

Genomic Diversity of Hepatitis B Virus Infection Associated With Fulminant Hepatitis B Development

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Context: After five decades of Hepatitis B Virus (HBV) vaccine discovery, HBV is still a major public health problem. Due to the high genetic diversity of HBV and selective pressure of the host immune system, intra-host evolution of this virus in different clinical manifestations is a hot topic of research. HBV infection causes a range of clinical manifestations from acute to chronic infection, cirrhosis and hepatocellular carcinoma. Among all forms of HBV infection manifestations, fulminant hepatitis B infection possesses the highest fatality rate. Almost 1% of the acutely infected patients develop fulminant hepatitis B, in which the mortality rate is around 70%.

Evidence Acquisition: All published papers deposited in Genbank, on the topic of fulminant hepatitis were reviewed and their virological aspects were investigated. In this review, we highlight the genomic diversity of HBV reported from patients with fulminant HBV infection.

Results: The most commonly detected diversities affect regulatory motifs of HBV in the core and S region, indicating that these alterations may convert the virus to an aggressive strain. Moreover, mutations at T-cell and B-cell epitopes located in pre-S1 and pre-S2 proteins may lead to an immune evasion of the virus, likely favoring a more severe clinical course of infection. Furthermore, point and frame shift mutations in the core region increase the viral replication of HBV and help virus to evade from immune system and guarantee its persistence.

Conclusions: Fulminant hepatitis B is associated with distinct mutational patterns of HBV, underlining that genomic diversity of the virus is an important factor determining its pathogenicity.

Keywords: Hepatitis B Virus; Liver Failure, Acute; Human Genome Project

1. Context

1.1. Fulminant Hepatitis

Fulminant hepatitis, also known as Fulminant Hepatic Failure (FHF), is a critical illness with a high mortality rate, reaching up to 80% (1). It is a rare condition, in which rapid destruction of the liver parenchyma leads to coagulopathy, altered mental status and subsequently multiorgan failure in an otherwise healthy individual (2). Owing to the advances in the field of liver transplantation, antiviral therapy and critical care management, survival rates have been substantially improved to more than 65% (3).

Over the years, there have been several different definitions of fulminant hepatitis or acute liver failure. The most widely accepted definition is proposed by the American Association for the Study of Liver Diseases (AASLD), which includes evidence of coagulopathy (International Normalization Ratio [INR] ≥ 1.5) and presence of encephalopathy in a patient without pre-existing cirrhosis and with an illness of less than 26 weeks duration (2). In 1968, Trey and

Davidson first introduced the term FHF to describe the onset of altered mental status within eight weeks of initial symptoms with no pre-existing liver disease (4). Two decades later, Bernuau et al. suggested that the term FHF would describe cases where encephalopathy developed within two weeks of the onset of jaundice and that sub-fulminant hepatic failure, cases where encephalopathy developed between 2 weeks and 3 months after the onset of jaundice (5). The acute liver failure (ALF) term was proposed by O'Grady et al. who further classified liver failure into three groups of (i) hyper-acute (onset within 1 week), (ii) acute (onset between 8 and 28 days) and (iii) sub-acute (onset between 29 days and 12 weeks). The latter classification was established based on the survival rate of the groups (6). Still, this classification is no more valid since a recent study has shown no prognostic significance distinct from the cause of the illness (3). In addition, patients in whom hepatic encephalopathy appeared between 8 and 24 weeks after the first symptoms are classified as having Late-Onset Hepatic Failure (LOHF) (7).

Some of the apparent factors that cause FHF are drug

induced liver injury, viral hepatitis, autoimmune liver disease and liver shock, whilst many FHF cases have no definable cause (3). In the present review, we assessed FHF caused by hepatitis B virus (HBV) infection, referred as fulminant hepatitis B (FHB).

1.2. Fulminant Hepatitis B (FHB) Infection

HBV infection leads to a wide spectrum of liver diseases, spanning from acute to chronic hepatitis, cirrhosis and Hepatocellular Carcinoma (HCC) (8). Only 1% of acutely infected patients develop fulminant hepatitis, which has a mortality rate of around 70% (9). Although there are few reports on the role of other hepatotropic viruses, HBV is probably the most common viral cause of FHF in most regions of the world (10). In developing countries, fulminant viral hepatitis, and particularly acute HBV infection, is the predominant cause of FHF. On the contrary, acetaminophen or other drug-induced hepatotoxicity is the most frequent cause of ALF in western countries (11, 12).

Acute exacerbation of chronic HBV infection can be also classified as a form of FHF. This acute deterioration can be related to precipitating events, such as chemotherapy or immunosuppressive agents, which can induce severe flares of hepatitis and lead to fatal fulminant hepatitis. To prevent HBV reactivation in inactive carriers, guidelines strongly suggest strict follow-up and careful monitoring before and during immunosuppressive therapy (13). FHF in the setting of HBV reactivation has a much higher morbidity and mortality rate than de novo fulminant hepatitis (14). In addition, Hepatitis D Virus (HDV) superinfection of a chronic HBV patient can also result in FHF (15). As soon as an individual shows signs of coagulopathy, jaundice or encephalopathy, he or she should be hospitalized and transferred to a Liver Transplantation (LT) center. Furthermore, antiviral therapy should be administered to the patient to prevent the recurrence of HBV after transplantation and then treatment has to be continued indefinitely (11). Deterioration of liver function in a patient with HBV-related liver cirrhosis belongs to a different disease entity, which is nowadays classified as "Acute-on-Chronic Liver Failure" (ACLF) (16).

1.3. HBV Genome Organization

HBV is a DNA virus with a genome length of only 3.2 kb (17). Its genome is a partly double-stranded circular DNA organized into four overlapping Open Reading Frames (ORF) including S for the surface gene, C for the core gene, P for the polymerase gene and X for the X gene. The S ORF encodes three envelope proteins, the large, medium and small (HBsAg), which are synthesized by starting transcription with pre-S1, pre-S2 or S gene, respectively. The C ORF encodes two proteins, "e" antigen (HBeAg) and core antigen (HBcAg) transcribed from precore (PC) RNA and pregenomic RNA, respectively. Production of both PC and pregenomic RNA is controlled by Basal Core Promoter (BCP) elements. HBeAg is a non-structural protein, which is secreted by infected he-

patocytes and serves as a marker of active replication and as an immune modulator. HBcAg is the nucleocapsid that encloses the viral DNA together with the polymerase. The X protein acts as a transactivator on HBV enhancer assisting virus replication (18, 19). The DNA molecule of HBV consists of a complete strand (L or minus), which is covalently attached to viral polymerase at the 5'-end and of an incomplete strand (S or plus), about two-thirds complete (19).

2. Evidence Acquisition

2.1. HBV Genome Diversity and its Sources

HBV DNA replicates via the Reverse Transcriptase (RT) enzyme, which lacks proofreading activity. Due to the error-prone nature of the RT enzyme, HBV has a relatively high substitution rate compared to other DNA viruses. The average daily production rate of HBV is up to 10^{11} virions per day, with an estimated error rate of $1.4 - 3.2 \times 10^{-5}$ nucleotide substitutions per site per year. Therefore, HBV exists as a quasispecies population, in which eventually a predominant strain is selected by endogenous (host immune system) and exogenous factors (antiviral therapy and vaccination). Likewise, many variations can be detected on nucleotide as well as on protein level across HBV genome (20, 21). The resulted HBV variants seem to contribute to viral pathogenic persistence and therapeutic limitations like antiviral treatment and vaccination (22). Because of this heterogeneity, HBV has a genetic classification, in which different HBV genotypes, subgenotypes and subtype (serotype) have been defined (23, 24).

2.2. HBV Classification

Human HBV is the prototype member of *Hepadnaviridae*, which include two recognized genera, the *Orthohepadnavirus* and the *Avihepadnavirus* (25). Phylogenetic analysis has classified HBV into eight genotypes (A - H), defined by an intergroup nucleotide divergence greater than 7.5% over the complete genome sequence (26). In addition, multiple subgenotypes have been recognized within genotypes (almost 40 subgenotypes), with more than 4% and less than 7.5% divergence over the full-length genome (19, 27). HBV "clades" are used as a subdivision within subgenotypes, presenting less than 4% nucleotide diversity over the complete genome sequences (23, 26). Due to several insertions and deletions among the genome, HBV genotypes have different genomic lengths (28).

HBV genotypes have a distinctive geographical distribution around the world (24). For example, genotype A is prevalent in northwestern Europe, USA and Africa. Genotypes B and C are restricted in Asia; genotype D predominates in the Mediterranean area, but is also found worldwide. Genotype E is prevalent in the African continent, genotype F in Latin America and genotype H in the indigenous population of Central America (29). The very rare reported genotype G has been recently found all around the world (19).

Prior to molecular classification, HBV strains were classified into four major HBsAg subtypes (serotypes) based on immunological heterogeneity of HBsAg (30). This subtype grouping is based on different HBsAg epitopes, present on the Major Hydrophilic Region (MHR), which spans between amino acid residues 99 - 169 (31). This part possesses a major immunogenic region called “a” determinant domain (amino acid 124 - 147) (19). The four main serotypes are *adw*, *ayw*, *adr* and *ayr* (32). A correlation between HBV subtypes and certain genotypes has been observed (33). In addition, genotype and serotype association often displays a characteristic geographical distribution (18, 34).

2.3. HBV Genotypes and Disease Progression

Recent studies suggested an association of different HBV (sub) genotypes with the clinical course of hepatitis. For instance, virus strains of subgenotype A2 have been frequently detected in acute forms of HBV infection (35). Other reports have shown that acute HBV infection with genotype A may increase the risk of progression to chronic hepatitis compared to other genotypes (36). Moreover, patients infected with genotype C have a greater frequency and severity of liver dysfunction compared to genotype B (27). It has been reported that subgenotype C2 increases the risk of HCC development compared to C1 (37). In general, genotypes C and D are found to be more often associated to liver cirrhosis and HCC than genotypes A and B (38). Among the genotype B infections, subgenotype B6 strains are commonly related with a mild clinical outcome, while B1 strains lead more often to acute and fulminant hepatitis B (39). Another interesting aspect is that patients infected with genotype F show lower survival rates than those infected with genotype A or D (40). In addition, genotype F infections detected in the Amazonian basin are associated with fulminant hepatitis in the context of HDV co-infection (41). Likewise, in the USA, genotype D infection has been identified as an independent risk factor for fulminant hepatitis (42).

Interestingly, a significant number of patients infected with genotype A or D were found to be anti-HBe antibody positive, while those infected with genotype D showed higher levels of serum Alanine Transaminase (ALT) (43). In addition, genotype D has been frequently reported among inactive carriers of HBsAg (44). Patients infected with genotypes A and B have reported to show a higher HBeAg seroconversion rate and normalization of serum ALT levels after interferon-alpha treatment than those infected with genotype C or D (45). Likewise, following pegylated interferon-alpha treatment, only genotypes A and B responded with HBeAg seroconversion and substantial decrease in HBsAg serum titers, while in patients with genotypes C and D, no change was observed (46). Moreover, it has been observed that patients infected with genotype A can develop an antiviral resistance earlier than genotype D. In a similar way, it has been re-

ported that genotype B strains are more likely to develop resistance against lamivudine antiviral than genotype C strains (27).

3. Results

3.1. Genome Diversities Related to Clinical Outcome

Most naturally occurring mutations on HBV genome are located at the C ORF. The dual BCP mutation A1762T + G1764A results in a down regulation of HBeAg synthesis, and the PC G1896A stop-codon mutation prevents the expression of HBeAg. Another PC mutation A1899G has been reported in combination with G1896A, which stabilizes the lower stem of the “ε” encapsidation signal and enhances the replication. The presence of both BCP and PC variants has been reported in severe liver cirrhosis and HCC. Several mutations, including deletions and insertions or stop codons have been reported in the S ORF of the HBV genome (47). Deletions or missense mutations in the Pre-S2 region can abolish the synthesis of the protein and alter B and T cell epitopes (19, 48). Moreover, HBsAg insertion or deletion or missense mutations can help the virus to evade host immune response (19). Truncated HBV surface proteins can contribute to the chronicity of HBV infections (49). Furthermore, a pattern of hotspot mutations located at the X region of the HBV genome (xI127T, xK130M, xV131I and xF132Y) are also known to be associated with transactivating function and HCC development (50).

3.2. HBV Genome Variations Related to Fulminant Hepatitis B Infection

It has been hypothesized that both viral and host factors play a role in the pathogenesis and clinical outcome of HBV infection and that FHF develops when there is an overwhelming immune-mediated lysis of infected hepatocytes (51). It has been suggested that FHB can be explained by three main virologic markers as (i) an increase in viral replication fitness, (ii) a change in viral gene expression and/or (iii) an alteration of B and T cell epitopes (52). These factors are discussed below in detail and summarized in Table 1.

3.2.1. (i) Pre-S and S Gene Variations Associated to FHB

During the course of fulminant hepatitis infection, several in vitro studies have isolated certain HBV variants with Pre-S/S gene mutations. A study on FHB strains identified a hepatitis B immune globulin (HBIG)-escape mutant sG145R on the HBsAg, being responsible for about 30% inhibition of virion secretion and increased replication compared to wild type strains (53). In addition, mutations in CAAT element of the S promoter have shown to cause a reduction in the S protein level and intracellular viral retention (54). Many other vaccine and/or HBIG escape mutations, as well as insertions in the “a” determi-

nant of the HBsAg, have been detected to cause a defect in viral particle secretion and an alteration of host immune system epitopes (53, 55). Moreover, an association between pre-S mutants and fulminant hepatitis has been reported. It is hypothesized that since B and T cell epitopes located on both pre-S1 and pre-S2 proteins, this may lead to an immune evasion of the virus and a more severe infection (56, 57). Of note, mutations along the pre-S/S gene may induce retention of the surface proteins in the Endoplasmic Reticulum (ER) of the hepatocytes, resulting in an ER stress that may induce oxidative DNA damage and genomic instability (58).

3.2.2. (ii) BCP, Precore and Core Gene Variations Associated to FHB

Many evidences suggest that FHB is strongly associated with HBV strains that do not produce HBeAg due to either BCP or PC mutations. During a study, HBV DNA clones were propagated from FHB patients and sequenced within the BCP and PC region. Interestingly, a significant number of clones carried the PC G1896A stop-codon and G1899A mutations as well as the dual BCP A1762T + G1764A mutation (59). The precursor protein of HBeAg decreases the encapsidation of the pregenomic RNA. Therefore, an absence or decrease of HBeAg synthesis can lead to enhanced viral replication and consequently increased host immune response (60). Investigators have shown that the double A1762T + G1764A mutation can slightly

increase viral DNA replication, while the C1766T + T1768A mutational pattern exhibits a 10-fold higher replication capacity than a wild type strain (61). Importantly, in vitro experiments indicate that mutations can synergistically influence HBV replication. For instance, BCP and PC can further enhance the replication of G145R and other “a-determinant” mutants (62).

Hepatocyte Nuclear transcription Factors (HNF) can regulate the function of HBV promoters and enhancers. They act on nuclear receptor binding sites, which are present in the Enhancer 1 (EN1), Enhancer 2 (EN2) and in the BCP region of the HBV genome. Thus, mutations in the enhancer elements and core promoter region can alter the HNF's binding sites or even favor the formation of new binding sites (63). In a Swedish study, both dual BCP mutations, A1762T + G1764A and G1764T + C1766G, were affected by the emergence of another BCP mutation at the position 1757. It has been suggested that both BCP variants form putative new binding sites Hepatocyte Nuclear Factor 3 and 1 (HNF3 and HNF1), respectively. As a consequence, patients who carried the above core promoter variants had higher viral load and serum aminotransferase levels than patients who did not (64). Similarly, in a case study of HBV reactivation after receiving immunosuppressive therapy, which was conducted in France, an 11-bp insertion (between 1775 - 1776) on the BCP region has been detected. The insertion has led to the formation of a novel HNF1 binding site and subsequently an enhanced viral replication and fulminant hepatitis (65).

Table 1. Potential Variations of the HBV Genome Associated With Fulminant Outcome and Their Effects^a

ORF	HBV nt or aa Variation	Effect
C		
BCP/PC	T1753C/A/G, T1754C/G	decreased HBeAg production , enhanced replication
	A1762T + G1764A	HNF3 binding site formation, decreased HBeAg production enhanced replication
	G1764T + C1766G	HNF1 binding site formation, enhanced replication
	C1766T + T1768A	enhanced replication
	11bp insertion: 1775 - 1776	HNF1 binding site formation, enhanced replication
	T1825C + A1827C	may affect the HBV life cycle
	G1896A + G1899A	abolished HBeAg expression, enhanced replication
	G1862A	decreased HBeAg production enhanced replication
Core	T1961C/A/G C1962D	T cell epitope alteration
	A2339G	T cell epitope alteration, enhanced replication
X	xI127T, xK130M xV131I, xF132Y	“hotspot” mutations may enhance or disrupt the replication
S		
Pre-S1/Pre-S2	Pre-S insertion, deletion or missense mutations	defective Pre-S proteins, T and B cell epitopes alteration, imbalance of S protein synthesis and intracellular retention, cytotoxicity
HBsAg	Immune escape mutations sM125T, sT127P, sG145R	vaccine and HBIG therapy failure, reduced virion secretion, enhanced replication
P	RT mutations pR/W153Q, pL180M + pM204V	restored HBV replication, LAM resistance

^a Abbreviations: ORF, Open Reading Frame; nt, nucleotide; aa, amino acid; RT, Reverse Transcriptase; LAM, Lamivudine.

Other mutations including T1753C/A/G, T1754C/G and G1862A in the BCP and PC have also been reported in FHB infection. It has been speculated that these variants cause fulminant hepatitis by increasing HBV replication and reducing HBeAg production (66, 67). An enhancement of HBV replication in parallel with reduced or abrogated HBeAg expression could probably trigger host immune response and lead to extensive hepatic injury, like fulminant hepatitis (68). Two nucleotide substitutions, T1825C and A1827C, situated on a well conserved 11 bp sequence of Direct Repeat-1 (DR1) were also reported to be implicated with FHB. These variants may affect the virus life cycle, since DR1 plays a pivotal role on the HBV replication (56).

Certain mutations in the core gene at nucleotides 1961 and 1962 were found to be associated with fulminant hepatitis as well. These mutations could change the amino acid S21, located in the HLA-A2 restricted Cytotoxic T Lymphocyte (CTL) epitope, spanning from amino acid 18 - 27, and favor the persistence of HBV (39). A mutation A2339G, which corresponds to core protein codon 147 located in the CTL epitopes, could also favor HBV persistence. The A2339G mutation was shown to enhance the replication of HBV in vitro and being associated with the inhibition of a furin-like protease. This results in a higher expression of complete core protein, which could function as a trans-acting regulator of HBV replication (69).

3.2.3. (iii) Pol Gene Variations Associated to FHB

The HBV genome is organized in a way that the envelope (S) gene is completely overlapped by the polymerase (P) gene. Therefore, substitution in the S gene has a corresponding alteration in the polymerase gene and *vice versa* (19). For instance, the escape mutation sG145R in the HBsAg protein corresponds in a change on the HBV RT region of the polymerase at codon rtR/W153Q. This RT mutation was found to partially restore the in vitro replicative capacity of a lamivudine-resistant rtL180M + M204V HBV strain (70). The emergence of multidrug-resistant HBV has been reported during antiviral therapy alone or in combination with HBIG therapy. These HBV resistant variants may lead to reactivation of the virus in a liver transplant recipient and development of fulminant hepatitis B infection (71, 72).

3.2.4. (iv) X Gene Variations Associated to FHB

Because of the overlap between HBV X and core region "hotspot" mutations (xI127T, xK130M, xV131I and xF132Y) affecting both proteins could arise. In detail, the mutations T1753C/A/G, A1762T, G1764A, C1766T and T1768A in the C region result in amino acid changes xI127T, xK130M, xV131I and xF132Y in the X protein, respectively (50). Since the X gene overlaps with the BCP and enhancer II complex, these "hotspot" mutations may enhance or disrupt the replication and expression of HBV and lead to fulminant hepatitis (73).

3.3. Fulminant Hepatitis B After Immunosuppression

HBV reactivation or exacerbation related to immunosuppressive therapy is an increasing clinical problem, which requires special attention (74). The recurrence of HBV infection can be observed in both inactive HBV carriers and occult, which are undergoing immunosuppressive chemotherapy for cancer. With the same manner, immunosuppressive agents given to patients with autoimmune diseases can also increase the risk of HBV reactivation (75). For instance, the anti-inflammatory drugs adalimumab (trade name Humira®) or infliximab (trade name Remicade®), which bind to tumor necrosis factor- α (TNF- α), have been approved in the USA by the Food and Drug Administration (FDA) for the treatment of several autoimmune diseases. Since TNF- α is an important pro-inflammatory cytokine of the immune system, adalimumab or infliximab treatment may lead to serious viral infections and reactivations, which can result to life threatening situations like fatal fulminant hepatitis B (76). Immunosuppression may allow the virus to proliferate, which extends to increased HBV DNA levels and HBeAg serum levels. Thus, stopping the therapy with immunosuppressive agents reactivates the host immune system and results in a rapid destruction of the infected hepatocytes. The emergence of anti-TNF- α agents as a key therapeutic option for autoimmune conditions has been associated with increased HBV reactivation cases (77). Based on this observation, current guidelines recommend preemptive therapy of anti-HBc positive individuals to prevent HBV reactivation as well as continuous antiviral treatment in patients with concomitant chronic HBV infection (78).

3.4. Fulminant Hepatitis B Treatment Options

FHB patients require strict virological surveillance to establish an early management of the severe condition and its complexities. As soon as an individual shows signs of coagulopathy, jaundice or encephalopathy, the patient should be managed in an Intensive Care Unit (ICU) and transferred to a Liver Transplantation (LT) center (11). Whether a patient would undergo liver transplantation depends on the probability of spontaneous hepatic recovery, which can be estimated by variables like the degree of encephalopathy, patients age and the cause of fulminant hepatitis (79). While no treatment is indicated for mild acute hepatitis B, there are compelling arguments that antiviral therapy (e.g., with lamivudine, tenofovir or entecavir) should be started in patients with signs of significant liver impairment (e.g., INR > 1.5). For instance, in severe, but non-fulminant hepatitis B, antiviral therapy may significantly accelerate the recovery (80, 81). Due to the rarity of fulminant hepatitis B, only limited data exist on the efficacy of antiviral therapy in FHB. Small studies on this condition, mostly conducted with lamivudine, indicated that antiviral therapy can be advantageous at least in a subgroup

of patients with FHB (82, 83). Even though antiviral therapy may not always alter the clinical course and avoid transplantation, it can still reduce the risk of reinfection in case of liver transplantation (84).

Orthotopic Liver Transplantation (OLT) is the only therapeutic option proven to improve the survival rates for patients already in advanced liver failure. During the last decade, OLT has helped to improve the survival rates of FHF patients from 15% to about 60 - 80% (3). The clinical outcome as well as the graft survival in HBV LT recipients has been improved. This significant step in LT is particularly an effective strategy of HBIG prophylactic therapy alone or in combination with preoperative antiviral agents followed by indefinite treatment (85). However, the emergence of nucleot(s) ide resistant mutants in the polymerase and HBIG escape mutants in the surface gene can lead to an adverse clinical outcome and a graft failure. Post-OLT follow-up studies have recognized a number of mutations related to HBV relapse and subsequent graft loss, like pI533L on the polymerase due to HBIG therapy and pL533L, pS559T, pM550I amino acid changes due to lamivudine antiviral therapy (71).

4. Conclusions

In summary, HBV infection is considered as one of the main causes of fulminant hepatitis in humans. Clinical manifestations of this severe type of infection are the consequence of interaction between virus and host immune system. In spite of the fact that HBV genotypes and subgenotypes show distinct clinical outcomes, all HBV variants have been reported in FHF context. FHB isolates present particular diversities within hepatitis viral genome, which may enhance viral invasiveness. These variants can lead to an increased HBV replication, altered immune epitopes and modified viral gene expression. Therefore, understanding HBV genomic diversity in FHB patients, will be useful for further studies regarding the management of FHB infections.

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Authors' Contributions

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