TOPIC HIGHLIGHT

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Clinical implications of hepatitis B virus mutations: Recent advances

Ivana Lazarevic

Ivana Lazarevic, Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia
Author contributions: Lazarevic I solely contributed to this paper.
Correspondence to: Ivana Lazarevic, MD, PhD, Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, Dr Subotica 1, 11000 Belgrade, Serbia. ilazarevic@med.bg.ac.rs
Telephone: +381-11-3643379 Fax: +381-11-3643366

Abstract

Hepatitis B virus (HBV) infection is a major cause of acute and chronic hepatitis, and of its long-term complications. It is the most variable among DNA viruses, mostly because of its unique life cycle which includes the activity of error-prone enzyme, reverse transcriptase, and the very high virion production per day. In last two decades, numerous research studies have shown that the speed of disease progression, reliability of diagnostic methods and the success of antiviral therapy and immunization are all influenced by genetic variability of this virus. It was shown that mutations in specific regions of HBV genome could be responsible for unwanted clinical outcomes or evasion of detection by diagnostic tools, thus making the monitoring for these mutations a necessity in proper evaluation of patients. The success of the vaccination programs has now been challenged by the discovery of mutant viruses showing amino acid substitutions in hepatitis B surface antigen (HBsAg), which may lead to evasion of vaccine-induced immunity. However, the emergence of these mutations has not yet raised concern since it was shown that they develop slowly. Investigations of HBV genetic variability and clinical implications of specific mutations have resulted in significant advances over the past decade, particularly in regard to management of resistance to antiviral drugs. In the era of drugs with high genetic barrier for resistance, on-going monitoring for possible resistance is still essential since prolonged therapy is often necessary. Understanding the frequencies and clinical implications of viral mutations may contribute to improvement of diagnostic procedures, more proper planning of immunization programs and creating the most efficient therapeutic protocols.

Core tip: Understanding the frequencies and clinical implications of Hepatitis B virus (HBV) mutations may contribute to improvement of diagnostic procedures, more proper planning of immunization programs and creating the most efficient therapeutic protocols. This paper brings an overview and latest research results on the most important, clinically relevant, HBV mutations. The covered topics include mutations involved in vaccine-escape phenomenon, resistance to currently available antiviral drugs, development of "occult" hepatitis, hepatocellular carcinoma, fulminant hepatitis and hepatitis B e antigen-negative hepatitis, as well as the problem of these mutations in HBV/human immunodeficiency virus and HBV/hepatitis C virus co-infected patients.

INTRODUCTION

Hepatitis B virus (HBV) infection is a major cause of acute and chronic hepatitis, and of its long-term complications. About 350 million people are currently infected with HBV worldwide and approximately 1 million die of liver failure, cirrhosis and hepatocellular carcinoma (HCC) each year[1].

HBV is the most variable among DNA viruses, mostly because of its unique life cycle which includes the activity of error-prone enzyme, reverse transcriptase, and the very high virion production per day (as many as 10^{12} viruses/d[2]). In last two decades, numerous research studies have shown that the speed of disease progression, reliability of diagnostic methods and the success of antiviral therapy and immunization are all influenced by genetic variability of this virus.

However, despite high mutational rate for DNA viruses (1.4×10^{-5}-3.2×10^{-5} per site per year[3]), HBV mutations that are clinically relevant arise slowly, mostly due to constraints imposed by overlapping reading frames in the genome and the host immune response.

HBV MORPHOLOGY AND LIFECYCLE

The human HBV is prototype member of the family *Hepadnaviridae*, which includes a variety of similar avian and mammalian viruses. Its partially double-stranded, circular DNA genome is contained in an icosahedric capsid, itself enveloped by a lipid bilayer bearing three different surface proteins: large (L), middle (M), and small (S). All known coding information is on the minus DNA strand and is organized into four open reading frames (ORF): longest *Pol* encodes the viral polymerase, three envelope proteins are encoded by the same region (Pre-S1, Pre-S2 and S region) but in a frame-shifted manner and partially overlapping with previous are core (C) and X ORFs.

The mechanisms by which HBV and other hepadnaviruses infect hepatocytes are still not well understood. The HBV lifecycle starts with virion attachment to an unknown specific receptor complex. It has been shown that the Pre-S1 domain is required for receptor binding and initiation of infection[4]. After entry and uncoating of virions, nucleocapsids are transported to the cell nucleus[5,6]. In the nucleus, viral DNA is repaired to yield a fully double-stranded DNA molecule which is then supercoiled to form covalently closed circular DNA (cccDNA). cccDNA has a very long half-life, despite the fact that it is not integrated into the cellular genome and it acts as the major transcriptional template for the virus[7].

HBV contain four promoters that control the transcription of 4 RNAs: the 3.5-kb pre-C and pregenomic (pg) RNA and the subgenomic 2.4, 2.1, and 0.8 kb RNAs, PreS1, S, and X, respectively[4]. These mRNAs are transported to and translated in the cytoplasm to produce viral proteins. The longest transcript of 3.5-kb is translated into HBV core antigen (HBcAg), solubile hepatitis B e antigen (HBeAg) and Pol protein. The three viral envelope proteins are translated from 2.4 and 2.1 kb RNAs and X protein from 0.8 kb RNA.

The 3.5-kb transcript (pgRNA) also serves as template for reverse transcription of the viral genome. This begins by binding of the polymerase to the packaging signal, epsilon, at the 5' end of the pgRNA. This reaction creates a signal for the assembly of nucleocapsids in which the reverse transcription will occur. The first step is negative strand DNA synthesis, while the pregenomic RNA is gradually degraded by the RNAse H activity of the polymerase. A positive DNA strand is then synthesized by the polymerase, using the negative strand as template. The synthesis of positive strand continues until it reaches 50%-70% of the length of the negative strand. The newly formed nucleocapsids either deliver their content into the cell nucleus or assemble with envelope proteins and enter the secretory pathway. The return of new DNA in the nucleus is used to yield additional cccDNA molecules[8]. However, most nucleocapsids assemble with envelope proteins and form mature virions either in the cytosol or in the endoplasmatic reticulum. The details of HBV virion assembly and release are not yet well understood[4], but it was suggested that complete virions are exported by exocytosis *via* multivesicular bodies[9].

HBs ANTIGEN MUTATIONS

The HBV surface antigen (HBsAg) is the major envelope protein and includes the regions involved in the attachment of the virus to the hepatocyte, and also the main epitopes recognized by neutralizing antibodies. Central core of HBsAg, comprising amino acids 99-169, which is referred to as the major hydrophilic region (MHR), is exposed on the surface and is involved in binding to antibodies directed against HBsAg. Antibodies found in vaccinated people and those used in immunoassays for HBsAg, are directed against this region; in particular, to a cluster of B-cell epitopes called the "a" determinant, which comprises two loops of amino acids 124-147[10].

When "a" determinant is affected by mutations, caused by selection or natural variation, important changes with regard to immunity and protection from HBV infection may arise. These changes could affect antigenicity of HBsAg and were shown to be responsible for: (1) false-negative results by some commercial assays for HBsAg; (2) evasion of anti-HBV immunoglobulin therapy; and (3) evasion of vaccine induced immunity.

The hepatitis B virus vaccine has become an essential part of global infant immunization programs. The current vaccine used in most programs is a yeast-derived recombinant HBsAg, containing only the small S protein. In 1988, the authors from Italy[11] reported results of a follow-up study of childhood vaccination and revealed that vaccinated children with strong antibody response to HBsAg could still become HBsAg positive. Soon other similar reports followed and the conclusion was that the incidence of this phenomenon was rare, involving about 2% of the children of HBsAg-positive mothers or HBsAg-positive family contacts[11,12]. More detailed analysis of virus from one patient revealed that "vaccine-escape"

phenomenon was associated with a point mutation, *i.e.*, substitution of glycine by arginine residue at position 145 (G145R)[11]. This mutation has since become the most widely reported "vaccine-escape" mutant[12-20]. It was shown that G145R was stable over time[11,21] and that its horizontal transmission was also possible[22,23].

Many other substitutions in the "a" determinant (T116N[24], P120S/E[17,18,25], I/T126A/N/I/S[19,25-28], Q129H/R[26,29,30], M133L[29,30], K141E[16,26,28], P142S[19,20,26,28], D144A/E[19,25,26,30], G145R/A[11-20]) have since been associated with escape from vaccine induced immunity. Interestingly, the number of studies reporting substitution of Gly by Ala rather than by Arg at position 145 is increasing in recent years[31-37].

The "vaccine-escape" mutants are more common in countries with high rates of endemic infection and universal immunization programs[14,31,32,38]. To date, the emergence of these mutations has not had a known negative effect on the immunization programs throughout the world[39] but it is conceivable to expect that mutant strains may become dominant in certain populations in more decades of vaccine utilization. Mathematical modeling predicts that it will take over 50 years before any "vaccine-escape" mutant become predominant[40]. Recent researches confirm that the risk of newly acquired HBV infection in vaccinated individuals is a rare event and that associated "vaccine-escape" mutations belong to "a" determinant region of HBsAg. Study from 2010[41] on efficacy of the universal HBV vaccination program in Taiwan reported only 0.009% HBV infected schoolchildren two years after vaccination. Vaccine escape was documented in only 3 isolates displaying mutations within "a" determinant (M133L, P134I). Similarly, authors from Australia[42] report only four cases (out of 138) of transmission to newborns from HBsAg positive mothers where only one was found to be associated with "vaccine-escape" mutant (D144E). In recent study from Iran[43], which included 6 newborns of HBsAg positive mothers, who developed HBV infection despite complete immunoprophylaxis, true vaccine escape phenomenon was observed in 2. The 2 isolates were considered "vaccine-escape" since they were not shown to be inherited from mothers (by transuterine transmission) and in both cases the mutations were found within the "a" determinant (Q129R in one and G145A in other).

In the era of sensitive techniques for detection of HBV DNA, a significant proportion of patients were reported to be HBV DNA positive in the absence of detectable HBsAg. The persistence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing HBsAg negative by currently available assays is termed occult HBV infection (OBI)[44]. One proposed mechanism of OBI development was infection with HBV mutants with defective replication activity or synthesis of S proteins[45]. However, in the majority of cases, "occult" HBV isolates were shown to be replication-competent viruses. The other explanation of OBI is infection with S gene variants ("diagnostic-escape"

mutants) which produce a modified HBsAg that is not recognized by commercially available detection assays.

OBI is one of the most challenging and widely investigated topics in the field of viral hepatitis in recent years. The clinical relevance of OBI is mostly associated with problems in transfusion medicine since the strains from OBI patients pose a potential threat for the safety of blood supply. The prevalence of OBI among blood donors from different European countries ranges from 0.0002% to 0.084%[46-50], while in China, a highly HBV-endemic region, it is as high as 0.18%[51]. Many studies do not provide enough information on mutations correlated with occult HBV infection because usually only limited parts of the genome is investigated and reported. According to recent study (2012)[50], the prevalence of specific OBI associated mutations ranged from 8.3% to 20.8% in patients with OBI, while it was only 0%-3.7% in patients with overt hepatitis. The comparison of mutational rates between the studies proved to be difficult because of number of differences in what is considered to be OBI-associated mutation and whether they include only "a" determinant or whole MHR.

In the study of Svicher *et al*[50], twenty mutations, mostly within "a" determinant but also in other parts of HBsAg, were found to strongly correlate to occult infection in genotype D strains (Y100S, Q101R, P105R, T115N, T116N, P120L, R122P, T123N, T126I, P127H/L, Q129P, M133T, Y134C, S143L, S167L, R169H, S174N, L175S, V177A). Since the reported mutations were completely different from those reported to be associated with OBI in genotypes B and C[52], it was suggested by the authors that OBI associated mutations are unique for each HBV genotype. Indeed, several studies comprising genotypes B and C identified G145R/A to be among major mutations responsible for OBI[51-53].

The results of study by Huang *et al*[51] suggested a potential association between MHR mutations and decreasing serum HBsAg levels. The study revealed that 4 mutations (C124R, C124Y, K141E, and D144A) strongly decrease the analytical sensitivity of seven commercial HBsAg immunoassays, and that 10 mutations (G119R, C124Y, I126S, Q129R, S136P, C139R, T140I, K141E, D144A, and G145R) significantly impair virion and/or S protein secretion *in vitro*.

The study by Cassini *et al*[54], which was conducted in order to better characterize the molecular basis of occult HBV infection, report C695T nucleotide change leading to stop codon at amino acid 181 of the *HBs* gene, in strains from liver samples. The novel *HBs* gene stop codon could be responsible for reduced production leading to undetectability of HBsAg. The authors recommend testing peripheral blood mononuclear cells and liver samples for diagnosis of serum-negative OBI because they found evidence of HBV low replication levels in both sites.

Finally, it was suggested that deletions in Pre-S region may also play significant role in OBI development since they can affect the expression, synthesis, and secretion of

Table 1 Hepatitis B virus mutations and their clinical implications

Type of mutation	Clinical implication	Mutation
HBsAg mutations	Vaccine-escape	T116N[24], P120S/E[17,18,25], I/T126A/N/I/S[19,25-28], Q129H/R[26,29,30], M133L[29,30], K141E[16,26,28], P142S[19,20,26,28], D144A/E[19,25,26,30], G145R/A[11-20,31-37]
	OBI	Y100S[50], Q101R[50], P105R[50], T115N[50], T116N[50], G119R[51], P120L[50], R122P[50], T123N[50], C124R/Y[51], T126I/S[50,51], P127H/L[50], Q129P/R[50,51], M133T[50] Y134C[50], S136P[51], C139R[51], T140I[51], K141E[51], S143L[50], D144A[51], G145R/A[51-53], S167L[50], R169H[50], S174N[50], L175S[50], V177A[50], Q181STOP[54]
Basal core promoter /precore mutations	HBeAg-negative hepatitis	T1753C[62], A1762T[59,60], G1764A[59,60], C1766T[62], T1768A[62], G1896A[63,64], G1899A[63,64]
	HCC	C1653T[68], T1753C[68], A1762T[66-68], G1764A[66-68], G1896A[68], G1899A[68]
	Fulminant hepatitis	A1762T[70], G1764A[70], G1862T[69,70], G1896A[70]
X gene mutations	HCC	3′-HBx deletion[82]
Antiviral drug-resistant mutations	LAM/L-dT-resistance	rtL80V/I[84], rtI169T[84], rtV173L[84], rtL180M[84], rtA181T/V[105], rtT184S/G[84], rtS202I[84], rtM204V/I[84], rtQ215S[84]
	ADF-resistance	rtA181T/V[96,105], rtI233V[97,98], rtN236T[96]
	ETV-resistance	rtL180M[85], rtT184G/S[85], rtS202I/G[85], rtM204V[85,108], rtM250V[85]
	TDF-resistance	rtP177G[103], rtA194T[102], rtF249A[103]

HCC: Hepatocellular carcinoma; OBI: Occult HBV infection; LAM: Lamivudine; L-dT: Telbivudine; ADF: Adefovir; ETV: Entecavir; TDF: Tenofovir; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

the S protein[53,55].

Clinically relevant HBsAg mutations mentioned in the text were listed in Table 1.

BASAL CORE PROMOTER AND PRECORE MUTATIONS

Both core and precore proteins are coded by the Pre-C/C ORF, precore starting from the first and core from the second initiation site. The precore protein therefore initially contains all of the core protein sequence plus 29 amino acids at its N-terminus. The first 19 of those 29 amino acids serve as the signal peptide to direct the protein to the endoplasmic reticulum where it is cleaved off. During transport to the cell surface, this protein is further matured and eventually secreted as a soluble antigen, HBeAg[4,10,56]. The role of this antigen is not yet understood but it is possible that it could be in modulating the immune response and facilitating the establishment of chronic infection.

Ever since serology markers became crucial for diagnosis of HBV infection, it was believed that the loss of HBeAg was a marker of the end of active viral replication and resolution of the hepatitis. However, it was first described in Mediterranean region[57] that HBeAg loss may be followed by active viral replication and progression of liver disease. Today, HBeAg-negative hepatitis is a worldwide known phenomenon[58] that does not represent a separate disease entity but arises due to selection of specific basal core promoter (BCP) and precore mutations in the HBV genome. Mutations occurring in BCP region modulate HBeAg secretion at the transcriptional level and those occurring in the precore region, block HBeAg production at the translational level.

A typical double mutation often found in the BCP, A1762T and G1764A, is responsible for decreased precore mRNA synthesis[59,60]. Despite the fact that HBe-negative hepatitis is characterized by several logs lower HBV DNA levels, BCP mutants were shown to enhance viral replication in cell culture[61,62]. The replication activity is further increased when they are accompanied with any of 3 additional BCP mutations (T1753C, C1766T, T1768A)[62]. HBeAg-negative hepatitis was often associated with more severe liver disease which may be the consequence of this enhanced viral replication in liver cells[61].

The most prevalent among precore mutations is a G to A switch at position 1896 (G1896A), that leads to a translational stop codon at amino acid position 28 in the sequence of HBeAg, resulting in the inhibition of the protein synthesis[63,64]. This mutation was found to be often accompanied by missense mutation of codon 29-G1899A. The prevalence of the 1896 stop codon mutation appears to be significantly associated with HBV genotypes harboring a T nucleotide at position 1858 (genotypes B, D, E and part of genotypes C and F) and this has been explained on a structural basis. The region essential for pgRNA encapsidation (packaging signal region, epsilon) overlaps with precore and has loop-like secondary structure. Therefore, a G to A mutation at nucleotide 1896 can stably pair with nucleotide at position 1858 in genotypes which posses a T at 1858 but not in those which posses a C[65]. This can also explain initially observed dominance of these mutants in Mediterranean region where genotype D is predominant.

Basal core promoter and stop codon mutants appear to be frequently associated. It was observed that BCP mutations were detectable in the late HBeAg positive phase of infection, whereas precore mutations emerged during anti-HBe seroconversion[62]. Therefore, the suggested order of events would be that the virus first reduces HBeAg expression through BCP mutations and then abolishes entirely HBeAg expression with precore mutations[10].

Many recent studies are addressing the possible association of BCP and Pre-C mutations with more severe outcome of liver disease, namely with appearance of ful-

minant acute hepatitis or development of HCC.

It is now believed that HBV-induced HCC involves a complex interaction between the viral factors and multiple host factors. HBV mutations associated with this disease outcome were mapped to different regions of the genome. Several studies demonstrated that patients who harboured A1762T/G1764A double mutation or mutations in the precore region were more predisposed to HCC than those with the wild type infection[66,67]. Recent meta-analysis[68] indicated that precore mutation G1896A is significantly associated with the increased risk of HCC and the progression of liver disease, especially among Asians. Also this study demonstrated correlation of other frequent precore mutation G1899A, common mutations in BCP- A1762T/ G1764A, T1753V and C1653T, as well as pre-S1 and Pre-S2 deletions with increased risk of HCC.

Traditionally, a fulminant course of HBV infection has been ascribed to abnormal host immune response rather than to the features of the virus. Nevertheless, all regions of viral genome are widely investigated for possible association with this disease outcome. Hou *et al*[69] indicated that one, not so commonly investigated, precore mutation G1862T was 5 times more often found in fulminant hepatitis patients than in chronic carriers. In the study by Inoue *et al*[70] the fulminant HBV strain, that was isolated from 5 cases of consecutive fulminant hepatitis, comprised BCP double mutation A1762T/G1764A, precore stop mutation G1896A and also G1862T, located within the bulge of the epsilon, packaging signal region. The study demonstrated in cell culture, that investigated HBV strain, retained the core particles and the core particle-associated HBV DNA in the cells. The authors concluded that all found mutations might work together for the observed retention and that these findings may have important implications for understanding the mechanism leading to fulminant hepatitis.

Clinically relevant BCP/precore mutations mentioned in the text were listed in Table 1.

X GENE MUTATIONS

X is an enigmatic HBV protein, necessary for efficient infection and replication *in vivo*. HBx is known to exhibit a transcriptional transactivation function for many viral and cellular genes and is therefore required for efficient transcription of viral RNAs from cccDNA in infected hepatocytes[4,71,72]. Since it has no DNA-binding activity, its transactivation function is manifested through interaction with cellular factors.

It was suggested that HBx protein had a role in HBV-induced carcinogenesis by number of different mechanisms[73-77]. However, the role of specific mutations in the X gene is not yet well characterized. Some of X gene mutants and also deletions of this region have been described in patients with hepatocellular carcinoma[78,79]. Integration of HBV genomic DNA into cellular chromosomes commonly occurs during the viral life cycle and is

observed in most chronic hepatitis samples and in 86.4% of HBV-related HCCs[80]. Upon integration of HBV DNA in host genome, the 3'-end of the HBx is often deleted[81]. This truncation of C-terminal region of HBx protein was shown to play an important role in HCC development through promotion of oxidative stress and induction of mitochondrial DNA damage[82].

ANTIVIRAL DRUG-RESISTANT MUTATIONS

The ultimate goal of antiviral therapy in chronic hepatitis B is to clear the virus from the host and to prevent the progression of liver disease to cirrhosis and HCC. However, chronic HBV infection cannot be completely eradicated due to the persistence of cccDNA in the nucleus of infected hepatocytes.

Reverse transcriptase (RT) domain of HBV polymerase represent a target for nucleoside/nucleotide analogs (NAs), drugs currently available to treat hepatitis B: lamivudine (LAM), telbivudine (LdT), entecavir (ETV), adefovir (ADV) and tenofovir (TDF).

All currently available NA drugs exert their anti-HBV actions by targeting at one or more of three steps: the priming reaction (synthesis of the short negative DNA strand), reverse transcription from pgRNA to negative DNA strand, or DNA-dependent DNA replication[83]. Nucleoside/nucleotide analogs have a similar structure to the natural nucleotides but with modification in the sugar ring or base group, so that the analog can compete with the natural nucleotides in binding to the polymerase.

The causes of viral persistence and resistance to antiviral drugs include combination of different viral and host factors where major are: long half-life of infected hepatocytes (30-100 d) and natural genetic variability of HBV that leads to formation of mutations and their archiving in cccDNA[84,85]. It is believed that single mutants exist in the overall viral population even before therapy begins. Different variants or mutants, *i.e.*, viral quasispecies, are selected within the same host in response to immune response or antiviral therapy during the course of infection[85]. Two types of mutations have been associated with resistance to NAs: primary resistance mutations, which are directly responsible for drug resistance, and secondary (compensatory) mutations, which promote or enhance replication competence of resistant strain.

The replacement of wild type virus in liver cells by a dominant mutant is a slow process. As resistant mutants mainly infect uninfected cells, the efficient spreading of the dominant mutant depends on the availability of free liver space for its propagation and replication[86]. This can partly explain the difference in incidence of resistance among different NAs. LAM has the worst resistance profile since resistance starts to develop within the first year of therapy and increases over time at an annual rate of 15%-25%[87] while it reaches > 80% after 5 years[88]. The rate of resistance for ADF and L-dT can be defined as intermediate: for L-dT it is 10%-25% after 2 years of

administration[89] and for ADF about 30% after 5 years[90] and even higher for patients already resistant to other NAs. The antivirals with the highest barrier for resistance mutations currently are ETV, with a resistance rate of 1.2% after 5 years[91], and TDF, with still no resistance reported after 4 years[92]. However, the chance of emergence of entecavir resistant HBV is as high as 51% in patients with pre-existing lamivudine resistant mutations after five years of entecavir treatment[93]. Also, in patients with LAM-resistant mutations, cases of viral breakthrough with TDF have been reported in HBV/human immunodeficiency virus (HIV) co-infected patients[94]. Modern techniques like ultra-deep pyrosequencing, reveal that low-prevalence resistance mutations can be found in patients much sooner than by direct PCR sequencing methods and probably years before they become clinically significant[95].

Investigation of molecular mechanisms reveals that resistance tends to be NA structure (sugar residue) specific. So far, five resistance pathways are recognized: (1) the pathway for L-nucleosides; (2) the pathway for alkyl phosphonates; (3) the pathway which is shared between the L-nucleosides and alkyl phosphonates; (4) the D-cyclopentane pathway; and (5) multidrug resistance.

L-nucleosides (LAM and L-dT) have a similar molecular structure and target site of action, and therefore similar patterns of antiviral resistance mutations. The L-nucleoside pathway is recognized by well-known substitution at position 204 of YMDD motif (rtM204V/I), the catalytic site within reverse transcriptase. This mutation alone is sufficient to result in resistance but is very often associated with compensatory mutations rtL80V/I, rtI169T, rtV173L, rtL180M, rtT184S/G, rtS202I and rtQ215S[84]. The role of compensatory mutations is to restore the viral polymerase function to near wild-type levels. When found alone these mutations are associated with very low level resistance *in vitro*. With various patterns of mutations leading to LAM resistance and very high resistance rates, this drug is no longer recommended as a first-line agent for naïve patients with chronic hepatitis B.

The pathway for alkyl phosphonates (ADF and TDF) is recognised by existance of mutations outside YMDD motif, in the B and D domain of RT. There are two primary resistant mutations belonging to this pathway-rtA181T and rtN236T[96]. Another mutation rtI233V has been identified in ADF-resistant HBV variants[97]. The true significance of this mutation remains contradictory since some authors confirmed[98] and some denied[99] its role in ADF resistance. It was reported by the latest study which was using three dimensional model of HBV polymerase/RT, that substitution of isoleucine to valine at position 233 did not appear to affect the catalytic sites of the RT and that it might not independently affect the antiviral action of adefovir[100].

The emergence of mutation rtA194T was associated with resistance to TDF, though clinical significance of this mutation is still unknown, as strains with this muta-

tion did not seem to confer resistance to TDF *in vitro*[101]. The subsequent *in vitro* study by Amini-Bavil-Olyaee *et al*[102] reported that rtA194T polymerase mutation was associated with partial tenofovir drug resistance but that it negatively impacted replication competence of HBV. Similarly, the newly published study by Qin *et al*[103] failed to detect sufficient replication capacity of HBV, in the presence of the rtA194T substitution, to be able to evaluate susceptibility to TDF, both *in vitro* and *vivo*. However, this study revealed that strains with aa substitutions rtP177G and rtF249A showed reduced replication capacity but enhanced resistance to TDF in both *in vitro* and *in vivo* analysis. The accurate molecular mechanisms leading to TDF resistance by these aa substitutions, remain still to be determined. On the other hand, consistent with previous evidence of no TDF resistance in clinical settings, Kitrinos *et al*[104] demonstrated that TDF monotherapy could maintain effective viral suppression for period of over 6 years of continuous therapy, without the selection of any mutation significantly associated with TDF resistance.

Mutations in the B domain of RT, rtA181T/V, were shown to confer resistance to both L-nucleosides and alkyl phosphonates[105]. The mutation rtA181T also encode a stop codon in the overlapping envelope proteins at aa 172 in the S region (sW172*) resulting in truncation of these proteins. As Warner and Locarnini (2008)[106] emphasized, the rtA181T/sW172* variant had a secretory defect and exerted a dominant negative effect on wild-type HBV virion secretion. This could result in a missed diagnosis of drug resistance if viral load was used as the only criterion for drug failure. Also, the accumulation of the truncated HBsAg in infected cells was demonstrated to favor the onset of hepatocellular carcinoma through the transactivation of cellular promoters[107].

The resistance to D-cyclopentane group, *i.e.*, ETV, can be manifested only in the case when at least three mutations are present: rtL180M + rtM204V and either rtT184G/S or rtS202I/G or rtM250V[85]. Therefore, mutations classically associated with lamivudine resistance are necessary for the development of virological breakthrough to entecavir. In the absence of LAM-resistant mutations, the ETV resistance develops very slowly. However, in case of ETV utilization in patients with LAM treatment failure, the development of ETV resistance is more to be expected. Recent study by Lee *et al*[108] (2013) confirm that patients who harbor M204V strains were significantly more prone to ETV resistance than those who harbor other LAM resistance mutational patterns -rtM204I or rtA181T.

Apart from rtA181T/V which is the example of single mutation involved in resistance to more than one NA, multidrug resistance including more than one mutation was described. Usually, it occurs in case of sequential monotherapy with different NAs when new resistance mutations are added to already resistant strain[109]. Analysis of clones obtained from patients with dual resistance (to LAM and ADV or ETV) has revealed the presence

of mutations conferring resistance to both drugs located in the same viral genome, resulting in resistance to both drugs. Also, a patient with triple-drug-resistant HBV strains was reported[110]. The recent study[111], involving clonal sequencing, revealed that the rtA181 mutation re-emerged in the majority of patients who had developed sequential resistance to LAM, ADV, and ETV during LAM plus ADV salvage therapy. The rtA181T/V mutation emerged after reversion to wild-type HBV from the strains harboring ETV-resistant mutations during therapy and existed in different genomes from LAM- or ETV-resistant mutations. The co-localization of multidrug mutants to the same genome of virus was rare.

Antiviral drug-resistant mutations mentioned in the text were listed in Table 1.

Finally, due to the overlapping of polymerase and envelope genes in HBV genome, NA therapy can lead to the emergence of complex HBV variants that harbor mutations in both the reverse transcriptase and the envelope proteins. Antiviral-drug-associated S gene mutations (ADASMs) can lead to three different consequences[112]: amino acid substitution mutations in the surface proteins, nonsense mutations resulting in truncated surface proteins and silent mutations. Also, there are ADASMs that cannot be explained by gene overlapping but which are probably the result of lower or reverse ratio of HBsAg to anti-HBs during long-term efficient viral suppression. As HBsAg mutations found in treatment naïve patients, the possible clinical impact of ADASMs is in altered antigenicity, viral fitness and oncogenic potential.

It was shown that triple HBV mutant (rtV173L + rtL180M + rtM204V) causes two amino acid changes in the overlapping surface gene (sE164D + I195M), that have an effect comparable to vaccine escape mutant sG145R[113,114]. Following LAM treatment, many patients harbor strains with S gene "vaccine-escape" mutations P120T and G145R. They produce changes in RT, rtT128N and rtW153Q, which were found to partially restore the *in vitro* replicative capacity of LAM resistant strain, therefore serving as compensatory mutations[113]. The latest study by Cento *et al*[115] identified additional 10 HBV RT mutations, beyond those currently known as classical resistance mutations (rtN53T, rtS78T, rtS85F, rtS135T, rtA181I, rtA200V, rtK212Q, rtL229V/F, rtM309K), which could contribute to the development of drug-resistance *in vivo*. Some of them showed a direct impact also on HBsAg-protein, inducing the development of mutations or stop-codons that might potentially affect HBV pathogenicity and oncogenic potential.

HBV MUTATIONS IN HBV/HIV AND HBV/HCV CO-INFECTED PATIENTS

HBV infection is common among HIV-infected individuals due to shared transmission routs of these viruses. So far, available data on the prevalence of clinically relevant HBV mutations in HBV/HIV co-infected patients is relatively scarce. This prevalence is believed to be higher than in HBV monoinfected patients since it is suggested that HIV interferes with the natural history of HBV infection by enhancing HBV replication[116]. Also, there is evidence that HIV infection and its associated immune suppression, may influence the molecular variability of the co-infecting HBV. Interestingly, HBV/HIV co-infected patients had lower HBV quasispecies diversity and evolutionary rate when compared to HBV monoinfected patients[117]. It was assumed, in a subsequent study[118], that low CD4 count might help the HBV isolates to retain their wild type characteristics.

The recent study from France (2013)[119], using prospective data from HBV/HIV co-infected patients during 9-year follow-up, reported incidence rates and characteristics of vast array of *Pol* and S gene mutations. An increase of incidence was observed in all mutation classes, especially in L-nucleoside-associated *Pol* gene. Therefore the subsequent ADASMs known to pose potentially serious public health problems approached over 10% of the study population by the end of follow-up. Among different investigated host and viral factors, LAM-treatment surfaced as a major risk factor for ADASMs since they were directly involved with the overlapping LAM-resistant *Pol* mutations. This link was less obvious when correlation of LAM-exposure and only immune-associated S mutations was analyzed. On the other hand, TDF-treatment was associated with a reduction in the occurrence of immune-associated S gene mutations. All the findings imply the need to use more potent antivirals and close follow-up for co-infected patients.

The problem of occult HBV infection in HIV-infected patients is still not well characterized. The reported prevalence of OBI among these patients varied significantly from 0%-89%[120-122]. Study conducted in Brazil[123] found that immune suppression in HIV-infected patients was not a determining factor for development of OBI. On the other hand, the previously mentioned study by Italian authors[54] showed that in HIV-infected patients, OBI was significantly more frequent when HBV DNA was searched for in samples other than serum.

The chronic HBV/HCV infection is generally considered a condition favoring the progression of liver fibrosis and the establishment of cirrhosis and also represents one of the most important risk factors for the development of HCC. Studies have suggested that an interplay must exist between the two viruses in cases of dual infection. It was shown that the HCV core protein can act as a gene-regulatory protein and negatively influence the expression of HBsAg[124,125]. Indeed, some authors confirmed that HCV co-infection can play an important role in development of OBI[126], while others reported absence of OBI in HCV co-infected patients[127].

The inhibitory effect of HCV on HBV can also limit the emergence of HBV mutations because the decreased replication would produce less mutant genomes. This was, so far, confirmed for BCP/precore mutations which were found less frequently in HBV/HCV co-infected than in HBV monoinfected patients[128-131].

CONCLUSION

Investigations of HBV genetic variability and clinical implications of specific mutations have resulted in significant advances over the past decade, particularly in regard to management of resistance to antiviral drugs. Determination of resistance profile is crucial in choosing the right antiviral agent to initiate therapy. In the era of drugs with high genetic barrier for resistance, on-going monitoring for possible resistance is still essential since prolonged therapy is often necessary.

The success of the vaccination programs has now been challenged by the discovery of mutant hepatitis B viruses showing amino acid substitutions in HBsAg which may lead to evasion of vaccine-induced immunity. However, the emergence of these mutations has not yet raised concern since it was shown that they develop slowly. It was shown that mutations in certain regions of HBV genome could be responsible for unwanted clinical outcome or evasion of detection by diagnostic tools, thus making the monitoring for these mutations a necessity in proper evaluation of patients.

Understanding the frequencies and clinical implications of viral mutations may contribute to improvement of diagnostic procedures, more proper planning of immunization programs and creating the most efficient therapeutic protocols.

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