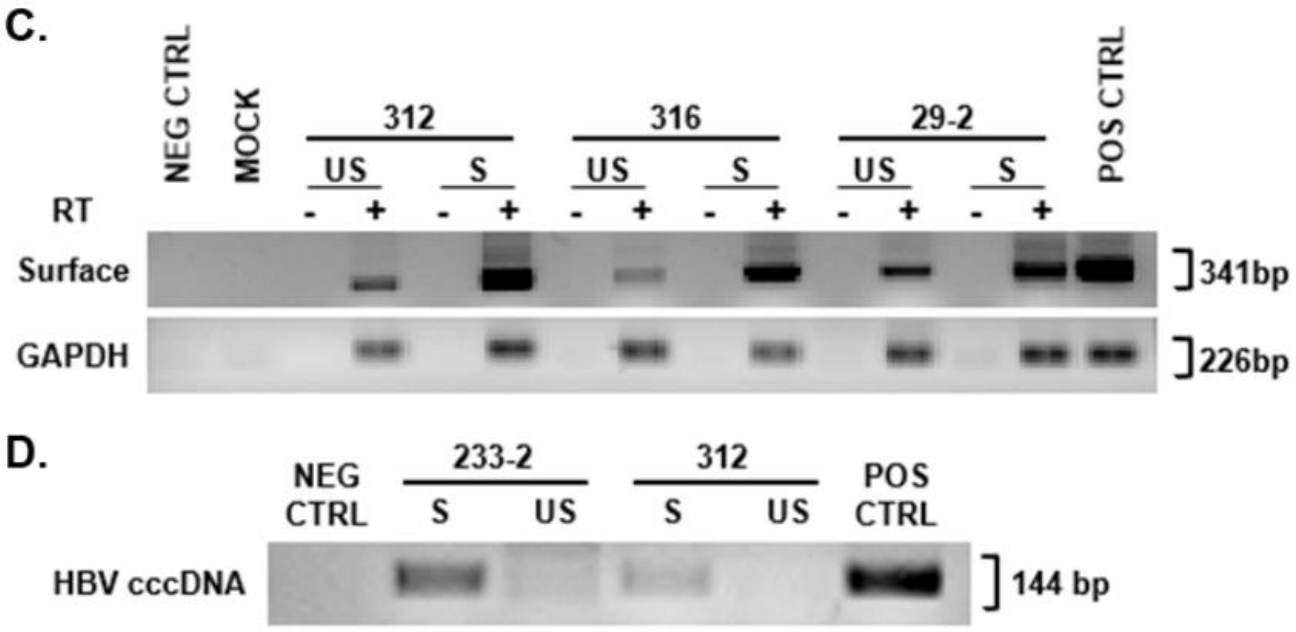


Figure 4.5. **PBMCs from CHB carriers were *ex vivo* stimulated with or without mitogens (PHA and PWM) followed by analysis of cultured cells and supernatant**. **(A)** HBV DNA levels in the supernatant of stimulated PBMCs shows significantly increased HBV DNA via qPCR analysis in seven different patients. The mean HBV DNA increase was 0.77 log copies/mL (Wilcoxon signed-rank test; **p = 0.0014). **(B)** PBMCs from NA-treated (n=3) and untreated CHB (n=4) carriers have a median fold-increase of 12 and 20, respectively, in HBV mRNA relative expression with mitogen stimulation (Mann Whitney; p = 0.06). **(C)** Similarly, RT-PCR in PBMCs from 3 CHB carriers (ID#s 312, 316 and 29-2) showed an increase in HBV mRNA after mitogen stimulation. US = unstimulated; S = stimulated. **(D)** Comparison of HBV cccDNA detection by nested PCR in PBMC from CHB carriers (ID#s 233-2 and 312). HBV cccDNA was detected in both stimulated and unstimulated PBMCs, and cccDNA level is increased after mitogen stimulation.

4.5 Discussion

Chronic HBV infection is the major cause of HCC and is implicated in extrahepatic malignancies, especially lymphoma. Prior studies have demonstrated the association of SNPs within the HBV genome and viral integration to HCC, but few have evaluated these viral features within the lymphoid cells/tissues. The current study evaluates liver, PBMC and a representative patient with a lymphoid cell malignancy for the presence of HBV genomes and oncogenic markers. Recently, new HBV biomarkers (i.e., serum RNA quantification) have been developed to assess HBV replication which we used along with in-depth molecular characterization (NGS, genotype, and integration) in clinical samples.

In the current study, the utmost care was taken to ensure that HBV genomes found associated with PBMC were not the result of cell-surface attached viruses. This was achieved by extensive proteinase and DNase treatments as established in the literature (228). We demonstrated that incubation of PBMC from HBV-naive/healthy individuals with plasma from a highly viremic CHB carrier, followed by cell-surface treatment successfully removed all extracellular viral particles (Figure S4.1). In addition, our findings indicate that viral replicative intermediates including HBV DNA and cccDNA are detectable in all samples and cohorts including CHB carriers without or with a history of hepatic or extrahepatic malignancy. Interestingly, we observed a higher detection rate of cccDNA within the PBMCs of CHB+HCC (16/23 or 69.6%) in comparison to CHB-only individuals (6/12 or 50%). Similarly, we demonstrate an overall trend of higher full-length and truncated HBV RNA present within the serum of CHB carriers with a history of HCC. As a promising clinical biomarker in development, serum HBV RNA has yet to be well characterized or studied in CHB carriers with malignancy (229). The current study

suggests that a higher HBV replication or transcription is occurring in individuals with HBV-related HCC.

Our in-depth molecular virological characterization demonstrates that factors associated with oncogenesis and HCC such as genotype, HCC-associated SNPs, and HBV integration are present within all samples. Most prior studies (136,138,141) of these viral factors evaluated serum/plasma or liver only, but we observed the pre-dominance of the oncogenic genotype C within the PBMCs of CHB carriers with or without HCC. The presence of HCC-associated SNPs was also noted within the PBMC. However, the oncogenic impact of genotype C or the evaluated SNPs found in the PBMCs is unclear. Differences in genotype and SNP frequencies were observed amongst the viral reservoirs. A prime example is the CHB+DCS case (ID# 420) as the genotype and SNP frequencies differed greatly amongst the plasma, PBMC, and DCS tumor (Table 4.2, Figure 4.2D). However, these observations were expected as previous studies by our group and others reported similar variations between the different reservoirs (126,127,148,189,214,230). Interestingly, our in-depth NGS results shows highly variable frequencies in SNPs present in all CHB cohorts assessed. Despite many studies associating these SNPs to malignancy, it still remains to be elucidated whether the proportion of variants within the HBV quasi-species, their long-term persistence, the stage of CHB, or host genetic variants are potential confounding aspects of these associations to HCC (136,231). Thus, the impact of the SNP frequencies observed in our study to HCC risk is challenging to determine.

HBV integration is an important causative agent in hepatocarcinogenesis, even in CHB carriers with low viremia or without cirrhosis. Indeed, Chen *et al.* (52) recently demonstrated the presence of HBV integration in patients with occult HBV infection and undetectable viral levels. Prior investigations of HBV integration in the lymphoid cells (160,181,232–234) typically lack

the host-viral junction analysis via sensitive use of AluPCR employed with clonal sequencing and are not performed with matching samples of PBMCs and hepatic or extrahepatic tumor tissue from HBsAg+ CHB individuals. Our current study is highlighted by an in-depth evaluation of viral integration in the circulating PBMC from CHB carriers, including a patient with extrahepatic lymphoproliferative malignancy. Using the well-established AluPCR technique, we detected large numbers of viral integration events within the PBMCs from all cohorts. Many of these HBV-host junctions are located at coding genes with strong associations to cancer, such as tumor suppressors and oncogenes. Further studies are required to analyse the functional consequences of these integrations, particularly with regards to immune cell function, malignant cell surveillance, and malignant transformation. In addition, the cellular and molecular biology underlying HBV integration in PBMCs such as the kinetics, frequency, and specific mechanism of integration would require investigation.

The presence of HBV within the lymphoid cells has been reported with a limited understanding of potential clinical implications (225). However, findings from prior studies suggests that extrahepatic viral replication is clinically relevant with regard to long-term HBV persistence. It was documented that extrahepatic HBV promptly infects donor hepatic tissue and persists for up to 15 years after liver transplant despite prophylaxis (148,189). Infection of lymphoid cells may adversely affect host immune responses, compromise antiviral surveillance, and contribute to viral persistence. In the current study, we found HBV replicative intermediates (DNA, RNA, and cccDNA), proteins (HBsAg and HBcAg), and integration in extrahepatic lymphoproliferative tumor (DCS). The HBV is present at low-levels, requiring sensitive PCR-based methods for cccDNA detection. Our study, limited by only one CHB patient with an extrahepatic malignancy, provides important “proof-of-concept” pilot data and a mechanistic

basis for epidemiological studies linking CHB with hematological malignancies, such as lymphoma and leukemia (175,177). Our observations are consistent with a recent study by Sinha et al. (182) who found HBV DNA, including A1762T/G1764A oncogenic mutants, within diffuse large B-cell lymphoma tissue from 21/24 patients.

We investigated the replicative potential of HBV within lymphoid cells which was evidenced by up-regulation of viral DNA in PBMC supernatant after *ex vivo* mitogen-stimulation. Increased viral replication was evident by increased levels of HBV cccDNA and enhanced viral mRNA expression. Similarly, independent studies by Bouffard *et al*. and Yan *et al*. reported that stimulation of PBMCs using mitogens, PHA and concanavalin A or IL-2, resulted in noticeable increases in HBV DNA, RNA, and HBsAg (169,235). These increases in HBV replication and expression after mitogen-stimulation could explain our findings of increased serum HBV RNA and higher rates of cccDNA detected in the PBMCs of CHB+HCC cases. Taken together, these data provide evidence that lymphoid cells from CHB individuals may support propagation of infectious HBV despite long-term suppressive NA-therapy. Our findings are also interesting from an oncological standpoint due to effect on mitogen-susceptible cellular pathways (236) leading to enhanced HBV replication in PBMCs undergoing malignant transformation. In addition to HBV (126,128), lymphoid cell infection was reported to be a common nature of other persistent viral infections, such as hepatitis C virus, herpesvirus, and human immunodeficiency virus type-1 by utilizing various entry mechanisms mediated through high or low affinity receptors, micropinocytosis and phagocytosis (237–240). Our data suggests HBV lymphotrophism as the molecular virological basis for HBV-related extrahepatic disease warranting additional clinico-pathological investigation. The current study is relevant in ongoing research efforts to develop a sterilizing HBV cure (50) and provides novel data on HBV

molecular biology (i.e., integration and replication) and HBV-related oncogenesis in liver as well as in lymphoid cells.

4.6 Supplementary materials and methods

4.6.1 Subjects from University of California San Francisco and Beijing YouAn Hospital, Capital Medical University

Clinical data on 7 CHB + HCC patients recruited from UCSF was previously published (Coffin et al, JVH 2011 (128)), all of whom received a liver transplant. 3 CHB + HCC cases were recruited from Beijing YouAn hospital (2 males, 2 on NA therapy, 3 HBeAg negative/anti-HBe positive, 1 detectable HBV DNA [477 IU/mL]; median [range]: age = 53 [19-63]; quantitative HBsAg = 845.3 IU/mL [164.4-4074]).

4.6.2 Genotype determination, SNP frequency determination, and quasi-species analysis

HBV genotype was determined using Sanger sequencing of S genomic region nested PCR amplicons and phylogenetic tree analysis along with representative reference sequences (226). Genetic variants within the HBV X/BCP/PC region including T1674C/G, A1752G, T1753V, A1762T, G1764A, T1768A, C1773T, A1846T, G1896A, and G1899A, were analyzed by next generation sequencing (NGS; Illumina Miseq) and SNP analysis of aligned reads. Processing of paired-end reads and identification of quasi-species was done as previously described (189). Haplotypes were defined using a threshold of 99% similarity in nucleotide sequence. Haplotype diversity within samples were determined using MEGA version X (241).

4.6.3 Evaluation of extracellular enzymatic treatment in removal of potentially adhered HBV

To demonstrate the efficiency of extracellular enzymatic (DNase/trypsin/DNase) treatment in removing extracellular HBV particles from cell surface and confirm detected virus, PBMC from

HBV-negative (healthy) individuals were co-incubated with plasma from a CHB carrier (case ID# 312) with high HBV DNA (8.2 log IU/mL). In brief, healthy PBMCs were cultured with viremic plasma diluted 1:1 with RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) for either 6 or 24 hours. Plasma from a HBV-negative individual was used as a negative control. The supernatant was collected after co-culture by centrifugation at 300g for 10 mins, followed by one DPBS wash. The PBMC pellet was then subjected to DNase I (10 μg, NEB), Trypsin (100 μg, Millipore-Sigma) and again with DNase I followed by two additional washes with 0.25% Tween 20 DPBS. All washes and the cell pellet were collected for DNA extraction by phenol/chloroform with subsequent HBV DNA detection using nested PCR with HBV surface genomic primers.

4.6.4 Quantification of full length and truncated HBV RNA in serum

HBV RNA in serum of CHB carriers was quantified as described by van Bömmel *et al*. (11). In brief, total RNA was isolated from 500uL of serum with TRIzol™ (Invitrogen, Carlsbad, USA) as per manufacturers' instructions and eluted into 30uL of RNase-free water. Specific amplification of total HBV RNA was accomplished using the Rapid Amplification of Complementary DNA (RACE) technique targeting HBV poly-A region. HBV RNA was subsequently quantified with the PerfeCTa FastMix II and dilution of a plasmid standard (Quanta Biosciences, Beverly, USA). Negative controls for qPCR include no template controls (water) and mock RNA extraction samples. Statistical analysis to compare mean serum RNA values were performed with two-tailed student t-test using a significance value of ≤ 0.05.

4.6.5 Direct nucleic acid hybridization for HBV cccDNA

A direct NAH of unamplified DNA was performed for Hirt extracted DNA from homogenized DCS tissue (case ID# 420). In addition, Hirt-DNA from explant non-tumor liver

tissues (case ID# CC06 and 405) and HCC tumor tissue (case ID# 405) were simultaneously collected. Hirt DNA was divided into 3 conditions: untreated; 85°C for 5 minutes followed by EcoRI digestion (from New England Biolabs [NEB], Ipswich, USA); or 85°C for 5 minutes followed by ApaLI digestion (NEB). The 85°C treatment would separate any potential relaxed circular DNA (rcDNA) into ssDNA strands. The restriction enzymes used were selected due to their single recognition site within the HBV thereby linearizing the cccDNA. Untreated and treated Hirt DNA was ran on a 1% agarose gel followed by transfer to a nylon membrane (Amersham Hybond N+). The membrane was probed with subsequent wash and detection using the Roche DIG Wash and Block Buffer set and DIG Nucleic Acid Detection kit. Probes for NAH were created with the Roche PCR DIG probe synthesis kit targeting the X/BCP/preC, preC/Core, and Surface genome regions (i.e., direct PCR primers). Equal proportions of these resulting probes were utilized for NAH. A NAH marker and positive control containing 3.2kb, 2.1kb, and 1.7kb fragments of HBV was created from restriction enzyme digestion of plasmids containing a full genomic HBV dimer. Negative controls for NAH includes mock Hirt extraction.

4.6.6 Western blot for HBsAg and HBcAg

Total protein was isolated from homogenized dendritic cell sarcoma (DCS) tumor tissue (case ID# 420) with a SDS-based lysis buffer followed by protein concentration determination using the Bio-Rad DC™ Protein assay as per the manufacturers' protocol (Bio-Rad Laboratories, Hercules, USA). 40ug of protein was loaded onto a 12% Sodium Dodecyl Sulfate Polyacrylamide (SDS-PAGE) gel and the subsequent resolved gel was transferred to a nitrocellulose membrane (Amersham Protran 0.45 NC). Membranes were blocked with 5% skim milk before exposure to primary antibody: 1:1000 anti-GAPDH mouse monoclonal (Invitrogen 39-8600); 1:100 anti-HBsAg mouse monoclonal (Santa Cruz sc-53299); and 1:500 anti-HBcAg

mouse monoclonal (Abcam ab8637). Secondary antibody used was anti-mouse IgG HRP conjugated (GE healthcare NA-931V) at 1:10000 (for GAPDH) or 1:1000 (for anti-HBsAg and anti-HBcAg). Blots were developed with Immobilon® Forte Western HRP substrate (Millipore Sigma, Burlington, USA).

4.6.7 Effect of mitogen stimulation on HBV in total PBMC from CHB carriers

Freshly isolated PBMCs from untreated and treated CHB carriers (n = 7, Table S4.3) were gently washed 3x with PBS followed by DNase/Trypsin/DNase treatment (as described above) to remove any extracellular HBV particles. The PBMCs were cultured in RPMI1640 medium supplemented with 10% FBS and 1% Pen/strep (Invitrogen). To stimulate T cells and B cells in the PBMC cultures, we used a cocktail consisting of phytohemagglutinin (PHA) and pokeweed mitogen (PWM) (5 μg/mL; Millipore-Sigma) along with interleukin-2 (IL-2) (20 U/mL; Millipore-Sigma) and interleukin-4 (IL-4) (1 ng/mL; Millipore-Sigma) to stimulate 2 x 10^6 cells per well (242). As a control (i.e., unstimulated PBMCs), only IL-2 and IL-4 was supplemented in cell culture. Both stimulated and unstimulated PBMC were cultured for 72 hours. Total PBMCs and cell supernatant were collected following *ex vivo* mitogen stimulation and analyzed for the presence of HBV cccDNA, DNA and mRNA.

4.6.8 HBV DNA quantification and mRNA detection after ex vivo mitogen stimulation

HBV DNA in *ex vivo* PBMC supernatant was quantified by qPCR assay using HBV surface specific primers (TaqMan with lower detection limit ~14 copies/mL, Table S4.4). All reactions were set up in duplicate using the PerfeCTa FastMix II (Quanta Biosciences) following the manufacturer's instruction. To detect HBV mRNA, total RNA was isolated from PBMC pellets using TRIzol™ reagent. 1μg of RNA was digested with DNase I (NEB) to remove DNA carry-

over followed by cDNA synthesis using the iScript™ Kit (Bio-Rad Laboratories). The subsequent cDNA was used for HBV S nested PCR as described above. Each cDNA synthesis step was performed in parallel with and without reverse transcriptase (RT) enzyme to confirm specificity for cDNA detection and not contaminating HBV DNA. Human glycerol-dehyde-3-phosphate dehydrogenase (GAPDH) expression level was examined in parallel (Supplementary Table 3) as an internal control for mRNA detection. In addition, RT qPCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) with HBV qPCR surface region and GAPDH as an internal reference gene (Supplementary Table 3). The $2^{-\Delta\Delta Cq}$ method was used for analysis of semi-quantitative qPCR data and to determine the change in surface mRNA expression after mitogen stimulation. All nucleic acid extractions were performed alongside mock extractions (i.e., PBMC or plasma from a healthy individual). PCR controls also included water (negative control) and HBV plasmid (positive control), as described above. Statistical analysis was performed using the non-parametric Wilcoxon signed-rank test was used to compare the means between the groups. A p-value of ≤ 0.05 was considered to be statistically significant.

4.7 Acknowledgements

The authors would like to thank all patients and staff of the Calgary Liver Unit and Ms, Gurmeet Bindra for assistance with sample collection.

4.8 Disclosures and Conflict of Interest

CSC has served as an advisory board member and/or has received research support from, Glaxo Smith Kline and Gilead Sciences. **KWB** has served as a speaker, advisory board member and/or has received research support from Bayer, Verlyx, Lupin, Astellas, Gilead, Amgen and Merck. The other authors have no relevant disclosures.

4.9 Funding/Financial Support

KCKL is supported by the Izaak Walton Killam doctoral scholarship.

4.10 Ethics ID

The study received approval from the local ethics review board committee at the University of Calgary (i.e., Conjoint Ethics Review Board, Ethics REB-16636).

CHAPTER 5: DIFFERENCES IN HBV REPLICATION, APOBEC3 FAMILY EXPRESSION, AND INFLAMMATORY CYTOKINE LEVELS BETWEEN WILD-TYPE HBV AND PRE-CORE (G1896A) OR BASAL CORE PROMOTER (A1762T/G1764A) MUTANTS

5.1 Abstract

Background: Chronic hepatitis B virus (HBV) infection is the leading cause of hepatocellular carcinoma (HCC) world-wide. HBV variants, particularly the G1896A pre-core (PC) and A1762T/G1764A basal core promoter (BCP) mutations, are established risk factors for cirrhosis and HCC, but the molecular biological basis is unclear. We hypothesized that these variants result in differential HBV replication, APOBEC3 family expression, and cytokine/chemokine expression. **Methods:** HepG2 cells were transfected with monomeric full-length containing wild-type, PC, or BCP HBV. Cells and supernatant were collected to analyze viral infection markers (i.e., HBsAg, HBeAg, HBV DNA, and RNA). Cellular APOBEC3 expression and activity was assessed by quantitative real-time (qRT)-PCR, immunoblot, differential DNA denaturation PCR, and sequencing. Cytokine/chemokines in the supernatant and in serum from 11 CHB carriers (4 non-cirrhotic; 7 cirrhotic and/or HCC) with predominantly wild-type, PC, or BCP variants were evaluated by Luminex. **Results:** HBeAg expression was reduced in PC and BCP variants, and higher supernatant HBV DNA and HBV RNA levels were found with A1762T/G1764A vs. G1896A mutant ($p<0.05$). Increased APOBEC3G protein levels in wild-type vs. mutant was not associated with HBV covalently closed circular DNA G-to-A hypermutations. Differences in cytokine/chemokine expressions, especially IL-13 were observed amongst the variants analyzed in cell supernatant. Noticeable increases of numerous cytokines/chemokines, including IL-4 and IL-8, were observed in *ex vivo* serum collected from

CHB carriers with PC mutant. **Conclusion:** HBV sequence variation leads to differences in HBV protein production (HBeAg) and viral replication in addition to altered host innate antiviral restriction factor (APOBEC3) and cytokine/chemokine expression.

5.2 Introduction

The hepatitis B virus (HBV) is a significant global pathogen with ~257 million chronic HBV (CHB) carriers worldwide (1). CHB can lead to cirrhosis, liver failure, and hepatocellular carcinoma (HCC). HBV chronicity is due to an ineffective host immune response and persistence of the intranuclear HBV minichromosome, covalently closed circular DNA (cccDNA), which are poorly targeted by currently approved reverse transcriptase inhibitors (nucleos/tide analog) therapies. (2). Due to the error-prone method of viral replication, the HBV exists as quasi-species within the host (3). HBV-related oncogenesis is complex and is influenced by viral characteristics such as genetic variants particularly within the X/basal core promoter (BCP)/pre-core (PC) region and integration into the host chromosomes contributing to genomic instability and hepatocarcinogenesis. Through next-generation sequencing (NGS), our group and others have demonstrated the variability of HBV within CHB carriers either with and without end-stage liver disease (cirrhosis and cancer) (4–6). Moreover, we have shown HBV genome integration in both liver and lymphoid cells in individuals with hepatic and extrahepatic malignancy (7,8).

HBV X/BCP/PC mutations (i.e., G1896A pre-core and A1762T/G1764A double mutants) are strong predictors of HCC risk (9–14) and frequently reported in large epidemiological studies of HBV-related HCC (3). However, there is a limited understanding of the underlying molecular mechanisms and cellular pathogenesis of viral sequence heterogeneity leading to end-stage liver disease. The G1896A pre-core mutation introduces a premature stop codon in the precore/core

HBV transcript resulting in abrogated HBeAg production (15). The A1762T/G1764 double mutation is located within the BCP region of the HBV genome which influences the expression of both the pre-core/core and the pregenomic (pg) RNA transcripts. As a result of the double mutation, pgRNA transcript synthesis is favored. HBeAg protein synthesis is reduced by ~30-50% whereas pgRNA expression and subsequent HBV genome replication doubles (3,15). Both the G1896A and A1762T/G1764A mutations are associated with the "HBeAg-negative hepatitis" phase of CHB. These mutants are frequently found in CHB carriers who experience hepatic flares and liver inflammation with HBeAg-negative serology (9,16). Clinically, HBeAg-negative CHB carriers are found to have significantly lower levels of HBV DNA (16). There is conflicting data from *in vitro* studies on the replicative capacity of G1896A or A1762T/G1764A HBV compared to wild-type HBV (17–19).

The innate antiviral restriction factors apolipoprotein B mRNA editing enzymes (APOBECs) serve to inhibit retroviruses, such as human immunodeficiency virus (HIV), due to their cytidine deaminase activity which results in G-to-A hypermutations. The human APOBEC3 family comprises of seven members (A, B, C, DE, F, G, and H) which vary in their localization, regulation, and substrate preferences (20). In both HBV infected patients and experimental models, the APOBEC3 family of proteins have been demonstrated to induce hyper-editing and degradation of HBV cccDNA with potential therapeutic applications (21–26). Interestingly, G-to-A hypermutations within HBV genomes due to APOBEC3 activity are disproportionately found in HBeAg-negative CHB carriers (27,28). In addition to the antiviral effects, APOBEC3 proteins are endogenous carcinogens as nucleic acid editing enzymes. Indeed, multiple cancers, including HCC, have been associated with APOBEC3 expression and mutational activity (29–31). Although many retroviruses encode specific viral products which functions to counteract the

effects of APOBEC3s such as the HIV vif proteins (32), a similar viral factor has been identified in HBV infection. We evaluated whether APOBEC3 activity and expression is altered by X/BCP/PC variants as a mechanism that increased risk of end-stage liver disease (i.e, HCC and cirrhosis) in CHB carriers harboring these mutants.

In the current study, we evaluated *in vitro* the functional properties of two clinically important genetic variants of HBV associated with HCC, the G1896A and A1762T/G1764A mutations. Our findings demonstrate that HBeAg expression is reduced, yet other markers of viral replicative capability are comparable between the mutant and wild-type HBV. It is noteworthy that A1762T/G1764A has a more robust viral replication than the G1896A HBV variant. Although HBV X/BCP/PC variants show reduced APOBEC3G expression, differential DNA denaturation (3D)-PCR and sequencing results show that APOBEC3 G-to-A hypermutation activity does not differ between the HBV variants and the wild-type virus. HBV infection leads to differential cytokine expression dependent on sequence in cell supernatant as well as in *ex vivo* tested serum samples from patients with and without end-stage liver disease. HBV PC/BCP mutations affects HBeAg expression, viral replication and APOBEC3 expression, but does not impact host innate antiviral genome editing *in vitro*.

5.3 Materials and Methods

5.3.1 Construction of full-length HBV plasmids and site-directed mutagenesis

Plasmid DNA containing a full genome copy of the HBV genome was constructed in-house. Briefly, HBV full genome was amplified from phenol-chloroform DNA isolated from a high viral load clinical sample (> 5×10^8 HBV copies/mL; genotype C) with high-fidelity Phusion DNA polymerase (New England Biolabs, Whitby, Canada). HBV FG P1 and P2 primers for amplification (Table S1) were derived from Günther *et al.* (33). PCR amplicons were purified

from a 0.9% agarose gel followed by digestion with HindIII (New England Biolabs) and ligated with T4 DNA ligase into the cloning vector pUC19 which was similarly prepared. Ligations were transformed into TOP10 *E. coli* chemically competent cells (Invitrogen, Carlsbad, USA) and successful clones were identified and confirmed via PCR and sequencing, respectively, with standard M13 primers thus generating the pUC19-HBV.wt plasmid (Table S1).

Generation of G1896A and A1762T/G1764A mutants were achieved using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, USA), as per manufacturer's protocol using the pUC19-HBV.wt and mutagenic primers (Table S1). Purified plasmids (pUC19-HBV.g1896a and pUC19-HBV.dmut) were isolated from selected clones and subsequently sequenced in-house to confirm the mutagenesis.

5.3.2 Cell culture and transfection of linearized HBV

HepG2 cells (Millipore Sigma, Burlington, USA) were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum and 1% pen/strep (Invitrogen) at 37°C and 5% CO_2. For transfection, cells were seeded into 6 well plates at 7.5×10^5 cells/well and incubated overnight. Each well of HepG2 cells were transfected with 266ng of linearized full-genome monomeric HBV (~7.5×10^{10} copies) using Lipofectamine™ 3000 (Invitrogen) as per manufacturer's protocols. Supernatant and cells were collected at 0.25, 0.5, 1, 2, 3, 5, and 7 days after transfection for analysis of HBV markers and cellular mRNAs or proteins. Linearized HBV was prepared by digestion of pUC19-HBV plasmids with BspQI (New England Biolabs). The desired 3.2kb HBV full-genome fragments were then purified from 0.9% agarose gels before use in transfections. All subsequent analyses included three independent transfections that were performed alongside 500ng of pcDNA3.1-GFP as a marker of transfection success and efficiency was evaluated by fluorescent microscopy.

5.3.3 Isolation and quantification of supernatant HBV DNA and RNA

HBV DNA in extracellular viral particles were isolated from 500uL of transfected cell supernatant using polyethylene glycol as previously published by Pollicino *et al.* (34). Subsequently isolated viral particles were then digested with DNase I (Quanta Biosciences, Beverly, USA) to remove any residual DNA from the transfection. Viral DNA was extracted from isolated particles using standard phenol-chloroform extraction and quantified using an in-house HBV DNA qPCR. In brief, the iTaq Universal SYBR Green (Bio-Rad Laboratories, Hercules, USA) was utilized alongside a plasmid standard for the purpose of HBV DNA quantification in supernatant. The PCR primers used spanned the ends of the linearized HBV DNA to reduce the detection of residual transfected DNA (Table S2).

Total RNA in the supernatant was isolated from 500uL of transfected cell supernatant with TRIzol™ (Invitrogen) as per manufacturers' instructions and eluted into 20uL of RNase-free water. Potential DNA contaminants were digested with DNase I (Quanta Biosciences) before specific amplification of HBV RNA using the Rapid Amplification of Complementary DNA (RACE) technique targeting HBV poly-A region (35). HBV RNA was subsequently quantified with the PerfeCTa FastMix II (Quanta Biosciences) and dilution of a plasmid standard.

Negative controls for qPCR include no template controls (water) and mock DNA/RNA extraction samples. Reverse transcriptase negative samples were also tested to confirm a lack of DNA contaminants in supernatant RNA experiments. All samples were performed in triplicate.

5.3.4 Detection of cellular HBV cccDNA

HBV cccDNA was isolated from transfected HepG2 cells by the Hirt extraction as previously described (4,36). Post-extraction T5 exonuclease digestion was performed prior to quantification

of cellular cccDNA using qPCR (21,37). In addition, our previously published nested PCR technique targeting the nicked region of HBV was utilized for detection of the cellular cccDNA (4). Approximately, 20-30ug of Hirt extracted DNA prior to T5 digestion was utilized for nucleic acid hybridization (NAH) by first size separation on a 0.9% agarose gel followed by transfer to a nylon membrane (Amersham Hybond N+). The membrane was probed with subsequent wash and detection using the Roche DIG Wash and Block Buffer set and DIG Nucleic Acid Detection kit. Probes for NAH were created with the Roche PCR DIG probe synthesis kit targeting the X/BCP/preC, preC/Core, and surface genome regions (Table S2). Equal proportions of these resulting probes were utilized for NAH. A NAH marker and positive control containing 3.2kb, 2.1kb, and 1.7kb fragments of HBV was created from restriction enzyme digestion of plasmids containing a full genomic HBV dimer. Negative controls for NAH includes mock Hirt extraction.

5.3.5 Analysis of supernatant HBsAg, HBeAg, and cytokines/chemokines

Quantification of HBsAg in supernatant was done with an in-house sandwich ELISA. In brief, 100ng of capture anti-HBsAg (Fitzgerald 10-H05H, clone M701077) was loaded into each well of protein binding plates (Corning, USA) in antibody binding buffer (Na_2CO_3/$NaHCO_3$ with pH 9.6) and incubated overnight at 4°C. Plates were blocked with 2% w/v BSA in PBS for 1 hour rocking at 37°C before addition of 100uL of sample supernatant and incubated overnight at 4°C. Captured HBsAg was detected with 1:2000 of anti-HBsAg HRP-conjugate (Fitzgerald 60C-CR2100RX) in 2% BSA incubated for 1 hour rocking at 37°C. Samples were then incubated with 100uL of TMB (ThermoFisher Scientific) followed by addition of an equal volume of HCl and measurement of sample absorbance at 450nm. A standard curve of HBsAg protein (Fitzgerald 30C-CP2019R) diluted in HepG2 cell culture media was constructed for

quantification of sample HBsAg. Positive signals were defined as 3 times the absorbance value of blank wells and all samples were performed in triplicates.

Presence of HBeAg within the supernatant was detected by the electrochemiluminescence EIA via the COBAS e411 platform (Elecsys; Roche Diagnostics, Laval, Canada). Cytokines and chemokines present in supernatant collected 12 hours post-transfection was analyzed using a 42-plex Luminex discovery assay (Eve technologies, Calgary, Canada).

5.3.6 Detection of viral proteins and APOBEC3G by Western blot

Total protein was isolated from transfected cells with a SDS-based lysis buffer followed by protein concentration determination using the Bio-Rad DC™ Protein assay as per the manufacturers' protocol (Bio-Rad Laboratories). 30ug of protein was loaded onto a 12% Sodium Dodecyl Sulfate Polyacrylamide (SDS-PAGE) gel and the subsequent resolved gel was transferred to a nitrocellulose membrane (Amersham Protran 0.45 NC). Membranes were blocked with 5% skim milk before exposure to primary antibody: 1:1000 anti-GAPDH mouse monoclonal (Invitrogen 39-8600); 1:100 anti-HBsAg mouse monoclonal (Santa Cruz sc-53299); 1:500 anti-HBcAg mouse monoclonal (Abcam ab8637); 1:1000 anti-APOBEC3G rabbit polyclonal (Abclonal #A1459). Secondary antibody used was anti-mouse IgG HRP conjugated (GE healthcare NA-931) at 1:10000 (for GAPDH) and 1:1000 (for anti-HBsAg and anti-HBcAg) or anti-rabbit IgG HRP conjugated (GE healthcare NA-934) at 1:1000 (for APOBEC3G). Blots were developed with Immobilon® Forte Western HRP substrate (Millipore Sigma). Densitometry analysis was performed using the Image Studio™ Lite software version 5.2 (LI-COR Biosciences, Lincoln, USA).

5.3.7 Semi-quantitative analysis of APOBEC mRNAs by qRT-PCR

Cellular mRNA was analyzed by isolating for total RNA with TRIzol™ (Invitrogen) as per manufacturers' instructions and eluted into 20uL of RNase-free water. Potential DNA contaminants were removed with DNase I (Quanta Biosciences) before cDNA synthesis using the qScript cDNA supermix (Quanta Biosciences). APOBEC3 mRNA fold change was calculated by amplification and normalization alongside the internal reference, GAPDH, using the iTaq Universal SYBR Green (Bio-Rad Laboratories, primer sequences can be found in Table S3). Negative controls for quantitative real-time (qRT)-PCR include no template controls (water), reverse transcriptase negative, and mock RNA extraction samples. All samples were performed in triplicate.

5.3.8 Evaluation of APOBEC3 hypermutation activity

3D-PCR was performed on the qPCR-amplified cccDNA products to identify the presence of HBV genome hyper-editing by APOBEC proteins based on the methodology of Xia *et al.* (37). Positive bands visualized on a 1.3% agarose gel were excised and sequenced in-house (University of Calgary Core DNA facility, Calgary, Canada). To further evaluate for APOBEC3 hypermutation activity, excised amplicons from the cccDNA nested PCR was inserted into the pGEM-T easy vector and transformed into TOP10 *E. coli* for clonal sequencing.

5.3.9 CHB patient sample collection and Luminex analysis

11 CHB carriers (4 non-cirrhotic, 1 cirrhosis and 6 with cirrhosis and HCC), enrolled from our previously published study (7,8) from the University of Calgary Liver Unit were recruited. This study was approved by the UofC conjoint health research ethics board, CHREB (Ethics ID# 16636). Whole blood was collected, from which serum was isolated for analysis in the 42-plex

Luminex discovery assay (Eve technologies). Published NGS sequencing data (7,8) showed the proportion of A1762T, G1764A, and G1896A mutants in the HBV quasi-species population allowed grouping of CHB carriers into three cohorts: predominantly wild-type (<50% of any mutant; n = 4), G1896A (>50% of only G1896A; n = 3), or A1762T/G1764A (>50% of only A1762T and G1764A; n = 4) (Table S4). Clinical data, laboratory assays, and HBV tests including HBV DNA, quantitative HBsAg, HBeAg and anti-HBe was determined with clinical PCR (Abbott or Roche TaqMan PCR) and commercial chemiluminescent microparticle immunoassays (Abbott Architect).

5.3.10 Statistical analysis

Quantifiable supernatant HBV markers (ie., HBsAg, HBV DNA, and HBV RNA) were compared using linear regression models. Comparison of densitometry relative expression, qPCR fold changes, and cytokine/chemokine concentrations were performed using one-way ANOVA with the post-hoc Tukey test. All statistical analysis testing used the GraphPad Prism version 6.0 with a level of significance of 0.05.

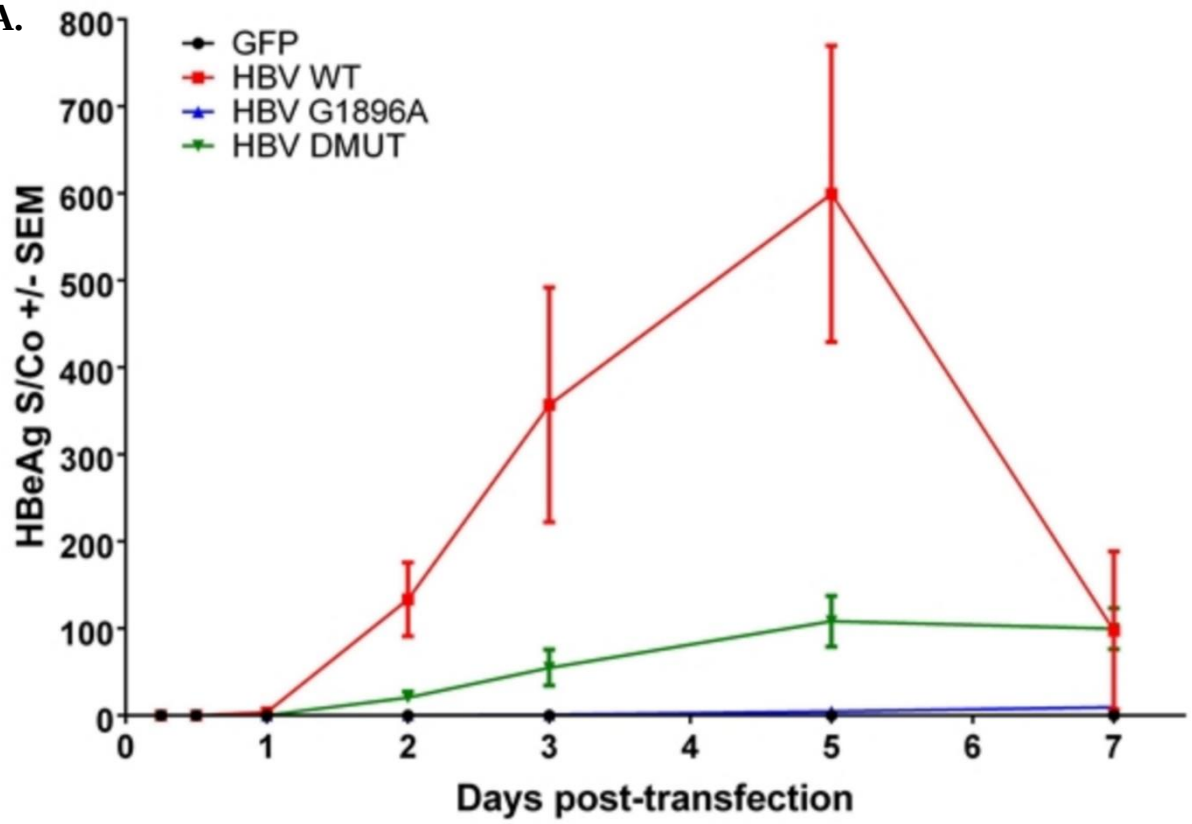
5.4 Results

5.4.1 Viral replication, with the exception of HBeAg, are comparable between X/BCP/PC mutants and wild-type HBV in cultured liver cells

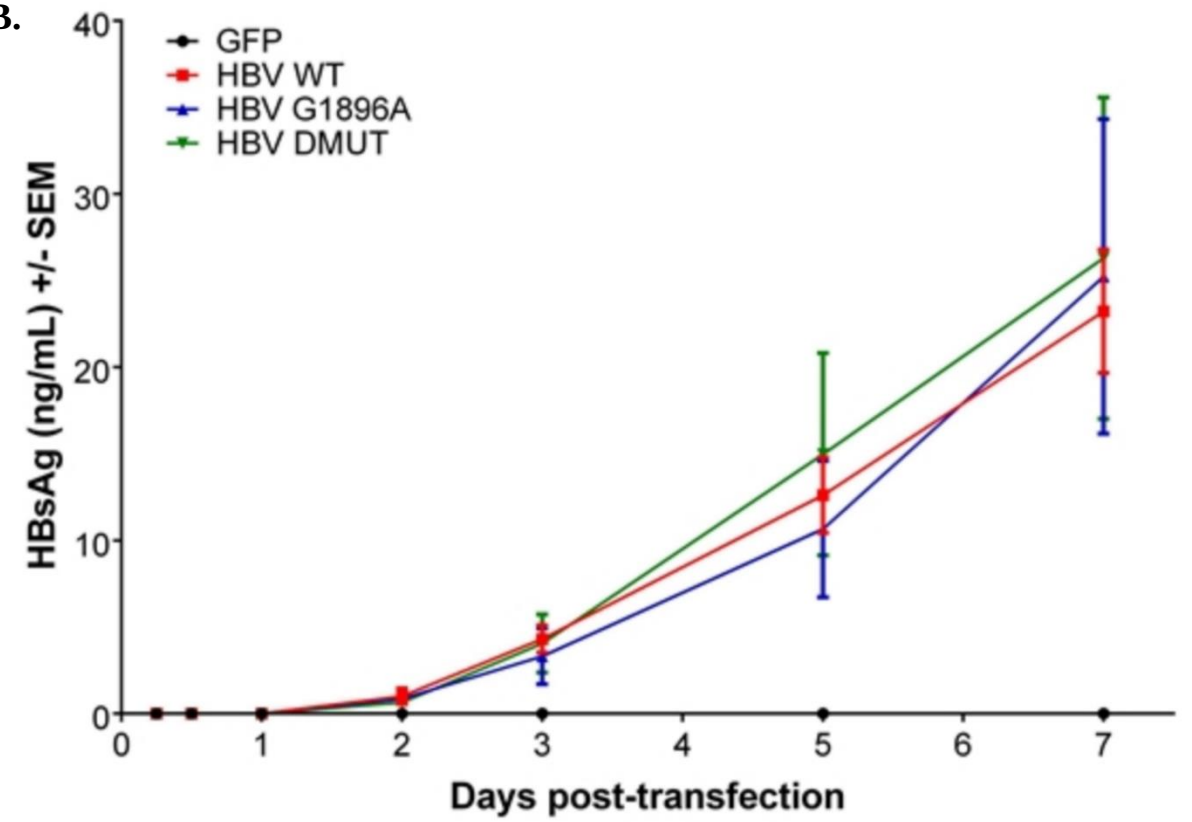
Viral replicative markers including HBsAg, HBV DNA, HBV RNA, and HBeAg in the cell supernatant were compared amongst each variant (Figure 1A-D). As expected, HBeAg expression differed with the greatest levels in the wild-type HBV and clearly reduced levels in the A1762T/G1764A double mutant (Figure 1A). Secretion of HBsAg is comparable between the tested HBV mutants and wild-type HBV. In addition, HBV DNA isolated from the

supernatant was quantified, with only minimal increases over the course of the transfection experiments (Figure 1C). Secreted HBV RNA species is a relatively novel clinical biomarker that have yet to be explored between HBV genetic variants *in vitro*. Here, we demonstrate that no significant differences are present between the X/BCP/PC mutations and the wild-type (Figure 1D). Interestingly, the A1762T/G1764A double mutation HBV has consistently higher levels of supernatant HBV DNA (p-value = 0.0363) and HBV RNA (p-value = 0.0220) in comparison to G1896A HBV variant.

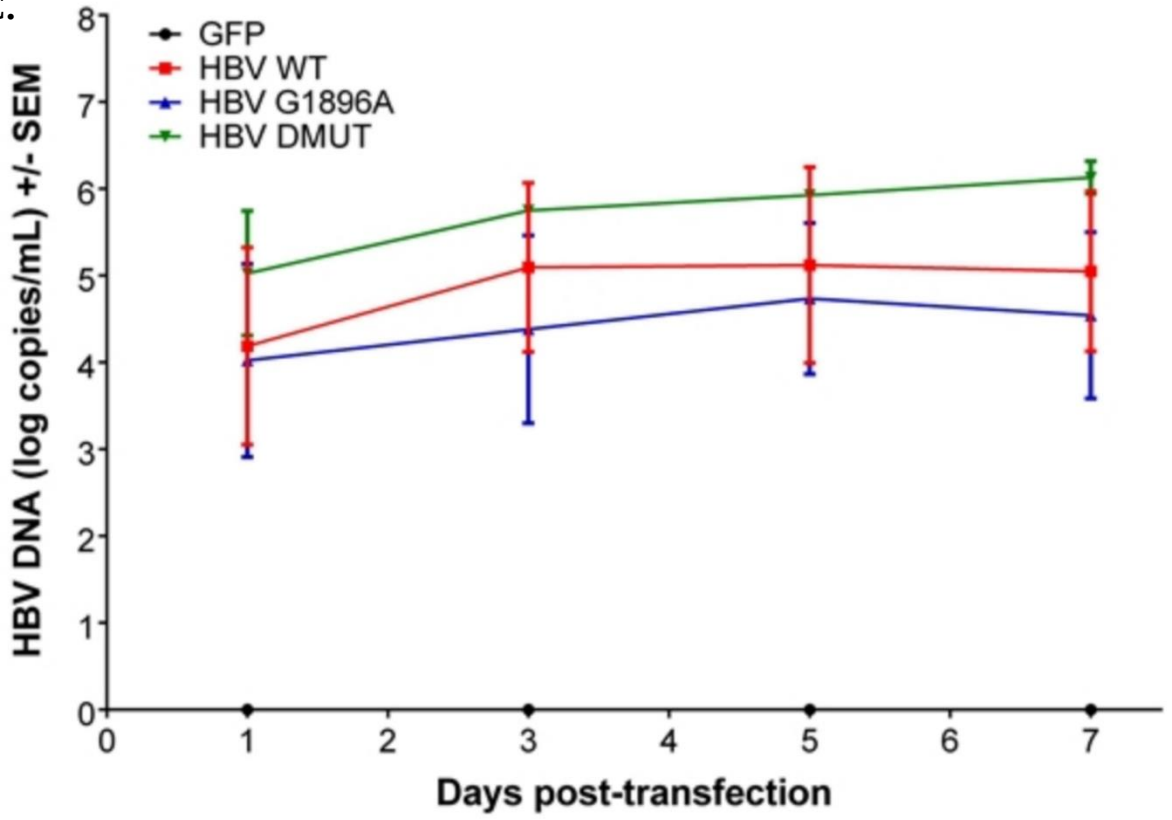
A.


GFP
HBV WT
HBV G1896A
HBV DMUT
HBeAg S/Co +/- SEM
800
700
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
Days post-transfection

B.


GFP
HBV WT
HBV G1896A
HBV DMUT
HBsAg (ng/mL) +/- SEM
40
30
20
10
0
0
1
2
3
4
5
6
7
Days post-transfection

C.


GFP
HBV WT
HBV G1896A
HBV DMUT
HBV DNA (log copies/mL) +/- SEM
0
1
2
3
4
5
6
7
8
0
1
2
3
4
5
6
7
Days post-transfection

D.

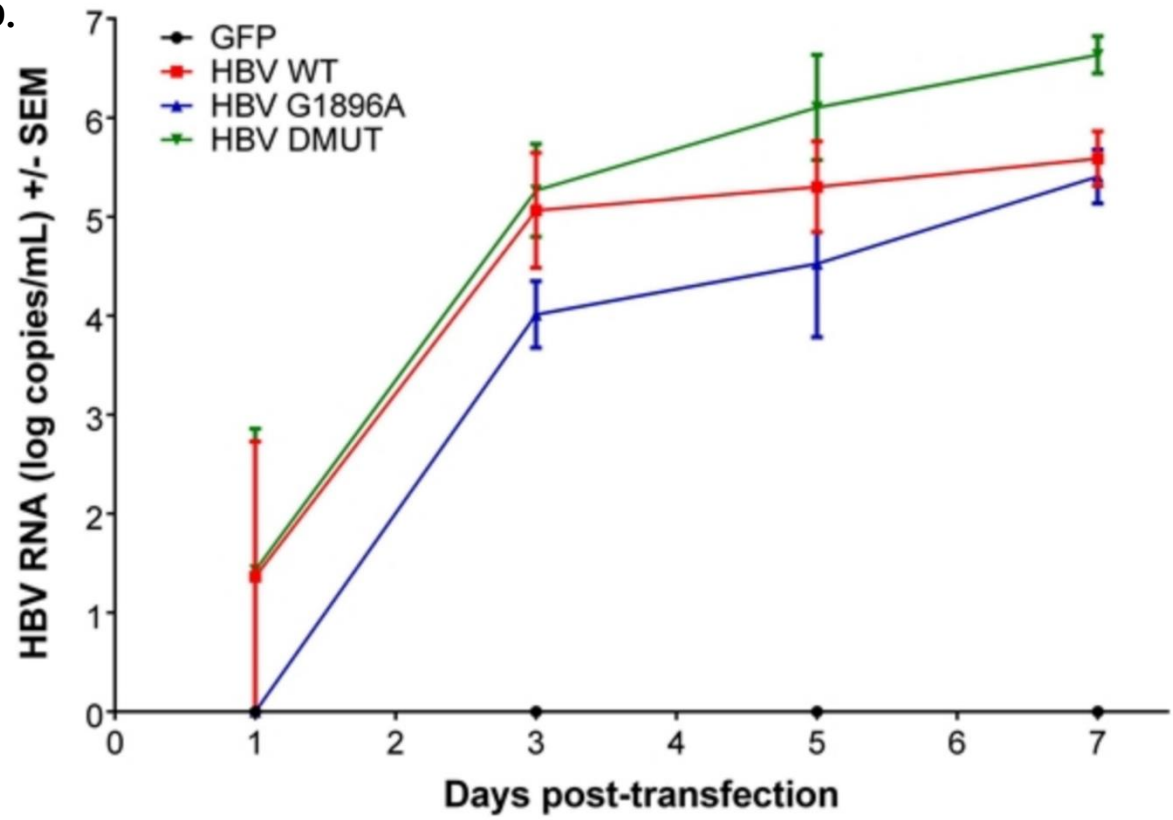

GFP
HBV WT
HBV G1896A
HBV DMUT
HBV RNA (log copies/mL) +/- SEM
0
1
2
3
4
5
6
7
0
1
2
3
4
5
6
7
Days post-transfection

Figure 5.1. **Differences in secreted HBV infection markers in HepG2 cell supernatant after transfection with wild-type vs. PC or BCP mutant HBV genomes.** HepG2 cells were co-transfected with pcDNA-GFP +/- linearized wild-type (WT), G1896A, or A1762T/G1764A (DMUT) HBV. Cell supernatant was analyzed post-transfection for **A.** HBeAg; **B.** HBsAg; **C.** HBV DNA; and **D.** HBV RNA. HBeAg was decreased in mutant compared to wild-type and differences in HBV DNA ($p = 0.0363$) and HBV RNA ($p = 0.0220$) was noted between G1896A vs. A1762T/G1764A mutants. Compiled results of three independent transfections.

Cellular HBV replication and protein markers were also assessed as well as transfection efficiency (Figure S1). In all cases and timepoints, HBV cccDNA was unquantifiable by qPCR, due to low level production. Similarly, direct NAH of Hirt extracts without T5 digestion only identified other forms of HBV DNA species (i.e., relaxed circular or double stranded linear DNA) (Figure 2A). Thus, in order to detect and compare HBV cccDNA, a nested PCR targeting the HBV nicked region effectively identified viral cccDNA samples extracted by Hirt with T5 exonuclease digestion (Figure 2B). Similar levels of cccDNA was observed between the different variants of HBV. Western blot analysis was used to identify intracellular viral protein including HBsAg species and HBcAg (Figure 2C). However, HBcAg was also undetectable, likely due to low level expression within the transfected cells.

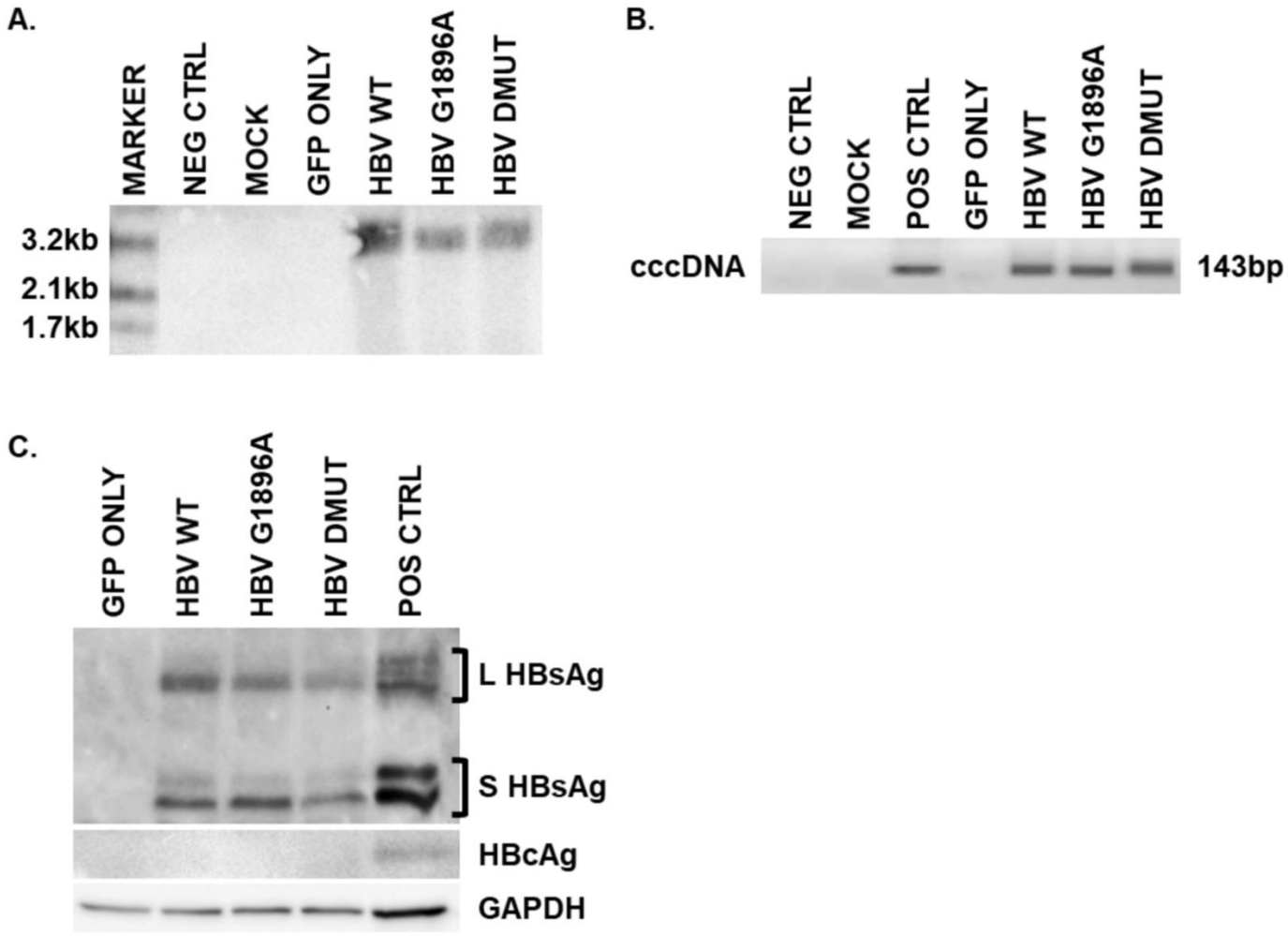


Figure 5.2. **Detection of intracellular HBV replication and infection markers following transfection with wild-type vs. mutant HBV genomes.** Representative images of 3 days post-transfection cellular cccDNA extracts shows undetectable cccDNA by **A.** direct nucleic acid hybridization (without T5 exonuclease digestion), but identification with the more sensitive **B.** nested PCR (with T5 exonuclease digestion) targeting the HBV nicked region. PCR positive control used was a full-length HBV plasmid. In addition, **C.** viral surface protein species were detected within the cellular lysates via immunoblot, but not HBV core proteins likely due to low levels**.**

Immunoblot positive control used was liver tissue protein extracts from a chronically infected HBV individual. Representative results of three independent transfections.

5.4.2 Differences in APOBEC3 family expression between wild-type vs. mutant HBV do not result in alterations in HBV cccDNA hypermutation in hepatoma cells

APOBEC3 family gene expression was evaluated by qRT-PCR in HepG2 cells transfected with linearized HBV DNA. The presence of the viral DNA or different variants resulted did not result in significant changes in APOBEC3 family mRNA induction (p-values > 0.5) (Figure S2A). Further, an evaluation of APOBEC3G protein levels by immunoblot showed increased amounts in wild type compared to the mock and HBV X/BCP/PC variants (Figure 3A) which was confirmed with densitometry analysis (p-values: vs. mock = 0.0233; vs. G1896A = 0.0117; vs. A1762T/G1764A = 0.0033). Subsequent analysis of APOBEC3 activity between HBV variants was assessed using 3D-PCR. Prior studies have found that HBV DNA species show G-to-A hypermutations via the APOBEC3 family members (21–26), however no visible differences in DNA denaturation was observed in the cccDNA 1 day post-transfection between the X/BCP/PC variants and wild-type HBV (Figure 3B). Indeed, sequencing data analysis showed similar levels of nucleotides (particularly G and A bases) present even amongst PCR products from lower denaturation temperatures (Figure S2B). This lack of hypermutation of the viral cccDNA was observed even at later time points including 3, 5, and 7 days post-transfection (Figure S3). To confirm the results of the 3D-PCR hypermutation assays, clonal sequencing of the cccDNA nested PCR products was performed and revealed a lack of HBV cccDNA G-to-A hypermutation (Figure S4).

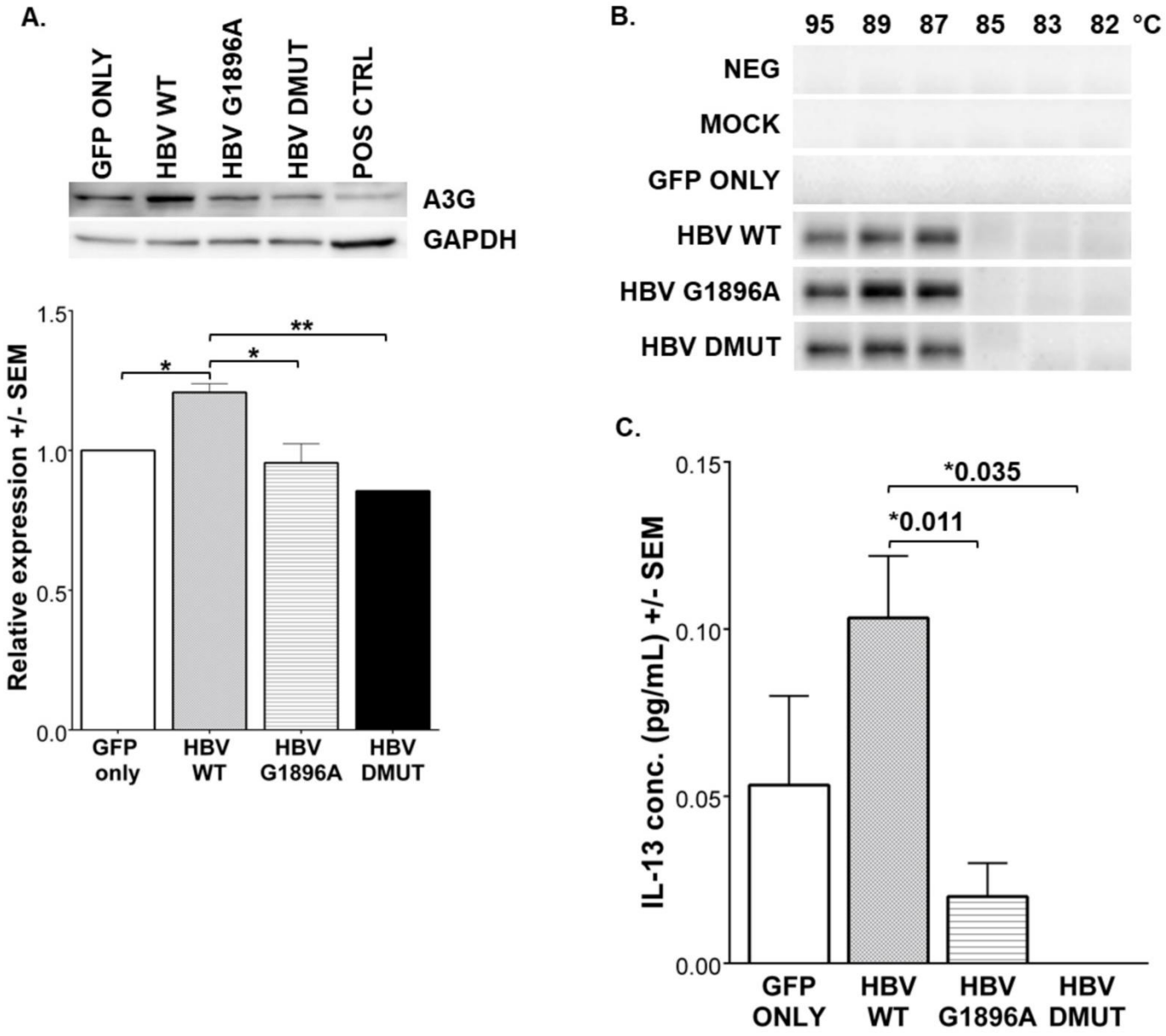


Figure 5.3. **Expression IL-13 and APOBEC3G (A3G) is reduced in HBV X/BCP/PC mutants, but do not result in any mutational changes in HBV cccDNA. A**. A3G protein was detected by western blot with densitometry analysis 1-day post-transfection. Reduced A3G protein expression was found in mutants vs. WT. Positive control was liver explant tissue from chronically infected HBV carrier. **B.** 3D-PCR was used to assess for APOBEC3 family hypermutation activity of HBV genomes

were evaluated 1-day post-transfection. **C.** Cell supernatant was collected 12 hours reveals that secreted immune factor IL-13 had differential expression patterns amongst the wild-type and mutant HBV. Relative expression and cytokine concentrations were analysed using one-way ANOVA with post-hoc Tukey test: *0.01 < p-value < 0.05; **0.001 < p-value < 0.01. Compiled results of three independent transfections.

5.4.3 Expression of cytokines/chemokines differs amongst HBV variants

Cytokines and chemokines within the supernatant were evaluated 12 hours post-transfection to identify differences in hepatocyte innate immune responses upon exposure to HBV. Analysis of cytokines/chemokines revealed differences in Interleukin (IL)-13 between the wild-type and variants tested. It is interesting to note that both of the X/BCP/PC variants lead to suppression of this cytokine in comparison to wild-type (p-values: vs. G1896A = 0.011; vs. A1762T/G1764A = 0.035, Figure 3C). Indeed, other cytokines/chemokines including IL-12P40, MDC, TGF-α, and sCD40L followed trends of differential expression levels amongst the wild-type and variant HBV (Figure S5). In addition, serum derived from both HBeAg positive and negative CHB carriers containing these mutations were analysed (Table S4). It is noteworthy that many cytokines/chemokines were noticeably elevated in the individuals with predominantly G1896A variant (Figure S6-7).

5.5 Discussion

HBV genetic variants have been identified with clinical implications including antiviral treatment response, immune escape, and increased risk to HCC (3). Prior epidemiological and population-based studies have demonstrated the associations of X/BCP/PC variants, especially

the G1896A pre-core and the A1762T/G1764A double mutation to cirrhosis and HCC (9–14). However, there is a limited understanding of the molecular mechanisms and pathogenesis underlying this association. There are few studies on HBV genetic variants and their functional impact on the novel viral biomarkers (i.e., serum RNA), APOBEC3 family, and host cytokine/chemokine responses. The current study evaluates these clinically relevant mutations using an *in vitro* transient transfection model in parallel with representative clinical samples with either wild-type, PC, or BCP mutants. Viral replication and infectious markers were assessed and clear differences between the X/BCP/PC variants were demonstrated. In addition, this model was used to evaluate whether these variants would induce APOBEC3 expression and activity and hence provide a plausible biological mechanism for carcinogenesis risk. Although we found reduced APOBEC3G protein expression in X/BCP/PC mutants compared to wild-type HBV, it did not impact APOBEC3 cytidine deaminase activity on the viral cccDNA.

In the current study, HBeAg expression was reduced and we found noticeable differences in the replication of G1896A and A1762T/G1764A mutations *in vitro*. The double mutation variant of HBV had more robust HBV replication, evident through the higher levels of supernatant HBV DNA and RNA. We also found increased HBV DNA levels and detection within the A1762T/G1764A mutant compared to the G1896A mutant in agreement with these *in vitro* results. Serum HBV RNA is a promising biomarker for cccDNA activity and expression that may have clinical utility for disease monitoring or predictor of treatment outcomes (38). Indeed, a report by van Bommel *et al.* demonstrated the association of serum HBV RNA to HBeAg seroconversion in patients treated with nucleos/tide analog therapies (35). More recently, a study by van Campenhout *et al.* (39) showed the association of serum RNA to established markers of HBV and liver inflammation including HBV DNA, HBeAg, and alanine transaminase (ALT).

Interestingly, the investigators also reported that the presence of BCP mutations (ie., A1762T/G1764A) correlated to lower levels of serum HBV RNA. However, these *in vivo* findings are in contrast with our *in vitro* results in which secreted HBV RNA from the A1762T/G1764A double mutant were not significantly reduced in comparison to the wild-type HBV. These differences might be due to the limitations of the transient transfection model used in our study and the inability to quantify cccDNA levels for correlation with HBV RNA secretion.

We used a well-established system with transfection of genotype C HBV into HepG2 cells. However, there are conflicting studies on the *in vitro* replicative capacity of the X/BCP/PC mutants which could arise from differences amongst the cell lines (i.e., Huh 7 and HepG2) used for the transfection by other investigators (17–19,40–42). It is also possible that the HBV DNA construct used for transfection (either a multimeric or monomeric genome) may impact *in vitro* findings as suggested by Samal *et al.* (18). Further, differences amongst the results of *in vitro* models may arises from other mutations, variants, and/or genotype of the HBV DNA. Indeed, independent studies by Sozzi *et al.* (43) and Cui *et al.* (44) presents genotypic differences amongst viral replication using Huh7 and HepG2 transfection models.

The APOBEC3 family members are increasingly recognized as carcinogenic when overexpressed and are frequently dysregulated or show mutations in cancer (29,30). Further, their association to retrovirus and HBV infection as innate restriction factors is well established (24,25,45). Thus, in our current study we assessed whether X/BCP/PC mutants and reduced HBeAg levels are linked to increased HCC risks via APOBEC3 overexpression and excessive activity. Despite the potential differences in APOBEC3G expression identified by immunoblot, no clear differences in hyper-editing activity of the cccDNA between HBV variants were found

by 3D-PCR. However, research largely from retrovirus (i.e., HIV) studies demonstrates that APOBEC3G is typically incorporated into new virions and the anti-viral effects are primarily noticeable in subsequently infected cells (46). The current transient transfection model of HepG2 does not allow subsequent HBV infection and transmission. Further, it is important to consider that APOBEC3 anti-viral activity might be more extensive than viral genome hyper-editing, particularly in HBV infection (47). Indeed, APOBEC3G with defective cytidine deaminase activity retains anti-HBV effects via inhibition of reverse transcription (48).

The strongest inducers of APOBEC3 expression are interferons (IFN) (49). In the current study, the levels of IFNα and IFNγ in cell supernatant may have been insufficient to induce APOBEC3 expression. Nonetheless, our findings demonstrate that cytokine/chemokine expression (i.e., IL-12P40, IL-13, and MDC) differs amongst the HBV wild-type vs. X/BCP/PC variants. To enhance the clinical relevance of the *in vitro* cytokine analysis, we evaluated serum derived from CHB patients with predominantly wild-type, G1896A, and A1762T/G1764A and found an increase in TNFα of the double mutant in comparison to the wild-type. This finding suggests a potentially higher level of inflammation within CHB carriers with predominantly A1762T/G1764A variants. It is intriguing to note that the consistent elevation of numerous immune markers, as well as elevated ALT, within the G1896A variant suggest overall heightened immune activity and hepatic inflammation within these individuals.

Due to the overlapping nature of the HBV genomes, the X/BCP/PC mutants evaluated would also induce mutations within HBx which has known carcinogenic effects. The A1762T/G1764A mutations are responsible for the K130M/V131I mutations found in the HBx. Recent independent reports by Siddiqui *et al*. and Chiu *et al*. linked mutant HBx to hepatocarcinogenesis (50,51), however these investigators studied an overexpression model using an HBx construct

without HBV infection and viral proteins/DNA. An intriguing connection of HBx to APOBEC3B was identified by Shen *et al*. (52) and demonstrated the up-regulation of DHX9 proteins by HBx. In their related study, Chen *et al*. (53) reported that DHX9 suppresses APOBEC3B and inhibited the anti-HBV effects. Additionally, HBx post-translational suppression of APOBEC3G has been shown within Huh7 cells (54). Overall, the pleiotropic effects of HBx may underly the impact of HBV genetic variants and APOBEC3 family activity.

The current study provides novel data on HBV genetic variants and their impact on viral fitness, potential association with innate antiviral restriction factors (i.e., APOBEC3 family), and host inflammatory cytokine/chemokine expression within a hepatocyte cell model system and in clinical samples from CHB carriers. HBV sequence variation impacts HBeAg expression, viral replication, APOBEC3 expression, and host immune response *in vitro*, and is supported by the analysis of representative clinical samples with wild-type vs. mutant HBV infection. The results of this study enhance understanding of HBV sequence variations and their impact on host-cell interactions, host innate antiviral restriction factors and the pathogenesis of HBV-related HCC.

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5.7 Disclosures and Conflict of Interest

No relevant disclosures.

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5.9 Ethics ID

The study received approval from the local ethics review board committee at the University of Calgary (i.e., Conjoint Ethics Review Board): Ethics REB-16636 and REB15-3137.

CHAPTER 6: DISCUSSION AND CONCLUSIONS

Chronic infection by HBV is responsible for a significant global disease burden. The WHO has an established goal of eliminating viral hepatitis by 2030, but recent reports advocate for additional work and funding to advance current progress towards achieving this goal (1,274). As the most common carcinogen for HCC, it is essential to elucidate the fundamental pathogenic mechanisms of HBV-related malignancy to identify potential preventative, diagnostic, and therapeutic opportunities. Much remains unknown regarding CHB carriers with HCC, particularly in the context of lymphotrophism and extrahepatic reservoirs of the virus. Additionally, OBI has been reported in association with HCC especially in association with comorbid liver disease (48–55). The research studies included in this thesis are significant in enhancing the current capability of detection and thorough molecular virological characterization of HBV. Further, this thesis advances the limited understanding of HBV lymphotrophism and oncogenesis, particularly within HBV-related HCC cases and *in vitro* functional analysis.

6.1 Significance of study findings

Detection of HBV can be particularly challenging, especially in CHB carriers with extremely low levels of the virus. Consequently, determination of HBV genotype is thereby a major obstacle which may limit effective CHB disease management. HBV genotype is clinically important for established links to the natural history and disease progression of HBV. Indeed, CHB carriers with genotype C are well-recognized for their increased risks of developing HCC (84,119). Further, genotypic differences are present in HBV treatment outcomes with genotype A experiencing the greatest responses to PEG-IFN treatment (38,68). Typically, the determination of viral genotype is clinically performed in with the INNO-LiPa kit, but this methodology is limited by the sensitivity of the kit (>10 IU/mL equivalent to ~50 vge/mL) (192).

In chapter 2, we demonstrate the effective use of our economical nested PCR technique in order to detect viral DNA from CHB carriers with ultra-low HBV viremia. The utility of this technique allows for accurate determination of viral genotype via free-to-use sequence alignment and phylogenic tree building software. The nested PCR approach has high sensitivity as the use of two consecutive PCRs allows for significantly enhanced DNA amplification. Our described methodology is technologically simple and cost-effective which provides advantages for large-scale clinical or research study use, particularly in developing countries with less resources. An additional benefit of the reported nested PCR is the capability to identify common clinically important genetic mutations of the virus associated with resistance to NA therapies.

The nested PCR and genotype determination methodology described in chapter 2 was effective for evaluation of persist HBV in post-liver transplant recipients for HBV-related end-stage liver disease. Plasma, serum, and PBMC samples were collected for use in the aforementioned assays, thus demonstrating the continual presence of HBV within the transplant recipients. To further characterize the genetic features of the virus isolated from the LT recipients, chapter 3 also incorporates NGS of the HBV X/BCP/PC, a genomic region with mutations frequently associated with cirrhosis and HCC. NGS effectively allowed for detailed HBV quasi-species analysis and the measurement of low frequency genetic variants within the X/BCP/PC region. Interestingly, our molecular analysis shows that oncogenic genotypes C and genetic HBV variants (G1896A, A1762T/G1764A, etc.) were ubiquitously present. These findings are particularly relevant in the context of potential end-stage liver diseases recurrence and continual management of HBV post-liver transplant. Prior studies by certain investigators have proposed the discontinuation of HBV prophylaxis, namely HBIG and NA, post-transplantation (150–153). However, our results would advise against this proposition as we

clearly demonstrate not only the continual presence of HBV many years post-transplant, but also the persistence of important viral genetic features associated with end-stage liver disease development. Our findings would suggest that significant risks of recurrence of overt HBV disease and cirrhosis or HCC remains, which should be controlled with continual use of HBV prophylaxis especially in the immunosuppressed post-liver transplant recipients.

The extrahepatic lymphoid reservoir is a site of HBV persistence that is poorly studied within HBV-related HCC individuals. Limited studies have explored the presence of established HBV oncogenic features such as viral genotype, genetic variants, and integration within this extrahepatic reservoir. Further, the presence of pro-oncogenic HBV may provide support towards the growing number of studies demonstrating increased risks of lymphoproliferative diseases such as DLBCL (172–179). Chapter 4 of this thesis describes an in-depth analysis of not only the blood (i.e. plasma, serum, and PBMCs), but also available liver, liver tumor, and DCS tumor from CHB carriers with or without HCC and one case with DCS. The findings reported in this chapter are particularly intriguing as pro-oncogenic HBV was detected in both hepatic and extrahepatic tissues within all cohorts. Viral integration, a crucial oncogenic feature of HBV, was identified using AluPCR in numerous coding genes with implications in carcinogenesis or cancer progression. These integration sites were present in liver, PBMCs, and both HCC and DCS tumor tissues. AluPCR is a well-established methodology for identification of HBV integration, particularly using liver tissue of CHB carriers. Although this technique is biased towards the location of Alu sites, it remains an effective, reliable, and technologically simple methodology to detect viral integration especially for evaluation of controversial reservoirs of HBV such as the lymphoid cells. Concerns with the low sensitivity of AluPCR was enhanced with the subsequent library production and clonal sequencing performed in this thesis. Viral proteins (HBcAg and

HBsAg) and replicative intermediates (DNA, RNA, and cccDNA) were detected within the DCS tumor tissues. Taken together, the findings described in this study are significant as they show HBV is present within extrahepatic lymphoid tissues and provides "proof-of-concept" data that HBV may contribute towards the development of malignant neoplastic diseases such as DCS.

An outstanding question regarding the detection of HBV within the lymphoid (ie., PBMC) cells was their potential to complete viral life cycle and produce new infectious virions. However, the work presented in chapter 4 contributes to address this valid question. Freshly isolated PBMCs derived from CHB carriers were stimulated with the use of mitogens (PHA and PWM). It is particularly noteworthy, that multiple viral replicative markers including HBV DNA, RNA, and cccDNA when the PBMCs were stimulated with exposure to mitogens. This finding provides crucial evidence demonstrating that HBV within the PBMCs are capable of upregulating and producing replicative intermediates essential in the viral life cycle. In addition, cells undergoing malignant transformation frequently have dysregulated or dysfunctional mitogen-susceptible cellular pathways (236). Therefore, these findings suggest that pre-cancerous or cancerous cells with overstimulated mitogen pathways may also have enhanced HBV replication as an unintended consequence.

An aspect of interest was the genetic variations present within the HBV X/BCP/PC region and their associations with cirrhosis and HCC. Indeed, numerous epidemiological reports have demonstrated clear links between the G1896A and A1762T/G1764A mutations with increased risks of HCC (38,84,136,138,141,198). Coincidentally, these mutations disrupt the expression of HBeAg, which was hypothesized to be a viral inhibitor of the innate antiviral restrictor factors – the APOBEC3 family. However, the study described in chapter 5 demonstrates that APOBEC3 expression is modulated compared to wild type HBV albeit with no impact on mutagenic activity

amongst the G1896A or A1762T/G1764A variants and wild-type HBV. Nonetheless, the findings of this research are significant in providing key evidence against the possible connection of HBV X/BCP/PC variants and carcinogenesis via the APOBEC3 family. However, additional work will be required to explore this connection particularly with regards to altered HBx that arise due to the X/BCP/PC variants. Further, this study suggested more robust viral replication within the A1762T/G1764A than the G1896A mutant, providing additional clarification to contrasting *in vitro* results of the HBV variants. These findings will require validation potentially through use of more sensitive techniques to characterize cellular HBV replicative intermediates and viral markers such as use of immunofluorescent for HBcAg detection or quantification of viral mRNA species.

The research studies described in this thesis contribute to an overall increase in the current capability of characterization and study of lymphotropic and pro-oncogenic HBV. Many of the techniques utilized are not only effective in the detection of extrahepatic HBV, but also in elucidation of the viral genotype, genetic features and host-cell interactions. Detailed analysis of this extrahepatic viral reservoir has confirmed the continual persistence of pro-oncogenic virus despite the use of HCC curative therapies (liver transplantation). This thesis also includes a thorough-to-date analysis of viral integration and genetic mutants within the PBMCs of CHB carriers. In addition, the in-depth highly sensitive techniques utilized demonstrates the presence of numerous HBV infection markers within tissue samples from extrahepatic lymphoproliferative disease (i.e. DCS). Lastly, the use of a hepatoma *in vitro* model was effective for the study of HBV genetic variants and to provide additional evidence regarding the functional significance of SNPs on viral replication and protein expression, impact on host cytokines, and APOBEC3 family expression and activity between the mutants.

6.2 Study limitations, outstanding questions, and future directions

Despite the advances to the understanding of HBV lymphotropism and extrahepatic reservoirs presented in this thesis, many outstanding questions remain. The research studies described in this thesis did not address any specific subset of PBMCs or lymphoid cells. With the use of flow cytometry, prior studies by Lee *et al.* and Chemin *et al*. detected of HBV genetic material and proteins within multiple subsets of PBMCs isolated including CD8+ T cells and CD56+ natural killer cells (166,168). In this thesis, in-depth NGS analysis of HBV genetic variants and identification of viral integration were not performed in the individual subsets of PBMCs. Therefore, future studies building off this research work should incorporate the exploration of the separated immune cell populations present within the PBMCs. It is possible that pro-oncogenic HBV are not present equally amongst the different subsets which contributes to different risks of extrahepatic oncogenesis. For example, if pro-oncogenic HBV is more frequently present within the B-cell populations, the virus may account for the increased risks of DLBCL observed clinically amongst CHB carriers.

The work described in this thesis is also limited by utilizing PBMCs as a representation of the lymphoid system. The full extent of HBV infection within the lymphoid reservoir remains unknown and could be much more extensive than observed within this thesis. In support of this possibility, Huang *et al.* and Shi *et al.* have identified HBV infected hematopoietic stem cells derived from the umbilical cord blood and bone marrow (170,181). Their findings would suggest that HBV infection expands beyond the PBMCs, and that infected hematopoietic stem cells which subsequently undergo differentiation may even be the source of HBV infected PBMCs or extrahepatic hematological malignancies. A unique lymphoid cell population of interest for future study are the liver-resident immune cells. The liver comprises of large numbers of resident

immune cells including Kupffer cells or natural killer cells (275). The liver-resident immune environment plays an essential role towards HBV-related HCC pathogenesis and warrants additional studies (276,277). Interestingly, no previously published studies have explored direct HBV infection of these lymphoid cells, which are likely the most exposed compartment of immunological cells due to their proximity to HBV infected hepatocytes. Thus, future experimentation studying the HBV lymphoid compartment, particularly in HBV-related HCC, should characterize the liver-resident immune cell population for HBV infection and subsequent functional consequences once infected. This could be achieved through isolation of the liver-resident immune cells using either flow cytometry or collagenase-perfusion based methodologies (278–280).

HBV cccDNA remains extremely elusive within the lymphoid cells and was only qualitatively identified using highly sensitive molecular techniques (i.e. nested PCR) in this thesis. Nucleic acid hybridization of unamplified cccDNA was unsuccessful in detecting viral cccDNA. Future experiments to validate the presence of HBV within lymphoid cells and extrahepatic lymphoproliferative diseases should incorporate novel highly sensitive tools for single cell cccDNA analysis. Most notably, the use of droplet digital (dd)PCR is promising for the sensitive detection and quantification of cccDNA within extrahepatic reservoirs. Recent studies have utilizing the ddPCR technology in order to analyze cccDNA on a single cell level with exciting results in established HepG2.2.15 cell lines, HBV-related HCC patients, HIV+HBV co-infected individuals, and OBI cases (281–285). Similarly, applying this novel technique to evaluate cccDNA within PBMCs and extrahepatic tumor tissues derived from CHB carriers would provide definitive data supporting HBV lymphotropism and extrahepatic carcinogenesis. Further, the benefits of quantification of cccDNA copies in each cell using

ddPCR could be harnessed to elucidate HBV biology and frequency of cccDNA within extrahepatic tissues.

Additional work will be required in order to validate the causative role of pro-oncogenic HBV in extrahepatic malignancies, particularly as this thesis was only able to evaluate one representative case. Indeed, thorough molecular analysis, similar to those performed in this thesis, within additional human extrahepatic tumor tissue would assist to confirm the oncogenic role of HBV. These tissues should be evaluated for the presence of oncogenic viral integration, genetic variants, and viral proteins known to impact carcinogenic cellular pathways (eg. HBx protein). A novel tool, multiplexed ion beam imaging (MIBI), could be employed for the in-depth analysis of collected extrahepatic tissues for HBV. This innovative technology is capable of simultaneous detection of a large number of proteins through antibody staining of tissue sections while retaining the tissue architecture and localization (286–288). Indeed, MIBI has also been applied to breast cancer tissues previously collected by standard formalin-fixed, paraffin-embedded (FFPE) to provide detailed cellular composition of the tumor microenvironment (286,287). Harnessing this technique for extrahepatic tumor tissues in CHB carriers could provide definitive evidence for the presence of HBV in these tumors. The viral proteins such as HBcAg could be targeted and tagged in order to identify the location and cell type of HBV infected cells. Further, this technique could also be applied to FFPE liver tissues historically collected from CHB carriers, such as for biopsy purposes, to identify any HBV infected liver-resident immune cells.

In addition to human extrahepatic tumor tissues, the woodchuck hepatitis virus (WHV) could serve as an important model system for validating the oncogenic role of hepadnaviruses in lymphoproliferative diseases. WHV frequently serves as a model system for the HBV as a close

family member of the hepadnaviridae (289,290). WHV is highly carcinogenic and can induce HCC in nearly all chronically infected woodchuck (*Marmota monax*) cases (289). In addition, numerous studies have demonstrated the lymphotropic nature of WHV which can replicate and produce infectious virions within the woodchuck lymphatic system (291–293). An exciting future direction would be to harness the woodchuck and WHV model for the study of extrahepatic malignancies induced by hepadnaviruses. However, the universal induction of HCC is problematic as it might mask the presence of lymphoproliferative diseases or would kill the animal prior to their development. To circumvent this situation, woodchucks could undergo curative HCC specific therapies (such as liver transplantation) followed by continual surveillance for lymphoproliferative and extrahepatic diseases.

The WHV and woodchuck model system could also be utilized to visually confirm hepadnaviruses lymphotropism. Utilizing intravital microscopy imaging, infection of lymphoid cells within the spleen or liver-resident immune cells could be visualized in real-time (294,295). Due to the overlapping nature of hepadnaviral genomes, insertion of fluorescent marker genes is extremely challenging. However, it could be worthwhile directly labelling the WHV virions with fluorescent dyes prior to animal exposure and visualization with intravital microscopy. Indeed, in an elegant technique described by Naumenko *et al.* (296), vesicular stomatitis virus was directly labelled with Alexa Fluor dye to produce viruses visible with intravital microscopy that retain infectious capability. Applying a similar methodology with WHV in experimental woodchucks could allow for real-time visualization of hepadnaviruses infecting lymphoid cells.

An aspect of this thesis which required further exploration is the functional impact of HBV infection and different genetic variants of the virus. The infection of HBV into host lymphoid cells will likely induce altered gene expression and may potentially impact immune function or

responsiveness. In addition, the host cells may respond differently to various X/BCP/PC genetic variants of HBV, likely due to either HBeAg or HBx protein differences. These host cellular changes in gene and protein expression could be further analyzed using in-depth RNA transcriptome and proteasome analysis. RNA sequencing using NGS platforms, such as the Illumina platform used in this thesis, could be employed in order to evaluate changes in host cell gene expression via presence and frequency of mRNAs (297). Expanding beyond the transcriptome, the cellular proteasome should also be explored post-infection with HBV using mass spectrometry approaches (298). Any changes in expression and levels of protein can be elucidated on a large scale to better identify host-pathogen interactions and the impacts of HBV infection. Harnessing these powerful technologies will provide fundamental information in understanding HBV pathogenesis, lymphotropism, and genetic variants.

In this thesis, HBV integration was demonstrated for the first time within an extrahepatic tumor and matching PBMC tissue. However, the functional and genetic consequences of the host-viral integration junctions observed were not thoroughly analysed. A better understanding of the host impact of HBV integration should incorporate detailed assessment of subsequent RNA and proteins produced. Indeed, by applying the HBV RNA capture methodology utilized by Tan *et al.*, integrated HBV-host chimeric mRNAs could be identified and analysed using NGS platforms (299). Alternatively, an exciting possibility could exploit the single-cell sequencing technologies available to simultaneously characterize both the genome and transcriptome of HBV integrated cells. Indeed, Macaulay *et al*. have elegantly described a detailed methodology to physically separate mRNA and DNA derived from single cells prior to sequencing (300,301). Prior identification of HBV infected cells via flow cytometry to target either extrahepatic tumor cells or PBMCs expressing viral proteins could first enrich single cell

isolations with cells potentially containing viral integration. The application of the innovative single cell technologies could effectively provide comprehensive data of HBV integration and their cellular impacts on gene expression.

Liver cancer, particularly HCC, is an increasingly global health concern. Incidence and mortality for the majority of cancers have been declining, but liver cancer cases are projected to continue to grow (302). Despite, the availability of an effective vaccine, HBV remains the major risk factor for HCC. HBV is traditionally described as a hepatotropic virus, but recent research utilizing highly sensitive viral detection and characterization approaches provides increasing amounts of evidence of HBV lymphotropism. Indeed, the work presented in this thesis using a variety of molecular approaches including nested PCRs, AluPCR, and immunoblots have demonstrated the persistence of pro-oncogenic HBV within the lymphoid reservoir. To date, this extrahepatic reservoir has poorly studied in HBV-related HCC patients. Elucidating the intricacies of the lymphoid reservoir could introduce a new conceptual framework in understanding how HBV induces liver cancer or extrahepatic malignancies. Novel HCC diagnostic or prognostic markers might be identified with potential utility in a liquid biopsy approach exploiting the minimal invasiveness of PBMC collection. Virally infected or induced neoplastic malignancies may provide innovative therapeutic opportunities such as the inhibition of overexpressed cellular pathways or immunotherapy possibilities via targeting mutant viral-host proteins. Exploration of hepatic and extrahepatic reservoirs in HBV-related malignancies may provide insight into the underlying pathogenesis of HBV-induced carcinogenesis.

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APPENDIX A: CHAPTER 2 SUPPLEMENTARY TABLES AND FIGURE

Table S2.1. Primers used for detection of HBV DNA, genotype determination, and cloning of the HBV full genome. HBV-specific primer binding locations derived from genome sequence of reference HBV genotype C (accession #AB031265).

PURPOSE	PRIMER NAME	SEQUENCE (5' TO 3')	HBV PRIMER BINDING SITE	ADAPTED FROM
preC/C direct	preCC DF	GCATGGAGACCACCGTGAACG	nt1606-1626	Coffin *et al.* (126,128)
preC/C direct	preCC DR	GAGGGAGTTCTTCTTCTAGG	nt2366-2385	
preC/C nested	preCC NF	TCACCTCTGCCTAATCATC	nt1825-1843	
preC/C nested	preCC NR	GGAGTGCGAATCCACACTCC	nt2267-2286	
X/BCP/preC direct	X/BCP/preC F1	GCATGGAGACCACCGTGAAC	nt1606-1625	Takahashi *et al.* (193)
X/BCP/preC direct	X/BCP/preC R1	GGAAAGAAGTCAGAAGGCAA	nt1955-1974	
X/BCP/preC nested	X/BCP/preC F2	CATAAGAGGACTCTTGGACT	nt1653-1672	
X/BCP/preC nested	X/BCP/preC R2	GGCAAAAAAGAGAGTAACTC	nt1940-1959	
Surface direct	HBPr134	TGCTGCTATGCCTCATCTTC	nt414-433	INNO-LiPa HBV Genotyping kit (Fujirebio US, Inc)
Surface direct	HBPr135	CARAGACARAAGAAAATTGG	nt803-822	
Surface nested / Genotype determination	HBPr75	CAAGGTATGTTGCCCGTTTGTCC	nt455-477	
Surface nested / Genotype determination	HBPr94	GGYAWAAAGGGACTCAMGATG	nt775-795	
Full genome amplification	HBV FG P1*	CCGGAAAGCTTGAGCTCTTCTTTTTCACCTCTGCCTAATCA	nt1820-1841	Günther *et al.* (194)
Full genome amplification	HBV FG P2*	CCGGAAAGCTTGAGCTCTTCAAAAAGTTGCATGGTGCTGG	nt1806-1825	
Cloning	M13 F	GTAAAACGACGGCCAGT	N/A	
Cloning	M13 R-17	CAGGAAACAGCTATGAC	N/A	

*Underlined nucleotides represents the recognition site of HindIII used for cloning

Table S2.2. Thermocycler conditions used for amplification and detection of HBV-specific preC/C, X/BCP/preC, and surface genomic region nested PCR for DNA extracts derived from clinically undetectable or low viremia chronic hepatitis B carriers.

HBV-SPECIFIC PCR*	DIRECT ROUND	NESTED ROUND
preC/C Direct: nt1606 - 2385 Nested: nt1825 - 2286	94°C - 4 minutes 40 cycles of: 94°C - 30 seconds 55°C - 30 seconds 72°C - 1.5 minutes 72°C - 10 minutes 4°C hold	94°C - 4 minutes 40 cycles of: 94°C - 30 seconds 55°C - 30 seconds 72°C - 1.5 minutes 72°C - 10 minutes 4°C hold
X/BCP/preC Direct: nt1606 - 1974 Nested: nt1653 - 1959	94°C - 5 minutes 35 cycles of: 94°C - 1 minute 55°C - 1.5 minutes 72°C - 2 minutes 72°C - 12 minutes 4°C hold	94°C - 5 minutes 35 cycles of: 94°C - 1 minute 55°C - 1 minute 72°C - 1.5 minutes 72°C - 12 minutes 4°C hold
Surface Direct: nt414 - 822 Nested: nt455 - 795	94°C - 4 minutes 40 cycles of: 94°C - 30 seconds 45°C - 30 seconds 72°C - 30 seconds 72°C - 10 minutes 4°C hold	94°C - 4 minutes 40 cycles of: 94°C - 30 seconds 45°C - 30 seconds 72°C - 30 seconds 72°C - 10 minutes 4°C hold
Full genome	98°C - 30 seconds 35 cycles of: 98°C - 10 seconds 62°C - 20 seconds 72°C - 2 minutes 72°C - 10 minutes 4°C hold	N / A

*Nucleotide positions derived from genome sequence of reference HBV genotype C (accession #AB031265).

Table S2.3. List of reference HBV genotypes and NCBI Genbank accession numbers used for phylogenetic analysis including sequence alignment and tree construction.

HBV GENOTYPE	NCBI GENBANK ACCESSION NUMBERS
A	AF297625 GQ184324 GQ331047 FJ692595
B	AB010290 EU139543 AB033554 AB100695 AB219428 DQ463787 AP011090 AP011096 GQ358146
C	AB031265 AB111946 X75656 AB241110 AB493837 EU670263 AP011107 AP011108 AB540583 AB554019 AB554018
D	AF151735 X72702 AB493846 U95551 HE974378
E	FN545821
F	AY090456 X69798 AB036908 AB365450
H	AY090460 AY090454

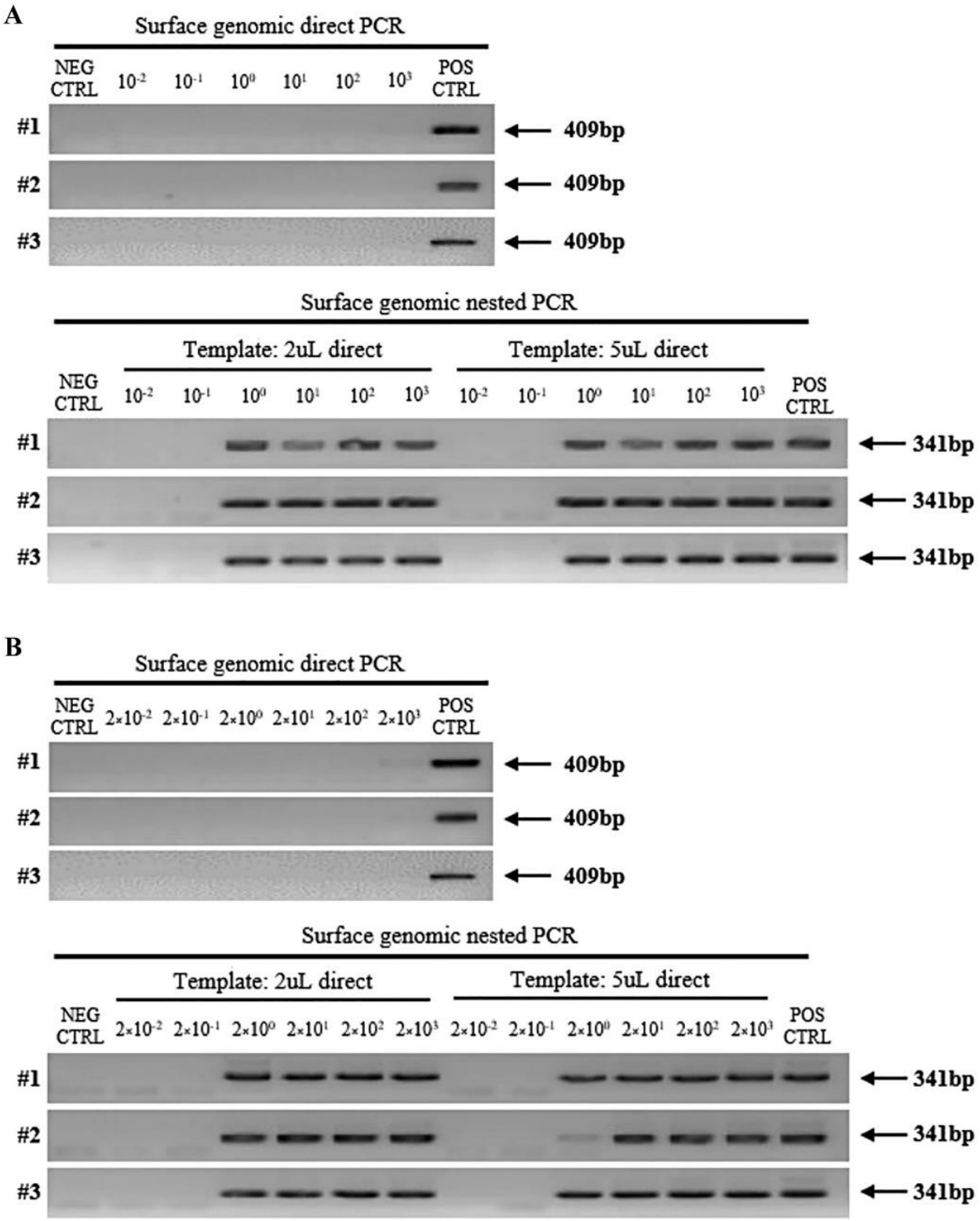

Figure S2.1. Surface genomic nested PCR was evaluated with house-made plasmids containing a single copy of the HBV full genome in 3 different sets of experimental replicates.

Direct and nested PCR products from the **A.** plasmid serial dilution 1 (10^3 to 10^{-2} copies/reaction) and the **B.** plasmid serial dilution 2 (2×10^3 to 2×10^{-2} copies/reaction) were analyzed on a 1% agarose gel. 2 μL and 5 μL of the direct round PCR was used as template in the nested round PCR. Negative and positive controls included a no template control (water) and 10^7 copies of the full genome plasmid, respectively.

APPENDIX B: CHAPTER 3 SUPPLEMENTARY TABLE

Table S3.1. Primers used for detection of HBV DNA, mRNA, and cccDNA and for nucleotide frequency analysis by NGS.

Primer	Sequence (5' to 3')
preCC DF	GCATGGAGACCACCGTGAACG
preCC DR	GAGGGAGTTCTTCTTCTAGG
preCC NF	TCACCTCTGCCTAATCATC
preCC NR	GGAGTGCGAATCCACACTCC
HBPr134	TGCTGCTATGCCTCATCTTC
HBPr135	CARAGACARAAGAAAATTGG
HBPr75	CAAGGTATGTTGCCCGTTTGTCC
HBPr94	GGYAWAAAGGGACTCAMGATG
X/BCP/preC F1	GCATGGAGACCACCGTGAAC
X/BCP/preC R1	GGAAAGAAGTCAGAAGGCAA
X/BCP/preC F2	CATAAGAGGACTCTTGGACT
X/BCP/preC R2	GGCAAAAAAGAGAGTAACTC
βglo F	TGCACGTGGATCCTGAGAACTTCA
βglo R	TGATACTTGTGGGCCAGGGCATTA
cccDNA F1	ACTCCTGGACTCTCAGCAATG
cccDNA R1	GTATGGTGAGGTGAGCAATG
cccDNA F2	AGGCTGTAGGCACAAATTGGT
cccDNA R2	GCTTATACGGGTCAATGTCCA
NGS-X/BCP/preC F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATAA GAGGACTCTTGGACT
NGS-X/BCP/preC R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGCA AAAAAGAGAGTAACTC

APPENDIX C: CHAPTER 4 SUPPLEMENTARY TABLES AND FIGURES

Table S4.1. Primers used for detection of HBV DNA, mRNA, and cccDNA and for nucleotide frequency analysis by NGS.

PRIMER	SEQUENCE (5' TO 3')
preCC DF	GCATGGAGACCACCGTGAACG
preCC DR	GAGGGAGTTCTTCTTCTAGG
preCC NF	TCACCTCTGCCTAATCATC
preCC NR	GGAGTGCGAATCCACACTCC
HBPr134	TGCTGCTATGCCTCATCTTC
HBPr135	CARAGACARAAGAAAATTGG
HBPr75	CAAGGTATGTTGCCCGTTTGTCC
HBPr94	GGYAWAAAGGGACTCAMGATG
X/BCP/preC F1	GCATGGAGACCACCGTGAAC
X/BCP/preC R1	GGAAAGAAGTCAGAAGGCAA
X/BCP/preC F2	CATAAGAGGACTCTTGGACT
X/BCP/preC R2	GGCAAAAAAGAGAGTAACTC
cccDNA F1	ACTCCTGGACTCTCAGCAATG
cccDNA R1	GTATGGTGAGGTGAGCAATG
cccDNA F2	AGGCTGTAGGCACAAATTGGT
cccDNA R2	GCTTATACGGGTCAATGTCCA
NGS-X/BCP/preCF	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATAAGAGGACTCTTGGACT
NGS-X/BCP/preCR	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGCAAAAAAGAGAGTAACTC

Table S4.2. Primers used for AluPCR to detect HBV-host integration sites.

PRIMER NAME	SEQUENCE (5' TO 3')*	HBV PRIMER BINDING SITE
Alu-sense	CAGUGCCAAGUGUUUGCUGACGCCAAAGUGCUGGGAUUA	N / A
Alu-antisense	AUUAACCCUCACUAAAGCCUCGAUAGAUYRYRCCAYUGCAC	N / A
Alu-sense TAG	CAAGTGTTTGCTGACGCCAAAG	N / A
Alu-antisense TAG	ATTAACCCTCACTAAAGCCTCG	N / A
HBV X1	ACAUGAACCUUUACCCCGUUGC	1131 – 1152
HBV X2	TGCCAAGTGTTTGCTGACGC	1174 – 1193
HBV X3	CTGCCGATCCATACTGCGGAAC	1258 – 1279
HBV C1	GAGUUCUUCUUCUAGGGGACCUG	2350 – 2328
HBV C2	AGTGCGAATCCACACTC	2288 – 2269
HBV C3	GGAAGGAAAGAAGTCAGAAGG	1978 – 1960
HBV S1	ACACGGCGGUAUUUUGGGGTGGAG	3042 – 3065
HBV S2	CAGGCTCAGGGCATATTGACAA	3070 – 3091
HBV S3	YCCTGCTGGTGGCTCCAGTTC	55 - 75

* **R**: A or G; **U**: dUTP; **Y**: C or T
Primer sequences derived from Murakami *et al*., 2005 (114)

Table S4.3. Summary of demographic and clinical information of CHB carriers used in evaluating the *ex vivo* mitogen stimulation of PBMCs.

CASE ID#	AGE / SEX / ETHNICITY*	HBV DNA[†] (log IU/mL)	ALT (U/L)	GENOTYPE[‡]	HBeAg[x]	anti-HBeAg[x]	qHBsAg[x] (log IU/mL)
29-2	46 / M / AS	U	54	B	-	+	3.1
233-2	32 / F / AS	U	35	C	+	-	5.1
300	45 / M / AS	7.4	58	D	+	-	N/A
302	49 / M / AS	U	64	B	-	-	2.4
309	36 / F / AS	6.5	42	C	+	-	N/A
312	41 / M / AS	8.3	45	C	+	-	N/A
316	31 / M / AS	8.8	103	C	+	-	4.6

***AS = Asian
[†] Determined by clinical PCR assay (Abbott Architect, LLOD = 10 IU/mL or 50 virus genome copies/mL); U = Undetectable
[‡] HBV genotype was determined by in-house HBV surface direct sequencing.
[x] Serology determined with commercial chemiluminescent microparticle immunoassays (Abbott Architect; Quantitative anti-HBc II and anti-HBs)

Table S4.4. Primers and probes used for HBV surface qPCR and human GAPDH in analysis of PBMCs stimulated with or without mitogens.

PRIMER NAME	SEQUENCE (5' TO 3')*	HBV PRIMER BINDING SITE
Surface qPCR FOR	ACTCACCAACCTSYTGTCCT	333 – 350
Surface qPCR REV	GACAMACGGGCAACATACCT	457 – 476
Surface qPCR probe	FAM - TATCGCTGGATGTGTCTGCGGCGT - BHQ1	368 – 391
GAPDH FOR	GAAGGTGAAGGTCGGAGTC	N / A
GAPDH REV	GAAGATGGTGATGGGATTTC	N / A

* **BHQ1**: Black Hole Quencher-1; **FAM:** 6-carboxyfluorescein; **M:** A or C; **S:** G or C; **Y:** C or T

Table S4.5. Detailed clinical and virological information of HBV+HCC cases recruited from the Calgary Liver Unit.

CASE ID#	AGE	SEX	ETH*	HBV VIRAL LOAD (IU/mL)†	ANTI-VIRAL THERAPY‡	CIRRHOSIS	HCC THERAPY**	CURRENT STATUS††	HBeAg	anti-HBeAg	AFP	ALT	Platelets	qHBsAg	AST
14	48	M	AS	U	TDF	Y	H; SOR	REM	NEG	NEG	42	33	137	73.51	49
14-2	49	M	AS	U	TDF	N	AS ABOVE	AS ABOVE	NEG	NEG	N/A	42	100	73.51	49
147	83	F	AS	200000	NT	Y	NT	DE	N/A	N/A	4.9	181	295	N/A	N/A
187	50	M	AF	310	ETV	Y	H; FOLFOX; ABX-EGF	DE	NEG	POS	4.5	14	221	311	12
191	68	M	AS	U	TDF	Y	RFA (2x)	REM	NEG	POS	0.8	13	176	629.59	17
261	55	M	AF	D	ETV	Y	TACE; RFA	REM AWAIT LT	NEG	N/A	3.1	40	112	N/A	36
261-2	55	M	AF	D	ETV	Y	AS ABOVE	AS ABOVE	NEG	N/A	3.0	32	124	N/A	30
265	73	M	AS	D	ETV	Y	H; RFA	REM	NEG	POS	12	10	205	N/A	20
266	73	F	AS	D	ETV	Y	TACE; H	REM	NEG	NEG	1.2	18	177	5.51	30
302	48	M	AS	D	ETV	Y	RFA; SBR; TACE	REC	NEG	NEG	16	38	98	N/A	63
302-2	49	M	AS	U	TDF	Y	AS ABOVE	AS ABOVE	NEG	NEG	1476.2	52	104	49.98	59
302-3	50	M	AS	U	TDF	Y	AS ABOVE	AS ABOVE	NEG	NEG	1750.4	37	125	N/A	44
305	42	F	AS	27	TDF	Y	H (2x); TACE	REM	POS	NEG	6.3	29	329	2617.32	32
307	56	M	AS	550000	TDF	Y	TACE	REM	NEG	POS	4.9	66	394	3231	54
313	38	M	AF	D	TDF	Y	H (2x)	REM	NEG	POS	265.3	59	143	770	50
319	55	M	AS	14	TDF	Y	RFA; TACE; LT	REM	NEG	NEG	4.9	44	117	1216	53
327	61	F	AS	994479	TDF	Y	N/A	UNK	NEG	POS	10170	90	137	2579	93
328	74	M	AS	U	TDF	Y	RFA; MWA; RFA	REM	NEG	POS	8	17	179	513	17
328-2	76	M	AS	U	TDF	N	AS ABOVE	AS ABOVE	NEG	POS	0.83	16	198	513.15	20
333	38	F	AF	>100000000	NT	Y	NT	REC	POS	NEG	6.7	30	356	11245.08	25
335	76	M	AS	D	ETV	Y	H (2x); RFA (3x); MWA; TACE	REC	NEG	POS	4.9	35	255	324	40
339	78	M	AS	U	ETV	N	RFA (2x)	REM	NEG	POS	13.5	35	157	N/A	35
345	42	M	AS	U	TDF	N	H; LT	REM	NEG	POS	119	17	208	1358	23
349	69	M	AS	D	ETV	Y	TACE; H	REM	NEG	POS	7	26	126	422	22
352	61	F	AS	D	TDF	Y	RFA; TACE (2x); PEI (3x); SBRT	DE	NEG	POS	6.6	40	72	1346.28	51
352-2	62	F	AS	D	TDF	Y	AS ABOVE	AS ABOVE	NEG	POS	N/A	25	81	N/A	36
405	58	M	AS	73	TDF	Y	H	REM	NEG	POS	47	19	151	168	20
418	59	M	CA	20	ETV	Y	RFA (2x)	REC AWAIT LT	NEG	POS	15.9	39	35	N/A	64

*__AF__: African; __AS__: Asian; __CA__: Caucasian †__D__: Detectable but not quantifiable; __U__: Undetectable **__ETV__: Entecavir; __NT__: No treatment; __TDF__: Tenofovir
**__ABX-EGF__: Panitumumab; __FOLFOX__: Folinic acid + fluorouracil + oxaliplatin; __H__: Hepatectomy; __LT__: Liver transplant; __MWA__: Microwave ablation; __PEI__: Percutaneous ethanol injection; __RFA__: Radiofrequency ablation; __TACE__: Trans-arterial chemoembolization; __SBRT__: Stereotactic body radiation therapy; __SOR__: Sorafenib
††__AWAIT LT__: Awaiting LT; __DE__: Deceased (from HCC); __REC__: Recurrence; __REM__: Remission; __UNK__: Unknown

Table S4.6. Measures of quasi-species complexity and diversity of the NGS analysis of X/BCP/preC from each compartment.

	CHB ONLY		CHB + HCC				CHB + DCS (n = 1)		
	PL (n = 19)	PB (n = 10)	PL (n = 20)	PB (n = 15)	LNT (n = 10)	LT (n = 3)	PL	PB	T
MEAN # OF READS	8341	10057	10563	7984	8213	9902	5180	3348	12121
MEAN # OF HAPLOTYPES	748	860	655	493	677	330	893	501	103
MEAN DIVERSITY (SEM)*	0.0382 (0.0019)	0.0390 (0.0013)	0.0400 (0.0036)	0.0461 (0.0101)	0.0364 (0.0022)	0.0340 (0.0034)	0.0268 (0.0025)	0.1432 (0.0114)	0.0369 (0.0042)

*Standard deviation is represented in the CHB + DCS cohort instead of SEM due to low sample number.

Table S4.7. All genes integrated with HBV identified by AluPCR within each cohort and tissue. Genes with red fill indicates those with implications in cancer development or progression.

COHORT	CHB ONLY	CHB + HCC			CHB + DCS	
TISSUE	PBMC (n = 14)	PBMC (n = 19)	NON-TUMOR LIVER (n = 11)	LIVER TUMOR (n = 4)	PBMC (n = 1)	T (n = 1)
CODING GENES DETECTED WITH HBV INTEGRATION	PAIP2	ROR2	NLGN4	NRF1	CLPB	Enhancer for LSG1
	KLF12	ZNF417	PTPRG	BCL2	P4HTM	PRKAR1B
	NEUROD6	KLF12	EIF3A	Enhancer for FSD1	IGH	ZFR2
	AMZ2	WDR62	NECTIN 2	HECA	SMG5	CGAS
	TIAM1	AMZ2	GSR	OSBPL5	RFPL1	PTPRT
	NDUFAF2	TRAPPC9	TMEM132D	AHNAK	DMD	TBC1D12
	CHAF1B	PDZD2	CDKN3	KAZN	TAFA2	COMMD1
	TBC1D31	LARGE1	CEP135	QDPR	TMIGD2	MIER3
	SIPA1L1	DNAH8	TRIM55	ABCB4	CNN2	ACACA
	FANCA	PEBP4	RYR2	LIX1L		Enhancer for PLK2
	SCAI	SCAI	FGF13	EPM2A		
	PDZD2	PLEKHA2	RAP1GAP2	WDR37		
	LARGE1	Promoter/Enhancer for FRMD8	RRP7A	TULP2		
	HMOX2	CDH13	SKAP1	GUCA1B		
	GPNMB	GRB2	TNFRSF10A	GOLGA5		
	NAA60	SUCLG2	SPDYE6			
	POGLUT3	ADCY5	MYLK			
	CPA1	RFPL3	PLEKHA2			
	SLC39A11	PTGS1	ZNF841			
	MGAM	GRIN2A	RUNX1			
	SBSN	CAMK1D	MYOF			
	IPO11	SCARB1	STK3			
	ARNT	NRTN	TPTE			
	CNNM1	KAZN	IPO11			
	PSMD14	MGAM	QDPR			
	SPECC1	TEX45	Enhancer for TM9SF4			
	SUGCT	ESPL1	LPCAT1			
	GOLGA8M	UBE2E1	SBSN			
	NTF3	PARD3	TRAPPC9			
	GGT1	OIT3	ESPN			
	MICB	HMOX2	IQCH			
	SYNE1	TRB	Enhancer of NOG			
	ADNP	RIMS3	ABCB4			
	SLC7A8	SEZ6L	RAD54B			
	NDST4	Promoter/Enhancer for RNF4	LHFPL4			
	FCGRT	NDUFAF2	ESRRB			
	Enhancer for TACC1	ARHGAP26	DCTD			
	BAZ1A	RPTOR	ZFR2			
		PCCA	TTLL3			
		SEPT9	ZNF568			
		ZNF695	SYCP2L			
		RENBP / NAA10	ULK4			
		FAM53B	UBR1			
		SYCP2L	GAB2			
		SMYD4	SEPP1			
		GOLGA5	FOXO3			
		PANK2	Enhancer for NMB			
		PALM2-AKAP2	RANGAP1			
		CAPN1				
		UBE3D				
		GLUD1				
		Enhancer for IL6ST				
		SULF1				
		VASP				
		AGAP1				
		TRAM2				
		PTPRN2				

Table S4.8. Total number of integrations and number in known coding genes detected in samples from case ID# 405 and 420

CASE ID#	TISSUE	TOTAL # INTEGRATIONS DETECTED	# INTEGRATIONS IN KNOWN CODING GENES
405 (CHB + HCC)	PBMC	32	18*
	NON-TUMOR LIVER	21	16
	LIVER TUMOR	23	7
420 (CHB + DCS)	PBMC	20	9
	DCS TUMOR	18	10

*2 of these detected integrations were in the same gene (NDUFAF2), thus only 17 unique genes were identified.

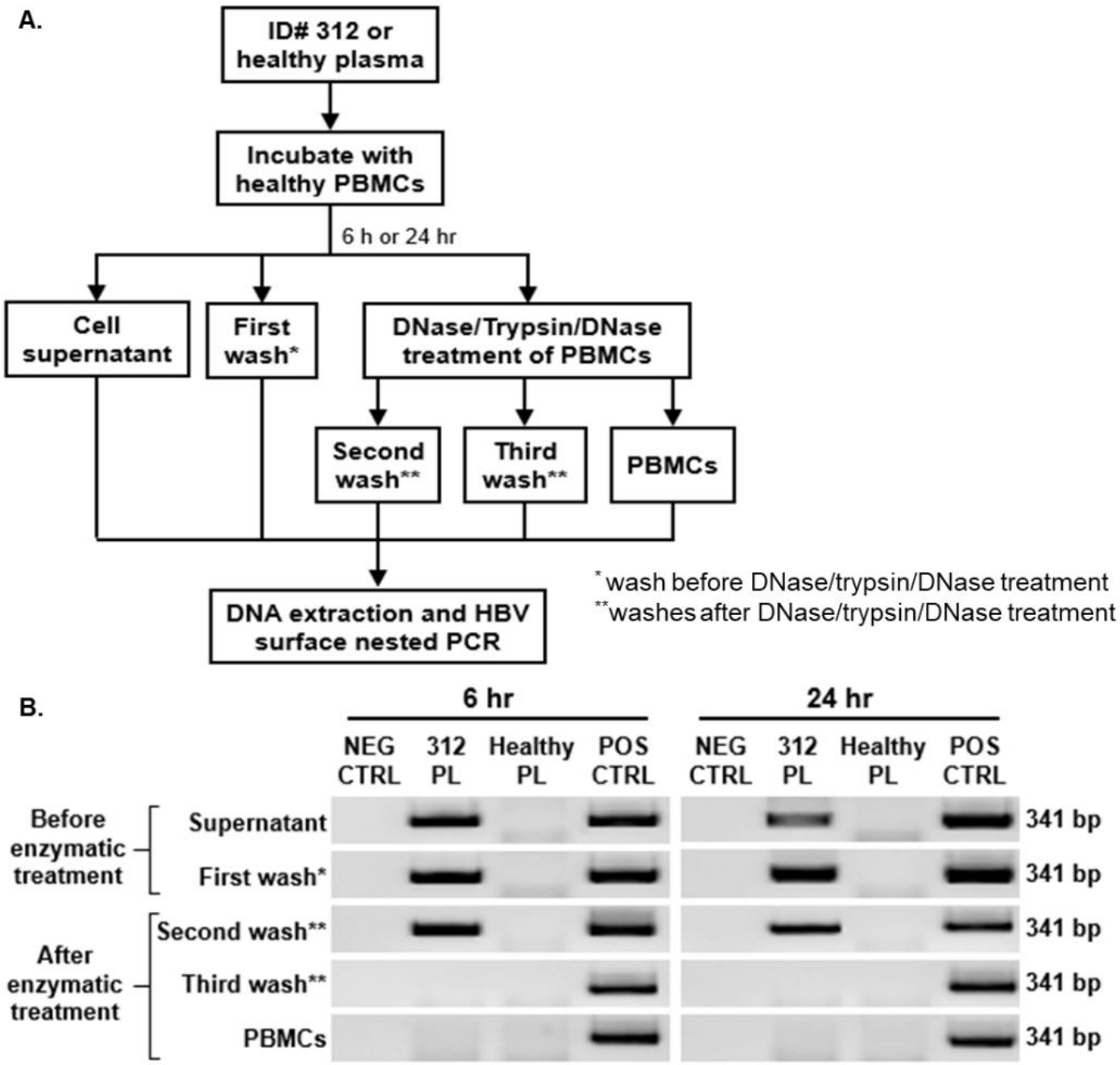

Figure S4.1. Extracellular enzymatic treatment of isolated PBMC with DNase/Trypsin/DNase effectively removes any potential extracellularly adhered HBV particles. To confirm that our detection of HBV in the PBMC were not from extracellular-surface adhered HBV and the effectiveness of our DNase/Trypsin/DNase treatment, HBV-negative healthy PBMC were incubated

with plasma from a highly viremic CHB carrier (case ID# 312). **A.** Flow chart of incubation of HBV-negative PBMCs with healthy plasma or plasma from a CHB carrier with high viremia (ID# 312; 8.3 log IU/mL) to demonstrate the efficiency of DNase/trypsin/DNase treatment in removing extracellular HBV particles. PBMCs were co-cultured *in vitro* with plasma diluted 1:1 with RPMI1640 + 10% FBS for 6 or 24 hours. Cell supernatant was collected, and cells were washed with DPBS before undergoing enzymatic treatment. After treatment, cells were washed twice and pelleted. All cell washes and final pellet were collected for DNA extraction and HBV surface nested PCR. **B**. Collected supernatant, washes, and PBMCs were analyzed with nested PCR targeting the HBV surface genomic region. At both the 6 and 24 hour time-points, DNase/trypsin/DNase treatment was effective at removing any potential extracellular (cell surface adhered) HBV particles.

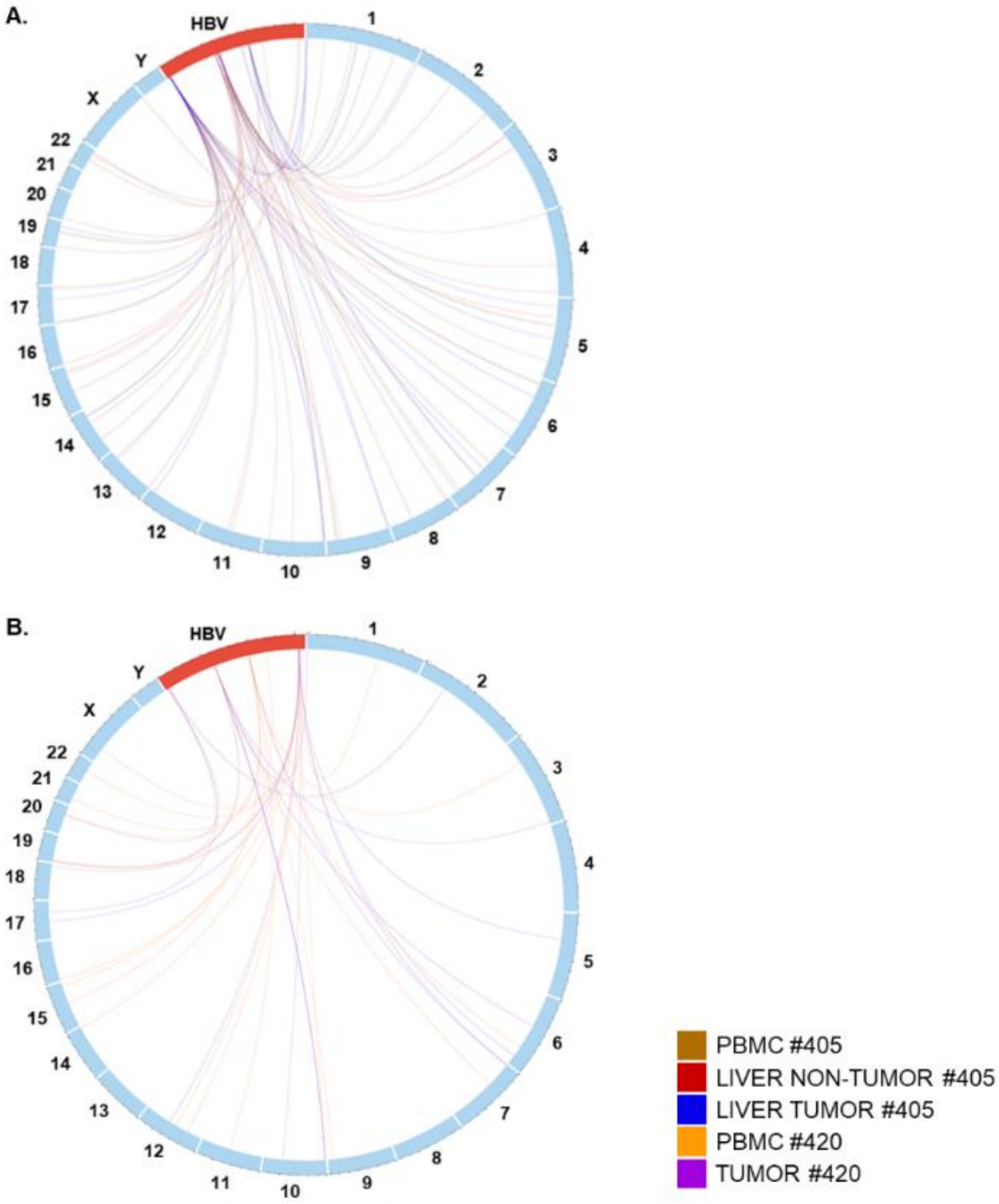

Figure S4.2. HBV-host integration sites identified with AluPCR followed by clonal sequencing in samples with matching tumor and PBMCs (cases #405 and

#420). The outside bands indicate the human chromosome (light blue) and the HBV chromosome (red, not to scale). Lines in the Circoplots shows the location of each integration junction in the viral and host genomes. **A.** PBMC (brown), Non-tumor liver (red), and liver tumor (blue) of CHB+HCC case ID#405; **B.** PBMC (orange) and DCS tumor (purple) of CHB+DCS case ID#420.

APPENDIX D: CHAPTER 5 SUPPLEMENTARY TABLES AND FIGURES

Table S5.1. Primers used for full genome amplification and site-directed mutagenesis of the HBV wild-type genome. Underlined nucleotides represent the recognition site of HindIII used for cloning and mutant nucleotides are enlarged and bolded.

PRIMER	SEQUENCE (5' TO 3')
HBV FG P1	CCGGAAAGCTTGAGCTCTTCTTTTTCACCTCTGCCTAATCA
HBV FG P2	CCGGAAAGCTTGAGCTCTTCAAAAAGTTGCATGGTGCTGG
M13 FOR	GTAAAACGACGGCCAGT
M13 R-17	CAGGAAACAGCTATGAC
A1762T/G1764A SDM-FOR	TGGGGGAGGAGATTAGGTTAA**T**G**A**TCTTTGTACTAGGAGG
A1762T/G1764A SDM-REV	CCTCCTAGTACAAAGA**T**C**A**TTAACCTAATCTCCTCCCCCA
G1896A SDM-FOR	CTGTGCCTTGGGTGGCTTT**A**GGGCATGGAC
G1896A SDM-REV	GTCCATGCCC**T**AAAGCCACCCAAGGCACAG

Table S5.2. Primers used for qPCR for quantification of HBV DNA, cccDNA, and RNA as well as nested PCR of cccDNA and DIG-probe synthesis.

TARGET	FOR SEQUENCE (5' TO 3')*	REV SEQUENCE (5' TO 3')	ASSAY
HBV DNA	CCGACCTTGAGGCGTACTTC	TACGGGTCAATGTCCATGCC	Supernatant HBV DNA qPCR
HBV RNA 3'RACE	ACCACGCTATCGCTACTCAC$(dT)_{17}$GWAGCTC	N/A	cDNA synthesis of supernatant HBV RNA
HBV RNA qPCR	CAACTTTTTCACCTCTGCCTA	ACCACGCTATCGCTACTCAC	Quantification of supernatant HBV RNA
cccDNA (92F and 2251R)	GCCTATTGATTGGAAAGTATGT	AGCTGAGGCGGTATCTA	HBV cccDNA qPCR
HBxin	ATGGCTGCTARGCTGTGCTGCCAA	AAGTGCACACGGTYYGGCAG	3D-PCR
cccDNA direct	ACTCCTGGACTCTCAGCAATG	GTATGGTGAGGTGAGCAATG	Direct round of cccDNA PCR
cccDNA nested	AGGCTGTAGGCACAAATTGGT	GCTTATACGGGTCAATGTCCA	Nested round of cccDNA PCR
X/BCP/preC	GCATGGAGACCACCGTGAAC	CATAAGAGGACTCTTGGACT	DIG-probe synthesis
preC/Core	GCATGGAGACCACCGTGAACG	GAGGGAGTTCTTCTTCTAGG	DIG-probe synthesis
Surface	TGCTGCTATGCCTCATCTTC	CARAGACARAAGAAAATTGG	DIG-probe synthesis

*__D__ = A, G, or T; __M__ = A or C; __R__ = A or G; __W__ = A or T

Table S5.3. Primers used for qPCR for cellular mRNA analysis.

TARGET	FOR SEQUENCE (5' TO 3')*	REV SEQUENCE (5' TO 3')	ASSAY
GAPDH	ACCAACTGCTTAGCCC	CCACGACGGACACATT	Cellular mRNA qPCR
APOBEC3A	GAGAAGGGACAAGCACATGG	TGGATCCATCAAGTGTCTGG	Cellular mRNA qPCR
APOBEC3B	GACCCTTTGGTCCTTCGAC	GCACAGCCCCAGGAGAAG	Cellular mRNA qPCR
APOBEC3C	CAACGATCGGAACGAAACTT	TATGTCGTCGCAGAACCAAG	Cellular mRNA qPCR
APOBEC3DE	ACCCAAACGTCAGTCGAATC	GCTCAGCCAAGAATTTGGTC	Cellular mRNA qPCR
APOBEC3F	CCGTTTGGACGCAAAGAT	CCAGGTGATCTGGAAACACTT	Cellular mRNA qPCR
APOBEC3G	GGTCAGAGGACGGCATGAGA	GCAGGACCCAGGTGTCATTG	Cellular mRNA qPCR
APOBEC3H	AGCTGTGGCCAGAAGCAC	CGGAATGTTTCGGCTGTT	Cellular mRNA qPCR

Table S5.4. Detailed clinical and virological data of CHB carriers analyzed by next generation sequencing analysis.

AGE / SEX / ETH / GENOTYPE* (CASE ID#)	HBV DNA, IU/mL† (Date)	qHBsAg, IU/mL (Date)	SERUM FULL-LENGTH / TRUNCATED HBV RNA, log copies/mL‡	ANTI-VIRAL THERAPY**	ALT	HBeAg/ Anti-HBe (Date)	PATHOLOGY / HCC THERAPY / FIBROSIS (FibroScan, kPa)††	% OF HBV QUASI-SPECIES‡ A1762T / G1764A / G1896A
Pre-dominant wild-type								
46 / F / CA / B (30)	**1300 (2009)** 550 (2010) 2900 (2011) 870 (2012) 2200 (2013) 686 (2014) 1397 (2015) 1281 (2016) 825 (2017) 1275 (2018)	2728 (7/2017)	0 / 1.84	NT	11	NEG/POS (03/2009)	LBx: (2009, 2013) Grade 2; Stage 1 Stage 0 (4.9kPa)	13.2 / 13.2 / 14.2 (2012)
35 / M / AS / D (83)	9.9×10^5 (2006) **D (2012)** D (2/2016) 8044 (7/2016) 9703 (5/2017) U (6/2017) D (6/2018) 425 (4/2019) U (11/2019)	1881 (2014) 1106 (2016) 860 (2017) 511 (2018)	2.02 / 1.61	TDF (2006)	23	POS/NEG (2006) NEG/POS (2013)	LBx: (2010) Grade 1; Stage 2 Stage 0 (4.8kPa)	21.5 / 21.3 / 13.7 (2012)
48 / M / AS / C (302)	150 (01/2013) 12 (02/2014) D (07/2014 to 01/2015) **D (07/2015)** D (01/2016-07/2016) U (01/2017)	387 (7/2014) 241 (7/2015) 91 (01/2016) 57 (08/2016) 49 (01/2017)	3.12 / 2.50	ETV (2013-2015); TDF (2015-present)	38	NEG/NEG (01/2013) NEG/NEG (01/2017)	Cirrhosis, HCC: RFA (2012); SBRT (2014); TACE (2015)	16.1 / 15.9 / 16.1 (2015)
38 / M / AF / E (313)	9.6×10^5 (2013) 38 (10/2013 D (1/2014) 18 (04/2014) 56 (07/2014) 29 (10/2014) U (01/2015) D (04/2015 -2015) **D (10/2015)** D (04/2016 - 10/2016) U (04/2017 - 12/2019)	770 (10/2014) **270 (04/2016)** 247 (10/2016) 105 (04/2017) 86 (10/2017) 81 (05/2018)	4.06 / 3.17	TDF (2010-present)	59	NEG/POS (10/2016)	Cirrhosis, HCC Resection (2014): Grade 3 poorly differentiated HCC; Stage 3 Fibrosis; Mild-moderate hepatitis Resection (2015): Grade 3 poorly differentiated HCC; vascular invasion; Stage 3 Fibrosis; Mild-moderate hepatitis Stage 4 (16 kPa, 2014); Stage 2 (8 kPa, 2018)	20.9 / 22.4 / 9.0 (2016)
Pre-dominant G1896A								
44 / F / AF / E (47)	**4900 (11/2010)** 1100 (5/2011)	N/A	2.47 / 1.95	NT	12	NEG/POS (2008)	LBx: (2009) Grade 0, Stage 1	2.9 / 3.6 / 61.0 (2010)

73 / F / AS / C (266)	1.2×10^6 (04/2008) 1600 (07/2008) 110 (09/2008) D (12/2008 - 06/2012) 33 (09/2012) D (01/2013 - 09/2013) 72 (01/2014) D (01/2014 - 03/2014) **U (10/2014)** U (01/2015) D (07/2015) U (01/2016 - 10/2019)	**5.2 (01/2015)** 5.5 (10/15) 5.0 (07/2017) 4.5 (10/2017)	3.11 / 1.71	ETV (2008-present)	18	POS/NEG (06/2007) NEG/NEG (10/2019)	Cirrhosis, HCC Resection (2014): Solid variant HCC; Severe fibrosis; Mild hepatitis	1.9 / 0.0 / 69.2 (2015)
67 / M / AS / C (CC15)	**U (10/2007)**	N/A	0 / 3.59	TDF	84	NEG/NEG	Cirrhosis, HCC: Liver transplant	0.5 / 0.7 / 98.3 (2007)
Pre-dominant A1762T/G1764A								
47 / M / AS / C (6-2)	2.3×10^6 (02/2007) 1.9×10^6 (9/2007) **D (9/2009)** D (03/2011 to 02/2014) U (10/2014) D (2/2015) 165 (8/2015) U (12/2015 - 11/2018) D (06/2019) U (03/2020)	452 (10/2014) 515 (2/2015) 378 (03/2017) 309 (4/2018) 294 (06/2018)	2.30 / 1.83	(TDF) ETV - present	40	NEG/POS (9/2010) NEG/POS (06/2019)	LBx: (2007) Grade 0, Stage 1 Stage 0 (3.0 kPa, 2015); Stage 0 (5.1 kPa, 2019)	94.9 / 95.7 / 3.4 (2009)
62 / M / AS / C (113-2)	3.6×10^7 (4/2011) 890 (8/2011) 110 (11/2011) 23 (02/2012) D (05/2012) 25 (8/2012) 40 (11/2012) 30 (02/2013) D (05/2013 to 11/2016) **D (11/2017)** U (5/2018) D (12/2018)	1288 (11/2014) 1140 (05/2015) 868 (5/2016) **980 (11/2017)** 835 (12/2018)	2.36 / 0.79	TDF (06/2011-present)	28	POS/POS (5/2011) POS/NEG (11/2017)	Cirrhosis	70.5 / 74.9 / 19.9 (2017)
56 / M / AS / C (307)	**5.5×10^5** (09/2015) U (06/2016) 13 (11/2016) D (03/2017 - 08/2017) U (03/2018) D (09/2018 - 03/2019) U (11/2019)	**3231 (09/2015)** 1338 (08/2017) 599 (11/2019)	2.51 / 2.90	TDF (09/2015-present)	66	NEG/POS (11/2016)	HCC TACE (2015); RFA (2016); Left hepatectomy (2016) Cirrhotic liver; post-cautery changes; no HCC, partial gastrectomy erosion to gastric wall (11/2016) Stage 1-2 (6.8 kPa, 2016)	87.8 / 88.6 / 8.7 (2015)
58 / M / AS / C (405)	2.6×10^6 (11/2012) 365 (11/2014) 975 (03/2017) 106 (03/2018) **73 (09/2018)** D (01/2019) U (12/2019)	379 (03/2017) 21 (12/2019)	0 / 4.59	TDF (2016 -present)	19	POS/POS (02/2007) NEG/NEG (11/2014)	Resection Moderately differentiated HCC, cirrhosis, no vascular invasion, Stage 4 (16.8 kPa. 2015); Stage 4 (15.8 kPa, 2016)	95.1 / 95.0 / 0.9 (2018)

***AF**: African; **AS**: Asian; **CA**: Caucasian; **F**: Female; **M**: Male

† Determined by clinical PCR assay (Roche TaqMan or Abbott Architect, LLOD:55, 20, or 10 IU/mL, respectively). **D**: Detectable but not quantifiable; **U**: Undetectable. Bolded dates are those within 6 months before sample collection.
‡ Serum HBV RNA, genotype, and quasi-species proportions determined by RT-qPCR, phylogenic tree analysis, and NGS, respectively, in previous study (7,8)
****ETV**: Entecavir; **NT**: No treatment; **TDF**: Tenofovir
††Based on pathology, liver biopsy, or Fibroscan. **FS**: Fibroscan; **HCC**: Hepatocellular carcinoma; **Lbx**: Liver Biopsy; **RFA**: Radiofrequency ablation; **SBRT**: Stereotactic body radiation therapy; **TACE**: Trans-arterial chemoembolization

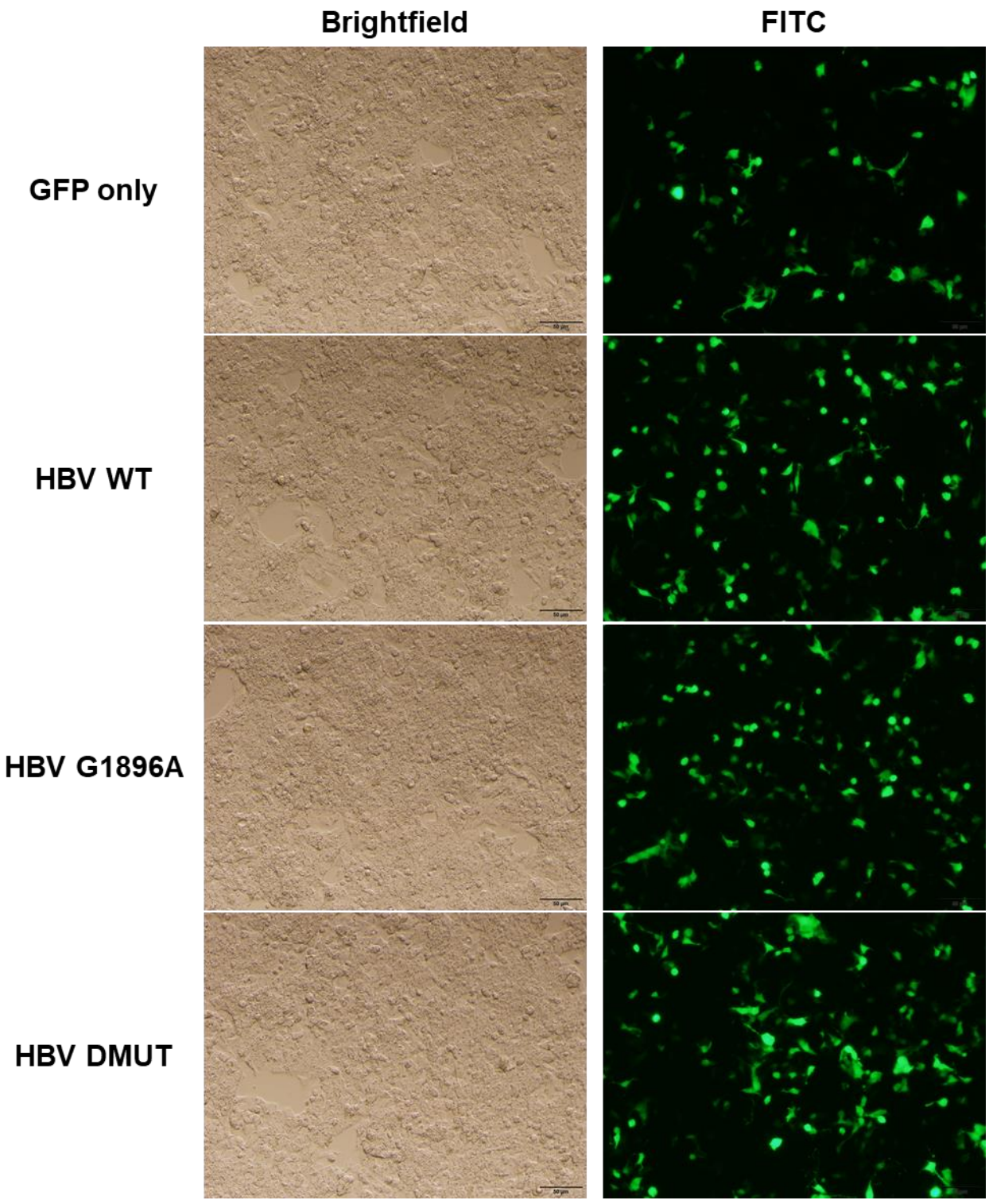

Figure S5.1. Representative images of transfection efficiency 3 days post-transfection using fluorescent microscopy. Using both brightfield and FITC filters, cellular

confluency and transfection efficiency was compared amongst the transfection conditions. WT = wild-type; DMUT = double mutant (A1762T/G1764A). Total magnification of 100X. Representative images of three independent transfections.

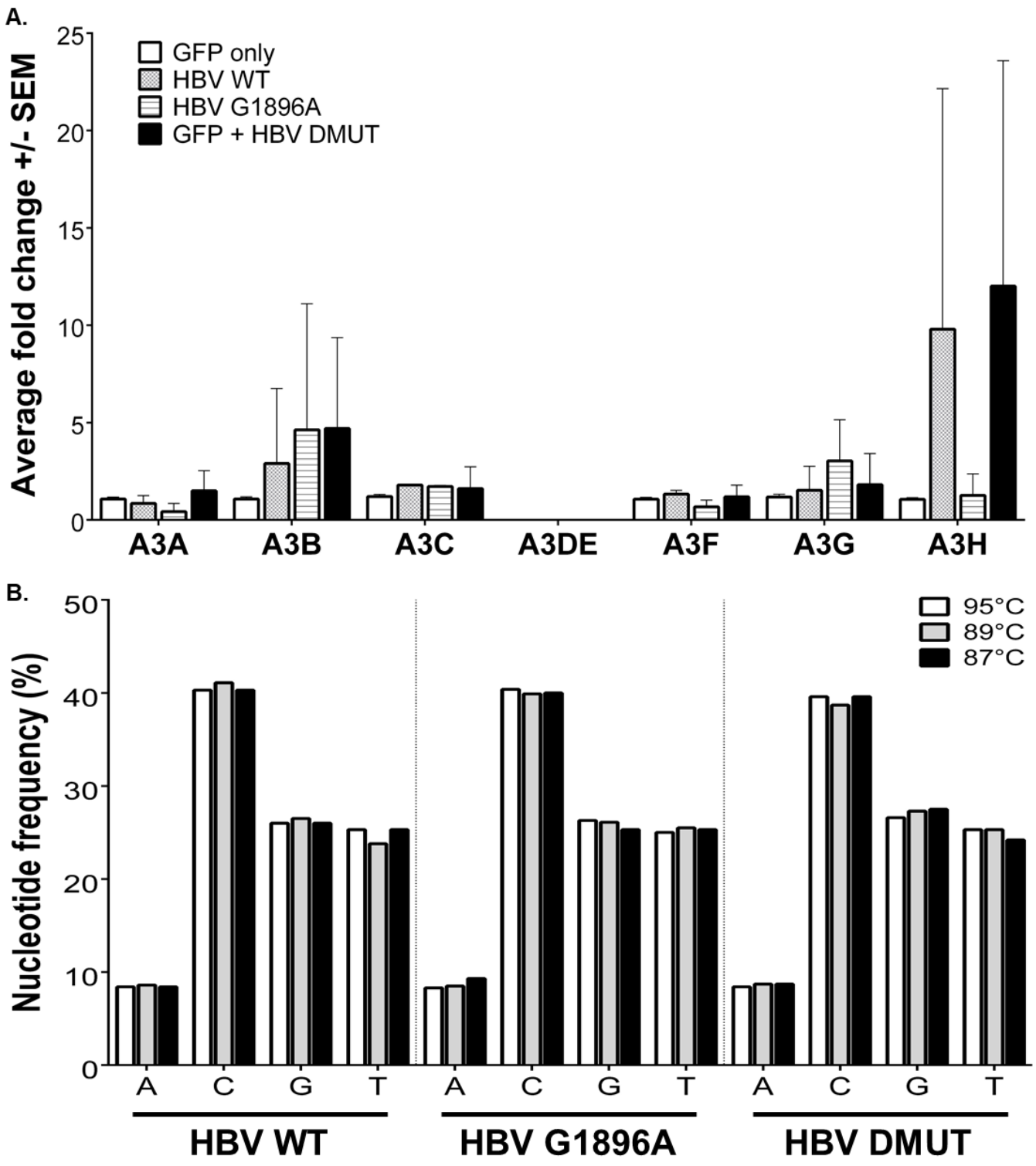

Figure S5.2. Reduced APOBEC3 (A3) cellular expression in HBV mutants and no HBV cccDNA hypermutation. Cellular A3 mRNA levels were evaluated 1-day post-

transfection using GAPDH as an internal reference gene. **A.** Virally transfected DNA resulted in minimal non-significant induction of APOBEC3B, 3C, 3G, and 3H; or relative decreases of APOBEC3A and 3F. Average fold changes were analyzed by one-way ANOVA with post-hoc Tukey tests. **B.** 3D-PCR products 1-day post-transfection were sequenced to assess for APOBEC3 family activity with no significant changes in HBV cccDNA.

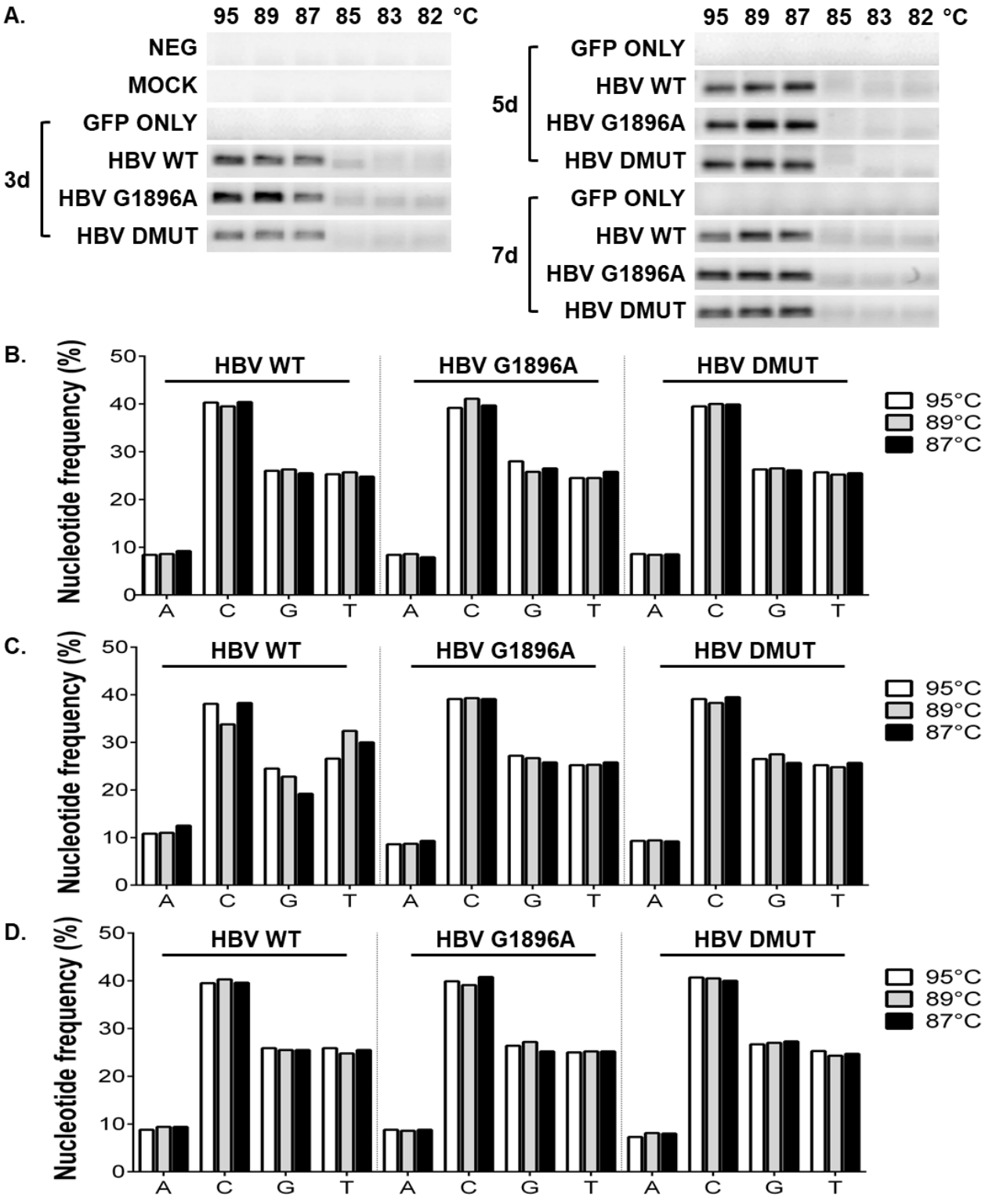

Figure S5.3. Lack of APOBEC3 hypermutation activity on HBV cccDNA even after 3-7 days post-transfection. Hypermutation of HBV genomes were evaluated 3, 5, and

7 days post-transfection to assess for APOBEC3 family activity by **A.** 3D-PCR, and subsequent sequencing of the excised bands to identify nucleotide frequencies from wild-type (WT), G1896A, and A1762T/G1764A (DMUT) at **B.** 3 days post-transfection; **C.** 5 days post-transfection; and D. 7 days post-transfection.

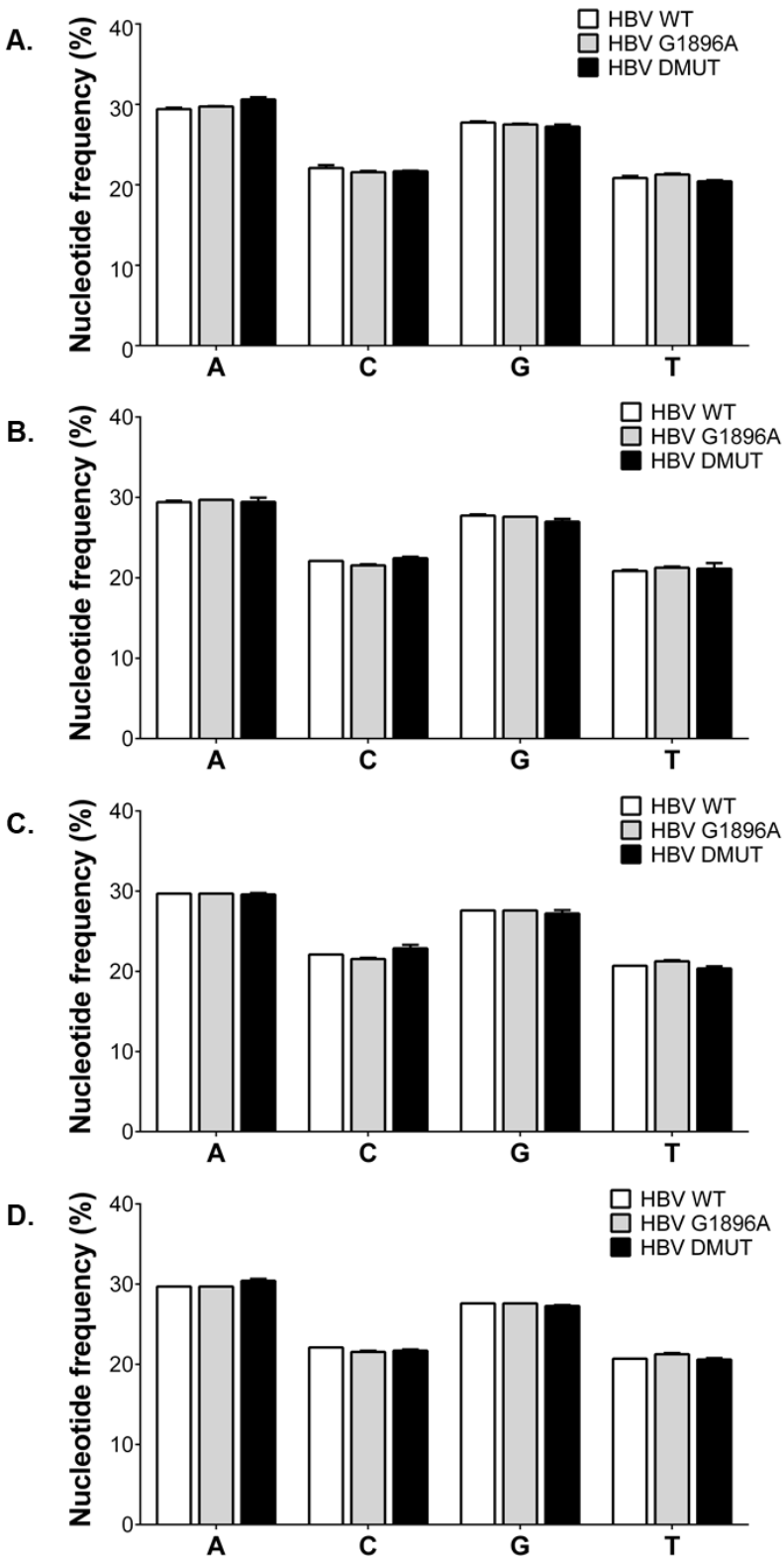

Figure S5.4. Evaluation of APOBEC3 hypermutation of HBV cccDNA by clonal sequencing. Nested cccDNA PCR products was used clonal sequencing by

insertion of gel excised products into the pGEM-T easy vector. Frequency of each nucleotide from sequencing was plotted from the **A.** 1 day; **B.** 3 days; **C.** 5 days; and **D.** 7 days post-transfection. WT = wild-type.

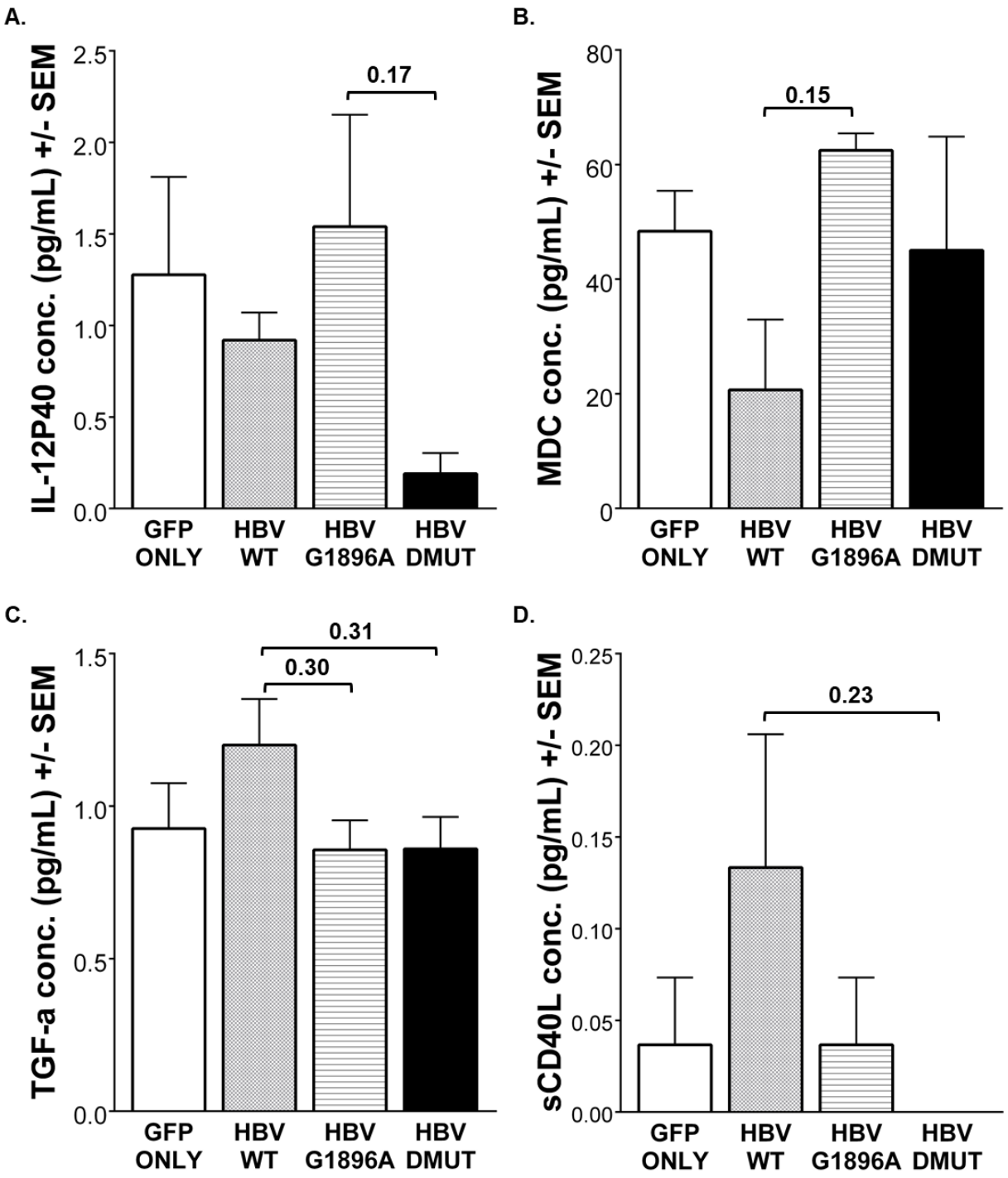

Figure S5.5. Cytokine/chemokine expression differs when transfected with different HBV variants. Supernatant collected 12 hours post-transfection was quantified for cytokines/chemokines by Luminex. The HBV variants resulted in different patterns of immune response induction as observed in many cytokines including: **A.** IL-

12P40; **B.** MDC; **C.** TGF- α; and **D.** sCD40L. One-way ANOVA with post-hoc Tukey tests were used for statistical analysis and numbers represent p-values.

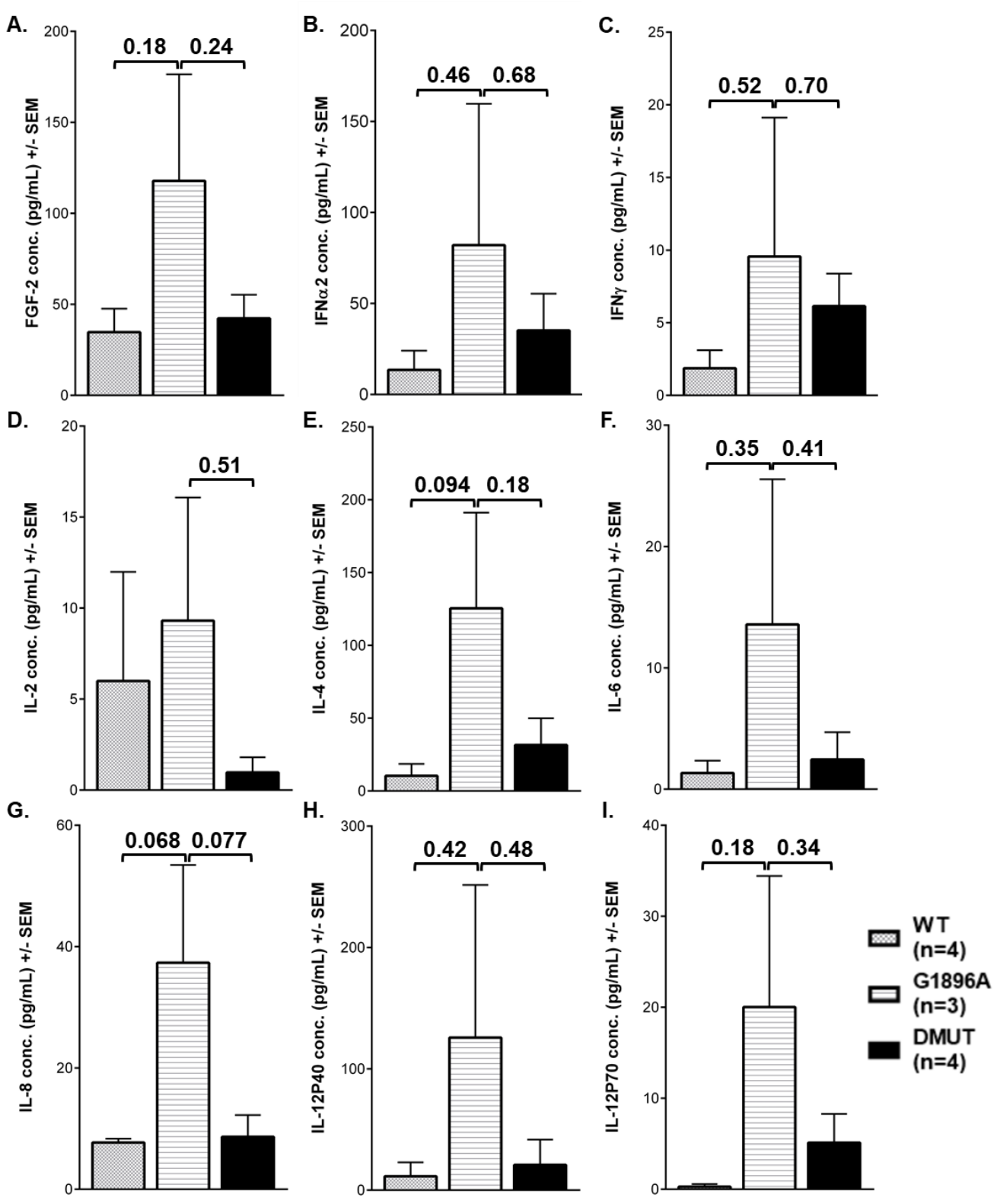

Figure S5.6. Elevation of cytokine/chemokine in CHB carriers with G1896A variant HBV.

CHB carrier serum was quantified for cytokines/chemokines by Luminex. A

general trend of elevated cytokines were observed in CHB carriers with G1896A variant including: **A.** FGF-2; **B.** IFNα2; **C.** IFNγ; **D.** IL-2; **E.** IL-4; **F.** IL-6; **G.** IL-8; **H.** IL-12P40; **I.** IL-12P70. Unpaired t-tests were used for statistical analysis and numbers represent p-values.

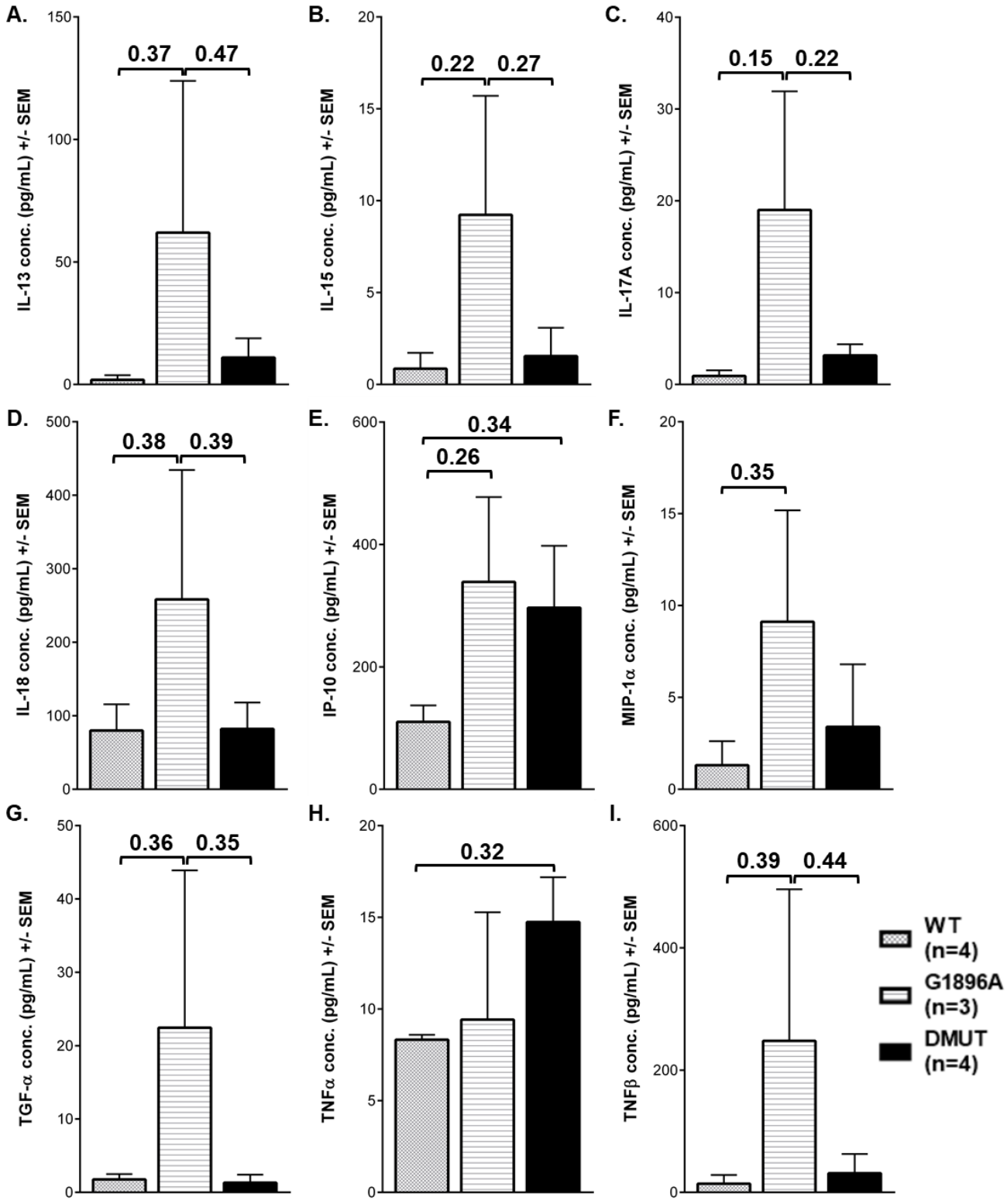

Figure S5.7. Elevation of cytokine/chemokine in CHB carriers with G1896A variant HBV.

CHB carrier serum was quantified for cytokines/chemokines by Luminex. A

general trend of elevated cytokines were observed in CHB carriers with G1896A variant including: **A.** IL-13; **B.** IL-15; **C.** IL-17A; **D.** IL-18; **E.** IP-10; **F.** MIP-1α; **G.** TGF-α; **H.** TNFα; and **I.** TNFβ. One-way ANOVA with post-hoc Tukey tests were used for statistical analysis and numbers represent p-values.

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E.1 CHAPTER 1

E.1.1 Figure 1.1A

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E.1.2 Figure 1.1B

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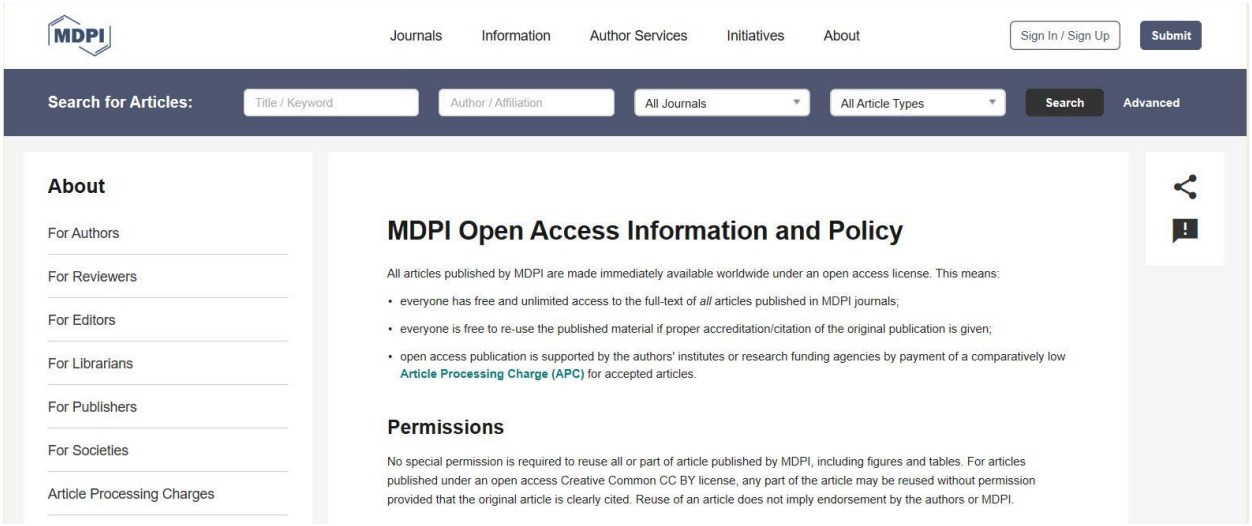

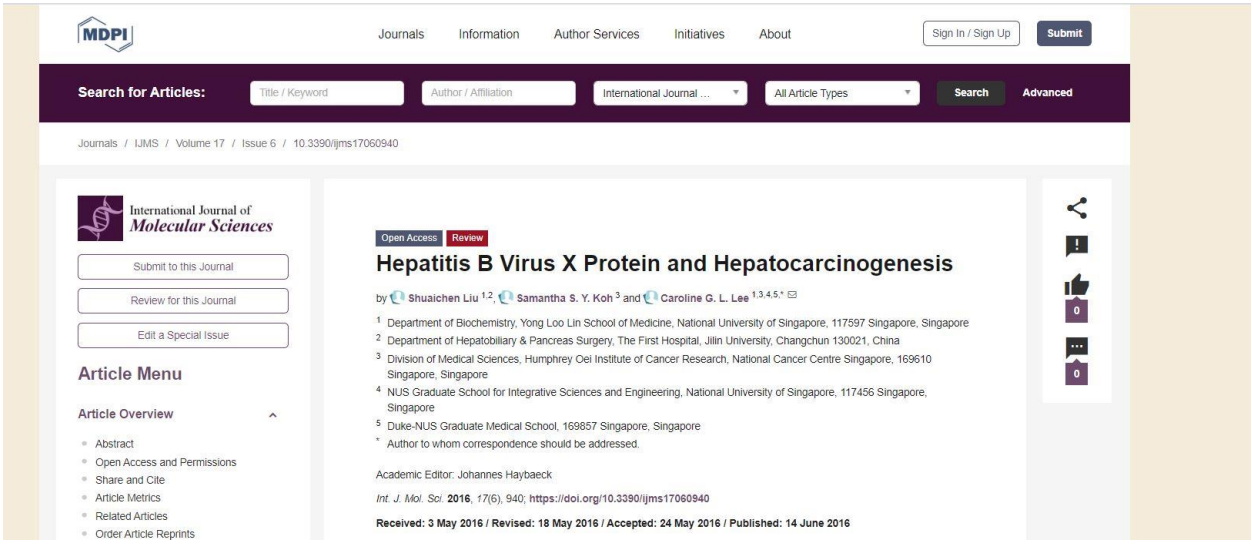

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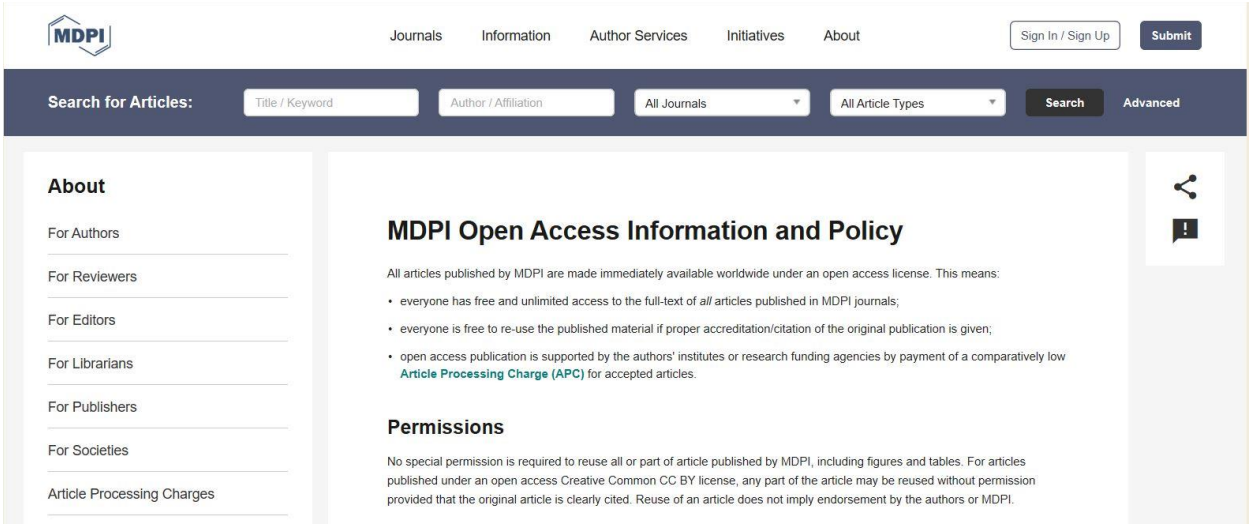

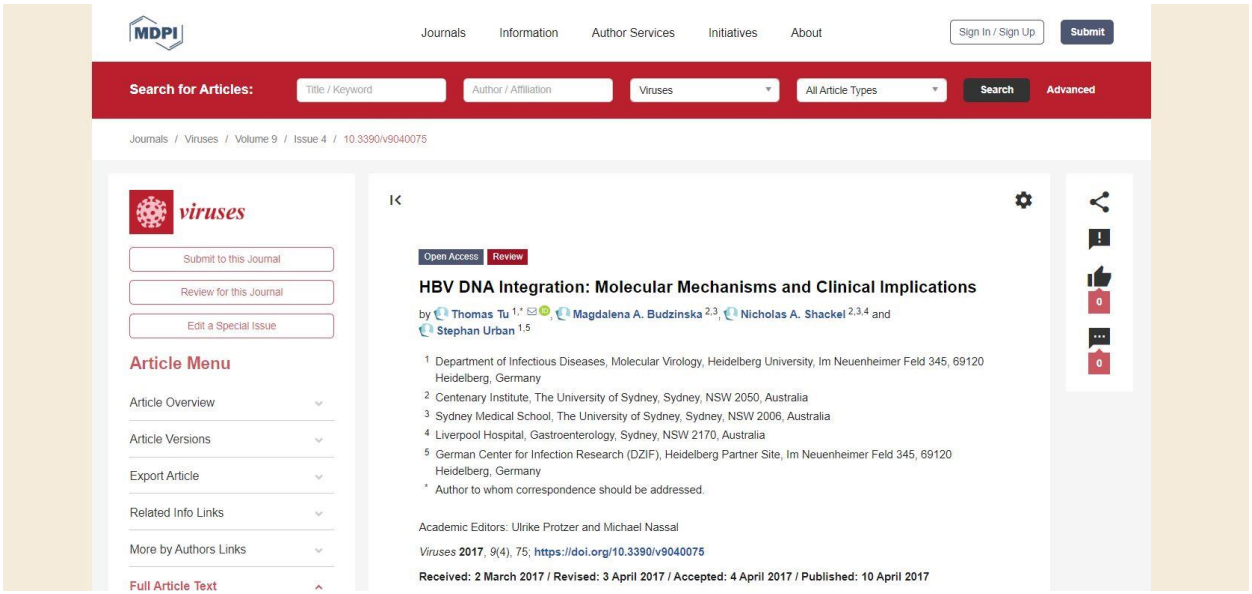

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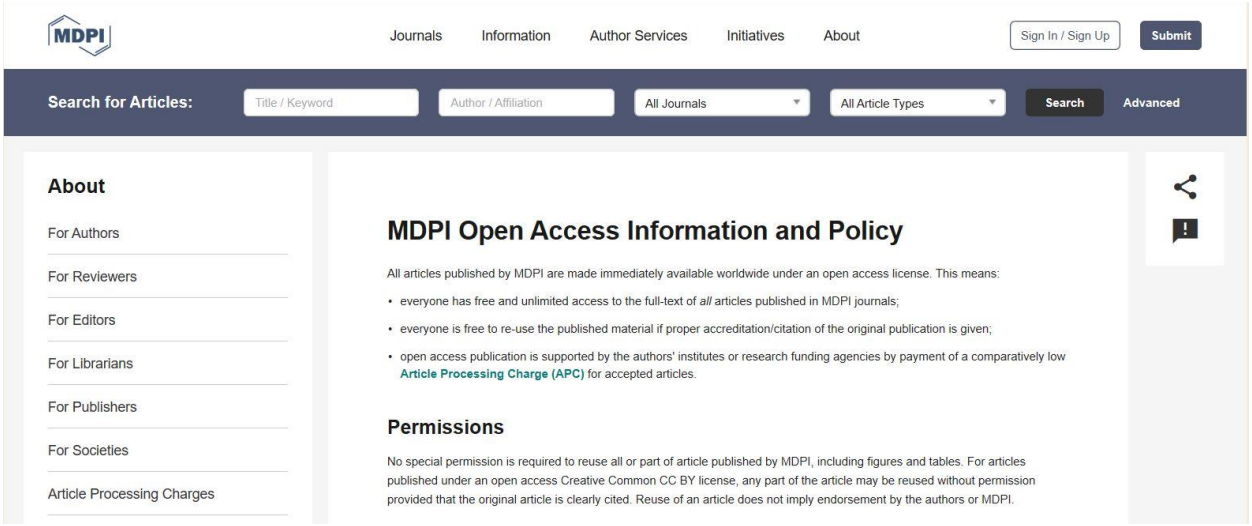

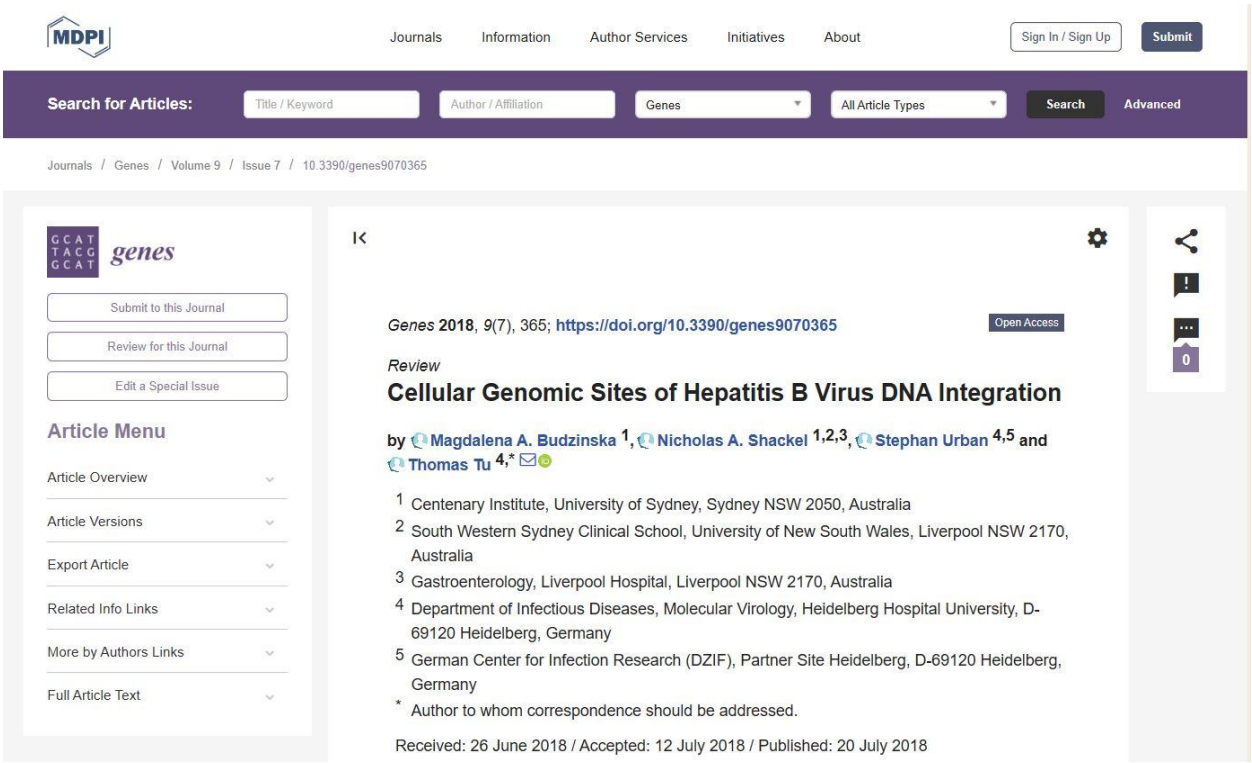

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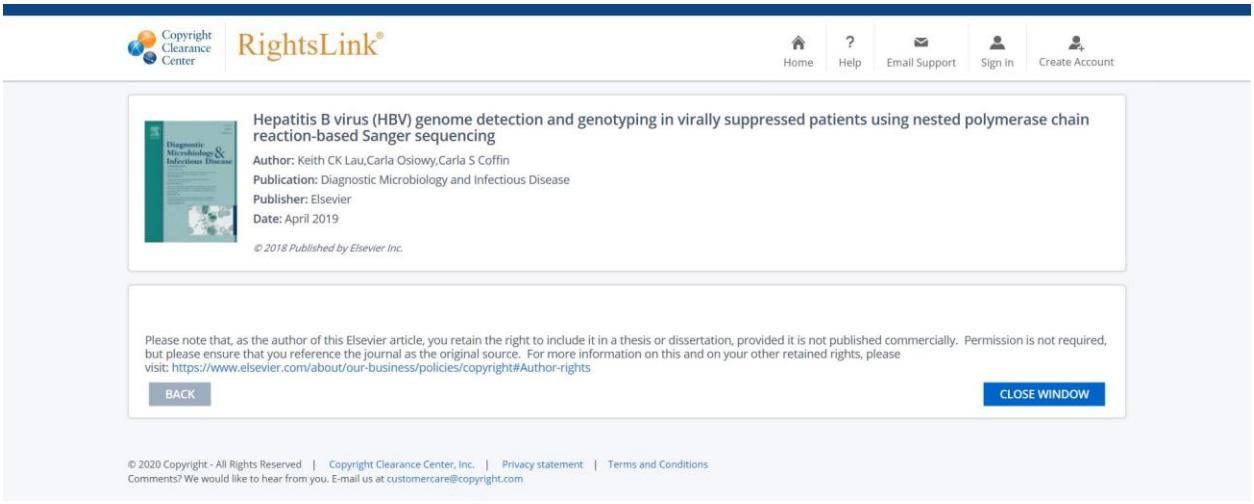

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Author: Keith CK Lau,Carla Osiowy,Carla S Coffin

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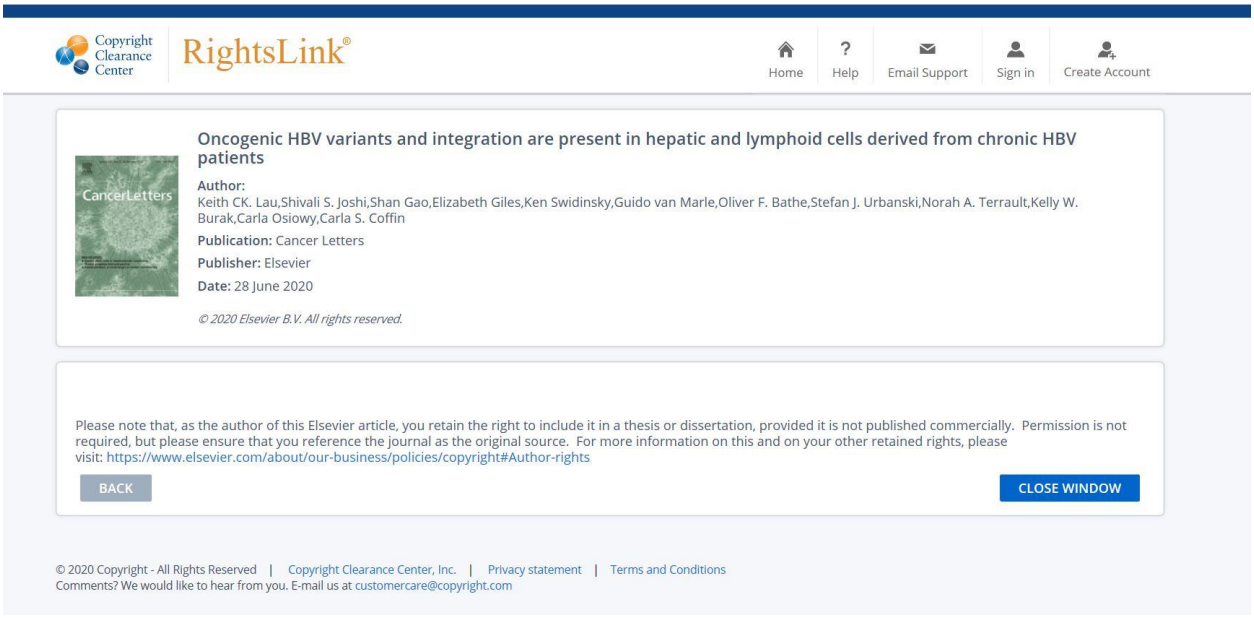

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Oncogenic HBV variants and integration are present in hepatic and lymphoid cells derived from chronic HBV patients

Author:
Keith CK. Lau,Shivali S. Joshi,Shan Gao,Elizabeth Giles,Ken Swidinsky,Guido van Marle,Oliver F. Bathe,Stefan J. Urbanski,Norah A. Terrault,Kelly W. Burak,Carla Osiowy,Carla S. Coffin

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SHIVALI JOSHI

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回复: Accepted_Cancer Letters

GS **Gao Shan** <[redacted]>
3:53 PM

To: Keith Chi-Kei Lau; Carla S. Coffin; Kelly W. Burak; osiowy Carla; Oliver F. Bathe; stefan Urbanski; Norah Terrault; Guido Van Marle; Shivali Joshi; Oliver B...

Dear Keith,

Sure. You have my permission 😊.

Best,
Shan

发件人: Keith Chi-Kei Lau <[redacted]>
发送时间: 2020 年 4 月 23 日 10:45
[redacted]
主题: RE: Accepted_Cancer Letters

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Co-author Printed Name

[redacted]
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Re: 回复: Accepted_Cancer Letters

NT **Norah Terrault** <[redacted]>
8:42 PM

To: Keith Chi-Kei Lau

You have my permission also!
Best,
N.

From: Gao Shan <[redacted]>
Sent: Thursday, April 23, 2020 2:53 PM
To: Keith Chi-Kei Lau <[redacted]>; Carla S. Coffin <[redacted]>; Kelly W. Burak <[redacted]>; osiowy Carla <[redacted]>; Oliver F. Bathe <[redacted]>; stefan Urbanski <[redacted]>; Norah Terrault [redacted]; Guido Van Marle <[redacted]>; Shivali Joshi [redacted]; Oliver Bathe [redacted]; Swidinsky, Ken (PHAC/ASPC) <[redacted]>; Giles, Elizabeth (PHAC/ASPC) [redacted]>
Subject: 回复: Accepted_Cancer Letters

Dear Keith,

Sure. You have my permission 😊.

Best,
Shan

发件人: Keith Chi-Kei Lau <[redacted]>
发送时间: 2020 年 4 月 23 日 10:45
收件人: Carla S. Coffin <[redacted]>; Kelly W. Burak <[redacted]>; osiowy Carla [redacted]; Oliver F. Bathe [redacted]; stefan Urbanski [redacted]; Norah Terrault [redacted]μ>; Guido Van Marle [redacted]; Shivali Joshi [redacted]; gao Shan <[redacted]>; Oliver Bathe [redacted]; Swidinsky, Ken (PHAC/ASPC) [redacted]; Giles, Elizabeth (PHAC/ASPC) <[redacted]>
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Best,

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