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Minireview

Human hepatitis B virus mutants: significance of molecular changes

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Abstract Human hepatitis B virus, the leading pathogen for hepatitis B, is a compact DNA virus with viral genes that largely overlap. An increasing number of mutations have emerged following human interventions such as vaccination and anti-viral therapy. While vaccine escape mutants are characterized by mutations on the antigenic hepatitis B surface antigen, those carrying mutations in other viral proteins are either resistant to anti-viral therapy or implicated in acute liver diseases. Molecular identification of these various mutants should shed new lights on the underlying mechanism of hepatitis B virus viral escape and resistance and provide helpful information on their effective eradication.

Key words: Human hepatitis B virus; Vaccine escape; Mutation; Anti-viral therapy; Hepatocellular carcinoma

1. Introduction

Hepatitis B remains a global health problem with a considerable morbidity and mortality. It is estimated that there are currently more than 350 million carriers worldwide. Hepatitis B virus (HBV) causes a variety of acute and chronic human liver diseases including fatal fulminant hepatitis, cirrhosis and hepatocellular carcinoma (HCC), that is one of the most common human cancers [1]. Belonging to the family of hepadnavirus, HBV is a partially double-stranded DNA virus that replicates through an RNA intermediate by reverse transcription [2]. The DNA genome of 3.2 kb is enveloped in a spheric virion that is composed of an outer envelope and an inner nucleocapsid. One particular characteristic of the HBV genome is its compact organization with four overlapping open reading frames (ORFs) encoding DNA polymerase, HBV surface antigen (HBsAg) core and HBX, respectively (Fig. 1). This compact genomic organization provides constraints on the number of mutations that occur naturally during viral replication, as some of them located within one ORF (e.g. HBsAg) generate concurrent mutations in the overlapping ORF (e.g. DNA polymerase) that may affect the viability of the virus.

Human interventions in preventing HBV have been focused on the preventive neutralization of the circulating viral particles and the therapeutic inhibition of the viral replication. While prevention was made possible by the HBV vaccines that induce protective anti-HBs antibodies targeting the most antigenic region of HBsAg [3], promising advances on viral inhibition have been made using nucleoside analogs that either inhibit the DNA synthesis by terminating the nascent proviral DNA or prevent the priming of HBV DNA polymerase to the RNA template [4].

These control measures have led to a marked reduction of the number of HBV carriers in the general population. However, various HBV mutants are being identified. These mutants are either capable of escaping neutralization by HB vaccines or are resistant to anti-viral agents. Unlike the naturally occurring HBV mutants that are often 'silently' present in chronic carriers, these newly discovered variants are located within functional domains of viral proteins and are now linked to various stages of liver diseases, including the acute hepatitis and the development of HCC.

Our review aims at giving a molecular overview of these mutants which in turn may provide helpful information on the design of a more effective vaccine and therapeutic agents.

2. Hepatitis B surface antigen and mutants

The HBV viral envelope is composed of cellular lipids and three related proteins, the large (L, preS1+preS2+HBsAg), middle (M, preS2+HBsAg) and small (S, HBsAg) surface antigens [5]. They are encoded by a single ORF of the HBV genome and the translation is initiated at separate in-frame ATG codons that are located at 118 and 55 amino acid residues from each other. The corresponding translated products are conventionally designated as LHBsAg, MHBsAg and SHBsAg and are completely contained within the HBV DNA polymerase (Fig. 2A).

The LHBsAg consisting of 400 amino acid residues contains the hepatocyte binding site (amino acid residues 21–47) and is involved in the attachment of HBV to hepatocytes [6,7]. The LHBsAg is also essential for virion assembly and the intracellular level of LHBsAg affects the secretion of viral particles [8]. Retention of the over-expressed LHBsAg in the endoplasmic reticulum may lead to the development of HCC in transgenic mice. Similarly, such a retention results in the 'ground glass' cells that are present in some human chronic HBV carriers ([5], Table 1). A role of LHBsAg in protective immunity is also suggested by the presence of both B- and T-cell epitopes [9]. Besides taking structural part in the HBV envelope, C-terminal truncated but not the full-length LHBsAg protein that is translated from HCC-integrated HBV appears to function as a general transcriptional activator and may have a role in the development of HCC [10].

On the other hand, the MHBsAg of 281 amino acid residues appears to be dispensable for HBV virion assembly. While rearrangement or deletion mutants have been identified in LHBsAg in chronic HBV carriers [11], a missense mutation at an initiation codon of MHBsAg is found in certain chronic

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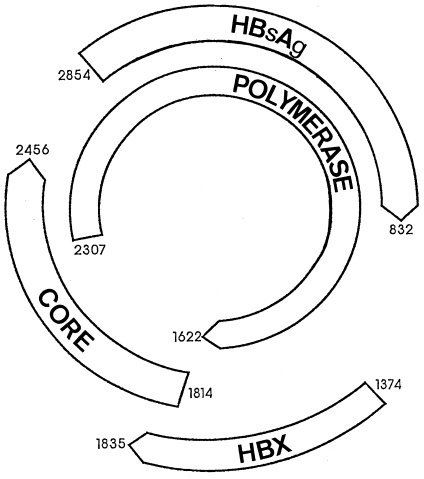

Fig. 1. Genomic localization of HBV viral proteins. The location of individual HBV viral protein (HBsAg, DNA polymerase, core and HBX) is shown on the circular HBV genome (adapted from [73] with permission). The arrows indicate the direction of translation and the numbers represent the nucleotide position of the start (e.g. 1374 for HBX) and stop (e.g. 1835 for HBX) codons. Numbering of the HBV genome (serotype *adw*) starts at the internal *Eco*RI site near the junction of preS1 and preS2.

carriers in Mediterranean countries [12] and also in patients with fulminant hepatitis [13] and HCC (Singapore patient of Oon strain, Table 1). Although HBV DNA polymerase overlaps the entire LHBsAg, it is interesting to note that preS1/S2 fall within the spacer domain and SHBsAg within the reverse transcriptase domain (Fig. 2A and B). This could explain the observed alterations within the preS region, as the concurrent changes in the spacer domain of the DNA polymerase that has no defined function would not affect much HBV viral replication.

The SHBsAg is a hydrophobic protein consisting of 226 amino acid residues and is the predominant component of the viral envelope. The topology structure of LHBsAg is assumed to display an outer (on the surface of the viral particle) or inner disposition of the N-terminus and an outer disposition of the C-terminus [14]. The major hydrophilic region of HBsAg has been defined from residue 100 to 160 in SHBsAg (or 275–335 in LHBsAg) [13]. This hydrophilic region has an assumed two-loop structure and is located on the surface of the viral particle (Fig. 3A). Defined as the 'a' determinant [15], this two-loop structure encompassing residues 124–147 in SHBsAg is conserved in all HBV genotypes and is directly involved in inducing neutralizing antibodies. It also constitutes the basis of current HB vaccines.

The clinical clearance of serum HBV following vaccination is characterized by the disappearance of HBsAg and a concomitant appearance of neutralizing anti-HBs antibodies, induced by the vaccine. However, incidence of co-existence of both HBsAg and anti-HBs has been observed following vaccination [16]. Emergence of HBV mutants capable of escaping the neutralization by anti-HBs is indicated by the persistence of HBsAg despite vaccination. The first case was reported from Italy with the presence of a Gly-145-Arg mutation on the HBsAg in the patient's serum [16]. A similar serological profile has subsequently led to the identification of this and other vaccine escape HBsAg mutants in other parts of the world [17–26].

In Singapore, for example, sequence analysis of serum viral DNA identified mutations on various positions of the 'a' determinant of HBsAg in 41 vaccinated infants with a breakthrough HBV infection [18]. These included Gly-145-Arg, Asp-144-Ala, Met-133-Leu, Gln-129-His and Ile/Thr-126-Ala [11]. The emergence of vaccine escape HBsAg mutants was also confirmed from Japan and China [20,22,27]. While the Gly-145-Arg mutants were identified in HBsAg and anti-HBs co-existing serum, Ile-126-Thr was detected either in infants without HBV infection or in those who developed acute hepatitis ([20], Table 1). In addition, the Thr-126-Ala mutation was also identified in a vaccinated Taiwanese infant who developed fulminant hepatitis ([19], Table 1). Vaccine escape HBsAg mutants have also been found in Spain [23], England [24], Gambia [25], Zimbabwe [26], Germany [17] but at a lower frequency as compared with many Asian countries.

Identification of vaccine escape HBsAg mutants following HBV vaccination from various countries indicated that muta-

Table 1
HBV mutants and liver diseases

Viral protein/promoter	Mutation	Liver diseases	Reference
SHBsAg	Gly-145-Arg	HCC	[37,38]
	Gly-145-Arg	Acute hepatitis	[19,36]
	Met-133-Thr	HCC	[37]
	Met-133-Leu	HCC	[28]
	Gly-130-Asn	HCC	[38]
	Ile-126-Thr	Acute hepatitis	[20]
	Ile-126-Ser	HCC	[38]
PreS1 in LHBsAg	Overexpression	Ground glass leading to HCC	[5]
	Gln-10-Lys	HCC	[38]
PreS2 in MHBsAg	Missense mutation at start codon	HCC	[38], unpublished data
	Missense mutation at start codon	Fulminant hepatitis	[13]
	Internal deletions	HCC	[38]
	Phe-22-Leu	HCC	[38]
Precore/core	Trp-28-stop codon	Acute hepatitis, HCC	[38,58]
	Pro-130-Thr	HCC	[38]
Core promoter	Nucleotide mutations (T^{1762}, A^{1764})	Fulminant hepatitis	[52]
	Nucleotide mutations (T^{1762}, A^{1764})	HCC	[38]
HBX	C-terminal insertions/deletions	HCC	[38], unpublished data
HBV genome*	Integration in host chromosomes	HCC	[74]

*HBV genomes have been found integrated in the host genomes of most HBV-related HCC.

tions were mainly occurring on the most antigenic 'a' determinant. Many of these mutations resulted in changes in their antigenic properties by computer-aided analysis [28], as illustrated in Fig. 3B for the most commonly reported mutation, Gly-145-Arg. Such changes in turn result in a decreased in vitro binding affinity to neutralizing antibodies [29].

Recent evidence from Singapore [30] and Japan [20] also suggested that some vaccine escape mutants carried mutations on other parts of HBsAg. The fact that these HBsAg mutants show an altered binding affinity to neutralizing antibodies [15,30] and the intriguing observation that preS2 containing vaccines induced mutations on the major hydrophilic region [31] appear to support the more complex structure of HBsAg. In such a complex structure, HBsAg displays clusters of antigenic epitopes, some of which are located in the region spanning amino acid 160–207 [32].

In addition to their detection in vaccinated infants, the HBsAg mutants may arise in patients with HBV infection but diagnosed negative for HBsAg and the recipients for orthotopic liver transplantation [33]. Naturally occurring HBsAg mutants have now been detected in a general population albeit that their incidence remains low [28]. Although it may take decades before the vaccine escape mutants become predominant over the wild-type HBV [34], an improvement of the current detection systems is needed as these mutants are able to cause liver diseases in both chimpanzees and humans [35,36] and are found in many HCC samples ([37,38], Table 1).

3. HBV DNA polymerase and mutants

While clinically relevant mutations on HBsAg are mainly related to vaccination, other significant mutations detected in HBV viral proteins include those in the HBV DNA polymerase following prolonged treatment with nucleoside analogs (i.e. lamivudine and famcyclovir) [4].

The HBV DNA polymerase consists of 832 amino acids and is composed of four functional domains [39]. The amino-terminal region (residue 1–178) is involved in priming the viral template. Separated by a spacer region of 158 amino acid residues (residues 179–336), the HBV DNA polymerase (spanning residues 337–680) possesses dual RNA- and DNA-dependent polymerase activity while the RNAse H domain (residue 681–832) is located at the C-terminus (Fig. 2B).

Unlike HBsAg mutants, mutations in the catalytic domain of the HBV DNA polymerase do not occur naturally (unpublished observations). Generally effective in suppressing HBV viral replication, long-term lamivudine (negative enantiomer of 2′-dideoxy-3′-thiacytidine, or (-)3TC) treatment has, however, led to the emergence of unresponsive HBV strains [40]. Mutations in the catalytic region of HBV DNA polymerase, in particular Met-552-Ile or Met-552-Val in the conserved 'Tyr-Met-Asp-Asp' ('YMDD') motif that comprises part of the active site (domain C) of the reverse transcriptase ([39], Fig. 2B), have been associated with the loss of inhibitory activity of lamivudine [40]. It is interesting to note that a mutation within the same 'YMDD' motif that is conserved in all reverse transcriptases [39] is also associated with viral resistance to (-)3TC in human immunodeficiency virus (HIV) [41]. On the other hand, the major famcyclovir resistant mutations (Val-521-Leu and Leu-528-Met) are clustered within the B domain of the DNA polymerase ([42], Fig. 2B). In

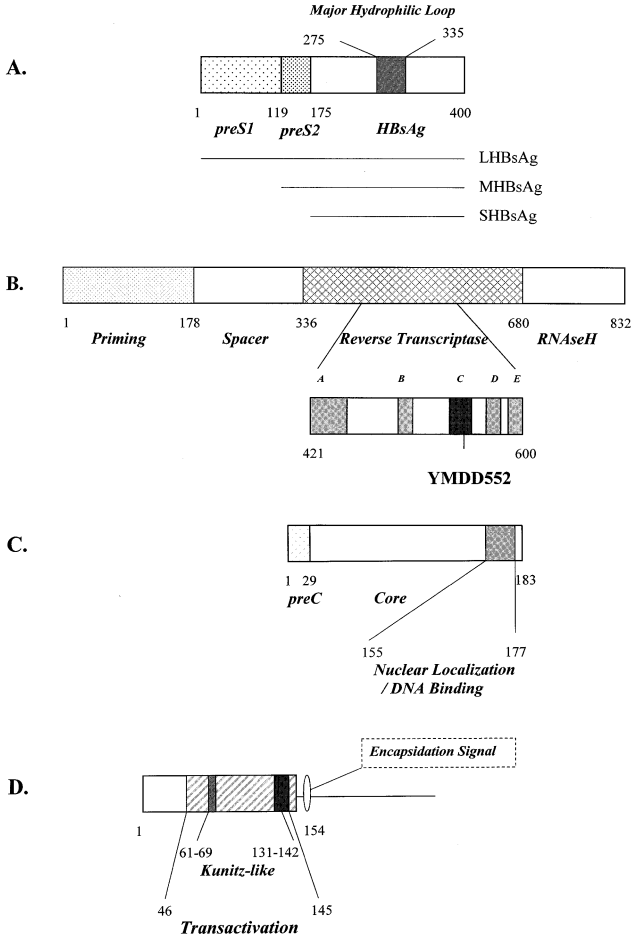

Fig. 2. Structural organization of HBV viral proteins. Functional domains of each HBV viral protein are shown by various boxes in shade. The positions of HBsAg (A) in relation to HBV DNA polymerase (B) and that between precore/core (C) with HBX (D) correspond to their genomic overlapping. (A) HBsAg. The size in amino acid residues is indicated (1–400). Positions of preS1 (1–118), preS2 (119–174) and the major HBsAg (175–400) are indicated below the box. The position of the antigenic major hydrophilic loop in relation to the full-length HBsAg is also indicated. The corresponding position of this antigenic loop within the major HBsAg is from amino acid 100 to 160. (B) HBV DNA polymerase. The size in amino acid residues is indicated (1–832). Positions of each of the four functional domains are indicated by numbers below the box: 1–177 (priming), 179–335 (spacer), 336–679 (reverse transcriptase) and 680–832 (RNAse H). In addition, the catalytic domain of the reverse transcriptase with the five conserved motifs (A–E in shade) is indicated in the enlarged box. The numbers below (421–600) the enlarged box represent the position of the catalytic domain and the position of the YMDD552 motif that is involved in viral resistance to anti-viral therapy (see text) is also indicated. (C) Precore/core. The size in amino acid residues is indicated (1–183). The position of precore (preC) is indicated in shade and numbered (1–29), while the position of core is represented by numbers (30–183) below the box. The nuclear localization and region involved in DNA binding are in shade and numbers from 155 to 177 within the core protein. (D) HBX protein. The size in amino acid residues is indicated (1–154). The box represents the HBX protein, while the adjacent solid line represents the DNA sequence of HBV DNA with the encapsulation signal (see text) in an oval form. The transactivation domain spanning residues 46–145 is indicated. The split Kunitz-like domain is also indicated (61–69 and 131–142).

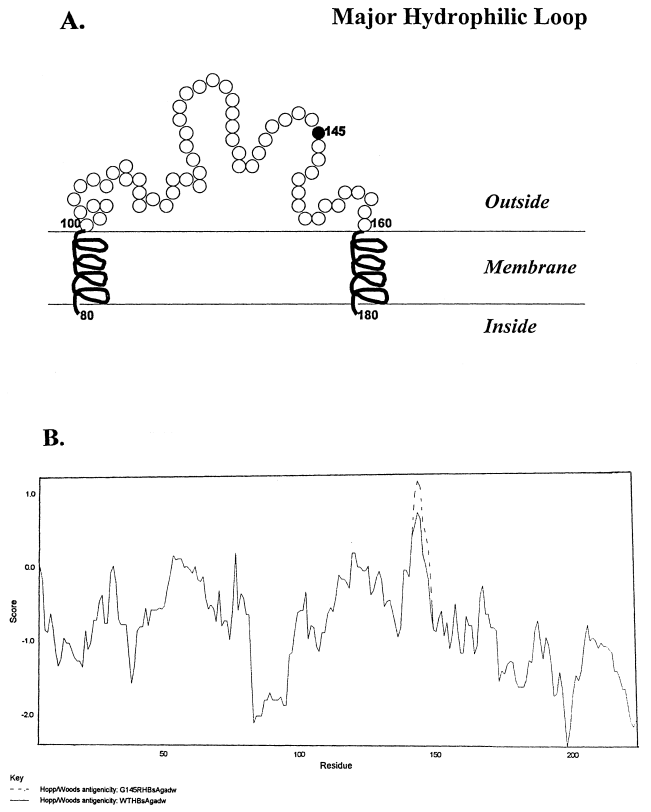

Fig. 3. Predicted features of the secondary structure of HBsAg. (A) Topological model of the major hydrophilic loop. The positions of the two predicted transmembrane helices upstream and downstream of the major hydrophilic loop are indicated by numbers (80–100 and 160–180, respectively). The amino acid residues forming the two-loop 'a' determinant are represented by open circles on the surface of the lipid membrane, while residue 145 where the most common vaccine escape mutation occurs is indicated by a black circle. The membrane represents the bi-layered lipid of the viral particle. (B) Predicted antigenicity profile of wild-type and Gly-145-Arg mutant HBsAg. The prediction analysis is according to the Hoop/Woods method (Omiga system), with seven amino acid residues (average size of an epitope) as the scanning window. Changes of antigenicity are seen in Gly-145-Arg HBsAg mutant and labelled in the discontinued line.

contrast to vaccine escape HBsAg mutants, recent reports indicate that these anti-viral resistant polymerase mutants are replication defective, probably due to the location of mutations within the crucial catalytic domain [43] and appear to be overtaken by wild-type HBV upon cessation of anti-viral therapy [44].

Despite their overlapping location on the viral genome, the major antigenic 'a' determinant on HBsAg is located in the variable linker region between the two conserved domains A (amino acid 410–426) and B (amino acid 498–528) of the DNA polymerase ([39], Fig. 2B). Many reported anti-viral resistant polymerase mutants give rise to concurrent HBsAg mutations outside the 'a' determinant (Glu-164-Asp, Trp-196-Ser/Leu and Trp-196-stop codon) [4]. Although these mutations do not result in alterations in the viral antigenicity, the generation of potential vaccine escape mutations has been identified in a lamivudine-treated Singapore patient who carries both the YIDD polymerase mutation and an independent Gly-130-Asp HBsAg mutation [45]. While these new lamivudine resistant HBV mutants are sensitive to other nucleoside analogs that inhibit the reverse transcription at a different level (i.e. gancyclovir) [45], their emergence should require further monitoring as they are capable of escaping detection/neutralization and are resistant to anti-viral therapy.

4. Precore/core and mutants

The structural nucleocapsid protein HBcAg consists of 185 amino acid residues plus an additional 29 amino-terminal residues (precore) ([46], Fig. 2C). It is involved in capsid formation, packaging of the pregenome reverse transcriptase complex, trafficking of the capsid in the cell and envelopment [47]. Serum appearance of antibodies against the nucleoside (anti-HBc) is indicative of the HBV infection. The HBe antigen, a 17 kDa secretory protein found in serum during active HBV replication, is encoded by the same core gene but lacks the arginine-rich C-terminal 35 amino acid residues [48]. Various motifs have been identified including nuclear localization and B- and T-cell epitopes [46].

While large overlaps can be seen among HBV DNA polymerase, HBsAg and HBX genes, a significant part of the core gene is not covered by any other coding regions (Fig. 1). There should therefore be decreased constraints for naturally occurring mutations in the core gene as compared with those found in overlapping genes (e.g. DNA polymerase and HBsAg) [49], which is reflected by the numerous naturally occurring mutations that are detected in chronic HBV carriers and those with fulminant hepatitis [50]. On the other hand, treatment with interferon that promotes viral clearance by immune stimulation also results in mutations on the B-cell epitopes of the core protein [51]. Mutations are also found in the precore promoter leading to an enhanced viral replication [52] and a precore stop codon mutation (G-A at nucleotide 1896) that results in an increased stability of the encapsulation signal (Fig. 2D) that is crucial for the pregenomic encapsulation and the initiation of HBV DNA synthesis [53].

While these precore stop codon mutants are frequently found in many Asian patients with fulminant hepatitis ([54–57], Table 1) including those from Singapore (unpublished data), their absence in European patients [58] suggests that other mechanisms may have contributed to the pathogenesis of fulminant hepatitis. Vertical transmission of such precore mutants with an enhanced viral replication activity has been found to cause hepatitis in newborns [59].

5. HBX and mutants

HBX, the smallest HBV viral protein with 154 amino acid residues, has been reported to interact with a wide range of cellular proteins [60], among which transcription activators that are involved in the activation of protooncogenes (c-*jun*) and growth control (Egr-1) [61,62]. In addition to the domains that are important for the transactivation and in vitro transforming activities [63,64], there is also a split but functional Kunitz-like serine protease inhibitor domain ([65], Fig. 2D). The function of HBX as a protease inhibitor has been further strengthened by the recent identification of the proteasome as its new target [66,67]. These findings suggest a more general role for HBX in inhibiting cellular processes of protein degradation.

Although the exact function of HBX in the pathogenesis of HBV-related HCC is not clear, it has been shown that HBX is

able to transform in vitro NIH3T3 fibroblasts and differentiated murine hepatocytes. When expressed in transgenic mice, HBX can induce HCC in certain lines [68] but it appears to assume an indirect action in cooperation with other cellular oncogenic factors in the liver oncogenesis [69,70]. Frequent mutations and deletions have been identified in the small HBX protein [60,71]. New mutations in HBX from Asian patients (including Thailand and Korea) were previously identified in a systematic study [72]. A novel class of HBX mutants has recently been identified in liver tissue of Singapore HCC patients (Table 1), some of these integrated HBV sequences also carried HBsAg mutations that may be associated with the development of HCC [37].

6. Conclusion

There is today an increasing understanding of the many mutations occurring in the hepatitis B viral genome. While some HBsAg mutants have appeared to be associated with vaccination or anti-viral therapy, others have existed naturally either in asympatomatic carriers or patients with serious liver diseases. Some of these mutations located in viral proteins such as HBsAg, precore and HBX have been found in acute fulminant hepatitis, cirrhosis and HCC. These human HBV mutants can be autonomous in behavior but capable of causing serious harm. Further molecular characterization of these various mutants should provide helpful information in developing better detection systems as well as more effective prophylactic and therapeutic agents against these mutants.

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