The Importance of Hepatitis B Virus Genome Diversity in Basal Core Promoter Region

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ABSTRACT

Hepadnaviruses employ an unusual strategy for the production of enormous number of virions during replication which makes rapid and substantial genetic sequence changes and alterations. The pathogenesis and clearance of hepatitis B virus (HBV) infection are engaged by the selection and expression of viral mutants during virus-host interactions. Mutations in regulatory regions such as the basal core promoter (BCP) which is thought to be related to lower production of hepatitis B “e” antigen (HBeAg) directly affects the clinical presentation of liver disease. However, the molecular structure of these mutations in chronic carriers has not been adequately evaluated. In this review we evaluate the molecular aspect and pathologic basis of basal core promoter mutations.

KEYWORDS
Hepatitis B virus mutation; Basal core promoter; HBV RNA; Protein x

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is associated with over 50% of hepatocellular carcinoma (HCC) incidence worldwide, and specific viral factors may increase the risk of HCC development.¹-² The HBV genome may undergo mutations during the course of chronic infection and accelerate progression of liver disease. Of the most important mutations in the context of HCC are those which occur in the basal core promoter (BCP) regions³ that may up-regulate HBV expression and have been found to be independent risk factors for HCC.⁴-⁶ HBV basal core promoter contains binding sites for a variety of transcription factors of the nuclear receptor super family. The significance of mutations in BCP has been thought to be related to loss of nuclear receptor binding sites and simultaneous gain of new liver transcription factor binding sites.⁷ Furthermore, BCP mutations not only change amino acids in the overlapping protein-x, they also structurally alter the stability of HBV RNAs and consequently its ability for HBV replication. The reason for selection of mutations such as the BCP mutations in the HBV genome is not fully understood. However viral and host factors, in addition to exogenous selection pressure, may partly be responsible for the predominant HBV species in an infected individual.
The absence of proof-reading ability of HBV accompanied by high rates of virus turnover provides the possibility for large numbers of quasi species of virus. Moreover the antiviral activity against HBV by the APOBEC3 family (an apolipoprotein B messenger RNA editing enzyme with cytidine deaminase activity) has been reported for hypermutation in the HBV genome. Structural analysis of BCP mutations can lead to better understanding of the clinical significance of these mutations and clarify the effect of viral factors in chronic HBV infection.

Current concept of HBV replication
The HBV genome compromises a 3.2 kb partially double-stranded DNA that replicates through an RNA intermediate, using its own encoded reverse transcriptase (RT). The HBV genome is organized into four overlapping open-reading frames that encode the envelope (preS/S), core (pre-core/core), polymerase (p) and protein-x (x). Once the virus is inside a cell, viral cores disassemble and the viral genome is delivered to the nucleus where it is repaired to become covalently closed circular DNA molecules (cccDNA). The HBV cccDNA molecule becomes a viral mini chromosome that is highly stable in hepadenavirus replication and is present in multiple copies in the nucleus of each infected cell. The HBV mini chromosome is the key template for transcription of four sets of HBV RNAs which is directed by several promoters in the viral genome.

In regulation of viral replication, enhancer II and core promoter of the HBV genome control the transcription of the x gene, pre-core mRNA and pregenomic RNA (Figure 1). Due to the overlap of the core promoter with the x gene, mutations in the BCP region potentially alter the structure and function of protein-x in developing chronic hepatitis.

Generation of HBV variants
A look at infected individuals and their older generation with the HBV variant shows that virions differ from one generation to another. Variability in the HBV genome is produced by many unknown functions, particularly by the correlation of viral replication which is the predominant power for natural selection of HBV variants in infected individuals.

HBV polymerase enzyme is an important factor in viral replication that does not have a proof-reading function. Consequently they are inherently error prone for the existence of the G to A hypermutated HBV genome that is likely to be generated by the substitution of U instead of C during reverse transcription of the pregenome RNA. Another reason for the high mutation rate in the HBV genome may be explained by high viral loads with turnover rates of about 25-50% of circulating virus per day along with reduced replication fidelity which seems to be related to alterations in the circulating pool of HBV. As mentioned before, the possible
importance of host cell conditions for the occurrence of viral mutation should also be noted. It has been demonstrated that the cellular cytosine deaminase activity of the family of APOBEC3 proteins induced G to A hypermutation in the HBV genome as a consequence of strong immunological host response against the virus for HBeAg clearance in addition to reduction of viral replication.\textsuperscript{13,19-20}

Structure and function of BCP

An important event in the HBV viral life cycle is regulation of transcription of the hepatitis B virus core promoter for viral replication. This promoter is composed of the BCP which is sufficient to direct initiation of both pre-core and pregenomic RNAs, and upstream regulatory sequence with a stimulatory effect on BCP activity.

Unlike many mammalian promoters, the BCP sequence failed to expose the presence of TATA or CAAT elements which is recognized by a short sequence of base pairs rich in adenine (A) and thymidine (T) residues and located about 25-30 nucleotides upstream of the transcriptional initiation site. However AT-rich regions located in the region of nucleotides 1744 to 1804 of the HBV genome play a role in the formation of a preinitiation complex containing the RNA polymerase holoenzyme.\textsuperscript{7,21}

BCP contains major nuclear binding sites which is recognized by a variety of the nuclear receptors super family, including hepatocyte nuclear factor 4 (HNF4) and the peroxisome proliferator-activated receptor $\alpha$-retinoid X receptor $\alpha$ (PPAR$\alpha$-RXR$\alpha$) heterodimer and a series of transcription factors such as CCAAT/enhancer binding protein (C/EBP) to regulate the transcription both the pre-core RNA and the core RNA.\textsuperscript{7,21-22} Different biological functions of pre-core and pregenomic RNA, as well as differential regulation of BCP by these transcriptional factors may have profound effects on HBV replication and pathogenesis.

Frequent mutations in BCP

Different HBV genotypes show various patterns of BCP mutations, of which a number of these mutations are frequently seen in some of the genotypes or subgenotypes. Substitution of G to A at nucleotide 1757 in the BCP region, has been found to affect the emergence of BCP double mutants and predict relative genotypic features in genotype D.\textsuperscript{6,23} The most frequent changes happen from G to T at nucleotide 1764 and from C to G at nucleotide 1766 and are generally in association with A1757 that affects the second AT-rich region.\textsuperscript{21,24} A double mutation (G1764T/C1766G) has been suggested to form a putative new binding site for the transcription factor HNF3. The frequent double mutant A1762T/G1764A in the BCP region is also the most common mutation prior to seroconversion to anti-HBe, which results in defective pre-core synthesis.\textsuperscript{7,25-26} These mutations create high affinity HNF1 sites and are highly prevalent in HBV patients with chronic liver disease who develop cirrhosis or HCC.\textsuperscript{25} Furthermore the T1753V mutation in BCP which has a lower frequency is associated with a statistically significant increased risk of HCC.\textsuperscript{25}

There are some rare types of BCP mutations, including point mutations alone or in combination with nucleotide insertions or deletions; mostly related to reduced HBeAg synthesis. A deletion of nucleotides 1763-1770 generates a low affinity HNF1 binding site and the C1766T/T1768A variants with very weak affinity for this binding site are predominantly found in asymptomatic and HBeAg negative patients.\textsuperscript{21}

Clinical aspect of BCP mutations

Core promoter mutants produce less pre-core mRNA and HBeAg both in individuals and in cell cultures.\textsuperscript{3,24} BCP mutations affect major binding sites usually for the generation of a novel HNF1 site, a strong liver-enriched
transcription activator, which could be considered as an efficient way to enhance HBV replication. On the other hand, G1757A substitution is widespread in HBV genotype D, more frequent in younger patients with lower ALT and viral load level and has been found that repressed the binding site HNF1 that is created by A1762T/G1764A. Therefore it is possible that early development of G1757A either directly or indirectly reduces the oncogenic potential of HBV.

As reported, A1762T/G1764A often occurs approximately ten years prior to the diagnosis of HCC and could be an early event in hepatocarcinogenesis, whereas T1753V mutations and combined mutations are late events. In a previous study we have shown that T1753V and A1762T/G1764A are increasingly more prevalent in chronic HBV infection with advanced liver disease. It was also revealed that the G1757A substitution might be a protective biomarker in chronic hepatitis B.

Among several mutational “hot spots” on the HBV genome, A1762T and G1764A mutations within the BCP region can be related to the severity of HBV infection, however fewer T1753C (A/G) mutations were noted in previous studies. A mutation at nucleotide 1753 was reported as a predictive factor for HCC among HBeAg-positive genotype C1 carriers. An in vitro study demonstrated a higher replication capacity of HBV variant A1762T/G1764A in conjunction with T1753C than the HBV variant with A1762T/G1764A. Molecular sequence analysis of the T1753C mutation with a bioinformatics tool showed the generations of pentanucleotide repeat sequence (JCV repeat like) in BCP region that could interact specifically with a protein with evidence of transcriptional regulation. The possible role of T1753C mutation to create the regulatory JCV promoter region needs to be clarified (Figure 2).

The other rare BCP mutations which produce HNF1 most likely enhance HBV transcription. The frequency of these mutations during chronic hepatitis seems to be similar and speculative to that of the A1762T/G1764A double mutation.

**BCP mutations in overlap with protein-x**

Among the proteins encoded by HBV DNA, protein-x is a key element of in vivo HBV infection and has been implicated in HCC development. Hepatitis B protein-x exerts most of its activities through direct interaction with TATA-binding proteins, leucine-zipper proteins and DNA repair proteins. This protein also transactivates a range of viral and cellular gene promoters by activating signaling pathways that are critical for viral replication and thought to contribute to oncogenesis. Protein-x increases HBV replication which

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**Fig. 2:** Part of the nucleotide sequence of the HBV genome in BCP region. The yellow arrow represents the T1753C mutation to create the JCV repeat.
leads to a higher frequency of HBV integration into the human host genome and induces a late G1 cell-cycle block.\textsuperscript{34} It was demonstrated that substitution of codons 130 and 131 of protein-x resulting from the double mutation A1762T/G1764A of the BCP region can physically and functionally interact with HNF1 to support higher HNF1 affinity on the BCP binding site.\textsuperscript{7} Moreover, these variants are thought to be responsible for a weak T cell response to an immunodominant HBx-derived epitope.\textsuperscript{35} Therefore the presence of protein-x mutants might account for the lack of T cell reactivity. These substitutions may also be associated with an additional mutation at position 127 in the protein-x which corresponds to T1753C mutation in BCP and has been identified in patients with HCC or fulminant hepatitis.

The T1753C HBV variant is also considered as one of the ‘hot spot’ mutations of protein-x encoding gene\textsuperscript{25} which is reported to increase the transactivation and antiproliferation activity of protein-x in HBV genotype D and, therefore contribute to carcinogenesis.\textsuperscript{29}

**Hepatitis B viral x mRNA secondary structure in basal core promoter mutations**

RNA molecules fold into secondary structures by matching base pairs. Therefore HBV genome mutations may have an important effect on RNA folding and the stability of HBV RNA structures. A putative secondary structure of RNA for the human hepatitis B virus x gene has been proposed by chemical and enzymatic methods in addition to the MFOLD computer program.\textsuperscript{36} The nature of HBV mutations on RNA transcripts of the HBV genome has not been characterized. It seems that BCP mutations potentially change the x mRNA and pre-genomic RNA secondary structures. Using the MFOLD computer program has indicated that the minimum free energy in the secondary structure of x mRNA is affected by a number of mutations. Accordingly, G1757A substitution in HBV genome increases the minimum free energy of x mRNA in nearly all variants while a mutation at nucleotide 1753 is related to a decrease in minimum free energy.

Figure 3 represents the minimum free energy dG = -160 and dG = -152 in variant A1762T/G1764A which is accompanied by a T1753V mutation in comparison with variant A1762T/G1764A accompanied with A1757G for x mRNA, respectively. Elevated minimum free energy could explain the more unstable RNA structure, thus RNAs have a limited time to be translated. However additional experimental studies to clarify the effect of mutations on RNA stability are required to determine its precise impact on disease development.

**CONCLUSION**

In this review we have attempted to summarize currently available data on mutations in
the BCP region with a common phenotype of moderate clearance of pre-core RNA and an increase of core/pregenomic RNA. It can be concluded that mutations in BCP are important in three levels: a) to change the binding sites for interaction with cellular transcription factors, b) the impact of these mutations on transactivity function of protein-x and c) the impact of these mutations in the BCP region on the stability of HBV RNAs. Thus more research is needed to answer the questions about HBV DNA function and its stability which may be affected by these mutations.

CONFLICT OF INTEREST
The authors declare no conflict of interest related to this work.

REFERENCES


