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Mini-review

Worldwide genetic diversity of HBV genotypes and risk of hepatocellular carcinoma

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ABSTRACT

Hepatitis B viruses (HBV) are responsible for over 50% of the worldwide attributable risk of hepatocellular carcinoma (HCC) and this figure increases even further in regions of high endemicity. Systematic sequencing of HBV genomes has identified that this common virus existed as eight distinct genotypes (denoted A–H), each regrouping variants with less than 8% divergence in their DNA sequence. These genotypes differ by their geographic distribution in populations around the globe. There is evidence that HBV genotypes also differ by their pathogenic properties, including their risk of persistence as chronic infection and their capacity to induce precursor disease or cancer. On the other hand, HBV genes may undergo mutations that become selected during the course of chronic infection and progressive liver disease. The most significant of these mutations in the context of HCC are those occurring in the pre-core (Pre-C) and basal core promoter (BCP) regions. These mutations may upregulate HBV expression and increase its virulence. These mutations may occur in all HBV genotypes but are more common in genotypes associated with more severe disease and cancer, in particular genotype C. Understanding the molecular basis of pathological variations between HBV variants is critical for prediction of disease severity. It will also be important to determine whether differences among genotypes may have an impact on the long-term protective efficacy of universal HBV vaccination.

1. Introduction

Hepatocellular carcinoma (HCC) often occurs as a sequel of chronic infection with hepatitis B virus (HBV); it is estimated that over 20% of the 400 million people with chronic hepatitis B infection will develop HCC [1,2]. In most HCC high-risk areas, the principal risk factors are HBV infection and consumption of AFB1-contaminated food [3].

The clinical course of HBV infection is variable, including acute self-limiting infection, fulminant hepatic failure, inactive carrier state and chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma (HCC) [4,5].

HBV chronic infection is characterized by persistence of Hepatitis B surface antigen (HBsAg) in the plasma over a time interval of 6 months. Although the HBsAg is detectable for several decades, the viral load decreases over the years, which coincide with the disappearance of Hepatitis B antigen e (HBeAg), a temporary increase in serum amino transferases levels and the outcome of anti-HBe. The evolution of the serological markers has been characterized

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by four phases depending on the activity of virus replication and specific immune response: immune tolerance, immune clearance, low or non replicative phase and reactivation phase [4,6] (Table 1). Chronic infection is a consequence of the disability of the immune system to control HBV infection and its frequency depends on age at which infection occurs and the route of transmission. In neonates infected perinatally, the frequency of chronic infection is as high as 90%; however, the frequency in children is 20–30% and in adults it is less than 10% [7]. Additionally, it has become evident that HBV may also persist in the form of serologically silent infections, leading to the concept of occult infection. Such infection may represent a non-negligible contribution to the population burden of HBV-related diseases and is a major concern for transfusional medicine and transplantation. In a recent statement, occult HBV infection has been defined as the presence of viral DNA in the liver, with absence of HBsAg and low titer or even absence of HBV DNA in serum [8]. Occult HBV infection occurs worldwide, and is not necessarily associated to mutations in the "a" determinant of the HBsAg [9]. Rather, it may represent an ultimate form of natural persistent infection. There is evidence supporting that occult HBV infection may accelerate the development of cirrhosis, particularly in HCV co-infected patients, but more studies are warranted to confirm this suggestion. This clinical presentation of the infection has also been associated to HCC, particularly in patients co-infected with HCV [10–13].

HBV exists as many distinct variants that differ by their capacity to become persistent and induce chronicity, as well as by the clinical manifestations of chronic infection including cancer. Prior to the definition of the genotypes, HBV strains were distinguished by serological analysis into nine hepatitis B HBsAg subtypes designated *ayw1*, *ayw2*, *ayw3*, *ayw4*, *ayr*, *adw2*, *adw4q−*, *adrq+*, and *adrq−*, determined by mutually exclusive amino acids substitution in positions 122 and 160 of the S region of HBV DNA. In 2004, Norder et al. used complete genome sequences of 234 HBV isolates as well 631 sequences of genes encoding HBsAg to assess the worldwide diversity of HBV [14]. This analysis confirmed the long history of co-evolution of HBV with humans and non-human primates. It also underlined the possible contribution of HBV variants defined on the basis of their genotypes to geographic and etiologic difference in the rate of progression of infection from acquisition of cancer to chronic liver disease and cancer. In this review, we summarize how recent knowledge of the genetic diversity of HBV has contributed to improve our understanding of the molecular epidemiology of infections and we discuss the emerging evidence of an association between some viral strains and increased frequency or rate of development of HCC.

2. HBV genome

The hepatitis B virus (HBV) belongs to the genus *Orthohepadnavirus* of the *Hepadnaviridae* family and shares with these viruses a circular genome of approximately 3.2 kb in length. It contains four overlapping open reading frames (ORF) encoding: ORF preS1/Pre-S2/S the surface antigens (HBsAg), ORF P the viral polymerase (Pol), ORF X the transactivator X protein (HBx) and ORF pre-core/core (Pre-C/C) the e Antigen (HBeAg) and Core protein (HBcAg) (Fig. 1A). Additionally, two viral enhancers (EnhI and EnhII) positively regulate transcription of the HBV promoters, including basal core promoter (BCP) that controls the transcription of both, the pre-core and core regions.

The partially double stranded DNA is generated from an intermediate RNA through reverse transcription (RT) activity of the Pol (Fig. 1B) [15]. The absence of proof reading capacity of the HBV Pol leads to a high mutation rate. On the other hand, the extreme overlapping of the ORFs of this viral genome represents a constraint for natural selection that limits the possibility of fixation of many of these mutations [16]. As a consequence of these opposite aspects, the substitution rate in HBV genome is intermediate between the ones of RNA and DNA viruses [17]. This genomic plasticity allows the generation of a quasispecies-like viral population [18], harbouring viral mutations that can occur and develop under particular selection pressures.

Several studies have pointed that recombination may play an important role in shaping the evolution of HBV [19,20]. In Human Immunodeficiency Virus (HIV), where recombination has been studied in greater detail and is a very frequent event; template switch occurring during reverse transcription seems to be the mechanism responsible for recombination. However, reverse transcription does not appear to be the event associated with recombination in hepadnaviruses, since, in contrast to HIV, it occurs after encapsidation of a single pregenomic RNA [15]. The exact mechanism of recombination of HBV genomes is not clear, but is likely to occur in the nucleus, by illegitimate replication [21] or by recombination with integrated HBV DNA [22].

Table 1
Clinical phases of HBV infection.

Phase	ALT	Viral load	HBeAg	Anti-HBe	Histological activity
Immune tolerance	Normal/low elevation	High	Positive	Negative	Normal/minimal histological activity
Immune clearance	Elevated	Fluctuating decreasing levels	Positive	Negative	Hepatic necroinflammation and variable fibrosis level
Low or non replicative	Normal	Undetectable or low levels	Negative	Positive	Inactive and minimal amount of fibrosis
Reactivation	Elevated	High	Negative	Positive	Moderate or severe necroinflammation with variable fibrosis

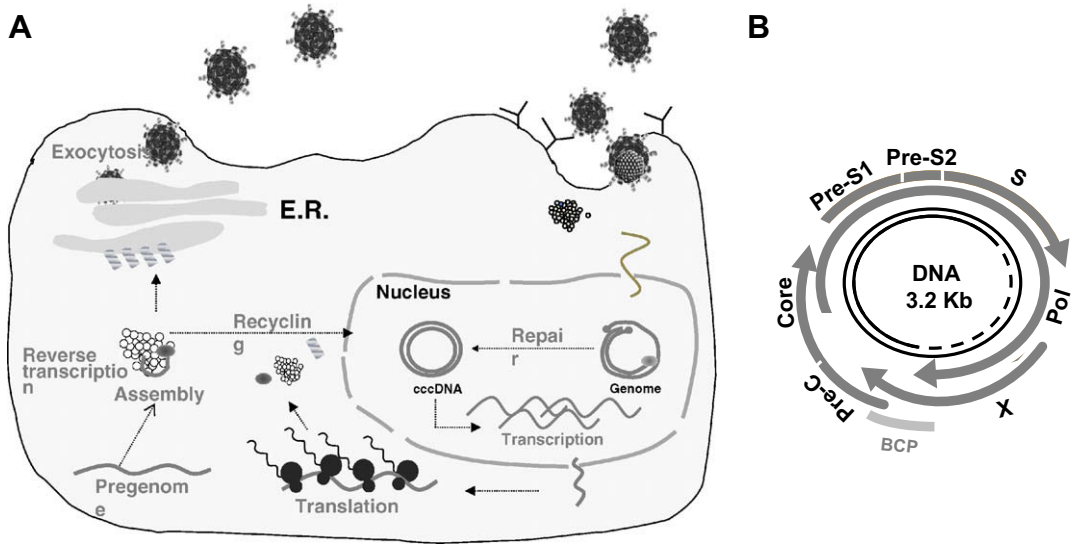

Fig. 1. (A) Life cycle of the hepatitis B virus. Enveloped virions infect the cell, releasing the nucleocapsids into the cytoplasm. HBV DNA is transported to the nucleus, and repaired to form cccDNA. Transcription of cccDNA by RNA polymerase II produces mRNAs and pregenomic RNA. Pregenomic RNA is encapsidated, together with P protein, and reverse transcribed inside the nucleocapsid. Part of these capsids is recycled to amplify the pool of intracellular cccDNA and the others are enveloped and released as virions. (B) HBV genome organization. The partially double-stranded, circular HBV DNA is indicated in black lines and the overlapped open reading frames in dark grey lines. In clear grey line the basal core promoter is shown (BCP).

3. Non-human HBV

HBV is found in several species of mammals, such as woodchuck (Marmota monax) and ground squirrel (Spermophilus beecheyi, Spermophilus parryii), in birds, such as duck (Anas domesticus), and grey heron (Ardea cinerea) [23–25]. Infection of woodchucks by woodchuck hepatitis B virus (WHV) may cause HCC. Indeed, like HBV, WHV can cause acute and chronic liver disease. Furthermore, chronic WHV infection in woodchucks usually leads to the development of hepatocellular carcinoma within the first 2–4 years of life. In liver tumours induced by chronic WHV infection in the WHV/woodchuck model of HBV infection, activation of genes of the myc family by WHV insertion has been well documented and differs from mechanisms involved in human HCCs.

Within the past 10 years, HBVs have also been characterized in the Old World great apes (orangutan, gibbons, gorillas and chimpanzees) and from a New World woolly monkey [26]. Each group of non-human primates appears to have a distinct strain of HBV that can be distinguished from human HBV based upon nucleotide sequence and selected amino acid changes in viral proteins. However, these HBV do not form a single HBV genotype. The Woolly monkey HBV is most divergent from other primate and human HBV sequences, while HBV sequences from the Old World great apes cluster together with separate branches for each group (see Fig. 2). In Non-human Primates infections by their respective HBV strains, no HCC development has been described.

4. HBV genotypes and subgenotypes

Due to its long history of co-evolution with humans and non-human primates, HBV has evolved in multiple genetic strains that are present at different rates in human populations. Based on a minimum divergence of 8% of the complete genome sequences, HBV has been classified in different genotypes consecutively identified as genotypes A–H. Within some HBV genotypes, subgenotype diversity has also been described, with a minimum genetic distance of 4%. Genotype diversity can also be detected by the sequencing of some regions of the genome, such as in particular the S gene; although subgenotype classification is not always accurate based on partial genomic sequencing [27].

During several years, any study was carried out in order to explore the relation between HBV genetic diversity and the complex course of liver disease; however latest researches have demonstrated associations between genotype and subgenotypes and disease severity and treatment outcomes of HBV infection [28,29].

4.1. Geographic distribution of genotypes

The eight HBV genotypes identified display distinct geographical and ethnic distributions (Fig. 3 and Table 2). For example, genotypes B and C are prevalent in Asia, while genotypes A and D are prevalent in Europe, United States and Central Africa.

Genotype A is classified into three subgenotypes, Aa/A1, Ae/A2 and Ac/A3 [30,31]. Subgenotype Aa/A1 is found in East and South Africa and South Asia and associated with mutation in the pre-core region and high prevalence of HCC in Africa [32,33]. Subgenotype Ae/A2 is mainly endemic in Europe and United States with increasing prevalence among young adults in Asian countries. Subgenotype Ac/A3 is mostly found among populations of West and Central Africa.

Genotype B can be classified into two major groups based on the presence or absence of recombination with

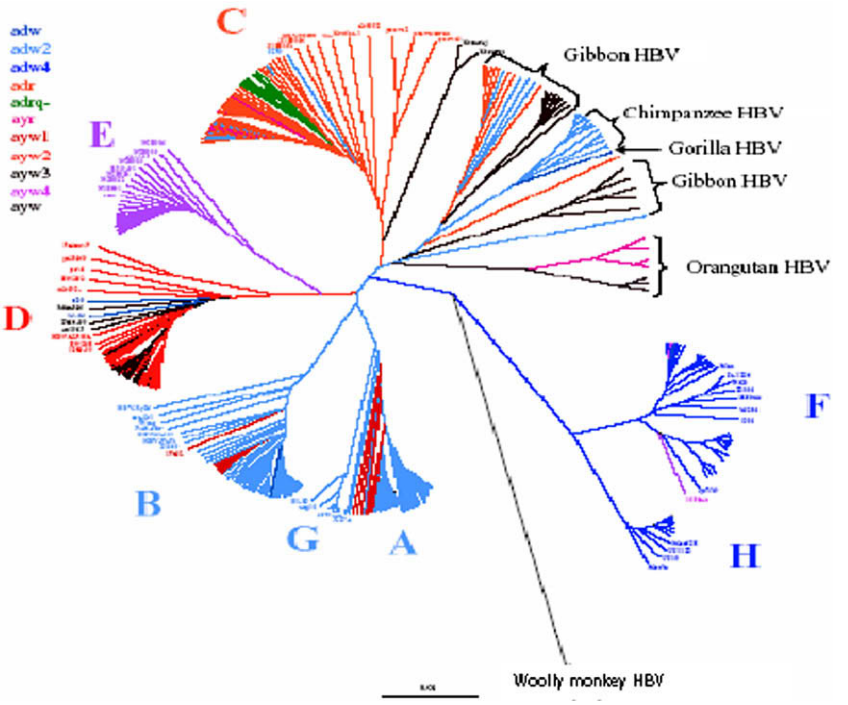

Fig. 2. HBV genotypes and primate strains. Genotypes are indicated on the branches, from [38] (PMID: 155,64,741).

Fig. 3. Geographic distribution of hepatitis B virus genotypes. The bigger size of letter corresponds to the prevalent genotype in each region.

genotype C in the core region [34]. Several subgenotypes had been reported, Bj/B1 is found in Japan and among indigenous populations of Alaska, Canada and Greenland (also with also B6). Subgenotypes with evidence of recombination ("Ba") are B2–B5; B2 is endemic in China and Taiwan, B3 in Indonesia, B4 in Vietnam and B5 in Philippines [35].

Genotype C is the most prevalent in Asia. Five subgenotypes have been described; subgenotype C1 (Cs) was found in Southeast Asia, C2 (Ce) in East Asia, C3 in Polynesia, C4

Table 2
HBV genotypes and serotypes geographic distribution (from Ref. [14]).

Genotype	Serotype	Major geographic distribution areas
A	*adw2, ayw1*[a]	North-West Europe, North America, South Africa
B	*adw2, ayw1*	South East Asia, China, Japan
C	*adw2, ayr, adrq−, adrq+*	South East Asia, China, Korea, Japan, Polynesia
D	*ayw2, 3 and 4*	Mediterranean area, Middle East, East Europe, India
E	*ayw4, adw2*[a]	West and Central Africa
F	*adw4q−*	South America, Alaska, Polynesia
G	*adw2*	France, United States, others?
H	*adw4*	Central America, Mexico, South United States, others?

[a] Serotypes rarely found in this genotype.

in Aborigines from Australia, and C5 in the Philippines and Vietnam.

Genotype D is the most widespread genotype and predominates in the Mediterranean area [36] and in the near and Middle East up to India. It was also found in Aboriginal populations of Indonesia and Papua-New Guinea. Phylogenetic analysis distinguishes four subgenotypes, D1–D5 [14,37,38]. The geographical distribution of the subgenotypes within D is less restricted than that of genotypes A, B and C. Strains from Middle East mainly belong to the D1, D2 major subgenotype also found in Russia and the Baltic region [39]. Strains from South Africa and Alaska mainly belong to the D3 subgenotype D4 includes strains from Oceania and Somalia. Subgenotype D5 has been described in India [37].

Genotype E is by far the dominant genotype in West Africa and has very low intra-genotypic diversity suggesting that this genotype has spread only recently. However, this genotype has been less extensively studied than others [40].

Genotypes F and H are endemic in the New World [41]. A HBV strain isolated from woolly monkey (a New World monkey) is closer to Genotype F with a noteworthy phylogenic distance from other strains of HBV, Humans or Non-Humans [14]. Several strains of genotype F have been isolated from Amerindian population in different countries in America; four subgenotypes have been described with genetic divergence among 4.3–6.1%. The subgenotype F1, in particular F1a have been found in Alaska, El Salvador, Guatemala, Costa Rica and Nicaragua; whereas F1b has been reported in Peru and Argentina. Strains of subgenotype F2 has been found in Costa Rica, Nicaragua, and Venezuela and Brazil. Subgenoype F3 is found in Colombia and Venezuela and F4 in Bolivia and Argentina [42–45].

Genotype G is mostly detected in co-infection with other HBV genotypes [46], mostly genotype A. This genotype is characterized by 36 nucleotides insertion within the core region and therefore is not able to process HBeAg. This genotype has no specific endemic area in the world.

Hybrid HBV strains resulting from genomic recombination between different genotypes are increasingly documented [9,19,47]. For instance, phylogenetic analysis revealed that hybrids between HBV genotypes B and C, which have sites of recombination over the pre-core/core region, were found in Asian countries, except for Japan [48,49]. Similarly, hybrids between HBV genotypes A and D have been reported in Italy and South Africa [50,51] and an aberrant recombinant between genotype C and a subgroup of genotype A was isolated in Vietnam [32]. More recently, hybrids of HBV genotypes C and D have been identified in Tibet and China [52,53]. A novel variant of hepatitis B virus was identified in Vietnam. This strain (HBV-VH24) had a novel intergenotypic recombination between genotypes A, C, and therefore was proposed as new genotype I [54] although the divergence with genotype C is borderline [55].

4.2. HBV genotypic variations and risk of HCC

Two main types of genotypic variations in HBV have been demonstrated to have an impact ion the risk and clinical course of HBV-related diseases. First, pathogenic differences have been reported in relation with some HBV genotypes. Second, specific variations in HBV have been associated with cirrhosis and HCC. These variations include in particular mutations in pre-core region (Pre-C, A1896G, inside the ε structure of the genome), in the basal core promoter (A1762T/G1764A) and in ORFs encoding preS1/PreS2/S and Pre-C/C (Table 3). In this paragraph we summarize key evidence demonstrating the impact of these variations on the risk of HCC. There is an overlap between Pre-C or BCP mutations and genotype, since these mutations appear to be more common in genotype C as compared to other genotypes [56]. Comparison between HBV isolates from different continents reported that the mutation rate of A1762T/G1764A was 64% for genotype C, 40% for genotype B and 35% for other genotypes [57].

Table 3
HBV most common variants and their clinical implications.

HBV genomic region	Mutations	Phenotypic association
Pre-core	G1896A	HBeAg negative mutants, severe forms of disease
Basal core promoter and enhancer region	C1653T	Rapid disease progression and HCC
	A1753T	Rapid disease progression
	T1762A, T1764C	HCC
	C1766T	Fulminant hepatitis
X	K130 M and V131I	HCC development
Pre-S	Deletions	Potential association with HCC
S	In a determinant	Vaccine escape mutants
Polymerase	In domains B, C and D of RT	Resistance to nucleos(t)ide analogue drugs

For references see text.

4.3. Genotype-related effects

Pathogenic differences in causing HCC have been reported among hepatitis B virus (HBV) genotypes. Among the first and largest studies on this question, those developed in Taiwan have contributed to establish that genotype C is associated with a more severe disease phenotype. The HCC risk of genotype C was explored in detail in two studies. The first study included 4841 male HBsAg carriers who were followed up for 14 years, during which 154 HCC cases were diagnosed. The risk of HCC for patients infected with genotype C (48.6%) was 5-fold the one of patients with genotypes A or B (representing, respectively, 1.4%, and 49.3%). The high viral load was also identified as a risk factor in this study; patients with plasma HBV DNA level of at least 10^4 copies/mL had a 2-fold increased HCC risk compared to patients with lower viral load, irrespective of age [58]. The second study included 2762 Taiwanese men and women seropositive for HBsAg and without HCC. The subjects were followed up for 15 year for a total of 33,847 person-years. A total of 153 HCC cases were diagnosed. The HCC incidence rates per 100,000 person-years were more than twice as high in carriers of C as in carriers of B genotypes (B carriers: 305.6 (95% confidence interval (*CI*) = 236.9–388.1); C carriers: 785.8 (95% *CI* = 626.8–972.9)). Moreover, among participants with a baseline HBV DNA level of at least 10^4 copies/mL, HCC incidences differed according to the presence of Pre-C or BCP mutations. Adjusted hazard ratio of developing HCC were 0.34 (95% *CI* = 0.21–0.57) for pre-core G1896A vs. wild type, and 1.73 (95% *CI* = 1.13–2.67) for BCP A1762T/G1764A vs. wild type [56].

The higher risk of genotype C of progression to terminal chronic liver diseases may be related to delayed spontaneous HBeAg seroconversion and longer duration of high HBV replication. Early seroconversion of HBeAg appears to be associated with a more favourable clinical outcome than late or absent seroconversion. The earlier seroconversion described for genotype B could be a consequence of a strong immunogenic stimulation during the clearance phase [59–63]. The impact of seroconversion has been analyzed for genotypes A and D in a prospective study in Spain. HBeAg positivity was significantly lower in cases infected by genotype D as compared to genotype A [64]. Overall, these results suggest seroconversion to HBeAg differ among HBV genotypes and that late or absent seroconversion could be related to progression of chronic hepatitis and liver disease.

An impact on pathogenicity has also been reported for genotypes other than C. A study in India demonstrated a higher impact of HCC in subjects infected with HBV genotype D as compared with genotype A [65]. Only genotypes A (46%) and D (48%) were found in the chronic HBV-infected patients, with a mixed infection with both genotypes observed in 6% of patients. Genotype D was associated with more severe liver diseases (61% vs. 30%, $P < 0.05$) and was more prevalent in HCC patients of less than 40 years of age, as compared to asymptomatic carriers (63% vs. 44%, $P = 0.06$). In a study of 258 Spanish patients with chronic hepatitis B, patients with genotype A hepatitis had a significantly higher rate of remission and clearance of HBV DNA and HBsAg than those with in genotype D. Death related to liver disease was more frequent with genotype F than with genotype A ($P = 0.02$) or genotype D ($P = 0.002$) hepatitis, further demonstrating that the long-term outcome of chronic hepatitis B is different in patients infected with HBV genotype A, D, or F [64]. The notion that genotype F, which is endemic to America, has a particularly severe phenotype has been well demonstrated in a cohort of 1176 Alaska Native people with chronic HBV infection, including 47 patients with HCC. In this cohort, genotype F was found in 68% of patients with HCC, vs. 18% of those without HCC ($P < .001$). For patients with genotype F, the median age at diagnosis of HCC was lower than that for patients with other genotypes (22.5 vs. 60 years, respectively; $P = .002$). In the same study, there was no difference in the number of subjects with basal core promoter and pre-core mutations between HCC patients and controls [66].

It should be noted that the risk of HCC may differ among subgenotypes. In a study comparing HBV C1 and C2 subgenotypes, HBV/C2 was found to be an independent was identified as an independent predictor of HCC. Moreover, the subgenotypes had specific patterns of BCP mutations, which were probably responsible for the increased risk of HCC associated with the C2 genotype [67].

4.4. Effects of mutations

Mutations in HBV genome occur due to spontaneous errors of viral polymerase, or as the consequence of selection pressure by the host immune system and/or by exogenous factors such as active or passive vaccination or drug therapy.

The Pre-C mutation (A1896G) prevents the production of HBeAg by introducing a premature stop codon into the ORF Pre-C/C that abolishes the production of HBeAg; however, HBV DNA synthesis persists and may cause liver damage with progression to cirrhosis and cancer. This mutation is frequently detected in patients with HBeAg negative chronic hepatitis B and in some patients with fulminant hepatitis B [68,69].

The BCP mutations affect the core promoter that regulates the expression of both HBeAg and the core protein. The common double mutation A1762T/G1764A results in a decrease in HBeAg expression with enhanced viral genome replication *in vitro* [70]. The BCP overlaps with the X region of the HBV genome and mutations in the amino acid sequences at positions 130 and 131 in this region (K130 M and V131I) have been proposed as prognostic markers for the development of liver cancer [71]. The effect of BCP mutations may be related to the higher rate of replication of genotype C. Substitutions in the BCP may increase genotype virulence by deregulating the transcription of pcARN/pgARN, increasing the risk of HCC in patients infected with genotype C [72]. However, BCP mutations also have an impact in the context of other genotypes. In a multivariate analysis in a cohort from Taiwan, Yang et al. (2008) reported a hazard ratio of developing HCC of 1.73 for double BCP mutant vs. wild type after adjusting for genotype [56].

Mutations in Pre-S have been recently reported in HCC cases compared to chronic or asymptomatic cases These

mutations consist into deletions in Pre-S in the integrated HBV DNA that impair the secretion of HBsAg, leading to increased endoplasmic reticulum and oxidative stress in hepatocytes [73,74]. Truncated forms of Pre-S2 have also been shown to interact with Cyclin A [75], a critical regulator of cell division cycle. Thus, deletions of Pre-S may contribute to hepatocarcinogenesis through several mechanisms.

5. Conclusions

Genetic variations in HBV are critical in the pathogenesis of liver disease, and provide clues about important molecular mechanisms by which HBV causes HCC. Thus, HBV genotypes and mutations represent candidate biomarkers for predicting risk of progressive disease and disease severity. In a recent study on a cohort of 820 patients, Yuen et al. (2008) [63] have formulated a predictive score for the development of HBV by integrating individual (gender, age) and viral (BCP double mutants, viral load and cirrhosis) parameters. It will be important to further investigate and compare the molecular patterns of genetic alterations in HCC that develop in association with different genotypes. Furthermore, it should be considered that the different genotypes may significantly differ in their responses to therapeutic intervention and to HBV vaccination. In the future, it will be important to assess the efficacy of current universal HB vaccination schemes with respect to protection against chronic infection and disease by different genotypes and HBV variants. It will also be important to determine whether vaccine strategies should be adapted in relation with the genotypes that are the most prevalent in a given population.

Conflict of interest

None declared.

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