CORE PROMOTER AND PRECORE MUTANTS OF HEPATITIS B VIRUS: PREVALENCE AND CLINICAL RELEVANCE IN CHRONIC HEPATITIS PATIENTS

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Abstract. The present study was conducted to determine prevalence and exact type, as well as nucleotide position of the precore/core mutations of hepatitis B virus found in Thai patients diagnosed with chronic hepatitis and/or cirrhosis in relation to the clinical parameters established with the respective patients. To that end, 24 HBeAg-positive and 56 HBeAg-negative individuals were selected at random from a cohort of altogether 256 chronic liver disease patients. DNA was extracted from their blood sera, amplified by polymerase chain reaction using semi-nested primers and subjected to direct sequencing. Clinically, the HBeAg-positive chronic hepatitis patients displayed significantly higher transaminase levels than those negative for HBeAg. Our results showed 2 of the 7 (28.6%) PCR-positive HBeAg-positive sera displaying double mutations in the core promoter region at position 1762/64. The nucleotide sequences obtained from the 24 PCR-positive HBeAg-negative sera revealed 18 (75%) mutations in the core promoter region (1762/64), and/or 7 (29.2%) mutations at position 1753, and/or 6 (25%) mutations of the start codon (1814), and/or 8 of (33.3%) nucleotide 1896 turning codon 28 into a stop codon and one sample (4.2%) displaying a deletion between nucleotides 1758-1772. It is suggested that the mutations observed have an impact on the DNA secondary structure in such a way that successful transcription of the HBeAg gene is rendered impossible. To what extent this mutation influences the severity of chronic liver disease remains to be elucidated.

INTRODUCTION

Hepatitis B virus infection represents a major burden on public health worldwide and is especially prevalent in Southeast Asia, China and sub-Saharan Africa where it is considered the ultimate cause of the majority of cases diagnosed with chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (Lee, 1997). The risk for any given individual infected to develop chronic liver disease is directly connected with the age at which infection occurs in that adults stand a much better chance to clear the virus than infants infected by carrier mothers during delivery (Popper *et al*, 1987).

Three clearly distinguishable phases attributable to virus-host interactions can be discerned in the natural history of chronic hepatitis B: virus tolerance usually associated with an asymptomatic disease course despite actively replicating HBV and HBeAg expression, virus clearance characterized by clinical exacerbation and remissions resulting from the host's cell-mediated immune response apparent by seroconversion to anti-HBe and finally, residual HBV integration with continued HBsAg expression leading to a variety of hepatic lesions as cirrhosis and HCC (Chu *et al*, 1985; Chen, 1993).

Recent advances in molecular biology have revealed remarkable genetic heterogeneity among hepatitis B virus (HBV) genomes, especially those isolated from chronically infected patients. To date, variants exhibiting mutations in almost all viral genes and regulatory regions have been identified. Among these, the precore mutants unable to synthesize hepatitis B e antigen (HBeAg) are those most frequently selected in nature (Lee, 1997). Previously, several studies have reported these mutants to predominantly occur in HBeAg-negative patients with chronic active hepatitis, as well as patients with fulminant hepatitis (Carman *et al*, 1989; Omata *et al*, 1991; Liang *et al*, 1991). However, more recent studies have shown the same mutants to be equally present in HBV carriers or mild

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forms of liver disease (Yotsumoto *et al*, 1992; Naoumov *et al*, 1992; Aye *et al*, 1994; Akarca *et al*, 1994; Karayiannis *et al*, 1995). Therefore, the exact impact of the precore mutants on clinical outcome has remained unclear.

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 (Lee, 1997). The A-1896 mutant is predominantly detected in certain parts of the world, such as in Mediterranean and East Asian countries, but is uncommon in North America and North Europe (Carman *et al*, 1989, 1992; Okamoto *et al*, 1990; Santantonio *et al*, 1991; Laskus *et al*,; 1993; Feray *et al*, 1993; Hawkins *et al*, 1994). The reported variability in their prevalence in different countries has been interpreted as a result of the deleterious effect of disruption of the encapsidation signal in certain predominant HBV genotypes (Li *et al*, 1993). Apart from this common variant, other precore mutations have also been reported, but less frequently (Okamoto *et al*, 1990; Santantonio *et al*, 1991; Fiordalisi *et al*, 1990; Tong *et al*, 1990).

Recently, double mutations of the core promoter at position 1762-1764, changing AGG to TGA, have been found in HBeAg-negative carriers and chronic hepatitis B (Okamoto *et al*, 1994). In an experimental study (Buckwold *et al*, 1996), these mutations reduced the transcription of precore mRNA by interfering with the binding of transcription factors, thus supporting the assumption that these mutations down-regulate HBeAg synthesis. These mutations were proposed to be associated with fulminant hepatitis B in Japan, (Sato *et al*, 1995), however, a more recent study from the United States has not reported detection of any of the core promoter mutations in such patients (Laskus *et al*, 1995). Moreover, it was also frequently found in both HBeAg-positive and HBeAg-negative chronic hepatitis (Takahashi *et al*, 1995).

Furthermore, a mutation at nucleotide 1753 changing the wild-type T into either C or G was often detected in anti-HBe positive patients along with the double mutation described above (Nagasaka *et al*, 1998).

The aim of this study has been to determine both prevalence and exact type, as well as nucleotide position of the precore/core mutations encountered among Thai patients with chronic hepatitis B. Previously, we had reported the preliminary data regarding the prevalence established, as well as the methods applied to that end (Poovorawan *et al*, 1999; Theamboonlers *et al*, 1999). In the present study, we have added various parameters and evaluated the clinical data regarding the severity of liver disease in these patients. As the prevalence and clinical relevance of the precore mutations seem to differ geographically, this study should provide additional useful epidemiological and clinical information from the region where HBV is hyperendemic and the data available are still limited.

MATERIALS AND METHODS

Patients

Two-hundred and fifty-six patients with clinical and/or histological evidence of chronic HBV infection attended the out-patient clinic, Gastrointestinal unit, Department of Internal Medicine, Chulalongkorn University Hospital between January and December, 1998. All patients were HBsAg positive as confirmed by ELISA (Auszyme, Abbott Laboratories, North Chicago, Ill, USA) and exhibited elevated alanine aminotransferase (ALT) levels. They comprised 189 patients with chronic hepatitis and 67 patients with cirrhosis. Patients with hepatocellular carcinoma (HCC) diagnosed by histology, imaging studies and/or serum alpha-fetoprotein levels were not included in the study. Regarding the presence of HBeAg in the sera, 112 patients were HBeAg-positive and the remaining 144 were HBeAg-negative (tested by ELISA; Hepanostika HBe, Organon Diagnostics, Boxtel, The Netherlands).

Of these 256 patients, 24 HBeAg-positive and 56 HBeAg-negative patients were randomly chosen for HBV DNA detection. They comprised 12 females and 68 males, whose age ranging from 21 to 69 years (mean 42.5 ± 13.1 years). 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 -70ºC until tested.

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 Collaborating Center, Nagasaki, Japan.

HBV DNA was amplified by semi-nested PCR in an automated thermocycler (Perkin Elmer Cetus, Branchburg, New Jersey, USA) as described elsewhere (Saiki *et al*, 1988). 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′ (nucleotide 1608-1625) and Ci1 with the sequence 5′-TTC CGG AGA CTC TAA GGC-3′ (nucleotide 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′ (nucleotide 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 94ºC, 55ºC and 72ºC for 1 minute each, followed by 30 cycles comprising a 30 second denaturation step at 94ºC, a 30 second annealing step at 55ºC, and a 1 minute extension step at 72ºC, each. The amplification was concluded by one cycle at 94ºC for 1 minute, 55ºC for 2 minutes and 72ºC 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 were loaded on a 2% Nusieve (FMC Bioproducts, Rockland, ME, USA) agarose gel stained with ethidium bromide on preparation. Electrophoresis was performed at 90 V for 70 minutes and the product band of 367 base pairs was visualized 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.

For determining the concentration of the amplified DNA, we measured the absorption at 260 nm of every sample in a UV spectrophotometer (Shimadzu UV 160 A). The concentration was calculated according to the formula that 1 OD 260 = 50 μg double-stranded DNA. Between 10 and 30 ng/μl (3-6 μl) of every respective DNA were subjected to cycle sequencing using dye-labeled terminators (8 μl and 3.2 pmole of specific primer at a final reaction volume of 20 μl) which represents a rapid and convenient method for performing enzymatic extension reactions for subsequent DNA sequencing on the ABI PRISM (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 96ºC for 10 seconds (denaturation), 50ºC for 5 seconds (annealing), and 60ºC for 4 minutes (extension). The reaction was concluded by cooling the thermal ramp to 4ºC. The extension products were subsequently purified from excess non-incorporated dye terminators by ethanol precipitation according to the manufacturer's specifications (Perkin Elmer Cetus) and subsequently prepared for loading on the ABI PRISM 310 Genetic Analyser.

For all the subsequent steps we referred to the ABI PRISM 310 Genetic Analyser user's manual (Perkin Elmer Cetus, Branchburg, New Jersey, USA).

Statistical analysis

The prevalence was expressed in percent related to the number of patients studied. Comparisons between groups were made by the χ^2 or Fisher's exact test for categorical variables and by the Mann-Whitney test or Student's *t*-test when appropriate for quantitative variables. A p-values below 0.05 were considered significant.

RESULTS

Patients were classified as two separate groups, those with chronic hepatitis and those with cirrhosis, which were again subdivided into two groups each depending on presence or absence of HBeAg. The transaminase (AST/ALT) levels in HBeAg-positive chronic hepatitis patients were signifi-

cantly higher than in those negative for HBeAg ($p < 0.05$). However, this difference was altogether absent among the cirrhosis patients, and with respect to demographic features, known duration of HBV infection, additional biochemical abnormalities and the severity of the respective liver lesions no significant differences were detectable between either chronic hepatitis and cirrhosis patients on the one hand, and HBeAg expression or lack thereof on the other hand (Table 1).

Table 1
Baseline features of patients with chronic hepatitis B and cirrhosis according to the presence and absence of HBeAg in serum.

Characteristics	Chronic hepatitis		Cirrhosis	
	HBeAg positive (N=78)	HBeAg negative (N=111)	HBeAg positive (N=34)	HBeAg negative (N=33)
Age (yrs)	35.9±11.7	44.1±10.5	41.9±12.4	49.6±13.7
Sex (male/female)	51/27	84/27	28/6	22/11
Known HBsAg positivity (yrs)	6.1±5.1	8.0±5.7	6.1±4.2	7.1±4.6
Biochemical liver function tests				
Bilirubin (mg/dl)	0.8±0.7	0.7±0.4	1.1±0.7	1.2±1.6
AST (IU/l)	80.3±69.3	50.5±29.6[a]	80.3±86.0	85.2±79.0
ALT (IU/l)	129.5±134.1	72.5±55.1[a]	72.6±83.1	78.0±101.6
AP (IU/l)	186.4±95.8	173.6±76.0	238.1±94.1	225.1±132.3
Albumin (g/dl)	4.6±0.4	4.8±0.7	4.3±0.7	3.5±0.7
Prothrombin time (sec)	13.2±1.5	12.9±2.0	14.6±2.6	19.1±14.4
Child A/B/C	-	-	28/5/1	19/9/5
Liver biopsy performed (N)	43	48	18	9
Degree of histological activity				
CPH	10 (23.3%)	25 (52.1%)	-	-
CAH	33 (76.7%)	23 (47.9%)	-	-

ALT: alanine aminotransferase; AST: aspartate aminotransferase; AP: alkaline phosphatase; CPH: chronic persistent hepatitis; CAH: chronic active hepatitis.
Quantitative variables are expressed as mean ± SD.
Categorical variables are expressed as n (%).
[a]$p<0.05$

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

Position of mutation	HBeAg-positive n = 7	HBeAg-negative n = 24
Core promotor		
1753 T-C	0 (0%)	7 (29.2%)[a]
1762 A-T, 1764 G-A	2 (28.6%)	18 (75%)
1758-1772 deletion	0 (0%)	1 (4.2%)
Precore gene		
Start codon	0 (0%)	6 (25%)[b]
1896 G-A	0 (0%)	8 (33.3%)[c]

[a]6 patients showed simultaneously mutations at 1762, 1764.
[b]2 patients showed mutations at 1762,1764 and start codon.
[c]5 patients showed mutations at 1762,1764 and 1896, 1 at the start codon and 1896.

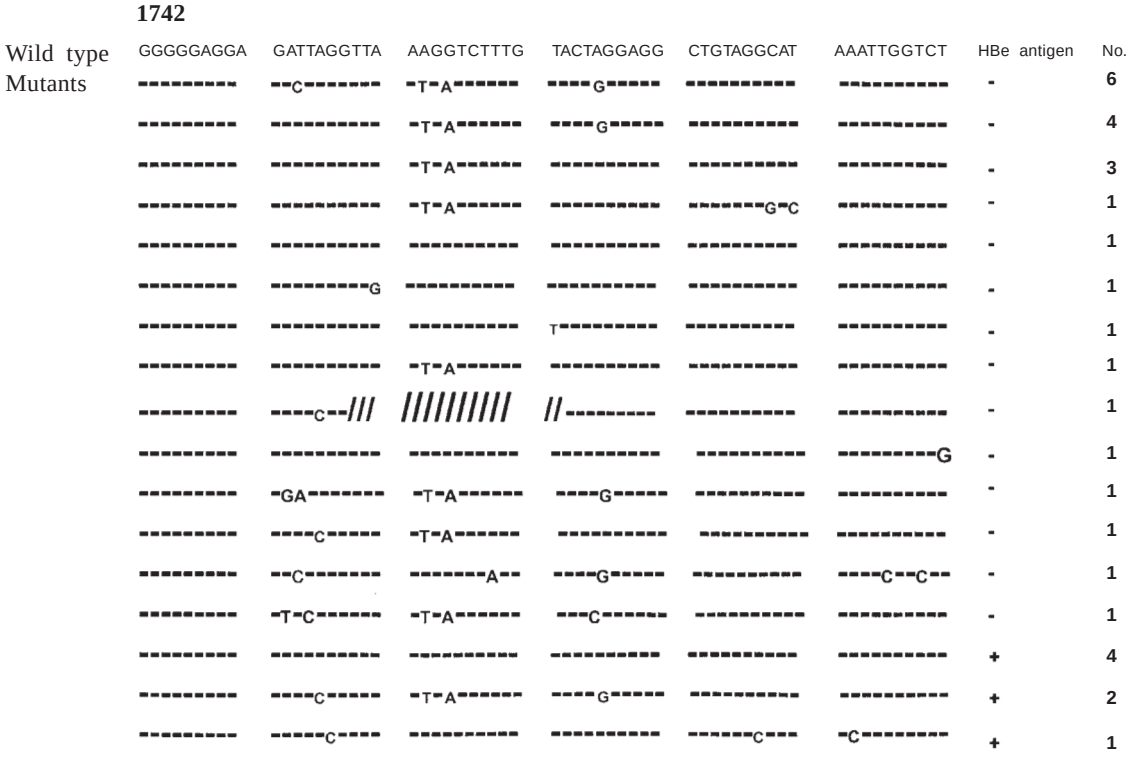

Fig 1–Nucleotide position and nature of mutations detected in the core promoter region of hepatitis B virus in relation to HBeAg expression. Top lane: HBV wild type; ---- identical to wild type sequence; /////// deleted sequence.

Of the 80 chronic hepatitis patients chosen, all sera were subjected to polymerase chain reaction using semi-nested primers covering the precore as well as the core promoter region of hepatitis B virus. Of the 24 HBeAg-positive sera, 21 (87.5%) were also found DNA-positive by PCR. Of those 21, 7 were randomly chosen for sequencing, two of which showed double mutations in the core promoter region at nucleotides 1762 and 1764, respectively. Of the 56 HBeAg-negative sera, 26 (46.2 %) were DNA-positive by PCR. Of those 26, the amount of serum necessary for sequencing was sufficient in 24. The nucleotide sequences thus obtained revealed altogether 18 double mutations at nucleotides 1762 and 1764 in the core promoter region, 6 of which simultaneously showed a mutation from T to C at nucleotide 1753, and/or 6 mutations of the start codon and/or 8 of nucleotide 1896 turning codon 28 into a stop codon. One HBeAg-negative serum had nucleotides 1758-1772 deleted. In some of the sera we detected double mutations, as well as various point mutations at loci different from those discussed the significance of which remains to be clarified. The details are depicted in Table 2 and Fig 1. The comparison between HBV precore gene sequences isolated from HBeAg-positive and HBeAg-negative chronic liver disease patients is shown in Fig 2.

As regards the severity of liver disease regardless of HBeAg status, we could not detect any significant association of the mutations at nucleotide 1753 or 1762 and 1764 in the core promoter with the liver damage in terms of elevated ALT levels and histology activity index (HAI) scores at the time of sampling (Table 3).

DISCUSSION

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 (Chu *et al*, 1985; Chen, 1993). The second phase is characterized by a clinical course of fluctuations in aminotransferase (ALT) levels as a result of the host's cell-mediated immune response. Consequently, HBV replication gradually

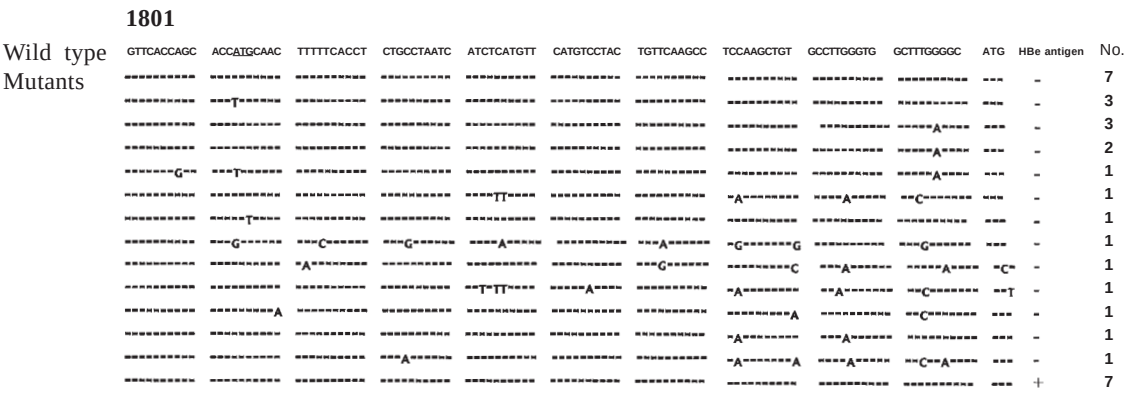

Fig 2–Comparison between HBV precore gene sequences isolated from HBeAg-positive and HBeAg-negative chronic liver disease patients.

Table 3
Relationship of core promoter mutations/deletions to ALT levels and HAI scores.

Core promoter mutations/deletions	N	Age (yrs)	ALT (IU/l)	HAI_{sum} scores	Known HBV positivity (yrs)
Nt 1762, 1764					
Positive	21	42.6±12.7	89.8±43.6	8.4±2.4	8.1±5.2
Negative	10	39.6±9.9	93.8±50.8	8.0±2.3	9.6±7.8
		NS	NS	NS	NS
Nt 1753					
Positive	7	44.3±10.1	95.9±43.8	9.3±1.7	8.1±5.0
Negative	24	40.9±12.3	89.7±46.6	8.0±2.4	8.7±6.4
		NS	NS	NS	NS

Nt: Nucleotide, ALT: Alanine aminotransferase
HAI_{sum} scores: The sum of the inflammation and fibrosis components of Histology activity index (HAI) scores.
Quantitative variables were expressed as mean ± SD.

decreases until altogether subsiding with subsequent HBeAg clearance and seroconversion to anti-HBe. Hence, patients negative for HBeAg tend to be associated with lower ALT levels compared with patients positive for HBeAg as demonstrated in the present study.

In contrast with some previous studies conducted in Asian countries, (Okamoto *et al*, 1990; Carman *et al*, 1992; Nagasaka *et al*, 1998; Hsu *et al*, 1995; Ehata *et al*, 1996) our data demonstrated the 1896 mutation occurring at a lower frequency in HBeAg-negative chronic hepatitis B. The reason for this low prevalence remains unclear despite all cases in this study bearing a T at position 1858. HBV reverse transcription requires the RNA intermediate to be folded in a secondary structure known as cis-acting encapsidation sequence (ε). The G-to-A mutation at nucleotide 1896 would disrupt the stable G-C base pair between position 1858 and 1896, resulting in significant reduction in packaging of pregenomic RNA and hence detrimental to viral replication (Lok *et al*, 1994). This may account for the low rate of this mutation detected in the United States, France and South Africa where the predominant HBV genotype has a C at nucleotide 1858 (Feray *et al*, 1993; Laskus *et al*, 1994; Kramvis *et al*, 1997).

Another mutation leading to the inability to produce HBeAg is the one affecting the start codon (ATG) of the precore gene and thus abolishing the start of protein synthesis. In the present study, we identified these mutations in 6 (25%) of the HBeAg-

negative patients with various discreet point mutations changing ATG into ATT (1 patient), AAG (1 patient), GTG (1 patient), and TTG (3 patients), respectively. Although the start codon mutations have been described previously to occur in diverse geographical areas, (Okamoto *et al*, 1990; Firodalisi *et al*, 1990; Kramvis *et al*, 1997) the overall prevalence seems to be rather low compared with our study.

For the synthesis and secretion of HBeAg, precore mRNAs have to be transcribed from HBV DNA and the precore and core regions have to be translated. Therefore, any mutations occurring in either the precore region or the core promoter and preventing the translation of precore mRNAs induce an HBeAg-minus phenotype. In the core promoter 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 promoter (Okamoto *et al*, 1994; Sato *et al*, 1995). 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 nucleotide 1762 and from G to A at nucleotide 1764, as detected in 75% of our HBeAg-minus patients, have been most frequently reported (Okamoto *et al*, 1994; Sato *et al*, 1995; Takahshi *et al*, 1995; Kurosaki *et al*, 1996).

In our study, the double mutation of T1762 A1764 in the core promoter was found more frequently in HBeAg-negative than in HBeAg-positive chronic hepatitis patients. This result was in agreement with some but not all of the previous studies (Okamoto *et al*, 1994; Nagasaka *et al*, 1998; Kurosaki *et al*, 1996; Kidd-Ljunggren *et al*, 1997; Lindh *et al*, 1998). Thus, it would be reasonable to conclude that the occurrence of HBV variants with core promoter mutations preventing the proper transcription of precore mRNAs result in a decreased expression of HBeAg. Unlike precore mutations which prohibit the synthesis and secretion of HBeAg completely, the effect of core promoter mutations to that end may be short of complete. In an experimental study performed in cultured cells, (Buckwold *et al*, 1996) the double mutation in the second AT-rich region of the core promoter has been found to prohibit the binding of HBV DNA with liver-enriched transcription factors. Moreover, the double mutation decreases the transcription of precore mRNAs and expression of HBeAg to approximately one third of the wild type. Recently, another experimental study from the same group found that this double mutation not only removed the nuclear receptor binding site but also created an hepatocyte muclear factor I (HNF1) transcription factor binding site. Thus, the specific suppression of precore RNA transcription by this frequent double-nucleotide mutation is the combined result of multiple factors (Li *et al*, 1999). It should also be kept in mind that, as HBeAg on the hepatocyte presents a target for cytotoxic T cells, (Milich *et al*, 1987; Pignatelli *et al*, 1987; Bertoletti *et al*, 1991) the mutation suppressing HBeAg expression may constitute an escape variant from cytoimmunity. Moreover, as the double mutation has been indicated to induce an increase in viral replication, replicating under host immune pressure might turn out beneficial for HBV (Buckwold *et al*, 1996).

We found a mutation from T to C at nucleotide 1753 in seven of the HBeAg-negative patients, most of which also displayed the double mutation at nucleotides 1762 and 1764, contrasting none among the HBeAg-positive samples. Furthermore, we could not find any possible mutations to convert the stop codon in the precore region accompanied these mutations in 6 HBeAg-negative sera. Hence, this particular combination of mutations might affect HBeAg expression. Along these lines, it has been suggested that there might be a secondary structure of pregenomic RNA from nucleotide 1742 to 1847 and that a structural change might be triggered by the double mutation at nucleotide 1762 and 1764, furthermore, that nucleotide 1751 to 1757 formed a stem structure and that mutations in this region would weaken the conjunction at the stem and hence open it for reverse transcription (Kidd-Ljunggren *et al*, 1997).

As yet, the clinical significance of the double mutation of T1762 A1764 has remained unclear. In recent studies, an association between these mutations and the appearance of higher ALT levels could be discerned (Takahashi *et al*, 1995; Kidd-Ljunggren *et al*, 1997). In addition, patients with these mutant strains showed more liver inflammation and fibrosis, as measured by histology activity index (HAI) scores than those with wild type strains (Kurosaki *et al*, 1996). Furthermore, a recent study from China demonstrated that the core promoter mutants were found more frequently in patients with hepatocellular carcinoma than in asymptomatic controls (Fang *et al*, 1998). However, no such association in terms of elevated ALT levels and histological severity was found in the present study. This discrepancy might reflect sequence variations

among geographical isolates of hepatitis B viruses. Hence, regarding clarification of its role toward the induction of severe liver disease further studies are certainly required.

As the core promoter 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 and might influence the activities assigned to this protein (Yuh *et al*, 1992). In that context, the double mutation from T1762 and A1764 changes the amino acids Lys at position 130 to Met and Val at position 131 to Ile, respectively. Since the transactivation domains and role of the X protein are largely unknown, the effect of these hot spot mutations on the transactivation activity of the X protein remains to be elucidated.

In conclusion, the 1896 stop codon mutation accounts for a minority of HBeAg-negative Thai patients with chronic hepatitis B infection despite the high incidence of the HBV genotype bearing a T at position 1858. In addition, we found that double mutations of T1762 A1764 in the core promoter were highly prevalent in patients negative for HBeAg but not in those positive for this marker. These mutations in the core promoter could possibly result in a decreased expression of HBeAg in the majority of HBeAg-negative chronic hepatitis B in Thailand.

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