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REVIEW

Clinical relevance of hepatitis B virus variants

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Abstract

The hepatitis B virus (HBV) is a global public health problem with more than 240 million people chronically infected worldwide, who are at risk for end-stage liver disease and hepatocellular carcinoma. There are an estimated 600000 deaths annually from complications of HBV-related liver disease. Antiviral therapy with nucleos/tide analogs (NA) targeting the HBV polymerase (P) can inhibit disease progression by long-term suppression of HBV replication. However, treatment may fail with first generation NA therapy due to the emergence of drug-resistant mutants, as well as incomplete medication adherence. The HBV replicates *via* an error-prone reverse transcriptase leading to quasispecies. Due to overlapping open reading frames mutations within the HBV P can cause concomitant changes in the HBV surface gene (*S*) and vice versa. HBV quasispecies diversity is associated with response to antiviral therapy, disease severity and long-term clinical outcomes. Specific mutants have been associated with antiviral drug resistance, immune escape, liver fibrosis development and tumorgenesis. An understanding of HBV variants and their clinical relevance may be important for monitoring chronic hepatitis B disease progression and treatment response. In this review, we will discuss HBV molecular virology, mechanism of variant development, and their potential clinical impact.

Key words: Molecular virology; Genetic heterogeneity; Quasispecies; Drug resistance; Immune escape; Viral lymphotropism; Hepatitis B virus

Core tip: The hepatitis B virus (HBV) has significant genomic diversity and some HBV variants are associated with antiviral therapy response, vaccine escape, diagnostic failure, liver fibrosis progression and hepatocellular carcinoma development. Understanding HBV molecular epidemiology as well as the clinical and pathological relevence of HBV variants during different disease phases may enable more accurate risk-stratification of individual patients at risk for serious sequelae of chronic hepatitis B infection.

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EPIDEMIOLOGY OF CHRONIC HEPATITIS B

Chronic hepatitis B virus (HBV) infection (CHB) is a serious global public health problem. There are an estimated 600000 deaths annually from complications of HBV-related liver disease. For over 3 decades, there has been a safe and effective HBV vaccine consisting of recombinant HBV surface (S) (*i.e.,* envelope) protein that has reduced infection rates in countries with widespread immunization programs[1]. The HBV is transmitted parenterally by contact with blood or body fluids of an infected person. In highly endemic areas, such as China, the incidence of HBV infection is greater than 8%, and is often acquired at birth or in early childhood from exposure to HBV infected mothers or family members. About 90% of unvaccinated infants born to mothers with CHB will became chronic carriers, and the risk of CHB is up to 30% in children infected at 1-4 years of age[2]. Despite implementation of widespread childhood vaccination programs, the incidence and mortality of HBV-related cirrhosis and hepatocellular carcinoma (HCC) continues to increase due to the enormous burden of chronically infected carriers worldwide.

NATURAL HISTORY OF CHB INFECTION

The HBV is a non-cytopathic virus and liver cell injury is due to a host immune mediated antiviral response to an infected cell. CHB is a dynamic disease, and the interplay between the virus and the host immune system influences disease course. In clinical practice, CHB is divided into four disease phases: immune tolerant, immune clearance, inactive, and reactivation phase[3]. The immune tolerant phase is characterized by persistently normal serum alanine aminotransferase (ALT) levels, high HBV DNA levels and presence of HBV e antigen (HBeAg), but with no evidence of liver injury. The immune clearance phase is characterized by presence of HBeAg, persistently high ALT and HBV DNA levels with some degree of liver inflammation. HBeAg seroconversion may occur at the late stage of the immune clearance phase. Thereafter, patients are likely to progress to the immune inactive phase characterized by normal ALT level, low/undetectable HBV DNA (< 2000 IU/mL or < 10^4 virus copies/mL), absence of HBeAg and presence of anti-HBe, as well as no/minimal histological injury. HBV reactivation can occur in some and is characterized by rebound viremia, presence of anti-HBe, elevated ALT levels and liver inflammation. This so-called "reactivation phase" may also occur due to the presence of preC/basal core promoter (BCP) mutations that abolish or downregulate HBeAg production leading to HBeAg negative CHB. There is recent data challenging the classification of these clinical phases. Imunological characterization of apparent immune-tolerant HBV-infected adolescents did not reveal any tolerogenic T-cell pattern[4]. Further, histologically active disease has been reported in CHB children considered to be immune tolerant[5,6]. Finally, analysis of HBV quasispecies (QS) in children with an immune tolerant clinical profile showed significant HBV diversity, which may be due to immune selective pressure[7].

In general antiviral therapy for CHB is recommended in patients with advanced liver disease (*i.e.,* cirrhosis) or prolonged immune active disease flares due to the risk of liver fibrosis progression. The currently approved anti-HBV therapies include interferon [*i.e.,* pegylated-interferon (Peg-IFN)], which has non-specific antiviral and immunomudulatory effects and nucleos/tide analogs (NA) targeting the HBV polymerase/reverse transcriptase (P/RT) region. There are five currently available NAs: lamivudine (LMV), telbivudine (LdT), entecavir (ETV), adefovir (ADF) and tenofovir (TDF). The second generation NA's (*i.e.,* TDF and ETV) are potent with a high genetic barrier to resistance and persistently suppress HBV replication. These drugs have a low reported risk of drug resistance or treatment failure despite years of sustained therapy[8,9]. In contrast older generation NA has an increased risk of treatment failure with long-term use due to drug resistance (Table 1)[10]. NA are very effective at reducing liver disease risk but must be used for prolonged periods as they do not offer a cure for CHB.

OVERVIEW OF HBV REPLICATION AND TISSUE TROPISM

The HBV is the prototype member of the *Hepadnaviridae* family which includes various avian and mammalian viruses sharing similar genome structure and organism trophisms[11]. It is a small DNA virus with approximately 3.2 Kb partially double stranded relaxed circular DNA (rcDNA) genome within a nucleocapsid surrounded by a lipid envelope. The full-length virus negative-strand has a approximately 7-9 nucleotide redundancy and the complementary positive-strand is approximately 50%-70% full genome length. The HBV genome consists of 4 overlapping open reading frame (ORF) encoding the polymerase gene (*P*), pre-S1/pre-S2/S gene (*preS1*/*preS2*/*S*), precore/core gene (*preC/C*) and *X* gene. Viral entry occurs after binding of the viral pre-S1 protein to its specific functional receptor, the recently identified sodium taurocholate cotransporting polypeptide[12]. The intact virion or "Dane particle" uncoats in the cytoplasm and the rcDNA genome is transported into the nucleus and repaired to covalently closed circular DNA (cccDNA) by host and viral polymerases. The presence of cccDNA indicates successful establishement of HBV

Table 1 Summary of clinically revelant hepatitis B virus variants

Location	Amino acid or nucleotide substitution (associated overlapping gene mutation)	Clinical impact
P (RT-A)	rtI169T (sF161L)	ETV resistance
P (RT-B)	rtL180M (sE164D)	ETV resistance
P (RT-B)	rtA181T/V	LMV, LdT, ADF/TDF resistance
P (RT-B)	rtT184S/A/I/L/G/CM	ETV resistance
P (RT-C)	rtS202C/G/I	ETV resistance
P (RT-C)	rtM204V/I	LAM resistance
P (RT-C)	rtM204I (sW196S)	LdT resistance
P (RT-C)	rtM204V (sI195M)	ETV resistance
P (RT-D)	rtN236T	ADF/TDF resistance
P (RT-E)	rtM250I/V	ETV resistance
P (RT-A)	rtL80V/I	Poor antiviral response to ADF with prior LMV resistant variants
P (RT-B)	rtF166L (sF158Y)	LMV-associated, compensatory
P (RT-B)	rtV173L (sE164D)	Compensatory mutation associated with LMV resistance (enhanced replication)
P (RT-B)	rtA194T	TDF resistance
S ("a" determinant)	sG145R (rtW153Q)	Antibody-associated escape mutation; reduced HBsAg level; restore LMV resistant HBV replication
S ("a" determinant)	sD144E/G145R (rtG153E)	Antibody-associated escape mutation
S ("a" determinant)	sP120T (rtT128N)	Reduced HBsAg level
EnhII	C1653T	HCC development (genotype C)
BCP	T1753V	HCC development (genotype B)
BCP	A1762T/G1764A	HBeAg production reduced by 50%; HBeAg seroconversion; escape anti-HBe immunity
Pre-C	G1896A	HBeAg seroconverstion; escape anti-HBe immunity; more severe course of disease; HCC development
S	W172* (rtA181T)	Cirrhosis and HCC development
Pre-S1/Pre-S2	Pre-S1/pre-S2 deletion (pre-S2 start codon and/or deletions in the 5'-terminal half of the pre-S2 region and pre-S1 3'-terminal half of the pre-S1 region)	More common in genotype C; progressive liver diseases; HCC development
Pre-S	Pre-S1 promoter mutation Pre-S2 promoter mutation	HCC development
X	K130M + V131I (double)	HCC development
X	V5M/L + K130M + V131I (triple)	HCC development

HCC: Hepatocellular carcinoma; BCP: Basal core promoter; HBeAg: HBV e antigen; HBV: Hepatitis B virus; ADF: Adefovir; TDF: Tenofovir; LMV: Lamivudine; HBsAg: Hepatitis B surface (S) antigen; LdT: Telbivudine; ETV: Entecavir; RT: Reverse transcriptase.

infection[13]. The cccDNA is transcribed to a 3.5 Kb pregenomic (pg)-RNA molecule with a unique stem-loop epsilon structure located at its 5′ end. Thus, HBV cccDNA is the "master" template for HBV negative-strand synthesis *via* reverse transcription, as well as hepatitis B core antigen or nucleocapsid protein and P/RT translation[14]. Additionally, the cccDNA is the template for four subgenomic messenger RNAs (mRNAs), which are translated into soluble or secreted HBeAg (from 3.5 kb precore mRNA), subviral S or envelope particles (2.4 kb and 2.1 kb mRNA) and X (0.8 kb mRNA). The HBV pgRNA is transported to the cytoplasm and binding of the viral polymerase to its 5′ end epsilon structure initiates encapsidation by HBV core particles[15]. Following encapsidation, the pg-RNA is reverse-transcribed and is gradually degraded by viral polymerase ribonuclease H (RNase H). The positive-strand DNA is then synthesized from the newly transcribed negative-strand DNA template[11,16]. Once the relaxed circular (rc) HBV genome synthesis is complete, the nucleocapsid interacts with envelope protein in the endoplasmic reticulum to form mature virions and they are secreted from the host cell. Alternatively, The rcDNA genome within the nucleocapsid core particles may also recycle to the cell nucleus to replenish the nuclear cccDNA pool. In summary, the HBV is a DNA virus but utilizes reverse transcription of an RNA intermediate to replicate its genome similar to retroviruses. This error-prone replication strategy combined with high viral replication rate (approximately 10^{12} virus/d) leads to significant viral variability or QS. The HBV genomic mutation rate occurring at each nucleotide of the HBV genome is estimated at approximately 10^{-5} base/site per cycle[13]. The long half-life of hepatocytes and cccDNA template play an important role in archiving spontaneously occurring and antiviral drug-associated mutants[17].

Although the HBV is predominantly a hepatotropic virus, there is increasing evidence documenting that the immune (lymphoid) system is also an important site for maintaining viral persistence[18]. In the closely related woodchuck animal model of HBV, woodchuck hepatitis virus (WHV) infection can be completely restricted to the lymphoid system and WHV invasion of lymphoid cells

is related to the viral load[19,20]. In human studies, HBV genomes are detectable in peripheral blood mononuclear cells (PBMC) from chronically infected patients despite long-term suppressive anti-HBV NA therapy[21], in patients after resolution of acute hepatitis B with HBV surface antigen (HBsAg) clearance[22,23], and in circulating transplacental PBMC from HBV positive mothers possibly leading to *in utero* infection of the neonate[24]. HBV antigens, mRNA, cccDNA and integrated forms have been detected in PBMC and extrahepatic tissues such as, bone marrow cells, spleen, and lymphoblastoid cell lines[25,26]. Additionally, upregulation of HBV replication in PBMC occurs following *ex-vivo* mitogen stimulation and the release of viral particles capable of further infection and replication from these HBV infected PBMC[27]. HBV genomes and viral proteins have been detected within a variety of immune cell subpopulations and, in some reports the virus appears to specifically target B cells and monocytes[28-31].

OVERVIEW OF HBV GENOTYPES

There are nine major HBV genotypes (A-I) worldwide, which are identified by greater than 7.5% divergence across the HBV full genome between each genotype[32]. There is also a tenth putative genotype "J" isolated from a Japanese individual[33]. In addition to HBV genotypes, at least 35 subgenotypes (*i.e.,* within genotype A, B, C, D, F, H, but not in genotype E, G) have been identified. The HBV genotypes/subgenotypes are ethnically and geographically distributed. For instance, genotype B and C are prevalent in Asia, while genotype A and D are most frequently seen in Europe, the Mediterranean region and the Middle East[34]. Certain genotypes may exhibit different mutations. The common HBV pre-core (pre-C) mutation more frequently exists in genotype B, C, and D than in genotype A[35,36]; genotype C tends to carry more mutations compare to genotype B[37]. In addition, genotypes are also linked to the natural history of CHB leading to distinct clinical outcomes and responses to therapy[38-40]. For instance, the cumulative rate of spontaneous HBeAg seroconversion with genotype B is higher than patients with genotype C infection[41,42]. Others report genotype-specific differences in NA response, resistance to older generation NA (*i.e.,* LMV or ADF) and, durability of HBeAg seroconversion (138.) Whilst this has less clinical relevance with the newer potent NA (*i.e.,* TDF and ETV), alternative therapy endpoints such as HBsAg loss and HCC potential may be identified. The role of genotypes in CHB management has been extensively reviewed[43-45]. In summary, clinically relevant features of HBV genotypes include: the rate and durability of HBeAg loss/seroconversion (A and D > B and C), the risk of developing aggressive HBeAg (-) CHB (C and D > A), spontaneous HBeAg loss (B > C), cirrhosis (C), HCC (C in Asians, F in Alaska Natives), and response to antivirals (A and B > C and D).

OVERVIEW OF HBV QUASISPECIES AND CLINICALLY RELEVANT HBV VARIANTS

HBV quasispecies

The HBV replicates *via* an error-prone RT leading to non-identical but a genetically closely related variants pool, which is known as QS. Both the wildtype and HBV QS are archived in the hepatocytes reservoir. In the process of Darwinian evolution, QS that survive selective pressue (*i.e.,* host immune response and/or NA therapy) may predominate. Thus, the HBV QS diversity may reflect host humoral response. It was reported that less HBV variants were found in patients in the immune tolerant phase compared to the immune active phase[46]. Recent studies have found that HBeAg seroconverstion was associated with dynamic changes in the HBV QS pool years before viral load drop, hence HBeAg seroconversion may be a slow process rather than a sudden immunological event[47]. In other studies, NA-associated HBV mutations were commonly found in CHB patients as minor populations even before the initiation of antiviral therapy[48]. It has been reported that NA treatment experienced patients, even without carrying a specific drug resistant mutation (*i.e.,* LMV-R), still demonstrate a high possibility to develop cross-resistance to a related drug[49]. Thus, it is possible that LMV-R mutations may pre-exist as a minor HBV QS strain. Further, HBV QS diversity and/or complexity 4 wk after initiation of antiviral therapy has been associated with response to treatment[50,51]. Due to the sensitivity of direct sequencing assays, some minor variants may not be detected, especially when the mutation proportion is less than 20%[52,53]. However, clonal sequencing and next generation sequencing assays can overcome these limitations and detect even minor QS variants.

HBV variants have been shown to be relevant to disease progression, development of HCC, reliability of diagnostic assay detection, vaccine failures and response to antiviral therapy[54,55]. We will summarize how specific mutations can impact the major functions of the 4 HBV gene products, highlighting variants associated with liver disease development (Table 1).

HBV preS/S variants (immune escape, diagnostic assay detection, and occult HBV infection)

The HBV envelope protein is encoded by *preS1*/*preS2*/*S* gene in a frame-shift manner generating three different envelope proteins: large (L), middle (M) and small (S). Detection of either the secreted or virion associated HBsAg for greater than 6 mo in serum confirms chronic infection. The HBsAg pre-S1 is involved in attachment to host cell receptor and neutralizing antibody binding. The antibodies predominantly target the hydrophilic region of major HBsAg protein, known as the "a-determinant", located at amino acid positon 99-170. Thus, "a-determinant" mutations may affect

HBsAg antigenicity, leading to vaccine escape, false-negative results by diagnostic HBsAg detection assays, and hepatitis B immunoglobulin treatment failures[56].

The transmission of HBV vaccine escape variants to susceptible individuals may have significant public health care implications[57]. The sG145R point mutation is the most widely reported "vaccine escape" mutant, which can infect anti-HBs positive individuals by reduced anti-HBs binding. The *sG145R* mutant is stable and can be transmitted horizontally in presence of high titer anti-HBs[58]. Furthermore, *G145R* mutant along with an insertion between 122 and 123 in the "a" determinant was reported in patients with fulminant reactivation of hepatitis B[59]. In addition to sG145R, the K141E, T131I variant, and insertion of three amino acids between 123 and 124 can significantly affect the structure of HBsAg[60]. More recently, other a-determinant substitutions were reported in association with vaccine escape (*i.e.,* T116N, P120S/E, I/T126A/N/I/S, Q129H/R, M133L, K141E, P142S and D144A/E). Although vaccine-escape mutations appear to be more common in endemic areas with universal immunization programs, to date these mutants have not caused any negative effect on global immunization programs since they appear to develop slowly[61].

Due to the overlapping ORF of the HBV *S* gene and *P* gene, NA targeting the HBV RT/*P* gene and induced antiviral mutations may lead to corresponding *S* gene mutation (and vice-versa), or so called antiviral-drug-associated *S* gene mutations (ADASM)[62]. The ADASM may influence clinical outcome by altering envelope protein antigenicity, viral fitness and oncogenic potential. For example, the *S* gene premature stop codon at position 172 (W172*), with a 55 amino acids missing at 3'-terminus, might result from the rtA181T mutation in the overlapping *P* gene. The W172* was shown to be associated with liver cirrhosis and HCC[63].

Occult HBV infection (OBI) is characterized by negative HBsAg in serum but with persistent HBV DNA in liver. According to the Taormina consensus conference definition, OBI is usually due to the presence of low-level replication competent virus in which viral HBsAg cannot be detected by standard commercial assays[64]. The viral DNA is only detectable in liver, serum, as well as PBMC but the viral load is usually very low (< 200 virus copes/mL). However, HBsAg negativity with ongoing moderate to high-level viral replication may be due to infection with HBsAg mutants that produce a modified HBsAg that cannot be detected by current commercial assays. Further, based on our groups studies it is speculated that during OBI, the HBV preferentially infects PBMC (compared to liver), especially at very low viral load suggesting a specifc selective mechanism involved in the course of OBI infection of the host immune system[21,65,66].

HBV preS1/preS2 deletion mutations

The *preS* gene represents the highest heterogeneity of the HBV genome[67]. The preS region mediates virus binding with hepatocytes, and interaction with B cells and T cells indicating that it plays an important role in the host immune response against HBV infection[68-71]. Thus immune pressure from vaccination as well as immunotherapy may induce the preS region mutation. Previous researchers have reported that the *preS* gene mutation can affect immune response, virus expression, synthesis and secretion[71-74]. It was found that preS deletion mutants often exist in CHB, especially in patients with HBV genotype C infection[75]. The *preS* deletion mutant strongly correlates with liver disease progression, possibly due to defective secretion, accumulation of HBsAg in the hepatocyte endoplasmic reticulum (ER), leading to ER-induced cell stress. The cell cytotoxicity can contribute to oncogenesis[76]. It was suggested that the preS deletion mutation together with another S point mutation is correlated with coexistence of HBsAg and anti-HBs, indicating specific immune selection pressure[77]. Additionally, the preS deletion mutation has been associated with the occurrence of HCC in several studies, which reported a 52%-62% incidence of *preS* deletion in patients who developed HCC[71,76,78-80]. The HBV genome can also integrate into human chromosome and play an oncogenic role. For instance, *preS2*/*S* genes were found with a 3' end truncation from integrated HBV DNA in HCC tissue. The truncated proteins may have transcriptional/transactivation potential leading to HCC development[81,82].

HBV P variants and drug-resistant mutations

The HBV P has 4 functional domains: a priming region, a spacer region, a catalytic region that plays a RNA-dependent RNA polymerase/DNA polymerase function, and a carboxy terminal region that has ribonuclease H activity. There are 7 domains in P/RT region: A-G. The YMDD (tyrosine, methionine, aspartate, aspartate) motif locates in catalytic site in the domain C. It is highly conserved in all genotypes and plays an essential catalytic role in HBV replication. Thus, YMDD mutations, such as YVDD (rtM204V, methionine to valine mutation) and YIDD (rtM204I, methionine to isoleucine mutation) mutations could lead to antiviral resistance and defective viral replication. As noted, NAs inhibit the HBV P/RT and both plus and minus strand HBV DNA synthesis. The NAs have a similar structure to natural nucleotides with a modified sugar ring or base group that competes with the natural nucleotides in binding to the HBV P, leading to chain termination. Compared to IFN, NAs are more commonly used due to their more favorable side effect profile. However they require prolonged treatment as they have minimal effect on the cccDNA pool. The molecular mechanism of drug-resistance is specific to the NA sugar ring structure. To date, four major drug resistance pathways have been identified[83]: (1) L-nucleosides pathway which is characterized by rtM204V/I mutation resulting in resistance to LAM and

LdT; (2) acyclic/alkyl phosphonate sugar pathway which is identified by presence of rtN236T substitution leading to resistance to ADF and reduced susceptibility of TDF; (3) the pathway which is shared by both L-nucleosides (LMV, LdT, reduced sensitivity to TDF) and ADF by emergence of rtA181T/V; and (4) the D-cyclopentante pathway which is characterized by presence of rtL180M and rtM204V/I mutations plus at least one substitution in one of the rtT184, rt202 and rtM250 amino acid (aa) positions. LMV has the worst resistance profile with an annual resistance rate of 15%-25% and > 80% after 5 years treatment[84]. The *rtM204V/I* mutant, which is located at position 204 of YMDD motif, can result in LMV and LdT resistance and is often accompanied with compensatory mutations (*i.e.,* rtL80V/I, rtI169T, rtV173L, rtL180M, rtT184S/G, rtS202I and rtQ215S)[85]. The compensatory mutations are able to restore HBV replication activity to near wild type levels. In addition, YMDD variants were also found in patients without prior NA exposure[86]. In recent study, the spontaneous YMDD variants were reported more frequently occurred in HCC patients with HBV genotype C, which might be the cause of greater oncogenesis of genotype C compare to genotype B[87]. Thus, it is important to monitor YMDD mutations in patients on NA therapy in order to adjust treatment regimen in time. The resistance rate to ADF is approximately 30% after 5 years treatment but may be higher in patients with pre-existing NAs-associated mutations[88]. Two primary mutations induced by ADF and TDF (rtA181T and rtN236T) belong to the acyclic/alkyl phosphonates pathway. The rtA194T variant has been reported to be associated with partial TDF resistance, and confer reduced HBV replication *in vitro*[89]. In clinical practice, however, TDF resistance and virological breakthrough has not been reported in patients after more than six years of treatment[8]. Similarly, rtP177G and rtF249A have also been shown to impact HBV replication and enhance resistance to TDF both *in vitro* and *in vivo*[90]. ETV also has a very high genetic barrier to the development of drug-resistant mutations; the rate of resistance occurrence is 1.2% after 5 years in treatment naïve patients[91]. The resistance to D-cyclopentante group (ETV) occurs only when at least three mutations are present: rtL180M + rtM204V and either rtT184G/S or rtS202I/G or rtM250V[17]. However, due to cross-resistance, the presence of LMV-resistant mutations can lead to ETV resistance and treatment failure. Of note the rtA181T/V mutation in domain B of HBV P, was reported to confer resistance to both L-nucleosides and acyclic/alkyl phosphonates[92]. Further, the rtA181T also encodes a stop codon at aa172 in the overlapping S region (sW172*) in a frame-shift manner, which leads to truncated S protein production. The rtA181T/sW172* mutation can cause defective secretion of HBV S and may play an oncogenic role leading to HCC by transactivation of cellular promoters[93].

Due to the overlapping ORF of HBV *P/S* gene, HBV P drug-resistance variants selected by NAs may lead to HBsAg amino acid change and altered antigenicity. Conversely, immune pressure on HBsAg is able to introduce variants that correspond with primary or compensatory drug-resistant mutations in the *P* gene[94], as noted above.

PreC/BCP mutations (HCC associated)

The HBV *preC/C* gene encodes both the HBV precore and core protein with distinct start codon (*i.e.,* preC initiates from the first start codon while core protein from the second). The preC protein encodes soluble HBeAg. It has an additional 29 aa at the N-terminus end, which serves as a signal to transport the pre-core protein to the cellular ER, the first 16 aa is cleaved, and the viral protein secreted from the cell as a soluble HBeAg antigen. HBeAg is believed to play an important role in immune tolerance and viral persistence. The HBeAg-negative CHB phase with active hepatitis occurs in association with a precore and BCP region variant[95,96]. The most prevalent mutation in preC region is G1896A, which generates a premature stop codon at aa 28 in the sequence of HBeAg, which affects the trafficking of the precore to the ER and subsequent HBeAg secretion. This mutation is significantly associated with HBV genotypes harboring a T nucleotide (genotypes B, D, E and part of genotypes C and F) rather than C nucleotide at positon 1858[95]. This is because this variant affects the stability of the pregenomic episilon structure, and the pregenomic encapsidaton signal. The preC mutation is more often observed in genotype D HBV infection (65%) compared to HBV genotype A infections (9%). It was found that the preC deletion mutation is often associated with more severe liver disease, but has also been found in inactive HBV carriers. In addition, the preC and BCP mutations are also related to response to IFN therapy: *e.g.*, the G1896A mutation was showed to be associated with poor response to IFN therapy independent of HBeAg status[97] while the presence of less mutations in BCP region are associated with a better treatment response[98].

The HBV BCP is located upstream of the *preC* gene, hence mutations that occur in the BCP region can downregulate preC mRNA transcription and inhibit HBeAg synthesis. The A1762T/G1764A double mutation in the BCP region, leads to preC mRNA reduction resulting in HBeAg seroconversion and a approximately 50% reduction of HBeAg levels[99,100]. Similar to preC mutations, BCP mutations also show genotype specific prevalence, and are more often seen in HBV genotype C and D infections[101]. One study demonstrated a significant temporal correlation between the relative increase in mutant concentration and HBeAg seroconversion. In HBeAg-negative hepatitis patients, viral load is usually several log lower compared to HBeAg-positive patients but the HBV replication capacity may be partially restored by BCP mutant, especially if accompanied with any of 3 additional BCP mutations (T1753C, C1766T, T1768A). The increased HBV replication may be associated with

disease progression[102]. The preC stop codon mutation and BCP mutations often appear together. Recent studies demonstrated that the combination of BCP and preC mutations and preS1, preS2 deletion mutants could lead to more severe liver disease including fulminant hepatitis and HCC. It is now believed that the development of HBV-induced HCC involves various factors in the interaction between HBV and the host. Multiple HBV mutations existing in different regions were shown to play an important role in HBV associated oncogenesis. For example, the BCP A1762T/G1764A double mutations and preC mutations are prone to HCC generation compared to patients with wild type HBV infection[37,103]. A recent meta-analysis concluded that HBV carriers, especially Asians, were significantly more likely to develop to HCC and severe liver disease with the presence of G1896A mutations. The other mutations in preC and BCP regions, such as G1899A, T1753V and C1653T are also associated with an increased risk of HCC development[104].

HBV X variants

The *HBV X* is the smallest gene, with an N-terminal negative regulatory/anti-apoptotic domain and a C-terminal transactivation/pro-apoptotic domain. The HBV X protein (HBx) is an unique regulatory viral protein since it does not bind to either viral or host DNA, however, it is able to activate transcription of viral and cellular genes by direct or indirect interaction with a variety of targets[105]. Thus, it is required for HBV persistence. Additionally, it can modulate various cellular functions, including active humoral and cellular immune responses which may ultimately result in HBV-associated hepatocarcinogenesis[106]. It was demonstrated that the HBx was a nuclear coactivator or could stimulat signal transduction by several pathways, such as nuclear factor-κB (NF-κB) signaling pathway. The NF-κB pathway was reported stimulated by HBx though direct acting on NF-κB itself, stimulating phosphorylation of NF-κB or interaction with upstream signal transduction pathway[107,108]. NF-κB is necessary for cell growth and viability; recent study showed the activation of NF-κB could prevent apoptosis. Thus, the HBx-induced NF-κB pathway activation may promote the survival of infected and mutated cells that favors the hepatocarcinogenesis[109,110]. Several *X* gene mutants and deletions have been reported in HCC patients. For instance, the existence of HBx130 + HBX131 double mutation and HBx5 + HBx130 + HBx131 triple mutation showed a significant risk for HCC development[111]. This was suggested due to the increasing activity of NF-κB by double HBx mutation and increased cell burden of triple HBx mutation and its potential influence on structure and NF-κB activity[111]. The HBV DNA integrates into host cellular chromosomes often with 3'-end deletion that may play an important role in HBV oncogenesis. Integrated HBV *X* gene sequences were found in liver tissue of most CHB patients and approximately 86% of HBV-related HCC patients[112].

CONCLUSION

The HBV has significant genomic diversity and some HBV variants are associated with antiviral therapy response, vaccine escape, diagnostic failure, liver fibrosis progression and HCC development. Understanding HBV molecular epidemiology as well as the clinical and pathological relevence of HBV variants during different disease phases may enable more accurate risk-stratification of individual patients at risk for serious sequelae of chronic hepatitis B infection.

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