Review

Mutants in the precore, core promoter, and core regions of Hepatitis B virus, and their clinical relevance

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SUMMARY

Molecular virology methods including polymerase chain reaction, sequencing and cloning were a revolution in our understanding about the viral genomes. In the case of hepatitis B virus (HBV), sequencing studies have resulted in the identification of a number of virus variants normally found during the natural course of infection, or arising as a result of medical intervention. Examples are the precore and the basic core promoter (BCP) variants, others affecting the enhancer II (EnII) region, as well as various deletion variants found in immunocompromised individuals. The appearance of precore/ core gene and BCP variants heralds the initiation of the seroconversion phase from HBeAg to anti-HBe. The above variants have been studied in detail in different settings of HBV infection such as acute or fulminant hepatitis, chronic hepatitis with high viremia levels and in immunocompromised individuals. The existence of the variants was associated with the clinical severity, prognosis of liver disease and the response to treatment. However, the mechanisms of their selection and clearance remain to be defined.

Keywords: Precore/Core Gene, HBeAg, precore variants, A1896, basic core promoter variants, fulminant hepatitis, chronic hepatitis, A1896; encapsidation signal, B-cell epitopes; CD4+ epitopes

1. INTRODUCTION

HBV isolates exhibit significant variation that may involve up to 12% of the virus nucleotide sequence. There are natural variants consisting the serological subtypes and genotypes of the virus.[1,2] Various populations of viral strains found in a single host are referred to as quasispecies. Such quasispecies populations are common in RNA viruses.[3] Although HBV is a DNA virus, it is similar to RNA viruses since it replicates through an RNA intermediate which is reverse transcribed. It is vulnerable to a high number of mutations, and it circulates as a population of quasispecies.[4] However, the potential for multiple mutations is limited by the strict genomic organization of the virus. The virus has 4 overlapping open reading frames (ORFs) and regulatory elements of transcription, replication and encapsidation within these ORFs. For example, the surface open reading frame (S) is entirely overlapped by that encoding the polymerase (P). Any nucleotide change that leads to loss of function of either protein will be deleterious to the virus and therefore lost. However, some point mutations may have not any obvious effect, either because the nucleotide change may not result in an amino acid change or because the change does not affect functional structures of the RNA or protein. Some of these silent mutations may be part of the quasispecies pool. Some of these variants are subsequently selected by the host immune response.

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2. GENOMIC CHARACTERISTICS OF THE PRECORE/CORE GENE

The precore/core ORF has two initiation (AUG) codons, which lead to the synthesis of two proteins. Initiation of translation at the first AUG results in the synthesis of the precore/core protein, the precursor of HBeAg, whilst the second initiation codon is utilised for the synthesis of the nucleocapsid or core protein (HBcAg). The two proteins are synthesised from different messages known as the precore mRNA and pregenomic RNA (pgRNA) respectively. Once it is synthesised, the precore/core protein is target-

ed to the endoplasmic reticulum (ER) by a 19 amino-acid signal peptide at its amino-terminus ([5]). Cleavage of this peptide releases the remainder of the protein into the ER lumen, where further processing of the carboxy-terminus results in the removal of about 34 amino-acids from this end. What remains is HBeAg, which is released from the cell. HBeAg retains 10 amino-acids from the precore region and shares the remaining 149 with HBcAg. The antigenic epitopes are different between the two proteins, because of conformational changes in HBeAg protein.

HBeAg is conserved within all members of the hepadnavirus family. In the neonate born to an HBV-infected mother, it has been suggested that HBeAg crosses the placenta and induces tolerance ([6]). HBeAg may also have an immunomodulatory function during infection in adult life as it suppresses T-helper cells (Th) so that elimination of HBV-infected cells does not occur.

HBcAg forms the nucleocapsid of the virus. The core protein assembles into particles that simultaneously encapsidate the pgRNA with a copy of the viral polymerase. Encapsidation is initiated by the binding of the polymerase to a stem-loop structure at the 5' end of the pgRNA known as the encapsidation signal or ε **(Fig. 1)**. This signal is essential not only for the packaging of the pgRNA but also for the initiation of reverse transcription.

3. GENOMIC CHARACTERISTICS OF THE CORE PROMOTER REGION

The core promoter (CP) of the viral genome plays an important role for HBV replication as it directs initiation of transcription for the synthesis of both the precore and pgRNAs. The CP consists of the BCP and upstream regulatory sequences.[7] The BCP overlaps with the 3' end of the X ORF and the 5'end of the precore region, and contains *cis*-acting elements that can independently direct transcription of the precore mRNA and pgRNA.[8] **(Fig.2)**. The pgRNA is translated into the core and polymerase proteins, but in addition serves as the template for the synthesis of the negative DNA strand of the virus by reverse transcription after encapsidation within the core particle. The precore transcript, which is slightly longer than the pgRNA and is initiated upstream of the precore start codon, is the template for the translation of the precore/core protein that produce HBeAg, as already described.

The enhancer II (EnII) element regulates the activity of the CP and partially overlaps with it and its upstream regulatory sequences **(Fig. 2)**. The region also contains nucleotide motifs constituting transcription factor binding sites.[9]

4. PRECORE VARIANTS

In Mediterannean countries, the majority of patients with HBV infection harbour the precore mutant virus.[10,11] A point mutation from G to A at position 1896 (G1896A or A1896) of the precore region which converted the TGG codon for tryptophan (codon 28) to TAG, a translational stop codon, abrogates hepatitis B e antigen (HBeAg) production whereas core antigen is normally produced. These patients are anti-HBe positive with high levels of HBV DNA.[12] This is by far the commonest substitution

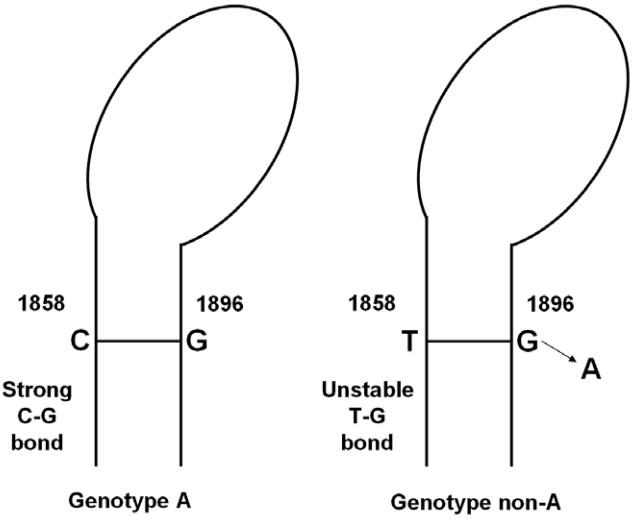

Figure 1. Location and nature of the mutation stabilising the secondary structure of pregenomic encapsidation signal in genotypes non-A.

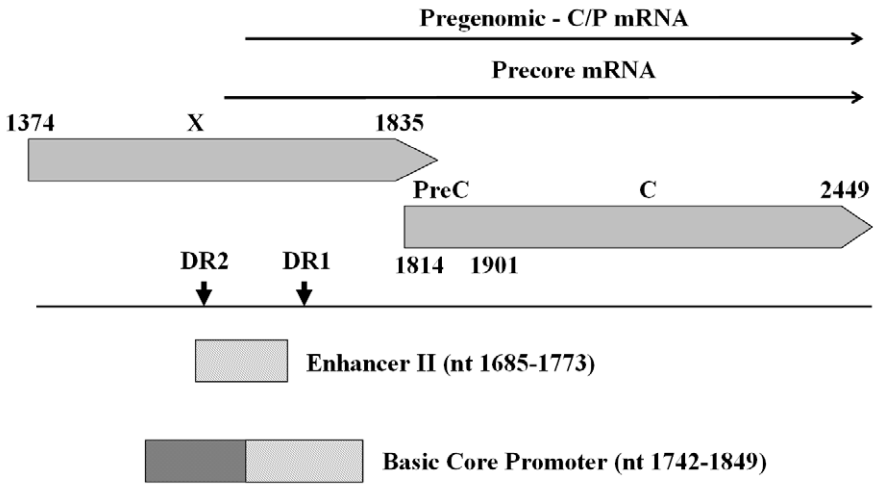

Figure 2. Transcriptional regulatory elements and the X gene. The promoters and enhancers involved in HBV replication are indicated. BCP, basal core promoter; DR, direct repeat.

encountered in anti-HBe positive patients. Other mutations that have the same phenotypic effect are loss of the precore/core protein translation start codon (ATG to ACG or CUG)[13,14] mutation of the second codon to a stop codon, and frameshifts and deletions resulting in the synthesis of nonsense proteins.[15,16] Further point mutations and stop codons are rare because such mutations would destabilise the secondary structure of the e encapsidation signal **(Fig. 2)**. Two further mutations, A1898[17] and A1899[18], are strongly linked with the T1856 and A1896, respectively. These associations are likely to be related to encapsidation signal secondary structure requirements, but whether they have clinical relevance remains unclear even after years of inquiry. A1899 is primarily seen (with rare exceptions) together with A1896, and in early studies, it was linked with severe disease. However subsequent work has failed to confirm this association.[19]

A significant proportion of chronic hepatitis patients in Mediterranean countries[20] and in the Far East[21] are infected with HBeAg-negative variants; it appears that this clinical picture has become the most common new presentation of chronic hepatitis B in Italy and Greece. In Gambia and Brazil, [22 23] as well as Northern Europe, there is low prevalence of the precore stop codon mutation in anti-HBe positive patients. These findings may relate to the prevalent HBV genotypes that circulate in these populations. Epidemiological studies of geographical distribution of genotypes demonstrated an association of mutant strains of HBV with certain genotypes.[2]

A part of e signal is base paired due to bond formation between nucleotide 3 of codon 15 (CC<u>C</u>) (nt 1858) and nucleotide 2 of codon 28 (T<u>G</u>G) (nt 1896)[24,25] **(Fig. 2).** However in genotype B, C and D, nucleotide 3 of codon 15 is a T (CC<u>T</u>) which in its turn favors the replacement of G by an A in nucleotide 2 of codon 28 (T<u>A</u>G). Thus, the conversion of TGG to a stop codon enhances the stability of the secondrary structure, which is required for the function of the e signal.[2] On the contrary, the base pairing C-G is disrupted in genotype A if the stop codon mutation develops and the stability of stem-loop structure decreases. This finding may explain why genotype A rarely circulates as HBe minus mutant and why D is the most common genotype among the pre-core minus mutants in Western countries.

It is believed that the pre-core stop codon mutation is selected during seroconversion from HBeAg to anti-HBe when there is a T at position 1858 (genotype non-A). The G-to-A mutation at position 1896 abolishes the production of HBeAg. On the other hand the mutations in the core promoter region namely the double mutation A-to-T at position 1762 and G-to-A at position 1764 of the basic core promoter are selected during seroconversion when there is a C at posistion 1858 (genotype A) because C at 1858 precludes the development of T1896A[26]. The mutations in basic core promoter reduce synthesis of HBeAg by suppressing the transcription of precore mRNA.

4.1. CHRONIC HEPATITIS

Following acute HBV infection, 5% of adults and almost 95% of children born to chronically infected mothers, become chronic carriers of the virus, probably because of

failure of induction or activity of cytotoxic T lymphocytes (CTL). In these patients despite the high level of viremia there is no inflammatory necrosis of infected hepatocytes (immune tolerant phase).[21] During the following years of infection, the immune tolerant phase is replaced by increased hepatitis activity presumably reflecting immune activation (immune clearance phase)([27]). Either because of CTL lysis of infected cells or of cytokine production by CD4-positive lymphocytes, hepatocytes infected with HBV are cleared during the seroconversion or immune clearance phase. About 5% of HBeAg positive chronic infections may seroconvert to anti-HBe per year. In the majority of these HBeAg(-)/anti-HBe(+) patients, HBs antigenemia and small amounts of HBV DNA are detected in the serum by polymerase chain reaction (PCR), despite normal liver function tests, indicating that viral replication is still occurring at a low level.[12]

In some patients, particularly those infected at birth or in the early years of life from infected family members, there is emergence of an HBeAg-negative variant. This virus, along with the HBeAg-positive strain, can be detected in virtually all patients after HBeAg/anti-HBe seroconversion.[12] The A1896 variant is present in very small amounts during the latter period of the HBeAg positive phase of the disease and is selected at, or after, seroconversion to anti-HBe, whilst the HBeAg-producing strain is gradually being cleared.[14] This process can take years, during which time a mixture of both strains is usually seen.[28] Some patients after seroconversion have high viraemia and develop further inflammatory liver disease.[29] Some component of the immune response is responsible for control of the virus during the anti-HBe positive phase; this is most probably a CD4+/CTL response to nucleocapsid proteins. Mutations within epitopes recognized by sensitized T cells, which are involved in control of the infection, may influence the re-emergence of the virus. It is noteworthy that during this phase of the infection, amino acid substitution in the core protein is frequent. It seems likely that the important selection pressure is the presence of anti-HBe in the absence of an adequate CTL response, as most of the variants so far described emerging in the late phase of the infection have a common phenotype, namely an inability to produce HBeAg.

4.2. FULMINANT HEPATITIS

There is strong epidemiological evidence linking A1896 with fulminant hepatitis B (FHB), suggesting that such a severe outcome may be due to viral factors rather than to strong immune response of the host.[30] There is evidence that both the fulminant case and the source of infection are infected with the A1896 variant[31-33] and both strains (FHB and source) have the precore mutant of similar or identical sequences.[34-37] It is evident therefore that the disease has been caused by the new host mounting a vigorous immune response to the particular strain of HBV. Many FHB cases are associated with infection by precore variants. However, FHB may also be associated with HBeAg-positive HBV infection.[31] Furthermore, even in anti-HBe-positive cases, the link to A1896 is far from clear; this variant has not been found in many anti-HBe-positive patients from several geographical regions.[38 39] It has been suggested that precore variants may replicate rapidly, spread throughout the liver and precipitate a strong immune response in the absence of HBeAg-mediated immune modulation. The result is a fulminant course due to rapid lysis of large numbers of HBV-infected cells. The development of FHB in neonates infected from HBeAg negative mothers and the observation that transmission of A1896 strains to children seldom gives rise to chronic carriage[40] may be partially explained by the absence of immunomodulation by HBeAg.

However, it appears that strains with A1896 are associated with different outcomes. Although A1896 is often found in FHB, this does not necessarily mean that it is the variant *per se* that is causing the fulminant hepatitis. The examination of cis-acting regulatory elements in sequences derived from isolates from Japanese patients with fulminant disease has revealed that there are several unique nucleotide mutations clustered in ENHII-CP (positions 1751-1768) and resulting in amino-acid changes in the X encoded protein (amino-acid 126-132)[41,42], regardless of their subtype. These changes located within the nuclear binding regions of the *cis*-acting elements of the basic core promoter[43] could potentially produce a modification in viral function. It was also demonstrated that several variations are associated with FHB with the commonest variants containing a double mutation (T1762/A1764) in the BCP. Transmission of an A1896 strain (with T1762 and A1764) to chimpanzees[41] resulted in more severe hepatitis than would be expected, although this was not accompanied by a higher than average level of viraemia. *In vitro* experiments have shown that, although an A1896 genome did not have higher replication efficiency, a genome from a FHB patient with A1896, T1762, and A1764 produced very high levels of HBV DNA[42]. Detailed phylogenetic analysis that included 20 epidemiologically unrelated FHB sequences[44] revealed six clusters (viral lineages) of fulminant strains each with distinct mutational patterns. It was found that sources of infection and index patients had highly related sequence patterns[34]. Mutations were clustered in the *cis*-acting regions and HBx protein but no

unique variant was specifically associated with this disease outcome. It was concluded that a combination of nucleotide variation of the enhancer/promoter regions plus aminoacid substitution in HBx were almost uniquely associated with the fulminant course.

Supporting the hypothesis that these strains are clinically relevant, the transcription efficiency *in vitro* of the BCP from A1896-associated FHB strains is two to three times greater than non-A1896 FHB and A1896 non-FHB sequences.[41] Finally, oligonucleotides containing variant sequences failed to bind to some nuclear transcription factors, implying that a central pathogenetic mechanism in FHB is loss of inhibition of transcription. Clusters and transcription efficiency were both strongly linked to mortality and rapidity of progression to fulminant hepatitis after infection.

Viral selection during the course of FHB has been demonstrated as well.[45] For example, in one infant-mother pair, the mother had a mixture of two subtypes and a mixture of quasispecies within each subtype, yet the infant was only infected by one subtype and a reduced number of quasispecies within that subtype. A1896 is the only precore variant (to our knowledge) associated with FHB, which leads to the interesting question of whether it is the functional effect (loss of HBeAg), or the background strain upon which A1896 is selected, that is the crucial factor associated with this clinical outcome. Hence, strains with mutations elsewhere in the genome, such as the BCP ones, have been linked with FHB in the presence or absence of A1896.

4.3. ACUTE NON-FULMINANT HEPATITIS

The incidence of the precore mutation during acute, non-fulminant, hepatitis is presently unclear. There is a controversy about what occur in acute hepatitis when transmission of a mixed population (both wild type and A1896 strains) takes place. Some investigators believe that during acute infection a clearance of HBeAg-negative virus takes place and HBeAg-positive virus dominates,[46] while others claim that precore variants need years to emerge after acute infection.[47] [32] A1896 was shown to dominate in both acute and chronic hepatitis cases of varing severity in Taiwan (58% in acute versus 70% in chronic),[48] but not so in a recent study from Japan.[47] Acute hepatitis in neonates born to anti-HBe-positive mothers is well described (40). The transmission of a homogeneous HBV population of A1896 leading to mild resolving acute hepatitis with seroconversion to anti-HBe in an adult without there having been an HBeAg-positive phase, shows that A1896 can lead to the same disease profile as wild type viruses.[49]

4.4. FIBROSING CHOLESTATIC HEPATITIS

Fibrosing cholestatic hepatitis (FCH) occurs occasionally in immunosuppressed host in conditions such as after liver transplantation,[50] after renal transplantation[51] and in HIV infection. These patients undergo a fulminant course and there is an abundant expression of both HBs and HBc antigens in the hepatocytes. Both precore[52] and HBc variants are often associated with FCH leading to intracellular accumulation of the antigens by a direct cytopathic effect, rather than immune lysis.

4.5. HBV REACTIVATION FOLLOWING CHEMOTHERAPY

Viral and host factors, as well as the type of chemotherapy given have been considered as predisposing factors for HBV reactivation[53]. The point mutation at position 1896 of the precore region (A1896) has been associated with severe exacerbation of chronic hepatitis B, and in immunocompetent individuals such exacerbations may run a fulminant course.[32]. A high rate of HBV reactivation has been documented in most studies of HBsAg(+)/HBeAg(-) patients carrying the A1896 mutation who have undergone cytotoxic chemotherapy compared with those carrying the wild type virus.[54] This finding was confirmed in different groups of individuals with HBV infection receiving immunosuppressive treatment such as patients with lymphoproliferative disorders[55], those receiving hematopoietic stem cell transplantation[56] and those with solid tumors.[57] With regard to the T1762/A1764 BCP mutations the findings are conflicting. Some studies suggested that these specific mutations could confer an increased risk of reactivation in HBV carriers undergoing hematopoietic stem cell transplantation[58] and others that these mutations alone do not appear to have any effect on promoter activity in carriers undergoing cytotoxic chemotherapy. It has also been documented that HBV with an A1799 mutation in the BCP region is prone to reactivate after chemotherapy in patients with solid tumours[32]. From a previous study in 8 patients who reactivated following chemotherapy we demonstrated that HBV reactivation could be attributed to the A1896 precore mutant virus in 7 of 8 patients and to a mutated BCP in 5 of 8 patients.[59] Both types of mutations result in defective synthesis of HBeAg and consequently the tolerogenic effect of HBeAg diminishes or disappears. However, we showed that the wild type virus may also be implicated less frequently in HBV reactivation, as documented in previous studies.[53]

5. BASIC CORE PROMOTER VARIANTS

Mutations in the core promoter region may have influences on viral gene expression and replication. A double mutation in the BCP leading to substitution of A for T, and G for A, at positions 1762 and 1764 respectively, has been described in various disease states of HBV infection.[60] The biological significance of the double A1762T/G1764A mutation is still under investigation, particularly in relation to the precore stop-codon mutation. The double BCP mutation has been shown to be associated with HBeAg negativity in some studies[60,61,62] but not in others.[26,63,64] The presence of the double mutation is clearly associated with downregulation of HBeAg production,[65,66,67] as demonstrated by transfection studies. The double BCP mutation results in decreased levels of the precore mRNA and therefore diminished production of HbeAg.[68,69] Despite the low levels of precore mRNA increased viral replication as a result of upregulation of pgRNA production, promoting encapsidation and core protein production was described.[70] Some investigators claimed that HBeAg negativity with the BCP mutations was associated with severe liver disease,[62,71,72] while this finding was not confirmed by others.[73] The double mutation has been detected with increased frequency in all settings of HBV infection including patients with fulminant hepatitis,[74] HBeAg and anti-HBe positive chronic hepatitis,[61] and hepatocellular carcinoma,[75] but less so in asymptomatic chronic carriers.[76] However, there is a controversy about the detection of the above mutations in all the settings described above in different geographical areas. For example, the 1762/1764 mutations were rarely seen in patients with FHB in the United States and Brasil.[77] This finding may be related to differences between prevalent genotypes in these areas.[78]

Chan and co-workers found that the double A1762T/G1764A mutation was significantly more common in genotypes which have C at nucleotide position 1858, while in contrast the precore stop-codon mutation was found in patients with a T at the same position.[26] The BCP mutations as mentioned before have been found in patients regardless of HBeAg status. However, in anti-HBe positive patients the double mutation was often accompanied by a change at position 1753, from T to C or G.[79] It was recently shown that CP mutations other than those at positions 1762/1764 could have a major impact on viral replication and HBeAg expression.[80]

Deletions within the CP region varying in length from 1-21 base pairs have been reported once again in different settings of HBV infection and are often characterised by low or high viraemia levels.[80] It appears therefore that the BCP and A1896 mutations determine replication rate, expression of HBeAg and pathogenicity in the context of the strain genetic background.

6. CORE GENE VARIANTS

HBcAg assembles into a capsid structure which encloses the pregenomic RNA together with the viral polymerase. The capsid protein can elicit B-cell activation and proliferation and produce antibodies both in a T cell-dependent and a T cell-independent manner.[81] These activated B cells present capsid derived peptides in the context of major histocompatibility complex class II antigens to T helper cells. Activated T cells are able to activate B cells specific for HBV antigens leading to increased antibody production.[82] It is evident therefore that capsid specific antibodies are produced by activated B cells, and B- and T-cell epitopes are important for their production. The immunodominant CD4+ T- cell recognition sites within HBcAg which have been found to induce significant T cell responses in a large proportion of patients irrespective of their HLA haplotype, span residues 1-20 and 50-69 or 1-25 and 61-85 and 117-131[83,84] in European and Far Eastern patients respectively **(Fig. 3)**. A major B-cell antigenic determinant is located around aminoacid position 80, namely 74-89,[85] a second one at residues 107-118[86] and a third one at aa 130-138.[85]

It is well established that infection with the precore stop codon variant of HBV causes chronic liver disease with acute exacerbations.[87] It has been shown that this variant exhibits a high prevalence of mutations in the core region.[88-90] Sequence variation is one of the most powerful

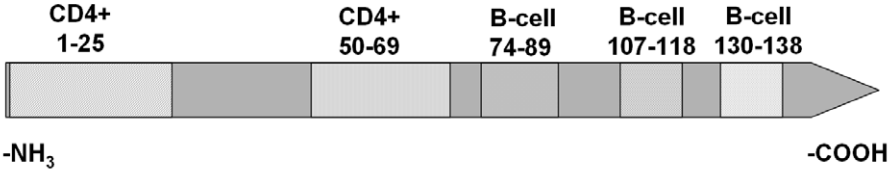

Figure 3. Immunologic epitopes of core protein. The CD4+ and B-cell epitopes are shown.

viral strategies for escaping recognition by the host's immune response. Core heterogeneity has been thoroughly investigated[88-90] and linked to virus persistence. It was previously demonstrated that a mixed viral population of various quasi-species circulates in the serum at any one time during chronic HBV infection.[88] In a study under publication, consecutive serum samples investigated and confirmed the presence of a mixture of core variants at different time-points during the course of chronic HBV infection. It has also shown that novel variants may emerge "de novo" during the course of the disease, which, were not detected at earlier time points samples. However, such variants may be present in the quasispecies pool but are not detectable due to their extremely small representation.[91] Some of the variants presenting as a minor population in the some initial serum samples appeared to have been selected and become dominant in subsequent sera. The core protein is a known target for B- and T-cell immune responses[82-86,91] and core mutations emerging de novo may alter core antigenicity.[91]. The replacement of a rather homogeneous core population by a varied population of quasispecies as evident from the sequencing results may also hamper HBcAg immune recognition. In addition, the clones in all the patients with active liver disease and who received no antiviral treatment demonstrated that the majority of the core protein aminoacid changes were concentrated in both B- and T-cell epitopes. Aminoacid changes in the core region have been previously associated with severe chronic liver disease and shown to affect equally both B- and T- cell epitopes. [88,89,92]

The emergence therefore of new mixed viral populations of core variants and the selection of new mutations that predominated subsequently at any one time point may be due to the substitution of aminoacids in B- and CD4+ T- cell epitopes. Thus, activation of B and CD+ T lymphocytes may be triggered by the altered target epitopes thus inducing a new immune response as evidenced by the high levels of IgM anti-HBc recorded in such cases.

Deletions within the core gene are variable in length and usually affect the central core region and carboxy-terminal region.[93, 94] Such deletions can be in frame, resulting in the production of truncated core species, or out of frame, terminating core synthesis prematurely. As deletion variants are unable to produce capsids or the capsids they produce are unstable, such variants co-exist with the wild type virus that provides the core protein for their encapsidation. Core deletion variants have been reported both in immune–competent[95] and immunosuppressed individuals.[96] In the former individuals, the detection of core internal deletion variants was associated with lower levels of viraemia and early seroconversion to anti-HBe, while in long-term immunosuppressed patients following kidney, or even liver transplantation, the presence of deletion variants was associated with increased risk of developing liver cirrhosis and end-stage liver disease.

7. CONCLUSIONS

A significant proportion of patients with chronic hepatitis in Mediterranean countries are infected with A1896 HBV mutant virus. A1896 is selected during seroconversion from HBeAg to anti-HBe because it enhances the stability of the secondary structure of e encapsidation signal in genotype non-A virus. For the same reason the mutations T1762 and A1764 of BCP are selected during seroconversion in genotype A virus. Whatever the nature of the variant, it results in the reduction or abrogation of HBeAg production. The removal of the tolerogenic effect of this soluble serum protein, leads to the "awakening" of the immune response. There is strong epidemiological evidence that precore A 1896 mutation as well as T1762 and A1764 of BCP are associated with fulminant hepatitis. It was suggested that the fulminant course was due to either vigorous immune response of the host or to high levels of HBV DNA or to high transcription efficiency of the virus. In addition a high rate of HBV reactivation after chemotherapy for leukaemia/lymphoma or solid tumors has been demonstrated in HBeAg negative patients carrying the A1896 precore mutant.

Infection with the precore A1896 mutant virus is often accompanied by the presence of mutations in the core region. Novel core variants may emerge de novo during the course of the disease or may circulate as a mixture of quasispecies. Since the core protein is a known target of B- and T-cell responses, the altered core antigenicity may help the virus to escape immune recognition and lead to virus persistence.

However, the wide range of chronic liver disease, from inactive carrier to liver cirrhosis and hepatocellular carcinoma, cannot be explained by the presence of precore and core variants. There may be an interactive mechanism between host genetic factors and virus variation within the core protein or other proteins. The exact clinical relevance of these variants needs further investigation.

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