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Effects of Hepatitis B Virus Mutations on its Replication and Liver Disease Severity

Abdulrahim Hakami[*,1], Abdelwahid Ali[2] and Ahmed Hakami[2]

[1]*Department of Medical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, Abha 61481, Saudi Arabia*

[2]*Department of Clinical Microbiology, College of Medicine, King Khalid University, Abha 61421, Saudi Arabia*

Abstract: Hepatitis B virus (HBV), nowadays, is one of the major human pathogens worldwide. Approximately, 400 million people worldwide have chronic HBV infection. Only 5% of persons infected during adulthood develop chronic infection. The reverse is true for those infected at birth or in early childhood, i.e. more than 90% of these persons progress to chronic infection. Currently, eight different genotypes of HBV have been identified, differing in nucleotide sequence by greater than 8%. In addition, numerous subgenotypes have also been recognized based on the nucleotide sequence variability of 4- 8%. It has invariably been found that these genotypes and mutations play a pivotal role in the liver disease aggravation and virus replication. The precore mutations (G1896A) and the double mutation (T1762/A1764) in the basal core promoter are important mutations that alter expression of the hepatitis B e antigen (HBeAg). The HBeAg is important for establishing viral persistence. The precore G1896A mutation abrogates the expression of HBeAg. Numerous other mutations alter the disease severity and progression. It is predictive that the infected patient has high risk of hepatocellular carcinoma if the genotype C is incriminated or if HBV possesses basal core promoter double mutation. Association of the remaining genotypes have been noted but with less degree than genotype C. Phenotypic assays of the different HBV protein markers with different molecular techniques illustrate the replication efficiency of the virus in cell lines. This review will discuss various mutations into their association with liver disease severity and progression as well as virus replication.

Keywords: HBV, Basal core promoter (BCP), Hepatitis B e antigen (HBeAg), Hepatocellular carcinoma (HCC), Liver cirrhosis (LC), Precore (PC), Wild type (WT).

INTRODUCTION

Hepatitis B virus (HBV) has high prevalence worldwide. More than 2 billion people have been infected with around 400 million individuals had chronic HBV infection representing approximately 5% of the world population with more than half million deaths annually due to liver cirrhosis or hepatocellular carcinoma [1, 2]; this indicates that the virus is highly contagious. HBV has four subtypes i.e. ayw, ayr, adw, and adr. At least 8 different HBV genotypes have been identified (A-H) where the nucleotide sequence varies by at least 8% [3, 4]. Recently, new genotype (I) has also been isolated from Laos, and genotype (J) was identified in Japan [5]. Many clinical and biological characteristics including transmission, seroconversion and frequency of mutations are completely dependant on HBV genotype (Table **1**). These different genotypes generally have restricted geographic distributions, with HBV genotype A is found in North America and Africa, genotype B and C are dominant in Asia, and genotype D present in Mediterranean countries and Europe [6-9] as well as some districts in western Japan [10]. Numerous subgenotypes that differ in nucleotide sequence by 4-8% have also been identified, and in some cases these too have a geographical limited distribution. For instance, subgenotype Aa (also known as A1) is generally restricted to Africa and Asia, whereas subgenotype Ae (A2) is more frequent in Europe. Subgenotype B1 is restricted to Japan, whereas subgenotypes B2-5 are found throughout China, Indonesia, Vietnam, Philippine respectively. Similarly, HBV genotype C is classified into C1 and C2 subgroups based on preS gene phylogenesis [11-13]. The genotype D subgenotypes have no specified geographic distribution, but D1 was derived from Middle East, D2 from Japan, and a recent subgenotype D6 has been described in Tunisia [14, 15].

HBV genome is approximately 3.2 kbp. There are four overlapping open reading frames (ORFs) organized in HBV genome. They are C ORF that encodes Hepatitis B core antigen (core or capsid protein; HBcAg) which is a non-structural form, also encodes HBe Ag, P ORF (Polymerase) which has a reverse transcription activity, S ORF (surface protein; HBsAg) and X ORF which was documented to play a role in the development of HBV- related hepatocellular carcinoma, HCC [16-19]. The basal core promoter (BCP) is located in the X ORF also [20]. There are two direct repeats, 11 nucleotides each; DR1 (1822-1832) and DR2 (1588-1598) that are involved in the viral replication. DR1 is located in the 3' end of RNA primer.

*Address correspondence to this author at the Department of Medical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, P.O. Box 3665, Abha 61481, Saudi Arabia; Tel: +966-72417090 ; Fax: +966-72417746; E-mail: abdu.lightpollution@hotmail.com

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DR2 is the complementary sequence and is close to the 5´ end of the negative DNA strand [21]. Pregenomic RNA (pgRNA) is the major transcription template, as well as the mRNA for core and polymerase. Encapsidation signal (ε) is a hairpin-like structure that consists of 5´UUC3'. It is the most common *cis*-element in the pgRNA. The mechanism by which HBV enters the cell is unknown; however, the replication cycle following entry is well characterized. After entering the cell, the viral envelope and protein coats are removed in the cytoplasm and the relaxed circular DNA (rcDNA) is converted to covalently closed circular DNA (cccDNA) in the nucleus [22] where it serves as RNA transcription template. Although HBV is a DNA encoding virus, the virus uses reverse transcriptase (RT) as part of its replication cycle. This reverse transcription uses the nuclear cccDNA as a template to produce virions [23]. After virus infection of hepatocytes, cccDNA is detected within 24 hours [24]. There are around 50 copies of cccDNA in the infected hepatocyte [25] that can be detected currently by PCR [26]. Two different RNAs of 3.5 kb (pregenomic RNA and precore RNA) and three other viral RNAs (2.4, 2.1 and 0.8 kb) are transcribed [27]. Transcription of HBV RNA as well as expression of polymerase and core protein is essential to create *in vitro* replication of HBV DNA and synthesis of its intermediates [28]. RNA transcription is mediated by host RNA polymerase II and regulated by two enhancers, four promoters and glucocorticoid-responsive element (GRE). Precore RNA and pgRNA are directed by the core promoter [29]. The pgRNA is then digested by RNase H activity of viral polymerase but leaving F undigested primer of around 17 nucleotides in the pgRNA 5' end from which (+)-DNA strand is synthesized after template translocation to DR2, then this strand is extended to the 5' end of the (-)-DNA strand. After primer circularization in a subsequent pathway, the rcDNA is made [30]. Polymerase uses 5´ end of internal bulge site in the encapsidation signal (ε) which plays a fundamental role in (-)-strand DNA synthesis and pgRNA encapsidation [31]. The closed-loop of pgRNA is formed by cellular eIF-4G by means of both cap and poly (A) binding proteins. The molecular chaperon heat shock protein 90 (HSP90) is required for RT-(ε) binding [32, 33]. The mechanism of capsid formation is unclear but recent results explained that HSP90 is necessary to form the capsid by binding to core protein dimmers [34].

It is laborious to propagate *in vitro* HBV due to the lack of a robust HBV cell culture and non availability of animal infection model. Cell lines usually lose then tropism to the virus few days after transfection but Huh7 and HepG2 (ATCC HB-8065) cell culture were established as standard HBV cell lines [22, 35]. For instance, HBV replication causes HepG2 apoptosis [36], therefore it can be valuable cells in the replication study task. Other animal models exist as hosts for human HBV, among which are Orangutan, Chimpanzee, and Gibbon [37]. Different HBVs replicate differently in different cell lines; however, hepatic cell lines QSG-7701 showed higher HBV DNA than HepG2 [38]. In terms of phenotypic characteristics, the replication products are measured in the cell culture supernatant and then undergo further analyses. Intracellular expression of HBV proteins and the pathogenicity of different genotypes have been compared [39]. Although a number of old and recent data exist, association of some genotypes with disease outcome is still not conclusive. There are some essential questions to be addressed, for example how do different HBV mutations involve in the severity of the disease?. What is the most virulent genotype and why do HBV variants yield different replication phenotype?. Since the discovery of HBV by Baruch Blumberg in 1965, scientists started to sequence its genome on a regular basis in an attempt to decipher some ambiguity of genomic correlation. The fact that HBV mutate slowly and continuously makes it complicated to fully understand the role of every mutation. This review is an attempt to correlate some mutations in the HBV genome to the virus replication and disease outcome.

HISTORY AND LIVER DISEASE PROGRESSION

The majority of adult-acquired HBV infection is acute and self-limiting. Around 5% of infected patients, during adulthood, will develop chronic hepatitis (CH), and may progress to liver cirrhosis (LC) or hepatocellular carcinoma (HCC). In some clinical cases, HCC can occur without cirrhosis [2]. In chronic carrier patients, HCC has an annual incidence of less than 1% for non- cirrhotic patients and 2-3% for those with cirrhosis [9]. Unfortunately, elevated HBV DNA in the serum has been associated with deaths in non-HCC patients [9]. Fulminant hepatitis is due to enhanced virus replication and occurs in 1% of infected individuals [27]. Certain HBV excretory proteins are used as markers for disease severity e.g., HBsAg positivity indicates disease chronicity whereas the marker of active viral replication is HBeAg. Elevated alanine transaminase (ALT) enzyme is also used as marker of viral hepatitis or liver damage. It is becoming increasingly apparent that HBV genotype influences HBV natural history and disease pathogenesis. Based on various studies, this genotypic diversity plays a crucial role in the pathogenesis and its prognosis [40]. HBV genotype A is predominant in asymptomatic individuals and the genotype most frequently associated with severe liver disease, including HCC is genotype C [41], which is associated with more serious liver illnesses compared to genotype B [42].

SOME INSIGHTS OF HBV MUTATIONS RELATEDNESS TO THE VIRUS REPLICATION

Variability of HBV genome is basically attributed to lack of proofreading during the replication process and the high copy number of the virus [43]. Characteristically, single mutation has different effects on viral replication and/or disease severity than double mutation. Virus mutation was predictive for liver disease but the role of precore (PC) mutation (A1896) in HCC is still not clear [44]. Furthermore, association of 1896 mutation with fulminant hepatitis has been documented [27]. The G1896A precore stop codon mutation at the codon 28 decreased HBV replication if paired with T1858 mutation [43]. Replicative intermediates were higher intracellularly for the PC mutant compared to wild type (WT) virus [45]. Intermittent and persistent replication *in vivo* is associated with dual PC/BCP mutation [46]. Furthermore, triple BCP mutation; T1762/A1764, and 1766 increases transcription of pgRNA whereas the double mutation 1762/1764 is less effective [47, 48]. This double mutation though increased the

Table 1. HBV Genotypes and the Comparison of Virological and Clinical Differences [9]

Clinical and Virological Characteristics	Genotypes			
	A	B	C	D
Mode of transmission	Horizontal	Vertical/Perinatal	Vertical/Perinatal	Horizontal
Tendency to chronic state	Higher	Lower	Higher	Lower
HBeAg seroconversion	Earlier	Earlier	Later	Later
LC and HCC outcomes	Better	Better	Worse	Worse
Frequency of PC (G1896A) mutation	Lower	Higher	Lower	Higher
Frequency of BCP (T1762/A1764) mutation	Higher	Lower	Higher	Lower

HBeAg= Hepatitis B e Antigen; LC= Liver Cirrhosis; HCC= Hepatocellular Carcinoma; PC= Precore; BCP= Basal Core Promoter.

genome replication *in vitro* but decreased HBeAg synthesis [20], and contributed to HBV pathogenesis *in vivo* [8]. PC mutation (G1896A) prevents HBeAg precursor translation whereas T1762/A1764 core mutation interfered with core mRNAs transcription leading to suppressed HBeAg synthesis [49] and was associated with high replication phenotype [50]. The prevalence of PC 1896 and BCP 1762/1764 mutations in hyperendemic districts is examined for the first time by Yang *et al*. 2008 [20]. A1899 mutation is reported in higher proportion in liver cirrhosis patients as well [46]. In relation to the association of genotypes and these common mutations, 1762/1764 is significantly higher in genotype A but lower in the genotype C. Genotype D has the highest A1896 mutation proportion [51]. Although PC and BCP were extensively analyzed, other mutations have been correlated with various effects, e.g. the bulge of ε signal is affected by G1862T mutation. These recent findings suggest the noticeable influence of genotypes on virus replication.

In a study of *in vitro* translation, G1862T mutation abolished precore/core proteins synthesis but not entirely impaired [52]. Inuoe *et al*. 2009 [27] found that G1862T has no effect on HBeAg expression. G1862T was also implicated in reducing DNA replication severely [53]. G1899A mutations increased DNA replication but the double mutation G1899A/G1862T was associated with considerably more replication compared to G1862T alone. Interestingly, U:G pair is converted into U:A in the lower stem (ε) in the two positions 1855/1899 if HBV strain possesses G1899A mutation. Using western blot analysis, core protein expression was reduced by G1862T mutation.

ASSOCIATION OF HBV MUTATION TO THE DISEASE OUTCOME

I. Precore (PC) and Double Basal Core Promoter (BCP) Mutations

Kobayashi *et al.* 2003 [49] showed that PC WT virus (G1896) was significantly more prevalent in patients who cleared the virus (negative HBeAg) compared to the G1896A precore mutant which persisted in patients who developed HCC. There were no discrepancies in the prevalence of T1762/A1764 double mutation in the BCP among the two groups of infected patients. Tsai *et al.* 2009 [48] confirmed that triple BCP mutation (1762/1764/1766) was associated with the highest increase in the transcription of pgRNA whereas double mutation was least effective. Biswas *et al.* 2011 [46] found that the double BCP mutation (1762/1764) was lower in genotype A than C but higher in D. Some patients with BCP mutants developed HCC [44]. The triple mutation (1753/1762/1764) was associated with severe liver illness [46]. C1753 mutation was associated with the previous BCP mutation in 42 patients whereas 38 patients with the BCP mutation did not have C1753, and 50 patients have had WT BCP [46]. Inactive carriers were associated with WT BCP; HBeAg status in these clinical cases did not play any role. 1762/1764 in chronic liver disease was only slightly significant. T1766/A1768 was not taken into account because of its very low prevalence. Patients with PC and BCP mutants showed consistent results as expected [44]. To illustrate this, 60% of (+)-HBeAg was detected in PC WT and 30% in PC mutants. On the other hand, 66% of patients with BCP WT were (+)-HBeAg compared to 55% for BCP mutants (Table **2**).

II. G1862T and G1896A Mutations

G1862 mutation was 5 times more common in fulminant hepatitis (FH) patients compared to chronic carriers [52]. Among 52 FH patients, G1862T was detected in 7 patients (Table **3**).

ESSENTIAL TECHNIQUES FOR MUTATIONS DETECTIONS

There are fundamental techniques to study viral replication and further to assess protein expression utilized by most scientific papers, among which are cell culture (mainly Huh7 and HepG2 cells) to transfect infectious cDNA clones of the virus, DNA purification, Southern blotting, determination of genotype, polymerase chain reaction (PCR), real time PCR, cloning, and sequencing. The HBV genome is often cloned into pCR-XL-TOPO, introduced into competent cells such as *E. coli* and kept at -70˚C for later studies. Site directed mutagenesis can be used to generate a specific virus mutant to be utilized to meet the criteria of the study being investigated. RT-PCR detects HBV virions in the cell culture supernatant. Günther *et al*. 1995 [54] introduced a novel technique to be able to analyse full HBV genome length in which genome is released from the clone by *Sap*I restriction endonuclease digestion, then transfected into HuH7 cell lines and this can generate an entire replication cycle with efficient viral protein expression. The technique was considered as a breakthrough in HBV molecular research. Clinical trials are

Table 2. Virological Characteristics of PC 1896 and BCP 1762/1764 Mutants [44]

Characteristics	PC Mutant (*n= 112*)	PC WT (*n= 270*)	BCP Mutant (*n= 124*)	BCP WT (*n= 156*)
Genotype A	2.1%	23.8%	9.9%	19.1%
Genotype B	56.9%	17.2%	14.9%	36.7%
Genotype C	40.9%	56.4%	74.4%	42.2%
Positive (+) HBeAg	30.1%	60%	54.8%	65.6%
Negative (-) HBeAg	69.7%	39.9%	45.2%	34.4%

PC= Precore, WT= Wild type, BCP= Basal Core Promoter.

valuable and may provide better prediction for the role of genotypes and mutations.

Table 3. Data of Some Serological Correlates and Disease Outcome and Genomic Mutations from 52 Patients with FH [52]

Characteristics	Group 1 (Survived) (*n= 18*)	Group 2 (Died) (*n= 34*)
(+)-HBeAg	5	12
PC (G1896A) mutants	7	17
BCP (1762/1764)	12	18
G1862T variant	1	6

FH= Fulminant Hepatitis.

RELATIONSHIP OF HBV GENOTYPES TO REPLICATION DISEASE OUTCOME

According to Sakamoto *et al.* 2006 [8], HBV/A prevalence was higher in CH patients compared to LC or HCC patients. HBV/C was significantly higher in LC and HCC. CH patients had higher HBeAg prevalence and they were mostly younger than LC patients. Frequency of PC mutation (A1896) did not show interesting difference among HBV/A. In HCC patients, HBeAg positivity, age and the frequency of PC mutation did not represent any significant differences. T1653 mutation was observed only in HBV/C (in Sakamoto *et al.* study) but not in HBV/A, and was higher in HCC than CH patients with HBV/C. 100 patients with different clinical status have been studied to show the relationship of different genotypes with the severity of liver disease (Table **4**).

In Sugiyama *et al.* 2003 study [39], HBV/C had the highest HBV DNA expression followed by Bj/Ba and then by genotypes A and D. In terms of phenotypic analysis, the genotypes Bj had the highest intracellular expression of core protein followed by HBV/C and subgenotype Ba. Extracellular expression of HBsAg was the highest for subgenotype Ae, then Aa and Ba using Northern Blot analysis. PreS/S mRNA expressed intracellularly in high level for Ae. Level of precore mRNA was high for Bj and C but lower in Ba. Intracellular DNA expression was significantly high for HBV/C but was very low for genotypes A and D, and lowest for Ae. HBV/A was significantly higher in asymptomatic patients whereas HBV/C is higher in HCC. Replication level in transfected HepG2 cells was high in genotype A than C and D [41]. Vietnamese LC patients were associated with genotype D.

Table 4. Comparison of Three HBV Genotypes and HBeAg Among Different Clinical Patients in Philippines [8]

Parameter	Genotypes			HBeAg
	A	B	C	
CH (*n=32*)	25 (78%)	4 (13%)	3 (9%)	30 (94%)
LC (*n=37*)	15 (41%)	7 (19%)	15 (41%)	21 (57%)
HCC (*n=31*)	11 (35%)	11 (35%)	9 (30%)	17 (55%)

DISCUSSION

HBV genotypes may induce varied liver disease but the virological differences are still to be defined and evidence behind this is becoming stronger nowadays. HBeAg secretion is affected by PC (A1896) mutation; this is why it is usually detected in HBeAg negative patients [20]. PC mutation prevalence was low and did not show any significant association with clinical status [46]; this is confirmed by Sakamoto *et al*. results [8]. It was also strongly evident that virus replication was not altered by PC mutation because HBeAg is not required for virus replication. Kobayashi *et al*. showed that patients infected with HBV PC WT are prone to clear the virus [49]. I perceive, the advantage of this is the large sample size (1077 patients) and long study duration (15 years). According to Tong *et al*. 2006 [44], 42% of the cirrhotic patients had PC 1896 mutant virus whereas the percentage of patients who developed HCC with PC 1896 mutation was 45% which is an indication of high risk to HCC. Significance of this mutation is still not obvious despite all related studies and further investigation is required. Table **2** elucidates that PC 1896 mutation is not 100% associated with negative HBeAg expression but its possible association with HBeAg negative status is verified. (+)-HBeAg was higher in PC mutants than PC WTs whereas (-)-HBeAg did not show significant variation among BCP mutants and WTs. The prevalence of PC WTs was higher in HBV/A. HBV/B on the other hand was associated with high PC mutant prevalence (Table **2**). Anti-HBe antibodies and (-)-HBeAg have given understanding of HBV biology. In (+)-HBeAg WT strain, anti-HBe can destroy hepatocytes and stop persistent infection; however, HBV can mutate into (-)-HBeAg to escape anti-HBe antibodies. Table **2** did not mention genotype D in its association with the prevalence of

either PC (1896) or BCP (1762/1764), but another study showed that BCP double mutation was less in HBV/D whereas A1896 was significantly higher in HBV/D compared to subgenotypes Aa and Ae [11]. All these data confirm the association of HBV mutations to disease severity and outcome.

BCP 1762/1764 mutants have strong evidence of its relation with HCC progression. This double mutation can be a bad indication to the infected individual due to its relation with HCC as presented in some previous studies [44]. Other reports showed that PC mutant is correlated with high viral load in (-)-HBeAg, and lower with BCP mutants [56]. Accordingly, Xu *et al.* 2011 exhibited opposite results in an investigation of 793 patients to understand the role of PC/BCP mutation [57]. Tong *et al.* 2006 [44] limitation is that BCP 1762/1764 analysis was not possible in some patients due to the low level of the virus DNA, but this does not weaken the evidence. As previously shown, data may not be agreeable. It is becoming apparent that triple BCP mutation is associated with higher replication efficiency and more severe illness than double mutation. This indicates the critical role of different patterns of mutations.

G1862T showed no effects on HBeAg expression [27, 53]. In Guarnieri *et al.* study [53] there was a reduction in the core protein expression. Conversely, they presented enhancement of the same protein for G1899A. Both 1862/1896 mutations reduced expression of HBeAg but the difference is that G1862T reduced virus replication; however, G1896A did not. Interestingly, association of 1899 mutation with G1862A greatly enhanced the replication. G1862T mutation should motivate researchers due to its vague effect by its own or by association with other mutations and because there are quite a few research on G1862T. This mutation can be associated with fulminant hepatitis (Table **3**). G1899A mutation has an interesting effect on the pair of 1855 position. These results suggest that investigation of the nucleotide position 1899 becomes mandatory in HBV molecular research due to its unfathomable influence on replication. Extracellular and intracellular expression among genotypes were not consistent according to Sugiyama *et al.* [39] and some observations may indicate that it is somewhat troublesome to link a particular genotype or mutation with certain degree of liver disease, but other mutations are confirmed by agreement and consensus though some mutations may show significant association, for instance T1653 mutation observed by Sakamoto *et al.* [8] is a predictive factor for HCC in patients infected with HBV/C. This factor can be added with genotype C as risk to HCC. Although pre-S mutation studies are limited, it is expected as a factor of infection chronicity [51]. For example, T3098C in the preS1 region and T53C were associated with HCC but further investigation is essential [40]. C1753 with 1762/1764 is indicative of severe liver disease [46]. Other stressors may negatively exacerbate the clinical status like the presence of C1753 with BCP double mutation. According to Biswas *et al.* 2011 observation, C1753 is becoming more common among newly infected patients. To analyze the difference between T1858 and C1858 mutations and their association with unanticipated factors, G1896 strengthens T1858 pairing [49] but replication capacity was equal in C1858 and T1858 [58]. The type of precore mutation detected is influenced by HBV genotype. G1896 is less common in HBV/A because the nucleotide at 1858 is a C. If 1858 is a T, the nucleotide at 1896 is able to mutate to an A, and maintain stability of the epsilon stem loop [55]. Interestingly, T1858 prevalence was 100% in patients infected with genotype D (*n= 61*) compared to 0% for subgenotypes Aa and Ae (*n = 54; n= 57*) respectively [11].

HBV/A expression was low in Sugiyama *et al.* study; on the other hand, replication level in HepG2 cells was high for genotype A according to Toan *et al.* [41]. The corresponding results for the same genotype were contradicted. Genotype A is becoming common in Vietnam [41] compared to previous studies showed that genotypes B and C are the most common in Asia including Vietnam. The limitations of Sugiyama *et al.* study [39] is the low sample size and did not use PC/BCP mutations, but they studied *in vivo* and *in vitro* experiments and it was a rare comparative study of many genotypes. Toan *et al.* [41] paper was significant because 460 patients were studied from different countries (Vietnam, Europe, and Africa). Although HBV/B encompassed the majority of participants in Yang *et al.* paper [20], they argued that HBV/C was associated with high risk of HCC. Yang *et al.* [20] discussed other factors like cigarette smoking and alcohol drinking. Their large sample size is another advantage of the study. Sakamoto *et al.* [8] exhibited that HBV/A and B were associated with 35% of HCC cases compared to 30% to genotype C in Philippines (Table **4**). There is a consensus that HBV/C is the most common genotype involved in HCC [19, 35, 50], thus, it is recommended to investigate genotype C especially in the field of antiviral research to prevent the fatal sequelae.

The association of HBV infection with other common viruses that are known to infect the liver such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) could have deceitful effect. It is very unlikely and seldom discussed in scientific HBV research albeit the role of some mutations alone is identified. The presence of other hepatitis viruses with HBV for long period of time might have a role in the replication and disease progression. One difference between *in vivo* and *in vitro* studies is the immune response in the infected individuals, and this should be taken into account. Another drawback is the infection duration difference i.e. the long period of infection inside human hepatocytes compared to few days of the *in vitro* infection model. The effect of viral load should be considered as well. Alcohol abuse, iron level, history of toxic or drug usage and impaired metabolisms must also be considered in the interpretation as factors for chronic liver disease. Some previous studies did not discuss the cell lines they are using as an interfering factor, for example, different cell lines produce different HBV DNA level for the same HBV variant, and thus it is preferred to compare the type of cell line in the replication studies. The subgenotypes are not discussed in some original reports; these subgenotypes within the same genotype (like Bj and Ba) have sometimes immense disparity in their effect on either replication or progression of infection. Skeptical observations lead to controversialism in some mechanism of the replication and the biologic function of HBeAg.

Comparative studies between distinct HBV genotypes are difficult due to environmental and host differences [55]. Another factor that makes these studies difficult is the various geographical distributions [50], the considerable mutations being analyzed and the emerging mutations such as A1899 and C1753. Inevitably, the very low prevalence of some newly emerging mutations is one of the interfering factors in HBV molecular studies. As can be seen, the exact effect of certain mutations could be perplexing, but future studies will provide full understanding. Classification is one significant issue in this context. HBV mutations change this classification, for example C1 and C2 subgenotypes classification were reported interchangeably by two studies [7, 32] and this will lead to confusion [12]. In addition, Norder *et al*. reported four subgenotypes for HBV/C, two years later; Sakamoto *et al*. showed five subgenotypes (C1- C5), therefore precise HBV designation is very significant [12].

In summary, HBV resolution can be facilitated by the presence of WT virus G1896 in CH patients with (+)-HBeAg. It can be concluded that BCP mutation (1762/1764) and genotype C are associated with high risk of HCC. As mentioned in the introduction, new genotypes and subgenotypes are being discovered, thus, it makes these studies difficult to accurately link specific genotypes and mutations with certain liver disease or *in vitro* replication study. HBeAg negativity might be associated with severe liver damage as in mutations affecting HBeAg expression. Future studies are valuable to determine the pathogenic outcome. More research will be needed considering more than one factor with more genotypes to reach agreement and to decipher uncertainties about the precise role of certain mutations and genotypes on either replication cycle or liver pathogenesis.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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