Overview of Hepatitis B virus Mutations and Their Significance

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ABSTRACT

Hepatitis B virus (HBV) is a partially double-stranded circular DNA virus that belongs to the Hepadnaviridae family. HBV mutations have been found in patients with acute and chronic infections and in all the four HBV open reading frames (ORFs- preS/S, polymerase, preCore/core, and X). The first escape mutant identified in HBV by genomic sequencing was the G145R mutation of S gene in 1988, after that other escape mutant have been detected.

The pol gene sequencing is mainly carried out to identify mutations causing resistance to nucleoside/ nucleotide analogues (NAs) which help clinician to plan effective therapeutic regime. Sequencing also helps to identify mutations, deletions and insertion in X gene which may have association with hepatocarcinogenesis, suppression of HBeAg secretion and increase of viral DNA synthesis. Though a lot of advancement has been there in last few decades in our knowledge of HBV lifecycle, genetic variability and pathogenesis, but still lot of factors are still unknown. There is need to understand the role of X gene and to know how much genotypes and sub-genotypes can influence the response to treatment and the risk of cirrhosis and hepatocellular carcinoma HCC.

KEYWORDS: HBV, Mutation, ORFs, Drug resistance.

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INTRODUCTION

The main part of the review should be a comprehensive but critical analysis of recent (last three to five years) developments, current problems, and future directions.

Hepatitis B virus (HBV) mutations have been found in patients with acute and chronic infections and in all the four HBV open reading frames (ORFs - preS/S, polymerase, preCore/core, and X). The preS/S ORF codes for three different surface molecules that form the surface antigen (HBsAg) that is the main antigen recognized by the immune system, HBsAg is responsible for the attachment of the virus to hepatocytes and the epitopes binding the neutralizing antibodies.\(^1\)^,\(^2\)\(^\text{1,}^2\)

The pol ORF codes for the reverse transcriptase (RT) domain of HBV polymerase that represents the target of the new antiviral agents belonging to the nucleoside/nucleotide analogues (NAs). Moreover, due to the overlapping S reading frame, mutations arising in the RT domain cause the appearance of mutations in the preS/S ORF.\(^1\)^,\(^2\)\(^1,\)^ PreCore/core ORF codes for the core nucleocapsid (HBcAg) and the e antigen (HBeAg) synthesis. Mutations in these sites mainly cause the well-known HBeAg negative hepatitis. Mutation A1762T and G1764A, responsible for the decreased preCore (PC) mRNA synthesis, have been detected in the specific basal core promoter (BCP) and described in HBeAg negative patients. X ORF encodes for a multifunctional non structural protein, the function of X protein is still unknown and unclear. It has been proposed to have a role in the establishment of infection and viral replication. Furthermore, a role of gene X in the HBV carcinogenesis has also been recently hypothesized.\(^1\)^,\(^2\)\(^1,\)^ In the present review we have discussed about HBV genetic variability and its significance.

EPIDEMIOLOGY

Transmission of hepatitis B virus results from exposure to infectious blood or body fluids. Possible forms of transmission include unprotected sexual contact, blood transfusions, re-use of contaminated needles & syringes, and vertical transmission from mother to child during child birth. HBV is endemic worldwide and in many parts of the world it is hyper-endemic also. The prevalence of HBV carriers varies from 0.1% to 2% in low prevalence areas, from 3% to 5% in intermediate prevalence areas and from 10% to 20% in high prevalence areas.

The wide range in HBV carrier rate in different parts of the world is largely related to differences in the age of infection, which is inversely related to the risk of chronicity. The rate of progression from acute to chronic HBV infection is approximately 90% for perinatally acquired infection, from 20% to 50% for infections acquired at 1 to 5 years of age and less than 5% for adult acquired infections.\(^3\)

Ten genotypes have been identified (A-J) on the basis of a sequence difference greater than 8% in the entire HBV genome or 4% in the S region. Each genotype is further subdivided into sub-
genotypes when differences in nucleotide sequences are more than 4% but less than 8%. Interestingly, both genotypes and sub-genotypes are related to clinical course, geographical distribution and mode of transmission. 4, 5

**MORPHOLOGY AND VIRAL GENOME**

HBV is a partially double-stranded circular DNA virus that belongs to the *Hepadnaviridae* family. The virus consists of the HBcAg, which contains circular DNA molecule approximately of 3.2 kb, and an outer envelope containing the HBsAg. One of the two strands is incomplete and associated with a DNA polymerase able to complete the strand. The longer strand of HBV DNA (L strand) is a complete circle, whereas the complementary strand is shorter (minus strand). Shorter DNA strand is the template for the synthesis of the viral mRNA transcripts. HBV DNA has a very compact coding organization with four partially overlapping ORFs that are translated into seven known proteins: polymerase protein (Pol gene); HBcAg and HBeAg (both from the C gene); large, medium, and small HBsAg (S gene); and the X regulatory protein (X gene). The overlap in the ORFs does not seem to limit variability since all HBV genes have variants. Non-coding regions are not present. 6-8

The first step in the HBV life cycle is its attachment to the hepatocytes through the interaction of its envelope proteins (pre-S1 region) with the host cell receptors. Then it penetrates into the hepatocytes, uncoating and the viral genome, organized as relaxed circular partially double stranded DNA (rc DNA), is sent to the nucleus and converted into covalently closed circular DNA (cccDNA). The cccDNA acts as template for transcription of four co-terminal mRNAs: pre-core and progenomic RNA, large surface mRNA, middle and small surface mRNA and X mRNA. pg RNA serves as a template for the reverse transcriptase and, after being transported to the cytoplasm, encodes viral capsid protein and viral polymerase, thus playing an important role in viral genome amplification and replication. 1, 2

The latter is transcribed into viral RNA gene products: HBV surface protein, structural core protein, non-structural core protein, X protein and viral polymerase. After this step the viral assembly occurs, followed by the virion secretion or there cycle of the newly generated nucleocapsid into the nucleus for conversion to cccDNA.

The permanence of cccDNA into the hepatocyte nucleus is a basic factor for viral persistence, because it allows for viral replication to restart, either during the antiviral therapy (resistance) or after the antiviral therapy is stopped (reactivation). 9, 10

**Hbv S-Gene Mutants**

The pre-S1/S2/S ORFs encode three envelope proteins which are determinant for virus assembly and virus attachment to hepatocytes. L protein is the substrate for viral receptor
attachment; M protein function is not well understood and finally S protein is commonly referred to as the HBsAg or Australia antigen. The small, the middle and the large proteins are detected as HBsAg. HBsAg protein contains the major B cell epitopes, the “a” determinant. HBsAg is the surface antigen targeted by the antibodies present in vaccinated people and by the antibodies binding to HBsAg in serological immunoassays. It is the major envelop protein, formed by 226 amino acids, it is highly heterogenic, but within the protein there are conserved areas defining the genotype.

The amino acid positions between 99 and 169 are called the major hydrophilic region (MHR), in which the “a” determinant is located, that is the main target of neutralizing B cell responses. Mutations causing a conformational change within the “a” determinant could affect the antigenicity of HBsAg, essential for inducing protective antibody and be responsible for escaping vaccine induced immunity, escaping anti HBV immunoglobulin therapy and providing false negative results in serological tests.

In 1988 HBV S-gene mutants were observed in Italian vaccinated children’s sera with the presence of both HBs antigen and anti-HBs antibodies. These children acquired infection from the mother and their S-gene sequences revealed glycine (G) to arginine (A) substitution at position 145, within the a-determinant of S-gene, causing conformational changes that allowed for the virus to escape the vaccine-induced response. G145R is the major vaccine-induced immune escape mutation and in the last years an increase of G145R detection has been reported by several studies, mainly in countries with high rate of endemicity and universal immunization program. Nevertheless, the risk of acquiring HBV infection is extremely low in a vaccinated subject. Other mutations later found were T116N, P120S/E, I/T126A/N/I/S, Q129H/R, M133L, K141E/P142S, and D144A/E.

In recent years, occult HBV infection (OBI) has been widely investigated. OBI is identified as the persistence of HBV DNA in HBsAg negative patient’s liver with or without other serological markers of previous HBV infection. The modified HBsAg molecules, most commonly, either cannot be detected by commercial available assays or very weakly exposed on the surface of hepatocytes due to a poor reorganization by the immune system. Finally, several authors suggest that host immune surveillance and epigenetic mechanisms are probably involved.

Other mutations that usually occur in Pre-S/S region seem to play an important role in inactivation of the preS2/S region promoter, resulting in interference with HBsAg secretion. As in this region there is the hepatocyte binding site they are associated with occult HBV status as well. Several studies dispute about the important role of pre-S deletions on the progression of liver disease. Above all, it seems that mutations in different genes are associated with the progression of liver disease. The regions involved are: pre-S, BCP and PC; moreover, it seems that the PC mutations precede the appearance of the others. Pre-S deletions, observed both in pre-S1 and pre-S2 regions,
cause a decrease in the synthesis and secretion of small surface antigen, which tends to accumulate in the hepatocytes and especially in the endoplasmic reticulum (ER). This supposedly causes an ER stress which in turn causes an oxidative DNA damage that induces mutagenesis and finally Hepatocellular carcinoma (HCC).

**Pol-Gene Mutants**

The goal of treatment in patients with chronic hepatitis B (CHB) is to eliminate the virus, thus reducing the risk of progressive liver damage that leads to the development of cirrhosis and HCC. However, due to the persistence of cccDNA forms in the infected hepatocytes nucleus, complete and definitive virus eradication is not achievable.

The currently available drugs, approved for treatment of CHB in many parts of the world, are 2 immuno-modulators (interferon α-2a and peginterferonα-2a) and 5 antiviral agents belonging to the NAs: Lamivudine (LAM-3TC), telbivudine (LdT), entecavir (ETV) and the acyclic nucleotide analogues Adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF). These last five drugs are inhibitors of RT domain of HBV polymerase. 19.

The viral polymerase/RT is encoded by the largest ORF. This protein arises from the translation product of the pre-core mRNA and pgRNA that serves as template for reverse transcriptional synthesis of viral DNA. Due to the absence of proof reading activity, the HBV polymerases/RT, leads to the introduction of random mutations into HBV genome. 20.

Earlier studies have suggested that LAM is the major cause of YMDD (tyrosine-methionine-aspartate aspartate) mutations (M204I/V) in the catalytic sites (C domain) within HBV P-ORF. 21. The mutations rtM204I/V (domain C), rtL180M (domain B) and rtA181T/V (domain B) confer resistance to LAM and LdT. M204I/V are often associated with compensatory mutations in other domains such as rtL80V/I, rtI169T, rtV173L, rtL180M, rtT184S/G, rtS202I, and rtQ215S which increase viral replication. 20-22, other proposed compensatory mutations are rtV84M, rt214, rtL217P, rtL229M, rtI233V and rtN238H. 21

Compensatory mutations emerge because the selection of resistance-associated changes in the viral polymerase is usually associated with some cost in replication fitness for the virus; these compensatory mutations are important in the setting of antiviral resistance because they “fix” the discriminatory primary drug-resistant mutations into the genetic archive of viral cccDNA, thus providing a “quasi species memory” 22.

ADV resistance is associated with two primary resistant mutations (belonging to the pathway for alkyl phosphonates) in the B and D domain, the rtA181T and the rtN236T. Furthermore, rtI233V is another mutation that has been identified in ADF-resistant HBV variants; its true significance
remains contradictory since some authors have confirmed and some have denied its capability to confer resistance\textsuperscript{24,25}.

Mutations in the B domain of RT, the rtA181T/V, were shown to confer resistance to LAM, LdT and ADV. The rtA181T mutation also encodes a stop codon in overlapping S reading frame, thus resulting in truncation of the HBsAg proteins. The rtA181T/sW172 variant has a secretory defect and exerts a dominant negative effect on the wild-type HBV virion secretion. This mutation is often present in patients with primary HCC\textsuperscript{26-28}.

Due to high genetic barrier, ETV and TDF are considered to be the most potent antiviral agents and there is a low risk of developing resistance, with the mutation incidence rate of 1.2% and 0%, respectively.\textsuperscript{29} Long-term monitoring shows HBV resistance to ETV in nucleoside-naive patients is rare. Multiple mutations are required to obtain high-level resistance to ETV. Those usually involved in ETV resistance are rtL180M + M204V and another among rtI169T, rt184G/S, rtS202I/G and rtM250V; actually, ETV resistance appears in LAM treated patients in which the rtL180M and M204V mutations were formerly present. There have not been confirmed reports of resistance selection during treatment of CHB with TDF in mono-infected individuals\textsuperscript{23}.

Recently, a complex TDF-resistance associated mutation pattern, including the rtR192PR substitution, those very close to the site of the rtA194T mutation has been found to confer TDF resistance \textit{in vitro}, and has been reported in a HIV-HBV co-infected individual failing TDF\textsuperscript{30}.

The common mutations that confers resistance to LAM and LdT, results in cross resistance to other L-nucleosides and reduce sensitivity to ETV but not against ADV/TDF. Conversely, mutations causing resistance to ADV and TDF generally do not give rise to resistance to L-nucleosides and ETV. Both the L-nucleosides and the alkyl phosphonates also select the mutation rtA181T/V, thereby making it a marker for multidrug resistance\textsuperscript{20-22}.

Further research has revealed that strains with YMDD mutations also exist in patients with chronic HBV infection not previously treated with Lamivudine\textsuperscript{31,32}, and the rate of viral breakthrough tended to be lower in patients without natural YMDD mutations than in those with natural YMDD mutations. Naturally occurring YMDD mutations are found in a large proportion of CHB patients who have not undergone anti-viral therapy. The incidence of YMDD mutations may be correlated with the HBeAg status and the HBV DNA level. These results also suggest that LAM therapy improves the clinical course in HBV patients with natural YMDD mutations\textsuperscript{33}.

The HBV polymerase (Pol) gene overlaps the HBsAg in a frame-shifted manner and results in drug resistant mutations in the HBV Polymerase gene, and directly affects the HBsAg and its function. Therefore, drug resistance mutations of polymerase gene may result in envelope gene stop
codon mutations, leading to modified viral secretion, infectivity and creating both viral escape to anti-HBs antibodies and modified HBsAg molecule not detected by screening tests 22.

Interestingly, neither the ADV-associated resistance mutation rtN236T nor the TDF-associated resistance mutation rtA194T selected only in vitro, cause changes in the HBV surface gene. HBV mutants carrying drug and vaccine resistance may represent a considerable individual risk and public health concern 34. According to international practice, guidelines for best treatment strategy after HBV resistance, recommend the use of a nucleoside/tide analogue with high antiviral potency and high genetic barrier, such as ETV or TDF 35.

**Pre Core/Core Mutants**

Pre-Core/Core region encodes for two proteins, one structural, the HBcAg, that forms the nucleocapsid, and the HBeAg that is a secretion protein. HBeAg is the marker of HBV replication and infectivity. In the natural course of HBV chronic infection, the loss of HBeAg expression and the appearance of antibodies directed against it (anti-HBe) usually represent the end of viral replication and the resolution of hepatitis 2, 36.

Mutations in the pre-core and core regions cause HBeAg-negative chronic hepatitis B with presence of anti-HBe, in which replicative infection continues and HBV-DNA remains detectable. Negativity in HBeAg is due to basal core promoter (BCP) and precore (PC) mutations that respectively modulate HBeAg secretion during transcription and stop HBeAg production 2, 11, 36.

Recent studies showed that BCP mutations and PC mutations produced a decrease in the HBeAg secretion and had a significant role in hepatocarcinogenesis 38. The A1762T and the G1764A, responsible for the decreased PC mRNA synthesis, were the typical specific BCP mutations detected and described, mainly together, in patients with HBeAg negative hepatitis. These two mutations were first found in a study of Baptista et al aimed at investigating the presence of mutations responsible for the HBeAg negativity and their possible role in hepatocarcinogenesis in the HBeAg negative patients 37.

The increased risk of HCC in patients harbouring a virus with the A1762T and the G1764A was confirmed by several studies but the mechanism of oncogenesis remains unknown 38-42. In addition to the A1762T and G1764A mutations, other BCP mutations have been identified: the T1753C, and the C1766T. Basically, these mutations reduce the HBeAg synthesis and enhance viral replications in liver cells, often in association with more severe and advanced liver disease 43.

Some of these mutations (T1753C, A1762T and 1764A), together with A1752G, A1846T, G1896A and G1899A, were significantly correlated with HBeAg seroconversion; in a recent work the authors showed significant differences between HBeAg positive and HBeAg negative paediatric patients groups. But the frequency of the mutations in HBeAg-negative paediatric patients were
significantly lower than in HBeAg negative adult patients, because the role of BCP/PC mutations are less important in the early phases of HBeAg seroconversion\textsuperscript{44}.

This mutation was often found in non-A genotypes associated with the mutation C1858T, whose onset is eased by typical viral structure of certain genotypes (B, D, E, C, F). Also these mutations have been first found in Mediterranean Countries, where the majority of patients are genotype D carriers. In a longitudinal study on 99 HBV- DNA positive patients, all genotype D, HBeAg negative and with PC G1896A mutation, Besharat observed that they still had a detectable HBV-DNA even after 7 years of monitoring, unlike the patients with the wild type PC sequence\textsuperscript{45}.

**X-Gene Mutants**

Gene X encodes for a multi functional non structural protein, originally defined X protein because its function was unknown and even now remains unclear. It has been proposed to have a role in the establishment of infection and viral replication. Furthermore, role of gene X in the HBV carcinogenesis has been recently hypothesized\textsuperscript{46}.

The HBX gene overlaps with the core promoter region and mutations here in this gene may alter the functions of the HBX protein, it plays an important role in HBV replication and hepatocarcinogenesis. Recent studies showed that, the HBX mutants linked with core promoter mutations may regulate p53 through S-phase kinase associated protein 2 (SKp2), promoting or preventing cellular transformation and proliferation. In HBX region, twelve mutations were associated with hepatocarcinogenesis, suppression of HBeAg secretion and increase of viral DNA synthesis\textsuperscript{47}.

**CONCLUSION**

Mainly due to molecular biology studies, in the last few decades we have acquired insights about HBV lifecycle, genetic variability and pathogenesis. In 1988, Zanetti et al discovered the G145R mutation within the “a” determinant of S gene, the first escape mutant identified by HBV genomic sequencing. After that other escape mutants have been detected and the relevant role has been established in immuno compromised patients and in OBI. Sequencing helps identify mutations able to confer resistance to the new NAs, identify the viral variants, genotypes and sub genotypes which help clinicians to plan the effective therapeutic regime. Moreover sequencing also identifies mutations related to hepatocarcinogenesis and the escape mutants or stop codons in the overlapping region of \textit{pol} gene with the S gene. Sequencing also helps identify mutations responsible of HBeAg negative chronic hepatitis B and finally to identify mutations, deletions and insertion in X gene probably associated with hepatocarcinogenesis, suppression of HBeAg secretion and increase of viral DNA synthesis. However further studies are required on HBV genetic variability, the role of X gene, how much genotypes and sub-genotypes could influence the response to treatment and the risk of cirrhosis and HCC.
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