Hepatitis B Virus Core Promotor and Precore Mutants in Thai Chronic Hepatitis Patients

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ABSTRACT In Thailand, hepatitis B virus infection along with its fatal sequelae liver cirrhosis and hepatocellular carcinoma constitutes a public health burden of endemic proportions. The second phase of the infection is usually characterized by cessation of HBV DNA replication and seroconversion to anti-HBe. However, persistent viremia with active liver disease has been encountered in some anti-HBe positive patients, a discrepancy explained by mutations in the precore/core region of the viral genome. The resulting failure to synthesize HBeAg may help the virus evade immune clearance, while HBV replication and expression of HBcAg proceed unchecked. Discreet types of mutation in the precore/core region have been found at varying prevalence depending on the predominant HBV genotype in the respective geographical areas. The purpose of the present study has been to elucidate both prevalence and type along with the nucleotide positions of the mutations prevailing among Thai chronic hepatitis patients. We subjected 68 patients to serological tests for HBeAg, as well as semi-nested PCR with subsequent DNA sequencing. HBV DNA was detected in 87.5% of HBeAg positive and 40.9 % of HBeAg negative patients, respectively. Mutations in the core promoter amounted to 28.6 % in HBeAg positive and 75% in HBeAg negative patients, in the start codon of the precore gene to 37.5% and in codon 28 to 18.8% in HBeAg negative patients. Our results have shown mutations affecting the core promoter of HBeAg to be by far the most prevalent in Thai patients, followed by mutations of the start codon whereas those changing codon 28 into a stop codon are less frequently encountered than has been reported from other areas indicating a distinct HBV genotype prevailing in Thailand.

KEYWORDS: hepatitis B virus, precore, chronic hepatitis.

INTRODUCTION

Hepatitis B constitutes a significant public health burden on a global scale with a particularly high prevalence in Southeast Asia, China and sub-Saharan Africa[1] where it is responsible for the majority of cases diagnosed with chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). The respective age at which an individual is infected appears to be of crucial consequence as, for example, adults are at a far lower risk to proceed towards chronicity than children infected either in the course of delivery by a carrier mother or otherwise during early childhood.[2]

The natural history of chronic hepatitis B is characterized by three phases attributable to virus-host interactions: virus tolerance, virus clearance and residual HBV integration resulting in a variety of hepatic lesions.[3,4] In the course of the first phase usually associated with an asymptomatic disease course despite high serum levels of HBV DNA and HBeAg expression, HBV is actively replicating. The second phase is characterized by a clinical course of exacerbations and remissions as a result of the host's cell-mediated immune response. Consequently, HBV replication gradually decreases until altogether subsiding with subsequent HBeAg clearance and seroconversion to anti-HBe. During the third phase, although some of the infected hepatocytes have been cleared, residual HBV DNA can become integrated in the host genome, and hepatocytes continue to express HBsAg. At this stage, a significant proportion of HBV carriers is prone to develop late sequelae, such as cirrhosis and HCC.

The sequence heterogeneity displayed by hepatitis B virus (HBV) has found increasing recognition as a characteristic crucial for HBV biology and pathogenesis. To date, variants exhibiting mutations in almost all viral genes and in regulatory regions have been identified. Variants unable to synthesize hepatitis B e antigen (HBeAg) are most frequently selected in chronically infected

patients undergoing seroconversion from HBeAg to HBe antibody (anti-HBe).[5,6] In some patients, these mutants appear to lead to severe chronic hepatitis[7] or, in case of transmission to an as yet uninfected host, even to fulminant hepatitis.[8]

Epidemiological and clinical studies have revealed a correlation between HBV replication and infectivity and the presence of HBeAg in that seroconversion to anti-HBe seems to lead to disappearance of serum HBV DNA and remission of liver disease as evident by the ensuing normalization of liver enzyme levels. However, several studies have shown persistent viremia with active liver disease in some anti-HBe positive patients,[9,10] a discrepancy explained by the discovery of mutations in the precore region whereby synthesis of HBeAg is prevented, while HBV replication and expression of hepatitis B core antigen (HBcAg) still proceed unhindered.[11,12]

The mutation most frequently encountered in the precore region is a G-A change at nucleotide 1896 resulting in a stop codon and hence leading to premature termination of the precore/core protein which is the precursor of HBeAg. Failure to synthesize HBeAg may be a means for the virus to evade immune clearance. Initial studies suggested these mutants to mainly occur in Mediterranean countries and Japan but more recently, stop codon mutants have been reported from diverse geographical areas.[8,13,14] The reported variability in their prevalence in different countries has been found related to the predominant HBV genotype as the stop codon mutation solely occurs in patients infected with genotypes bearing a T at nucleotide 1858.[15,16]

In Thailand, data available on hepatitis B precore/core mutants and more specifically, on the most frequently encountered type of mutation are still limited. In order to further elucidate these issues we performed the present study which was aimed at establishing both prevalence and exact type as well as nucleotide position of the mutations encountered among Thai chronic hepatitis patients, as well as at comparing our findings with those from various other countries.

Materials and Methods

Population studied

Sixty-eight patients who attended the out-patient clinic, gastrointestinal unit, Department of Internal Medicine, Chulalongkorn University Hospital, and had been clinically and biochemically diagnosed with chronic hepatitis were included in the study. They comprised 9 females and 59 males, their age ranging from 21 to 69 years (mean age 43 ± 12). All patients were HBsAg positive as confirmed by ELISA (Auszyme, Abbott Laboratories, North Chicago, Ill.) and exhibited elevated alanine aminotransferase (ALT) levels. After having obtained the patients' informed consent as to the purpose of the study, venous blood samples were taken, the sera separated by centrifugation and stored at –70°C until tested.

Laboratory Methods

HBeAg screening

All 68 sera were tested for the presence of HBeAg by ELISA (Hepanostika HBe, Organon Diagnostics, Boxtel, The Netherlands) according to the manufacturer's specifications.

HBV DNA extraction

DNA was extracted from 50 µl of serum, twice per sample, with proteinase K/SDS in Tris buffer, followed by phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in 20 µl of sterile water and directly subjected to the polymerase chain reaction.

HBV DNA detection

The selection of primer sets for HBV DNA amplification was based on sequence data provided by Dr M Yano at the WHO Collaboration Centre, Nagasaki, Japan.

HBV DNA was amplified by semi-nested PCR in an automated thermocycler (Perkin Elmer Cetus, Branchburg, New Jersey, USA) as described elsewhere.[17] Briefly, 5 µl of the respective DNA sample were added to a reaction mixture containing 1 U of Taq polymerase (Perkin Elmer Cetus, Branchburg, New Jersey, USA), each of four deoxynucleotide triphosphates (Promega Corp, Madison, WI, USA) at a concentration of 200 µM, primer pair RMD 26 with the sequence 5'-ATG GAG ACC ACC GTG AAC-3' (nt. 1608-1625) and Ci1 with the sequence 5'-TTC CGG AGA CTC TAA GGC-3' (nt. 2038-2020) for the first amplification round, and primer pair RMD 26 (as above) and PC 1 with the sequence 5'-GGA AAG AAG TCA GAA GGC-3' (nt. 1974-1957) for the second amplification round, respectively, each primer at a 1 µM concentration, 10 mM Tris buffer and 1.5 mM $MgCl_2$ at a final volume of 50 µl. The first amplification round consisted of one cycle at 94°C, 55°C and 72°C for 1 minute each, followed by 30 cycles comprising

a 30 second denaturation step at 94°C, a 30 second annealing step at 55°C, and a 1 minute extension step at 72°C, each. The amplification was concluded by one cycle at 94°C for 1 minute, 55° C for 2 minutes and 72°C for 10 minutes. For the second amplification round 5 µl of the first PCR product were added to the reaction mixture and amplification was performed in a manner identical to the first round. 10 µl of each amplified DNA sample was loaded on a 2% Nusieve (FMC Bioproducts, Rockland, ME, USA) agarose gel stained with ethidium bromide on preparation. Electrophoresis was performed at 120 V for 45 minutes and the product band of 367 base pairs was visualised on a UV-light box.

DNA purification and sequencing

The PCR product was purified for sequencing using the QIAquick PCR Purification Kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's specifications and subsequently subjected to 2 % agarose gel electrophoresis in order to ascertain its purity. The concentration of the amplified DNA was determined spectrophotometrically (Shimadzu UV 160 A). Between 10 and 30 ng/µl of every respective DNA were subjected to cycle sequencing using dye-labelled terminators which represents a rapid and convenient method for performing enzymatic extension reactions for subsequent DNA sequencing on the ABIPRISM™310 Genetic Analyser (Perkin Elmer Cetus, Branchburg, New Jersey, USA). This round of amplifications was performed according to the manufacturer's specifications using primer pair RMD 26 and PC 1 to amplify the particular DNA strand of interest for further sequencing. Cycle sequencing consisted of 25 cycles at 96°C for 10 seconds (denaturation), 50°C for 5 seconds (annealing), and 60°C for 4 minutes (extension). The reaction was concluded by cooling the thermal ramp to 4°C. The extension products were subsequently purified from excess un-incorporated dye terminators by ethanol precipitation according to the manufacturer's specifications (Perkin Elmer Cetus) and subsequently prepared for loading on the ABIPRISM™ 310 Genetic Analyser. For all the sub-sequent steps we referred to the ABIPRISM™ 310 Genetic Analyser user's manual (Perkin Elmer Cetus, Branchburg, New Jersey, USA).

Results

Of the 68 chronic hepatitis patients tested by serology for the presence of HBeAg, 24 (35.3%) were found positive and 44 (64.7%) negative (Table 1).

Table 1. Hepatitis B virus DNA in chronic hepatitis patients.

	HBeAg+ve n = 24	HBeAg –ve n = 44
HBV DNA +ve	21 (87.5%)	18 (40.9%)
HBV DNA - ve	3 (12.5%)	26 (59.1%)

Table 2. Hepatitis B virus core promotor and precore mutans in chronic hepatitis.

Position of mutation	HBeAg+ve n = 7(%)	HBeAg-ve n = 16(%)
Core promotor		
1762 A-T, 1764 G-A	2 (28.6)	12 (75)
Precore gene		
Start codon	0 (0)	6 (37.5)
Codon 28		
1896 G-A	0 (0)	3 (18.8)

Note: 2 patients showed mutations at promotor and start codon, 2 at promotor and codon 28 (turned to stop codon), 1 at the start codon and codon 28 (turned to stop codon).

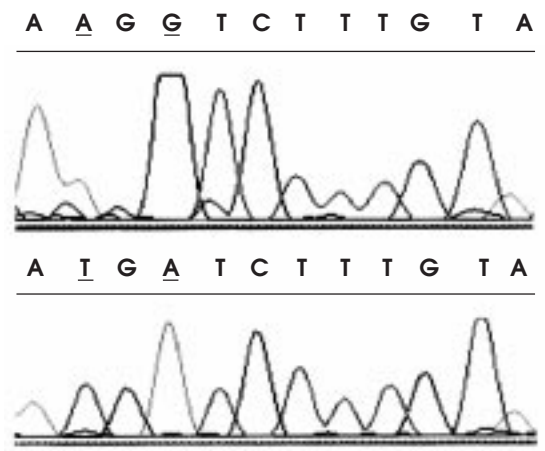

Fig 1. Electropherogram of nucleotide positions 1761-1774 indicating the double point mutation at positions 1762 (A to T) and 1764 (G to A). The wild-type strain is shown at the top.

Irrespective of these results, all sera were subjected to polymerase chain reaction using semi-nested primers covering the precore as well as the core promotor region of hepatitis B virus. Of the 24 HBeAg-positive sera, 21 (87.5%) were also found HBV DNA-positive by PCR. Of those 21, 7 were randomly chosen for sequencing, two of which showed mutations in the core promotor region at nucleotides 1762 (A-T) and 1764 (G-A), respectively (Table 2). Of the 44 HBeAg-negative sera, 18 (40.9 %) were HBV DNA-positive by PCR. Of those 18, the amount of serum

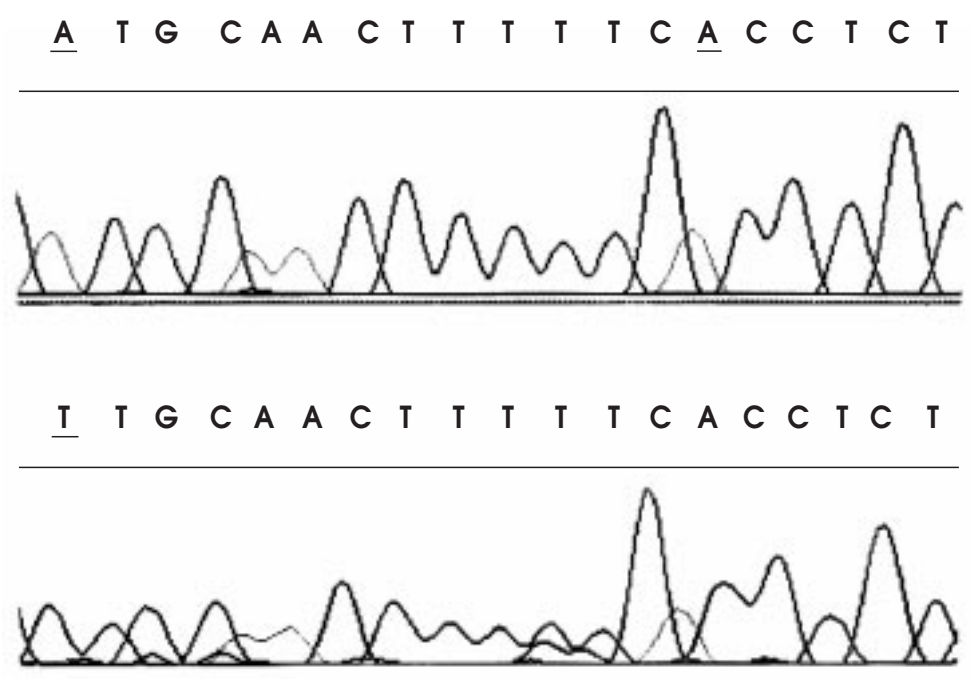

Fig 2. Electropherogram of nucleotide positions 1812-1833 indicating the point mutation at nucleotide 1814 which changes the precore start codon (ATG) into TTG and hence abolishing initiation of translation. The wild-type strain is depicted at the top.

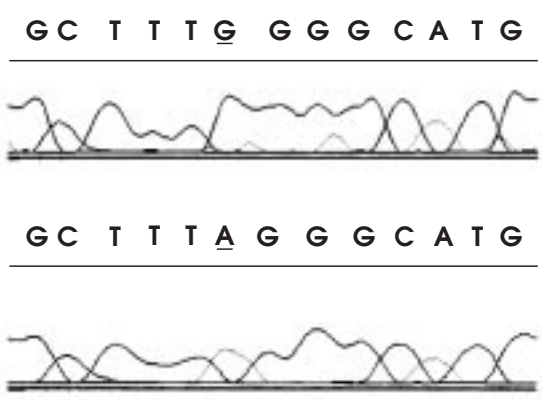

Fig 3. Electropherogram of nucleotide positions 1890-1904 indicating the point mutation at nucleotide 1896, which changes codon 28 (TGG, tryptophane) into a stop codon (TAG). The wild-type strain is shown at the top.

necessary for sequencing was sufficient in 16. As depicted in Table 2, the nucleotide sequences thus obtained revealed altogether 12 mutations in the core promotor region (Fig 1), 6 mutations of the start codon (Fig 2) and 3 of nucleotide 1896 turning codon 28 into a stop codon (Fig 3). In some of the sera we detected double mutations. More specifically, 2 patients displayed simultaneous mutations at the promotor and start codon, 2 at the promotor and codon 28, and 1 at the start codon and codon 28. The clinical relevance of these respective mutations has been described elsewhere.[18]

Discussion

As mentioned earlier, the natural course of HBV infection is characterized by decreasing and eventually ceasing viral DNA replication, as well as by clearance of HBeAg and seroconversion to anti-HBe during the second, so-called clearance phase. Hence, the majority of patients negative for HBeAg will also be negative for HBV DNA in their serum, that is, they will not display free replicating virus. Yet, the virus may have integrated its DNA into the host genome and thus, viral proteins are expressed just as naturally as those encoded by the host's own DNA, with one crucial difference in that they elicit the host's immune response. The ensuing symptoms include first and foremost a pathologic elevation of the liver enzymes and in cases of chronicity, liver damage in the form of cirrhosis and ultimately, hepatocellular carcinoma.

For the synthesis and secretion of HBeAg precore mRNAs have to be transcribed from HBV DNA and the precore region and the core gene have to be translated. Therefore, any mutations occurring in either the precore region or the core promotor and preventing the translation of precore mRNAs induce an HBeAg-minus phenotype.

In the core promotor of all HBV isolates described to date, irrespective of genotypes or subtypes, three

AT-rich regions located 20-30 bp upstream of the transcription start site are recognized by transcription factors with binding sites in the core/pregenome promotor.[19,20,21] Various mutations in the first and second AT-rich region have been observed in asymptomatic HBV carriers or patients with acute or chronic hepatitis B. Of those, the paired mutations from A to T at nt 1762 and from G to A at nt 1764, as detected in 75 % of our HBeAg-minus patients, have been most frequently reported.

As the core promotor is located within the coding region of the X gene, mutations in it have been shown to induce amino acid changes in the carboxyl-terminal part of the X protein. In that context, the double mutation from T^{1762} and A^{1764} changes the amino acids Lys at position 130 to Met and Val at position 131 to Ile, respectively. These as well as other amino acid conversions exclusively occur in a sequence of Glu-Ile-Arg-Leu-Lys-Val-Phe-Val, which represent amino acids 126-133 of the X protein reported to bear an HLA-A2-restricted cytotoxic T cell epitope.[22]

With respect to decreased synthesis and secretion of HBeAg, mutations in the core promotor are not as powerful as in the precore region. Yet, they would gradually induce the eventual seroconversion of the host by shifting the balance in favor of anti-HBe. Nonetheless, the virologic explanation most frequently encountered regarding the HBeAg negative profile of hepatitis B comprises mutations within the precore region of HBV DNA which block translation of the protein.[23,24] Of the numerous mutations identified to date, the one responsible for more than 90% of HBeAg-minus hepatitis patients is a point mutation from G to A at nt 1896 which changes the tryptophane (UGG) codon into a translation stop codon (UAG) in the complementary mRNA.[5,11] This mutation causes premature termination of the precore/core protein thereby preventing synthesis of HBeAg which may be expressed on the membrane of hepatocytes and thus serve as a target for cellular immune response. Hence, failure to produce HBeAg may help evade immune clearance, which could account for the frequent development or selection of the stop codon mutant. In the present study we detected this particular mutant in a mere 19% of the patients tested. This low prevalence may be attributable to the fact of the mutation only occurring in patients infected with HBV genotypes bearing a T at nt 1858[25,26] which initially were believed to be restricted to Mediterranean countries and Japan. Although the stop codon mutants have meanwhile been detected in additional geographical areas[27], they still might occur at a rather low frequency in Thailand. Actually, we have detected C instead of T at position 1858 in all of our patients tested, which renders a mutation from G to A at position 1896 highly unlikely.

Another mutation leading to the inability to produce HBeAg is the one affecting the initiation codon (ATG) of the precore gene and thus abolishing the start of protein synthesis. In the present study, we identified various discreet point mutations changing ATG into ATT (1 patient), AAG (1 patient), GTG (1 patient), and TTG (3 patients), respectively.

Anti-HBe positive chronic hepatitis predominantly caused by precore mutants of HBV has become the most frequently encountered form of chronic hepatitis B in most Mediterranean countries and many parts of the world.[28] The exact role of these mutants in the pathogenesis, as well as in determining the clinical course of the disease and its response to interferon therapy has remained a matter of controversy. For example, the results obtained by a Japanese group indicate that patients with HBeAg negative chronic hepatitis B might respond better to interferon therapy than HBeAg positive patients and hence, that the precore stop codon mutant might be sensitive to interferon.[29] In contrast, another group reported hepatic infections caused by a precore mutant of HBV to be more resistant to interferon therapy than wild-type infection (25 % vs. 46 % responders, respectively).[30] A study performed in Italy even suggests the shift from wild-type to precore mutant pattern of infection to be the consequence of interferon treatment or spontaneous HBeAg seroconversion, as the wild-type pattern was observed to predominate among the non-responders (8 vs. 3) thereby suggesting the long-term response to interferon to be associated with the take-over of precore mutants[31].

Experimental data thus far obtained suggest a unique pathway of cytogenicity accompanied by reduced recognition of infected cells and subsequent behavior as virus escape mutants, which might explain the resistance to interferon therapy encountered in a larger proportion of anti-HBe positive compared with anti-HBe negative patients.

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