PREVALENCE OF CORE PROMOTOR AND PRECORE MUTANTS OF HEPATITIS B VIRUS IN THAILAND BY RFLP AND SEQUENCING

Apiradee Theamboonlers[1], Pisit Tangkijvanich[2], Podchanad Jantaradsamee[1], Petra Hirsch[1], and Yong Poovorawan[1]

[1]Viral Hepatitis Research Unit, Department of Pediatrics; [2]Department of Biochemistry, Faculty of Medicine, Chulalongkorn University and Hospital, Bangkok 10330, Thailand

Abstract. Hepatitis B virus has been known to frequently undergo mutations of its genome at various sites, mostly due to it employing a reverse transcriptase devoid of proofreading capacity in the course of its replication. The purpose of the present study has been to screen 257 HBsAg-positive chronic liver disease patients, more specifically 78 cases chosen at random out of those negative for HBeAg and 33 of the HBeAg-positive cases serving as controls for three discreet point mutations in the precore/core region of hepatitis B virus. To that end, HBV DNA extracted from sera was amplified by polymerase chain reaction (PCR) using semi-nested primers and subsequently subjected to restriction fragment length polymorphism (RFLP) analysis, 36 HBeAg-negative versus 30 HBeAg-positive sera, respectively, as well as to direct sequencing in some samples randomly selected to corroborate the RFLP results. Our results showed double mutations at positions 1762 and 1764 of the core promoter in between 25/36 (69.4%) and 19/25 (76%) of the sera tested, a missense mutation of the start codon in between 8/36 (22.2%), and 5/25 (20%) and a mutation turning codon 1896 into a stop codon in between 9/36 (25%) and 6/25 (24%) determined by RFLP and sequencing, respectively. These data indicate the double mutation at positions 1762 and 1764 to be the most prevalent among HBeAg-negative chronic hepatitis patients in Thailand whereas, in contrast to reports from other Asian countries, the mutation at nucleotide 1896 occurred in a mere 25%, while on the other hand the mutation abolishing the start of protein synthesis was observed to occur at a higher frequency than determined in several other geographical areas.

INTRODUCTION

Hepatitis B virus (HBV) infection constitutes a public health problem on a global scale accounting for the majority of chronic hepatitis cases with their fatal sequelae cirrhosis and hepatocellular carcinoma (Lee, 1997). Furthermore, genetic heterogeneity is a common feature of the virus and can influence the outcome and course of the infection. For example, vaccine failure might be attributable to mutations reported for HBV, caused by the virus employing a reverse transcriptase without proofreading capacity for part of its replication (Lanford *et al*, 1997), particularly those affecting the *a* determinant of the surface antigen (HBsAg) gene leading to immune escape mutants (Poovorawan *et al*, 1998a). However, these particular escape mutants as yet do not appear to impair the prevention effected by vaccination. Additional mutations frequently encountered affect the core gene of HBV coding for hepatitis B e antigen (HBeAg) in that point mutations in nucleotides 1762 and 1764 of the core promotor have been shown to lead to highly enhanced viral replication as a result of increased viral encapsidation of pregenomic RNA into the core particles (Kidd and Kidd-Ljunggren, 1996; Baumert *et al*, 1998). Alternatively, mutations in the gene encoding the precore/core protein (precore stop codon mutants) cause loss of HBeAg and seroconversion to anti-HBe despite persistent HBV replication (HBeAg minus mutant). Mutations in the core gene may also lead to "immune escape" due to a T cell receptor antagonism (Hosono *et al*, 1995).

Various methods have been developed in order to facilitate detection of these mutations in order for patient care to keep one step ahead of the virus's ingenuity to escape detection, for example, single-strand conformation polymorphism analysis (SSCP) capable of identifying mutant DNA comprising as little as 3% of the total gene copies in a polymerase chain reaction (PCR) mixture (Hongyo *et al*,1993). Allele-specific competitive blocker PCR (ACB-PCR) constitutes an even more powerful

Correspondence: Prof Yong Poovorawan, Vital Hepatitis Research Unit, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University and Hospital, Bangkok 10330, Thailand.
Tel: +662-256-4909; Fax: +662-256-4929; E-mail: Yong.P@chula.ac.th

method able to detect mutants at a frequency of 10^{-5} (Parsons and Heflich, 1998a), and in combination with MutEx enrichment this method can identify mutations at mutant fractions as low as 10^{-7} (Parsons and Heflich, 1998b). Our own group has recently applied direct sequencing in order to screen chronic liver disease patients suspected of underlying HBV infection despite seroconversion to anti-HBe for mutants in the precore/core region employing the method of direct sequencing (Poovorawan *et al*, 1999).

The present study has been aimed at evaluating the prevalence of these mutations among Thai patients diagnosed with chronic liver disease and seropositive for HBsAg by means of restriction fragment length polymorphism (RFLP), another powerful tool for detection of mutations.

MATERIALS AND METHODS

Study Population

Initially, the population studied comprised 257 patients diagnosed with chronic hepatitis, who either attended the out-patient clinic or had been admitted to Chulalongkorn University Hospital between January and December 1998. Upon having received their informed consent as to the purpose of the study, venous blood was obtained, the sera separated by centrifugation and kept at -70°C until tested.

Laboratory methods

Serology: The 257 sera were tested for the presence of HBsAg and HBeAg by ELISA (Auszyme, Abbott Laboratories, North Chicago, Ill. and Hepanostika HBe, Organon Diagnostics, Boxtel, The Netherlands, respectively) 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: HBV DNA was amplified in an automated thermocycler (Perkin Elmer Cetus, Branchburg, NJ, USA) using semi-nested primers 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, NJ, USA), each of four deoxynucleotide triphosphates (Promega Corp, Madison, WI, USA) at a concentration of 200 (M, primer pair eP1-1 with the sequence 5′-GCA TGG AGA CCA CCG TGA AC-3′ (nt. 1606-1625) and eP1-2 with the sequence 5′-GGA AAG AAG TCA GAA GGC AA-3′ (nt. 1955-1974) for the first amplification round, and primer pair eP1-1 (as above) and Xi3 with the sequence 5′-AAG CCT CCA AGC TGT GCC-3′ (nt. 1866-1833) 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. For the purpose of the subsequent restriction reaction with StuI (see below) two specific primers were used for the second round amplification in order to accommodate the restriction site recognized by the enzyme. Their sequences were for the sense primer 2C 5′-CCA TGC AAC TTT TTC ACC TCT G-3′ (nt. 1812-1833) and for the anti-sense primer 2D 5′-ATA CGG GTC AAT GTC CAA GGC C-3′ (nt. 1897-1918), respectively. 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 2 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% agarose gel (FMC Bioproducts, Rockland, ME, USA) stained with ethidium bromide on preparation. Electrophoresis was performed at 90 V for 80 minutes. The product band of 369 base pairs for the first and 288 base pairs for the second PCR, respectively, were visualized on a UV transilluminator.

DNA purification and sequencing: Some of the PCR products were purified and subjected to direct sequencing as described elsewhere (Poovorawan *et al*, 1999).

Restriction fragment length polymorphism (RFLP): The PCR products analyzed by direct sequencing were also subjected to RFLP analysis using the DNA restriction enzymes Sau 3AI to investigate the site at codons 1762-1764 (Takahashi *et al*, 1995) and Rsa I to examine the start codon, respectively. To that end, 15 U of Sau 3AI were added to the first round PCR products (369 bp), and 15 U of Rsa I to the second round products (278 bp), respectively, and subsequently incubated at 37°C for 3

hours, 30 minutes. In order to determine if nucleotide 1896 had undergone a G-A mutation leading to a stop codon, the products of the second round PCR performed with primer pair 2C/2D were incubated with 5 U of Stu I at 37°C for 3 hours 30 minutes (Niitsuma *et al*, 1995). The RFLP products were again subjected to electrophoresis on a 2% Nusieve agarose gel and their respective sizes compared to those of a suitable nucleotide size marker (100 bp DNA ladder, Promega Corp, Madison, WI, USA). The sizes expected were 369 bp for the 1762/1764 wild type, 212 bp and 157 bp for the 1762/1764 mutant (Fig 1), 206 bp for the 1814 wild type and 278 bp for the 1814 mutant (Fig 2), 105 bp for the 1896 wild type and 85 bp for the 1896 mutant (Fig 1), respectively.

RESULTS

Of the 257 patients initially tested for HBsAg by ELISA, all were found to be positive. The subsequent ELISA test for the presence of HBeAg showed 144 (56%) HBeAg negative and 113 (44%) HBeAg positive cases. Of the 144 negative samples, 78 were chosen at random and subjected to PCR, revealing

Fig 1–2% Nusieve gel of RFLP pattern. From left to right: M = 100-base pair DNA ladder; W = core promoter wild type, undigested; m = core promoter wild type digested with Sau 3AI; W = precore stop codon wild type, undigested; m = precore stop codon wild type, digested with Stu I.

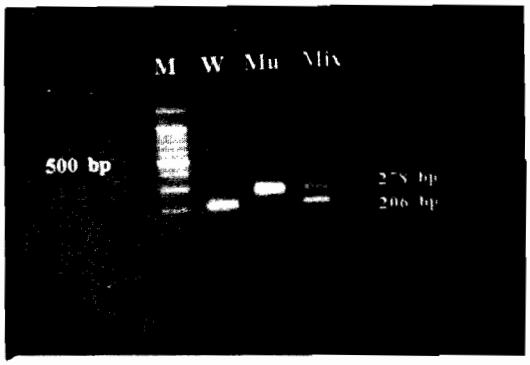

Fig 2–2% Nusieve gel of RFLP pattern. From left to right: M = 100 base pair DNA ladder; W = precore start codon wild type, digested with Rsa I; Mu = precore start codon mutant, undigested; Mix = wild type/mutant mixed population, digested with Rsa I.

Table 1
Prevalence of mutations in the core/precore region of HBV - comparison between direct sequencing and RFLP analysis.

Mutation site	HBeAg+ve				HBeAg-ve			
	Sequencing n=16		RFLP n=30		Sequencing n=25		RFLP n=36	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)
1762/64 (A-T, G-A)	4	(25)	4	(13.3)	19	(76)	25	(69.4)
1814 (ATG - ATT,TTG,GTG)	0	(0)	0	(0)	5	(20)	8	(22.2)
1896 (G-A)	0	(0)	0	(0)	6	(24)	9	(25)

NB: Among the HBeAg-negative samples, double mutations were found at sites:
1762/64 and 1814 in 3 cases (8.3%), 1762/64 and 1896 in 2 cases (5.6%), 1814 and 1896 in 1 case (2.8%), and in addition, one mixed population of wild type and 1814 mutation (Fig 2).

One specimen of HBeAg-negative showed 15 bp deletion at the promotor area (nt. 1758-1772) by direct sequencing.

42 HBV DNA negative samples and 36 cases, which expressed HBV DNA. All 36 samples were subjected to RFLP analysis and 25 of the 36 samples, again chosen at random, had the RFLP results confirmed by direct sequencing. Of the 113 HBeAg positive samples, 33 were chosen at random for PCR analysis showing 30 HBV DNA positive and 3 HBV DNA negative cases, respectively. The 30 DNA positive samples were analyzed by RFLP and 16 of the 30 were chosen at random for confirmation by direct sequencing. The results of the RFLP and sequence analyses, respectively, are shown in Table 1.

DISCUSSION

The purpose of the present study has been to screen patients diagnosed with chronic hepatitis due to HBV infection as affirmed by serological tests for the presence of HBsAg, and who furthermore had been proven to be HBeAg negative, for mutations in the precore/core promotor region of HBV DNA, especially as the HBeAg-negative state which in cases of wild-type viral infections would indicate seroconversion to anti-HBe tends to induce the assumption that the patient has succeeded in clearing the virus. Whereas in a previous study (Poovorawan *et al*, 1999) we had tested chronic hepatitis patients for these mutations by means of direct sequencing, in the present one we extended the number of sera to be screened and applied the method of restriction fragment length polymorphism (RFLP) in comparison with direct sequencing among a randomly selected number of samples. The results obtained by both techniques proved to be comparable as confirmed by the lack of mutations discerned by either method at the start (1814) and stop (1896) codons among the HBeAg-positive samples, as well as by the comparable mutation percentages obtained in the core promotor region with both groups and at the start and stop codons among the HBeAg-negative sera, respectively. Hence, the method of restriction fragment length polymorphism has been found adequate to substitute for the more expensive and time consuming technique of direct sequencing.

As to the prevalence of the particular mutations detected, we have found the one affecting nucleotide 1896 by a change from G to A and thus preventing HBeAg production by introducing a TAG stop codon in 25 % of the HBeAg-negative patients, a prevalence much lower than that reported in some other studies previously conducted in Asian countries (Okamoto *et al*, 1990; Carman *et al*, 1992; Ehata *et al*, 1996; Lok *et al*, 1994). In the course of the reverse transcription step forming part of HBV replication cycle the RNA intermediate has to acquire a secondary structure known as cis-acting encapsidation sequence (ε). A G-to-A mutation at position 1896 would interfere with the stable G-C base pair between nucleotides 1858 and 1896 and the ensuing drastic reduction in packaging of pregenomic RNA on its part would impair viral replication (Lok *et al*, 1994).

Another mutation leading to an HBeAg-negative phenotype abolishes the start of protein synthesis by altering the start codon of the precore gene from ATG to any combination of the three nucleotides effected by a single point mutation and not coding for methionine. To illustrate this point, we have detected by direct sequencing ATT, AAG, GTG and TTG, respectively, in place of the wild type ATG. In comparison with the rather high frequency amounting to between 20 and 22% discerned in the present study, the overall prevalence of the start codon mutations reported to occur in various geographical areas (Okamoto *et al*, 1990; Fiordalisi *et al*, 1990; Kramvis *et al*, 1997) appears to be quite low.

The mutation type found by far at the highest frequency among our HBeAg-negative sera is the double mutation occurring in the core promotor sequence at nucleotides 1762 and 1764 located at the 3′ terminus of the second of the three AT-rich regions 20-30 base pairs upstream of the transcription start site (Okamoto *et al*, 1996). These AT-rich sequences are recognized by transcription factors with binding sites in the core promotor (Okamoto *et al*, 1994; Sato *et al*, 1995) and especially in the first and second of these regions various mutations have been detected among asymptomatic HBV carriers, as well as patients with acute or chronic hepatitis B, with the double mutation at positions 1762 and 1764 representing the one predominantly encountered.

In agreement with some previous studies (Okamoto *et al*, 1994; Nagasaka *et al*, 1998) which reported the double mutation at positions 1762 and 1764 to occur more frequently among HBeAg-negative than HBeAg-positive chronic hepatitis patients, it might be concluded that core promotor mutations impacting proper transcription of precore mRNAs lead to decreased expression of HBeAg. Moreover, it should be noted that, since HBeAg on the hepatocyte presents a target for cytotoxic T cells (Pignatelli *et al*, 1987), a mutation suppressing HBeAg expres-

sion may offer an escape route from cytoimmunity to the virus and, as the double mutation has been implied in inducing an increase in viral replication, replicating under immune pressure exerted by the host might be advantageous for HBV (Buckwold *et al*, 1996).

In conclusion, in an area notorious for chronic HBV infection with vertical transmission constituting the rule rather than the exception (Poovorawan *et al*, 1998b), the high prevalence of precore/core region mutations should not come as a surprise, in particular as their frequency has been shown to depend on the duration of chronic HBV infection (Hamasaki *et al*, 1994), but rather ought to constitute a warning in that the HBeAg-negative state does not necessarily indicate virus clearance as previously assumed. It should also be kept in mind that therapy with interferon-alpha, which has been considered the only effective treatment for chronic hepatitis B, has been shown to induce, though infrequently as yet, the appearance of precore/core mutants (Laskus *et al*, 1995). Also, among patients exhibiting interferon-induced seroconversion to anti-HBe mixed viral infection of precore mutant and wild-type HBV could still be observed in sera of HBV carriers obtained within one year after seroconversion suggesting the precore mutants to prevail over the wild type in HBeAg-negative carriers for several years after seroconversion (Karasawa *et al*, 1995). Likewise, the comparatively novel drug lamivudine, designed to inhibit viral replication, has already been reported to induce the virus to mutate within the YMDD motif located in the gene coding for the polymerase and thereby rendering it resistant to treatment (Tipples *et al*, 1996).

Therefore, as due to the virus's ingenuity efficient medications increasingly appear to elude the physicians' grasp vaccination against hepatitis B virus appears to represent the only reasonable approach to combat this public health threat, especially in areas of high endemicity.

ACKNOWLEDGEMENTS

We would like to express our gratitude towards the entire staff of the Viral Hepatitis Research Unit, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University Hospital, for their tireless effort in this research project. We also would like to thank the Thailand Research Fund, Senior Research Scholar and the Molecular Research Project, Faculty of Medicine, Chulalongkorn University for supporting this research.

REFERENCES

Baumert TF, Marrone A, Vergalla J, Liang TJ. Naturally occurring mutations define a novel function of the hepatitis B virus core promoter in core protein expression. *J Virol* 1998; 72: 6785-95.

Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promotor on the precore gene expression and viral replication. *J* Virol 1996; 70: 5845-51.

Carman WF, Ferrao M, Lok AS, Ma OC, Lai CL, Thomas HC. Precore sequence variation in Chinese isolates of hepatitis B virus. *J Infect Dis* 1992; 165: 127-33.

Ehata T, Yokosuka O, Imazeki F, Omata M. Point mutation in precore region of hepatitis B virus: sequential changes from 'wild' to 'mutant'. *J Gastroenterol Hepatol* 1996; 11: 566-74.

Fiordalisi G, Cariani E, Mantero G, *et al*. High genomic variability in the pre-C region of hepatitis B virus in anti-HBe, HBV DNA-positive chronic hepatitis. *J Med Virol* 1990; 31: 297-300.

Hamasaki K, Nakata K, Nagayama Y, *et al*. Changes in the prevalence of HBeAg-negative mutant hepatitis B virus during the course of chronic hepatitis B. *Hepatology* 1994; 20: 8-14.

Hongyo T, Buzard GS, Calvert RJ, Weghorst CM. Cold SSCP: a simple, rapid and non-radioactive method for optimized single-strand conformation polymorphism analyses. *Nucleic Acids Res* 1993; 21: 3637-42.

Hosono S, Tai PC, Wang W, *et al*. Core antigen mutations of human hepatitis B virus in hepatomas accumulate in MHC class II-restricted T cell epitopes. *Virology* 1995; 212: 151-62.

Karasawa T, Aizawa Y, Zeniya M, Kuramoto A, Shirasawa T, Toda G. Genetic heterogeneity in the precore region of hepatitis B virus in hepatitis B e antigen-negative chronic hepatitis B patients: spontaneous seroconversion and interferon-induced seroconversion. *J Med Virol* 1995; 45: 373-80.

Kidd AH, Kidd-Ljunggren K. A revised secondary structure model for the 3′-end of hepatitis B virus pregenomic RNA. *Nucleic Acids Res* 1996; 24: 3295-301.

Kramvis A, Bukofzer S, Kew MC, Song E. Nucleic acid sequence analysis of the precore region of hepatitis B virus from sera of Southern African black adult carriers of the virus. *Hepatology* 1997; 25: 235-40.

Lanford RE, Notvall L, Lee H, Beames B. Transcomplementation of nucleotide priming and reverse transcription between independently expressed TP and RT domains of the hepatitis B virus reverse transcriptase. *J Virol* 1997; 71: 2996-3004.

Laskus T, Rakela J, Persing DH. Nucleotide sequence analysis of precore and proximal core regions in patients with chronic hepatitis B treated with interferon. *Dig Dis Sci* 1995; 40: 1-7.

Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733-45.

Lok AS, Akarca U, Greene S. Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. *Proc Natl Acad Sci USA* 1994; 91: 4077-81.

Nagasaka A, High S, Marutani M, *et al.* Prevalence of mutations in core promotor/precore region in Japanese patients with chronic hepatitis B viral infection. *Dig Dis Sci* 1998; 13: 2473-8.

Niitsuma H, Motoyasu I, Saito Y, *et al.* Prevalence of precore-defective mutant of hepatitis B virus in HBV carriers. *J Med Virol* 1995; 46: 397-402.

Okamoto H, Miyakawa Y, Mayumi M. Mutations in the hepatitis B virus core promoter for a decreased expression of hepatitis B e antigen. In: Rizzatto M, Purcell RH, Gerin JL, Verme G, eds. Viral Hepatitis and Liver Disease 1996; 121-6.

Okamoto H, Tsuda F, Akahane Y, *et al.* Hepatitis B virus with mutations in the core promotor for an e-antigen negative phenotype in carriers with antibody to e antigen. *J Virol* 1994; 68: 8102-10.

Okamoto H, Yotsumoto S, Akahane Y, *et al.* Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J Virol* 1990; 64: 1298-303.

Parsons BL, Heflich RH. Detection of a mouse H-ras codon 61 mutation using a modified allele-specific competitive blocker PCR genotypic selection method. *Mutagenesis* 1998a; 13: 581-8.

Parsons BL, Heflich RH. Detection of basepair substitution mutation at a frequency of 1 x 10(-7) by combining two genotype selection methods, MutEx enrichment and allele-specific competitive blocker PCR. *Environ Mol Mutagen* 1998b; 32: 200-11.

Pignatelli M, Waters J, Lever A, *et al.* Cytotoxic T-cell response to the nucleocapsid proteins of HBV in chronic hepatitis. *J Hepatol* 1987; 4: 15-21.

Poovorawan Y, Theamboonlers A, Chongsrisawat V, Sanpavat S. Molecular analysis of the a determinant of HBsAg in children of HBeAg-positive mothers upon failure of postexposure prophylaxis. *Int J Infect Dis* 1998a; 2: 216-20.

Poovorawan Y, Sripattanawat R, Theamboonlers A, Chongsrisawat V, Nuchprayoon I. Hepatocellular carcinoma: significance of HBV vertical transmission. *Asian Pac J Allergy Immunol* 1998b; 16: 93-103.

Poovorawan Y, Theamboonlers A, Jantaradsamee P, *et al.* Hepatitis B virus core promoter and precore mutants in Thai chronic hepatitis patients. *J Sci Soc Thai* 1999 (in press).

Saiki RK, Gelfand DH, Stoffel S, *et al.* Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988; 239: 487-91.

Sato S, Suzuki K, Akamatsu K, *et al.* Hepatitis B virus strains with mutations in the core promotor in patients with fulminant hepatitis. *Ann Intern Med* 1995; 122: 241-8.

Takahashi K, Aoyama K, Ohno N, *et al.* The precore/core promoter mutant ($T^{1762}A^{1764}$) of hepatitis B virus: clinical significance and an easy method for detection. *J Gen Virol* 1995; 76: 3159-64.

Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine *in vivo*. *Hepatology* 1996; 24: 714-7.